Link-N Peptide: A potential therapeutic agent for biological repair of early degenerated Human Intervertebral Discs

Rahul Gawri

Division of Surgical Research,
McGill University, Montreal

July 2013

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

© Rahul Gawri 2013
This work is dedicated to the loving memory of Sonam Gawri, beloved daughter and sister, who will remain in our hearts forever.
ABSTRACT

Chronic low back pain is a disease affecting a big portion of the population with 70% having at least one episode of low back during their lives. Intervertebral disc (IVD) degeneration is the most common cause of low back pain. It is associated with degradation and loss of proteoglycans, mainly aggrecan. Currently main treatment modalities offered for treating IVD degeneration are surgical and mainly target end stage disc disease. Medical therapies are being developed to treat and retard IVD degeneration and growth factor therapy is one such upcoming modality. Link protein is a component of IVD matrix. Link-N is a 16 amino acid peptide, cleaved from the N terminal of Link protein. It is found in the matrix of degenerating IVDs and is thought to have an effect on IVD metabolism including stimulation of proteoglycan synthesis. To test the regenerative potential of Link-N in degenerating human discs, IVD cells were exposed to the peptide. Link-N exposure resulted in a dose dependent increase in proteoglycan synthesis, stimulated proteoglycan synthesis and modulated protease production in an inflammatory environment. Organ culture models are commonly used tools for understanding disease process and action of potential therapeutic agents. There was no ideal model for studying IVD pathophysiology in humans; therefore a whole organ culture model was developed. This model maintained cell viability up to 4 months and Link-N peptide was able to stimulate sustained proteoglycan synthesis in the discs. In order to ensure a sustained effect of treatment, sustained activity is important. The stability of Link-N peptide was evaluated in the presence of IVD cells. Link-N was processed by IVD cells generating a new peptide retaining the bioactive properties of the parent peptide. Thus, the present study establishes Link-N peptide as a promising bioactive agent for treating IVD degeneration by regenerating degenerated discs and by retarding the ongoing degenerative process.
RÉSUMÉ

La lombalgie est une maladie chronique affectant 70% de la population de plus de 60 ans. La dégénération des disques intervertébraux (DIVs) est la principale cause de lombalgie. Elle est associée à la dégradation et la perte de protéoglycans, principalement de l’agrégane. Les traitements présentement offerts, comme la chirurgie, visent les stades avancés de la maladie. Des facteurs de croissance ont aussi été utilisés pour traiter et/ou retarder la dégénération des DIVs. La protéine Link est une composante de la matrice des DIVs. Link-N est un peptide de 16 acides aminés produit par le clivage de la section N-terminale de la protéine Link. Link-N est retrouvé dans la matrice des disques en cours de dégénération et notre hypothèse est qu’il aurait un effet positif sur le métabolisme des DIVs. Afin de vérifier le potentiel régénérateur de Link-N, des cellules des DIVs ont été exposées au peptide. Nos résultats démontraient que Link-N induisait la synthèse de protéoglycans de façon dose-dépendante et modulait la production de protéases dans un environnement inflammatoire. Il n’existe pas de modèle idéal pour étudier la physiopathologie des DIVs humains. Nous avons donc développé un modèle de culture de DIVs entiers. La viabilité cellulaire a été maintenue jusqu’à 4 mois dans ce modèle. Aussi, Link-N fut capable de stimuler une synthèse soutenue de protéoglycans dans le disque, condition essentielle afin d’assurer un effet soutenu d’un traitement. Les études de la stabilité de Link-N dans les IVDs démontaient que le peptide était transformé et que le nouveau peptide généré par l’activité cellulaire conservait les propriétés bioactives du peptide parent. La présente étude établie donc le peptide Link-N comme un agent bioactif prometteur dans le traitement de la dégénération des DIVs et le ralentissement du processus dégénératif en cours.
ACKNOWLEDGEMENTS

I would sincerely like to thank my supervisors Dr. Lisbet Haglund, Dr. John Antoniou and Dr. Fackson Mwale for giving me the opportunity to join the Orthopaedics Research Laboratories at Royal Victoria Hospital (now relocated to Montreal General Hospital) and Lady Davis Institute for Medical Research, Jewish General Hospital for conducting the research work presented in this thesis. I sincerely express my heartfelt gratitude and appreciation for providing me with excellent mentorship, excellent training facilities and for the superb mentorship during the laboratory work and the preparation of this thesis.

I would also like to thank my thesis supervisory committee members, Dr. Maria Petropavlovskaiia, Dr. Peter Roughley and Dr. Jean Ouellet for their constructive inputs and monitoring my timely progress.

I am also thankful to:-

— Late Dr. Dick Heinegard, Lund University, Sweden, for hosting me in his laboratory and letting me use the facilities available to his research team.

— Dr. Patrik Önnerfjord, Lund University, Sweden, for introducing me to the techniques of proteomics and for helping me to conduct the experiments and analyze the results from Link-N mass spectrophotometry experiments. I am also grateful to him for all the wonderful time I spent in Sweden and for all the advice received, work related and non-work related, and above all for being my friend.
— Dr. Peter Roughley, McGill University, for his generous gift of anti-G1 antibody and for allowing me to use scintillation counting facilities at Shriner’s Hospital for Children, Montreal.

— Dr. Thomas Steffen for allowing me to use the biomechanics part of his laboratory.

— Dr. Alain Petit, Lady Davis Institute for Medical Research, Jewish General Hospital for teaching me basics of basic science research and for all the help extended for translation of English text to French from time to time and for being one of my first friends in Montreal.

— Dr. Ovidiu Ciobanu, Lady Davis Institute for Medical Research, Jewish General Hospital for helping me with the figures used in this manuscript and for allowing me to use some of his excellent medical illustrations. Many thanks for showing me around Montreal in a different way, for giving me tips on photography, for countless rides to the airport and for being an excellent friend.

— Transplant Quebéc and its coordinators for organising the calls for spine retrievals through the organ donation program.

— Members of McGill Scoliosis and Spine Research Group, Dr. Jean Ouellet, Dr. Michael Weber, Dr. Michele Parolin, Dr. Waleed Awwad, Dr. Jacob Matthews, Dr. Rajeet Singh Saluja and Dr. Felipe Rossel for helping me with the spine retrievals.

— Members of the lab, Laura Epure, Janet Moir, Bashar Alkhatib, Scott McGrail, Lorne Beckman, Rajashree Sen, Padma Madiraju and Hongtian
Wang for helping me with experiments and providing an excellent working environment.

The presented thesis work was funded by Canadian Institutes of Health Research (CIHR), AO Spine, and North American Spine Society (NASS). Additional salary support came from internship program of MITACS through Acceleration-Quebec and research fellowship from Research Institute of McGill University Health Center (RI-MUHC).

A note of thanks to all my friends and relatives, continents and oceans away, for keeping in touch, giving me moral support and checking on my sanity every now and then.

I would also like to briefly thank all my critics for not believing in me and for their constant discouragement, which kept me strongly motivated and focused.

Finally I would like to express my deepest love and gratitude to my parents, Mr. Chandra Prakash Gawri and Mrs. Savita Gawri and to our furry kids, Viktor and Rocky for loving me, inspiring me, comforting me and for everything words can’t express. Thank you for giving me a blessed and wonderful life, for a wonderful education, for encouraging me and for believing in me and my vision, when no one else did......
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>i</td>
</tr>
<tr>
<td><strong>RÉSUMÉ</strong></td>
<td>ii</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGEMENTS</strong></td>
<td>iii</td>
</tr>
<tr>
<td><strong>TABLE OF CONTENTS</strong></td>
<td>vi</td>
</tr>
<tr>
<td><strong>LIST OF ABBREVIATIONS USED</strong></td>
<td>xiv</td>
</tr>
<tr>
<td><strong>LIST OF FIGURES</strong></td>
<td>xix</td>
</tr>
<tr>
<td><strong>LIST OF TABLES</strong></td>
<td>xxiii</td>
</tr>
<tr>
<td><strong>Introduction:</strong></td>
<td>xxv</td>
</tr>
<tr>
<td><strong>Chapter 1: (Literature Review)</strong></td>
<td>1</td>
</tr>
<tr>
<td>1. Focused clinical problem: Lower Back Pain</td>
<td>2</td>
</tr>
<tr>
<td>1.1 Low Back Pain (LBP):</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Incidence and prevalence of LBP in general population:</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Financial impact on healthcare system:</td>
<td>4</td>
</tr>
<tr>
<td>2.1 Anatomical structure and function of human spine:</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Structure and function of healthy human IVD:</td>
<td>11</td>
</tr>
<tr>
<td>2.3 Blood supply and nutrition of IVD:</td>
<td>14</td>
</tr>
</tbody>
</table>
2.4 Spinal Column: The Phylogenetic Overview: .......................................................... 18
2.5 Embryological development of human intervertebral disc (IVD): the notochordal cells: ........................................................................................................... 19
2.6 Cells of Intervertebral Disc: .................................................................................. 22
   3.1 Aggrecan: .............................................................................................................. 28
   3.2 Collagen: ............................................................................................................. 30
   3.3 Link Proteins: .................................................................................................... 32
   3.4 IVD extracellular Matrix Metabolism (ECM): .................................................. 34
   3.5 Link-N Peptide: ................................................................................................. 36
   3.6 State of ECM health and disease: ....................................................................... 37
4. IVD culture models: ............................................................................................... 41
5. Regenerative Medicine approaches in regeneration and repair of degenerated IVD: ..................................................................................................................... 43
   5.1 Cell based therapies: ........................................................................................... 44
   5.2 Use of growth factors in IVD: ............................................................................. 45
   5.3 Other modern approaches: ................................................................................ 46
6. Conclusions: .............................................................................................................. 46
Chapter 2 (Methods and Materials) ................................................................. 48

1. Source of Materials: .................................................................................... 49

1.1. Materials: ................................................................................................. 49

1.2. Antibodies: ............................................................................................... 50

1.3. Instruments and Softwares: ..................................................................... 51

2. Sources of Tissues: ....................................................................................... 51

2.1. Bovine Intervertebral Discs (IVD): ......................................................... 51

2.2. Human Lumbar Intervertebral Discs (IVD) Retrieval: ......................... 52

3. Radiology and grade of degeneration assessment on procured human lumbar
spines: ............................................................................................................. 56

3.1. X-ray of procured human lumbar spines: .............................................. 56

3.2. Grade of degeneration assessment of retrieved spines: ...................... 58

4. Definition of mediums and wash buffers used: ........................................ 59

5. Preparation of Link-N solution: ................................................................. 60

6. Isolation and culture of bovine and human intervertebral disc (IVD) cells in
alginate microspheres: ................................................................................... 62

6.1. Preparation of alginate solution: ............................................................. 62

6.2. Dissection of bovine and human intervertebral disc (IVD): ................. 62

6.3. Isolation of bovine and human intervertebral disc (IVD) cells: .......... 63

6.4. Embedding of disc cells in 1.2% alginate and culture in 3D microspheres: 64
6.5. Exposure of bovine and human intervertebral disc (IVD) cells to Link-N:...66

7. Analysis of gene expression by disc cells exposed to Link-N: .......................... 67

7.1 Exposure of intervertebral disc (IVD) cells to Link-N:................................. 67

7.2 Isolation and preparation of RNA from beads harvested from Link-N and Link-N+ IL-1β experiments: ............................................................................. 68

7.3 Reverse transcription and real time PCR (RT-PCR) analysis of prepared RNA: .................................................................................................................. 69

8. Preparation of human and bovine intervertebral discs (IVD) for no endplate (NEP), cartilaginous endplate (CEP) and bony endplate (BEP) culture models: ... 70

9. Culture of bovine and human intervertebral discs: ....................................... 73

10. Live/Dead® analysis: ...................................................................................... 74

10.1 Live/Dead® analysis of alginate beads: ...................................................... 74

10.2. Live/Dead® analysis of punches from CEP intervertebral discs (IVDs):.... 75

10.3 Preparation of slices from punches for confocal microscopy:.................... 76

10.4 Confocal microscopy of the tissue slices prepared from intervertebral disc (IVD) punches: .................................................................................................... 77

11. Injection of Link-N in CEP human intervertebral discs (IVDs) in culture:...... 78

12. Analysis of $^{35}$SO$_4$ incorporation in newly synthesized proteoglycans (PGs) under the effect of Link-N: ................................................................. 81
13. Diffusion and distribution of Link-N injected in intervertebral discs (IVDs) prepared with CEP method: ................................................................. 82
14. DMMB assay for GAG content estimation: .............................................. 86
15. Western blot analysis to probe for G1 domain in CEP intervertebral discs: .... 86
16. Mass spectrophotometry: ........................................................................ 87
17. Statistical analysis: .................................................................................. 88

Chapter 3 (Results) ....................................................................................... 91

1. Effect of Link-N on proteoglycan synthesis and protease production by bovine and human IVD cells cultured in non inflammatory and inflammatory environment: ........................................................................................................ 92

1.1 Effect of Link-N on proteoglycan synthesis by bovine and human IVD cells: ........................................................................................................ 92

1.1.1 Dose response curve for proteoglycan synthesis by bovine and human IVD cells: ........................................................................................................ 92

1.1.2 Variation in response to Link-N exposure in human IVD cells from different donors: ........................................................................................................ 95

1.1.3 Evaluation of response to Link-N in human IVD cells from a very young donor: ........................................................................................................ 96

1.1.4 Evaluation of specificity of Link-N sequence in stimulating proteoglycan synthesis in bovine and human IVD cells: .............................................................. 98
1.2 Effect of Link-N on gene expression of aggrecan and proteases by bovine and human IVD cells cultured under non inflammatory and inflammatory environment: ........................................................................................................................................ 100

1.2.1 Effect of Link-N on aggrecan and proteases gene expression by bovine and human IVD cells under non inflammatory environment: ......................................................... 100

1.2.2 Effect of Link-N on gene expression by bovine IVD cells cultured under inflammatory environment: .............................................................................................................. 103

1.2.3 Effect of Link-N on proteoglycan synthesis by bovine and human IVD cells cultured in an inflammatory environment: ...................................................................................... 105

2. Development of a whole organ culture model for long term culturing of IVD: 108

2.1 Evaluation of the swelling potential of bovine and human IVD prepared with NEP and CEP isolation techniques: ........................................................................................................ 108

2.2 Evaluation of effect of surface tension on direction of swelling in NEP IVDs: ...................................................................................................................................................... 111

2.3 Evaluation of effect of IVD proteoglycan on swelling potential of CEP model: ..................................................................................................................................................... 113

2.4 Evaluation of effect of isolation method on cell viability of human IVDs in short term culture: ........................................................................................................................................ 114

2.5 Effect of different nutritional levels on cell viability in IVDs prepared with CEP method: ........................................................................................................................................ 118
2.6 Effect of culture conditions on metabolic state of human IVD prepared with CEP method: ........................................................................................................ 120

2.7 Effect of prolonged culture periods under different nutritional states on cell viability in IVDs prepared with CEP method: .............................................. 122

3. Effect of Link-N injection in CEP model human IVDs in culture: ............... 124

3.1 Evaluation of $^{35}$SO$_4$ diffusion and equilibrium post injection in human IVDs prepared with CEP method: ........................................................................ 124

3.2 Effect of modified culture technique on cell viability in human IVDs prepared with CEP method: .................................................................................. 126

3.3 Effect of Link-N injection on cell viability in human IVDs prepared with CEP method: ................................................................................................. 128

3.4 Effect of Link-N injection on proteoglycan (PG) synthesis in human IVDs prepared with CEP method: ........................................................................ 129

3.5 Evaluation of stimulation of proteoglycan synthesis in areas adjacent to Link-N injection site: ...................................................................................... 133

3.6 Evaluation of diffusion of Link-N injected in human (IVDs) prepared with CEP method: ..................................................................................................... 135

3.6.1 Diffusion of injected Link-N through endplates of IVDs prepared with CEP method: ................................................................................................. 135

3.6.2 Evaluation of binding of injected Link-N to the matrix of human IVDs: . 136
3.6.3 Evaluation of specific binding of 5-TAMRA conjugated Link-N in bovine IVDs through the Link-N moiety: ................................................................. 139

3.7 Evaluation of sustained stimulation of proteoglycan synthesis in human IVD injected with Link-N: ................................................................. 141

4. Metabolism of Link-N by IVD cells and evaluation of biological effect of generated metabolic products: ................................................................. 143

4.1 Stability of Link-N peptide in culture and its modulation by nucleus pulposus (NP) and annulus fibrosus (AF) cells: ............................................. 143

4.2 Detection of generated cleaved by products of Link-N modulation by human annulus fibrosus cells: ................................................................. 145

4.3 Evaluation of biological activity of Link-N (1-8) peptide by assessing stimulation of proteoglycan production in human and bovine IVD cells exposed to the sequence in a non-inflammatory environment: .......................... 147

4.4 Evaluation of biological activity of Link-N (1-8) peptide by assessing stimulation of proteoglycan production in human and bovine IVD cells exposed to the sequence in an inflammatory environment: .............................. 151

Chapter 4 (Discussion) ............................................................................................................. 155

1. Advantages of cartilaginous endplate (CEP) model of human IVD culture: .... 157

2. Link-N as a therapeutic agent for treating IVD degeneration: ............................. 164
3. Differences in response to Link-N by IVD cells reported by other recent studies and difference in response by NP and AF cells: ............................................................. 170

4. Degradation of Link-N by human AF cells and discovery of minimum sequence which is still bioactive: ............................................................................. 172

5. Variation in response to Link-N by different human donors and selection of candidate cohort for Link-N therapy: ................................................................. 175

6. Possible modes of delivery of Link-N to human IVDs in vivo and its clinical relevance: ................................................................................................................. 178

7. Conclusions: ................................................................................................. 181

Chapter 5 (Contributions to Knowledge) ................................................................ 182

Reference List: ..................................................................................................... 194

Appendix ............................................................................................................... 224

Appendix 1(A): Questionnaire to evaluate whether the harvested spines had associated pain symptoms (English version). ......................................................... 225

Appendix 1(B): Questionnaire to evaluate whether the harvested spines had associated pain symptoms (French version). ......................................................... 226

Appendix 2: Harvest form citing inclusion criteria for the harvested spine ............ 227

Appendix 3: Permission from copyright holder of figures used in the thesis .......... 230
LIST OF ABBREVIATIONS USED

ACN- Acetonitrile
ADAMTs- A Disintegrin And Metalloproteinase with Thrombospondin Motifs
AF- Annuls Fibrosus
AMBIC- Ammonium bicarbonate
BEP- Bony Endplate
BMP- Bone morphogenetic protein
cDNA- cyclical deoxyribonucleic acid
CEP- Cartilaginous Endplate
CHAD- Chondroadherin
COMP- Cartilage oligomeric matrix protein
Conc. – Concentration
CPM- Counts per minute
CS- Chondroitin Sulphate
CS-1- chondroitin sulfate-1 glycosaminoglycan attachment domain
CS-2- chondroitin sulfate-2 glycosaminoglycan attachment domain
Ct- threshold cycle
Da- Daltons
DDD- Degenerative Disc Disease
DMEM- Dulbecco's modified Eagle medium
DMMB- Dimethylmethylene blue
DNAase- Deoxyribonucleotidase
DTT- Dithiothreitol
ECM- Extracellular Matrix
EP- Endplate
EtOH- Ethyl alcohol
FA- Formic acid
FGF- Fibroblast like growth factor
G1- N-terminal globular domain 1 of aggrecan
G2- globular domain 2 of aggrecan
G3- C-terminal globular domain 3 of aggrecan
GAG- Glycosoaminoglycans
GDF-5- Growth and development factor 5
Gms- Grams
HA- Hyaluronic Acid
HBSS- Hank’s Balanced Salt Solution
HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HH- High glucose high FCS media
HL- High glucose low FCS media
IAA- Iodoacetamide
iAF - Inner Annulus Fibrosus
IGD- Interglobular domain
IGF-1- Insulin like growth factor -1
IL-1 - Interleukin-1
ITS- Insulin- Transferrin- Selenium
IVD – Intervertebral Disc/ Discs
KS- Keratan Sulphate
LBP – Low Back Pain
LC ESI MS- liquid chromatography on-line with electrospray-iontrap mass spectrometry
LH- Low glucose high FCS media
LL- Low glucose low FCS media
LP-1- link protein component 1
LP-2- link protein component 2
LP-3- link protein component 3
MALDI-TOF- Matrix-assisted laser desorption/ionization-time of flight
MMP- Matrix Metalloproteinase/ Metalloproteinases
MRM- multiple reaction monitoring
MSCs- Mesenchymal stem cells
MW- Molecular weight
NEP- No endplate
NP- Nucleus Pulposus
O₂- Oxygen
oAF- Outer Annulus Fibrosus
PBS- Phosphate Buffer Saline
PG - Proteoglycan/ Proteoglycans
psi- Per square inch
QTOF-LC MS/MS - liquid chromatography tandem mass spectrometry with quadruple time of flight

RNA - Ribonucleic acid

RNAase - Ribonucleotidase

RT - Reverse transcription

RT-PCR - Real time polymerase chain reaction

SDS/PAGE - Sodium dodecyl sulphate/ polyacrylamide gel electrophoresis

siRNA - Small interfering RNA

SLRPs - Small leucine rich repeat proteoglycans

TAMRA - Tetramethyl-6-carboxyrhodamine (dye)

TGF - Transforming growth factor

ΔΔCt - Difference between the Ct values of 18S from the Ct value of each target
LIST OF FIGURES

Figure 1: Views of Spinal Column................................................................................. 7

Figure 2: The Vertebral Body.......................................................................................... 9

Figure 3: Function of IVD............................................................................................. 11

Figure 4: Macroscopic Structure of IVD......................................................................... 13

Figure 5: Nutritional Supply of IVD............................................................................... 16

Figure 6: Nutrient Gradients in IVD............................................................................... 17

Figure 7: Embryonic Development of IVD...................................................................... 21

Figure 8: Cells of IVD.................................................................................................... 24

Figure 9: Matrix of IVD.................................................................................................. 27

Figure 10: Structure of Aggrecan.................................................................................... 29

Figure 11: Interactions between Hyaluronic acid and Link protein................................. 33

Figure 12: Enzymatic Cleavage sites in Aggrecan.......................................................... 35

Figure 13: Generation of Link-N peptide......................................................................... 37

Figure 14: Change in IVD matrix composition with age.................................................... 39

Figure 15: Retrieval of Lumbar Spine............................................................................. 53

Figure 16: Radiology of Harvested Lumbar Spine............................................................ 58
Figure 17: Grades of Degeneration of Harvested Spine ........................................ 59

Figure 18: Different IVD Culture Models .......................................................... 71

Figure 19: Preparation of Different IVD Culture Models .................................... 72

Figure 20: Preparation of IVD cores for confocal microscopy ............................ 77

Figure 21: Autofluorescence of IVD matrix autofluoresced in UV range (double arrows) and distinctly had no contribution ................................................. 83

Figure 22: Proteoglycan synthesis dose response curve in bovine and human IVD cells ........................................................................................................... 94

Figure 23: Variation in response to Link-N in IVD cells from different human donors ................................................................. 96

Figure 24: Evaluation of proteoglycan synthesis in human IVD cells from young donors ........................................................................................................ 97

Figure 25: Evaluation of Link-N sequence on neo proteoglycan synthesis by bovine and human IVD cells ................................. 99

Figure 26: Gene expression analysis in bovine IVD cells exposed to Link-N in non-inflammatory environment ........................................ 102

Figure 27: Gene expression analysis in bovine IVD cells exposed to Link-N in an inflammatory environment ........................................ 105
Figure 28: Evaluation of Link-N sequence on neo proteoglycan synthesis by bovine and human IVD cells. ................................................................. 107

Figure 29: Evaluation of swelling potential in NEP and CEP IVDs .................... 110

Figure 30: Evaluation of direction of swelling in bovine IVDs ......................... 112

Figure 31: Correlation of proteoglycan content to swelling potentials of human IVDs ................................................................................. 114

Figure 32: Evaluation of cell viability with different isolation methods for IVD whole organ culture. ................................................................. 117

Figure 33: Evaluation of cell viability in human IVDs CEP model cultured in different conditions for 4 weeks. ....................................................... 119

Figure 34: Evaluation of the effect of varying nutritional states on the metabolism of disc matrix in human IVDs in culture for 4 weeks. ................. 122

Figure 35: Evaluation of cell viability in human intervertebral discs (IVDs) CEP model cultured in different culture conditions for 4 months. ........... 123

Figure 36: Evaluation of 35SO4 diffusion and equilibrium injected in human IVDs prepared with CEP method .............................................................. 126

Figure 37: Evaluation of effect of culturing human IVDs prepared with CEP method in gas permeable bags on cell viability: ........................................... 127
**Figure 38:** Evaluation of effect of Link-N injection on cell viability in human IVDs prepared with CEP method ................................................................. 129

**Figure 39:** Evaluation of stimulation of proteoglycan synthesis in human CEP IVDs injected with Link-N ............................................................................. 130

**Figure 40:** Evaluation of interspinal and intraspinal variation in stimulation of proteoglycan synthesis in human CEP IVDs injected with Link-N ............... 132

**Figure 41:** Evaluation of stimulation of proteoglycan injection at sites adjacent to the site of Link-N injection ........................................................................... 134

**Figure 42:** Evaluation of diffusion of Link-N injected in human IVDs .......... 136

**Figure 43:** Evaluation of binding of injected Link-N to IVD matrix. .......... 138

**Figure 44:** Residual binding of Link-N to IVD matrix ................................ 139

**Figure 45:** Evaluation of specific binding through Link-N moiety in 5-TAMRA Link-N conjugate injected in IVDs prepared with CEP method .................. 140

**Figure 46:** Evaluation of sustained effect of Link-N injection on proteoglycan synthesis in human IVDs ................................................................. 142

**Figure 47:** Evaluation of stability of Link-N cultured with and without human IVDs .......................................................................................................... 145

**Figure 48:** Identification of sequence of cleaved peptide generate by modulation of Link-N by human AF cells ................................................................. 147

xxii
Figure 49: Evaluation of the effect of Link-N (1-8) on proteoglycan synthesis in bovine and human IV) cells in a non-inflammatory environment: .......................... 150

Figure 50: Evaluation of effect of Link-N (1-8) on proteoglycan synthesis in bovine and human IVD cells in an inflammatory environment ........................................... 153
LIST OF TABLES

Table 1: Tabulation of IVD donors with age, sex, cause of death and IVD levels used in the study. ............................................... 56

Table 2: List of genes evaluated post Link-N exposure ........................................... 70

Table 3: Table showing cell viability in IVDs cultured in different culture conditions for 4 weeks .................................................... 120

Table 4: Table showing cell viability in IVDs cultured in different culture conditions for 4 months .................................................... 124
Introduction:

(Rationale and Objectives of the Research)
Introduction:

The intervertebral disc (IVD) is a fibrocartilaginous tissue forming a cushion like structure between two vertebral bodies and providing ports of exit for spinal nerves arising from the spinal cord and imparting flexibility to the spinal column, enabling a high degree of flexibility in movement.

Low back pain (LBP) is a fairly common problem with one year prevalence rate at 56% in individuals less than 60 years of age [1]. LBP is strongly associated with degeneration of IVD giving rise to what is called degenerative disc disease (DDD). The components of the matrix namely proteoglycans (PG) and collagens are degraded in DDD and the IVD loses its ability to retain water due to loss of negatively charged PGs providing the cushioning effect and thus leading to collapse of the adjacent vertebral bodies on each other and compressing the nerves arising from spinal canal leading to neural and neuromuscular symptoms.

Treatment of LBP is symptomatic and the treatment options available for advanced DDD are symptomatic including physiotherapy and for end stage disc disease, surgical. Tissue engineering and regenerative medicine approaches employed in treatment of DDD aim at restoring PG levels within the IVD in order to enable IVD to retain water and provide mechanical support to the vertebral bodies. Many approaches have been employed in this direction ranging from cell based therapies to use of growth factors and gene therapy. Amongst all the approaches, growth factor
therapy is perceived as the most viable option due to its relative ease of administration and cost effectiveness when compared to other therapies [2-4]. Use of recombinant growth factors though could be limited by their high cost of production and procurement. This problem has led to discovery and evaluation of growth factor like bioactive peptides. One such candidate is Link-N peptide, which is a cleavage product of Link protein found in the extracellular matrix (ECM) of IVD, and has shown stimulation of PG synthesis in human IVD cells and rabbit IVD injury model.

Cell cultures, organ culture models and animal models are commonly employed in conducting research in IVD regeneration. IVDs from lower animals such as rats and mice are generally used for research studies and these are not the best models to study human IVD degeneration due to presence of embryonic notochordal cells throughout life and different biomechanics to human spinal segments. Thus, if stimulatory response to growth factors or growth factor like agents can be shown in human degenerated IVDs, which is a naturally occurring process spanning decades, it will push us one step closer to conducting human trials. For this, the need for an organ culture model for human IVD is warranted. Development of an organ culture model has not been successful for human discs to date and previous attempts to create such a system were only aimed at biomechanical studies, which were not fit for biological evaluations as both short term and long term viability of the IVD were not achieved, thus limiting the expansion of knowledge base.

**Objectives:**
The specific objectives of the current doctoral research thesis project were:-

Firstly, to evaluate the effect of Link-N peptide on stimulation of PG synthesis in cells isolated from nucleus pulposus (NP) and annulus fibrosus (AF) regions of human and bovine IVDs. The dosage of Link-N peptide at which optimal response is seen was determined and its potential to stimulate PG synthesis in an inflammatory milieu at this dose was evaluated, which is important if a growth factor is to be established as an injectable therapeutic agent for use in human subjects.

Secondly, to evaluate the effect of Link-N in conditions closest to in vivo human conditions, intact free loading whole disc organ culture model was developed with success and this model was able to sustain cell viability over prolonged culture periods. This has never been attempted and achieved and this organ culture model is unique and first of its kind for human discs. The regenerative potential of Link-N peptide was evaluated in this organ culture model and its sustained effect in this system studied. The distribution and localization of injected Link-N peptide in cultured human IVDs was also studied.

Thirdly, the metabolism of Link-N peptide by IVD cells was evaluated and a novel peptide discovered and its biological potential in IVD regeneration was assessed.
Chapter 1:
(Literature Review)
Literature Review:

1. Focused clinical problem: Low Back Pain

1.1 Low Back Pain (LBP):

Low back pain (LBP) affects almost all individual in one form or another, be it a direct effect or an indirect effect due to back pain sufferer amongst family or friends. LBP can be classified as acute, episodic or chronic forms based on pattern and duration of occurrence [5]. It can also be classified on the basis of causation namely traumatic, metabolic, psychogenic, age related etc. Another classification which is based on the probable source of pain in patients suffering from LBP classifies the disease as discogenic, neurogenic or musculogenic. Acute back pain is a single episode of incidence generally not lasting more than 6 weeks [International Classification of Disease (ICD) 9 definition] and generally doesn’t require surgical intervention and is treated symptomatically. Episodic or recurrent LBP, on the other hand is associated with events or activities leading to LBP which generally resolves by itself in few days. Examples of causes of episodic LBP are (and not limited to) rigorous exercise without proper warm up, painful menstrual cycles due to irritation of peritoneal by maturing ovarian follicles (known as Mittelschmerz), which generally is a type of referred pain to the lower spinal segment. Andersson (1999) quotes various guidelines and recommendations and define chronic LBP as “Back pain that lasts for longer than 7–12 weeks. Others define it as pain that lasts beyond the expected period of healing, and acknowledge that chronic pain may not have well-
defined underlying pathological causes. Others classify frequently recurring back pain as chronic pain since it intermittently affects an individual over a long period” [6]. Neurogenic LBP is sequelae of compression of dorsal nerve root compression at intervertebral foramen because of collapse of intervertebral space due to intervertebral disc (IVD) degeneration. Musculogenic LBP arises from stiff muscles which are in the state of sustained clonic contraction, where patients locate and describe the pain to arise from around the area of lower lumbar spine due to nerve distribution related localization of pain. Discogenic LBP is an area of debate where the origin of pain in otherwise asymptomatic LBP is thought to be due to degeneration induced innervation of IVD and neuritis and radiculitis due to disc rupture [Source: International Classification of Disease (ICD-9), disease code 722.22]. Chronic LBP is among the most common chronic illnesses and according to one review, is ranked 6th amongst top non-life threatening chronic illnesses affecting humans [7]. Chronic LBP is mainly a disease associated with ageing process but its incidence has a very broad age spectrum involving very young subjects. Outside of normal or accelerated ageing, chronic LBP maybe caused due to trauma, excessive stretches leading to rupture of IVD fibers, abnormal loading states such as pull by second and third trimester gravid uterus etc.

1.2 Incidence and prevalence of LBP in general population:
Low back pain (LBP) is a disease with widespread prevalence across all age groups. Majority of the cases of LBP are seen in the age group from 40-60 years [8]. Every 4 in 5 adults have at least one episode of LBP in their lifetimes [9]. LBP is the most
common cause of disability in persons below age 45 and 2nd most common cause (after arthritis) in 45-65 years age group. Reported one year prevalence rates vary between 24-60% [10-12]. 15-45% of adults suffer from LBP per year and 1 in 20 adults present with a new episode every year. 70% of the population past age 60 will have experienced at least one episode of low back pain [13]. In U.S. the lifetime incidence of LBP is between 50-80% [8]. The incidence of LBP is higher in females (47.0 per 1000 person-years) compared to males (42.2 per 1000 person-years) [14]. According to a Danish study, females reported more cases of LBP than males but the difference was insignificant [1]. Whereas according to Statistics Canada, in Canada there is no difference in incidences in males and females (http://www.statcan.gc.ca/pub/82-619-m/2006003/4053542-eng.htm). According to one study, in U.S., Caucasian population is more affected than other racial groups [15]. Around 2% of all doctor visits are due to LBP according to American Medical Association (AMA, 2005). There is evidence that an episode of LBP in early adulthood can serve as a risk factor to developing chronic LBP later in life [16]. Incidence of LBP is strongly associated with type of occupation with occupations requiring strenuous activities are more prone to developing LBP [17]. Such high incidence and prevalence across a wide range of age groups makes it a disease that requires a fairly good share of healthcare budget to be treated.

1.3 Financial impact on healthcare system:

LBP has profound financial impact on healthcare systems all around the world due to direct and indirect incurred costs. Direct costs are related mainly to treatment
modalities, both therapeutic and symptomatic whereas indirect costs are incurred due to loss of productive man hours and payments made under workman’s compensation [18]. The cost of treating LBP, both direct and indirect costs, in United States alone has remained steady at around $100 billion per year according to estimates done in 1991 and 2004 respectively [19]. Another 2003 study puts the total cost of treating LBP at close to USD 6000 per patient per year at the prevalence rate at any given point of time [20]. Data from other developed countries has also put the expenditure in billions of dollars with Sweden (2001) at €1.8 billion [21], Australia (2001) at $9.1 billion [22], UK (1998) at £ 12.3 billion[12] and Germany (2009) at € 7000 per patient per year [23]. In Canada, the estimates have been put around at 30 billion dollars but no systematic review has been published due to provincial nature of healthcare system. Quebec’s La Commission de la santé et de la sécurité du travail (CSST), the agency for workman’s compensation, has put the cost of treatment at around CAD 6000 per claim per year (Sherbrooke Model) [24].

Workman’s compensation board of British Columbia puts the median of claims at CAD 6981 (www.worksafebc.com/health_care_providers/Assets/PDF/posterpresentations/low_back_pain1987-2001.pdf). Clearly Canada too faces a heavy burden on healthcare system due to high incidence and prevalence of low back pain and also due to high cost involved with symptomatic and therapeutic management of it. Focus is now shifting to diagnose and treat the disease at an early stage and to find medical treatments for low back pain, since surgical modalities are the only option available for treating disc disease. Expanding research in the LBP in the field of
regenerative medicine involving growth factors, bioactive peptide, cell and gene based therapies will thus help in decreasing morbidities and prevalence of LBP and reduce the burden on the exchequer.

2. Anatomy and Functionality of Human Spine:

2.1 Anatomical structure and function of human spine:

Human spine follows the common structural pattern found in class *mammalia*. Human spine is divided into 5 regions, namely cervical, thoracic, lumbar, sacral and coccygeal. There are 4 curves found in human spine corresponding to their anatomical location, namely cervical (ventral curve), thoracic (dorsal curve), lumbar (ventral curve) and sacral (dorsal curve) [Figure 1].
Figure 1: Views of Spinal Column: Dorsal (posterior), ventral (anterior) and lateral (left) views of human spine depicting the segments of human spine and showing the location and direction of the spinal curves. Image obtained from www.netterimages.com. Image also printed in Atlas of Human Anatomy 5th Ed., by Frank H. Netter.
The main functions of the human spine include maintaining the upright, erect, bipedal stature of the human skeleton. The curvatures of human spine make it “neutral” to ensure that the spine doesn’t crumble under the axial weight of the human body and the atmospheric pressure experienced by it. The human spine is composed of following number of vertebrae; cervical (7), thoracic (12), lumbar (5), sacral (5) and coccygeal (4). Human spinal column is composed of 33 bony segments during fetal stage but at birth the sacral and the coccygeal segments fuse to form one segment and post natal human spine comprises of cervical (7), thoracic (12), lumbar (5), sacral (1) and coccygeal (1), a total of 26 bony segments. The bony part of the spinal column are called vertebrae which as stated are 26 in number and considerably vary in shape and size as one progresses down from cervical to sacral region. All the vertebrae in human spine are functional except for coccygeal segment as it is vestigial in human.

The mechanical functional unit of spine is called motion segment and is composed of two adjacent vertebral bodies with the intervening intervertebral disc [25].

Typical vertebral body (with exception of 1\textsuperscript{st} and 2\textsuperscript{nd} cervical vertebrae) is composed of the centrum (body), pedicle, transverse process, spinous process and superior and inferior articular facets [Figure 2 (A)]. The notch on the inferior border of the pedicle of the cephalic vertebrae and the notch on superior border of pedicle of caudal vertebrae together form vertebral foramen which serves as an outlet port for the dorsal nerve radicals arising from the spinal cord [Figure 2 (B)].
Figure 2: The Vertebral Body: (A) Cephalic end view of lumbar vertebrae showing various anatomic landmarks and features. (B) Lateral view of lumbar vertebrae showing various anatomic landmarks and features including the position of vertebral foramen. Figures obtained and modified from Gray’s Anatomy of the Human Body, 20th Ed. U.S. These images are in the public domain because its copyright has expired.
due to publishing prior to 1918. This applies worldwide. [Information source: https://en.wikipedia.org/wiki/File:Gray93.png].

The IVDs are named depending on the vertebral bodies it separates, for example, L1-L2 IVD means IVD lying in between L1 and L2 vertebrae. Thus all the 23 IVDs are named in this way except C1-C2 as there is no cartilage in between C1 and C2 vertebrae.

The spinal column is stabilized by a series of spinous and inter-spinous ligaments. External structural support is provided by various spinal and paraspinal muscles. The vertebral body along with the intervertebral disc forms the anterior spinal segment whereas the remaining parts comprise what is called the posterior segment of the human spine. Together these anterior and posterior segments form the spinal canal and enclose the spinal cord.

The vertebral bodies are separated by a fibro cartilaginous tissue known as intervertebral disc (IVD). IVDs serve as a cushion and shock absorbers and intercalate between the vertebral bodies and prevent them from collapsing on each other. It is because of IVDs that the vertebral bodies have a viable space between them, the vertebral foramen, as described previously described, which is the entry and exit port for spinal, parasympathetic and sympathetic nerves. This near perfect biomechanical design of the human spine enables us to maintain a bipedal upright
posture and withstand close to 400kgs/ psi of atmospheric pressure without collapsing

[Figure 3].

![Figure 3: Function of IVD: Figure depicting the location and function of IVD. Note the exit of spinal nerves from spinal cord through transverse foramen. Without the IVD the vertebrae will crash on each other and compress on nerves. (Figure modified from http://www.mmi.mcgill.ca/mmimediasampler/).](image)

2.2 Structure and function of healthy human IVD:

Intervertebral discs (IVDs) are the fibro-cartilaginous structure lying between two adjacent vertebral bodies. The IVDs are separated from the vertebral bodies by 3-4
mm of hyaline cartilage called endplate. The endplate has two thinly demarcated parts, the one towards the vertebral body is called bony endplate (BEP) and the part towards the disc is called cartilaginous endplate (CEP). The endplate is considered as the part of the intervertebral disc. The collagen fibers from the annulus fibrosus anchored and are closely interwoven to the endplate. The outer part of the disc is not covered by endplates and is called the ring apophysis. The intervertebral disc has roughly two zones. Central gelatinous nucleus pulposus (NP) and the concentric lamellar surrounding fibrous region called annulus fibrosus (AF). [Figure 4(A)]. The AF region is divided into inner annulus fibrosus (iAF) and outer annulus fibrosus (oAF). With maturity from fetal to adult, the endplates decrease in diameter and thickness and cover only NP and iAF in adults [26].

Recent studies have shown the endplates to be single layered in some individuals and double layered in others. The double layered endplates are biomechanically more stable and with better adjacent disc health [27, 28].
Figure 4: Macroscopic Structure of IVD: Schematic representation of macroscopic structure of Intervertebral Disc. (B). Depiction of NP and AF regions of IVD. Proteoglycans (PG) gives jelly like consistency and tends to spread out which is restricted by AF. The lamellae of AF are arranged in crisscross fashion giving it additional structural support. (A) Reproduced with permission from www.chirogeek.com, (B) Modified from http://www.mmi.mcgill.ca/mmimediasampler/)

Endplate over the NP region has collagen fibres arranged in a random orientation pattern and encloses proteoglycans (PGs) within its fibrils. In AF region, the collagen fibres are arranged in an organized manner and oriented horizontal and parallel to the vertebral body. The collagen fibres of AF lamellae are continuous and blend with the collagen network of endplate to form a continuous structure [29-31].
As stated, IVD has two clearly demarcated regions, NP and AF. NP is central in location and is mainly composed of PG. The main function of NP is weight bearing and providing shock absorbing properties to the disc. In healthy discs, NP is gelatinous in nature due to its high water retention capacity owing to high PG content. AF is mainly fibrous and is composed of concentric lamellae of collagen. The main function of AF is providing structural strength to the disc and to prevent the extrusion of NP when excessive loads are applied on the disc. It also restricts unrestricted swelling on high water attracting NP. The AF also serves to redistribute the dissipate energy and load applied on the disc following Pascal’s law and prevents structural failure of the disc when subjected to physiological and non-physiological loads. In an adult disc there are around 25 such lamellae. The collagen fibres in these lamellae are oriented 60º to the horizontal. The direction of the adjacent lamellae is opposite to each other. This provides extra strength and restricts the tears of the AF fibers in one direction. If this was not the case and AF fibres were aligned in one direction, the AF would be all torn if excessive torsion or flexion-extension forces were applied in one plane. This crisscross design is nature’s way of restricting damage and tears in AF [29, 30, 32, 33]. [Figure 4(B)].

2.3 Blood supply and nutrition of IVD:

Intervertebral disc is the largest avascular organ in the human body [34]. This is nature’s way of protecting the unique composition of the intervertebral disc. The intervertebral disc is only vascular in the outer 1/3 region of the annulus fibrosus. The nutrition of the disc is mainly maintained through the endplates through passive
diffusion of nutrients. Out of the two established routes, diffusion through endplates is the major route of nutrition [35-37]. The capillary vasculature forms tufts beneath the subchondral bone below the endplate and from there the nutrients leak out of the capillaries through endothelial gaps in the capillaries and provide nutrition to the disc by diffusing inside the disc by following a diffusion gradient [Figure 5]. The avascular nature of the intervertebral disc makes it a very bad candidate for repair and regeneration due to lower clearance of accumulated proteases and cytokines but is also a protective mechanism as it prevents the entry of inflammatory cells namely the lymphocytes and neutrophils thus ensuring there is no fibrosis caused as a repair response and only the proteins native to disc matrix are replenished.

The nutrition of the IVD is through concentration driven gradients across the discs. The nutrition of disc cells is dependent on the porosity of the subchondral endplate and vertebral bodies. The state of the arterioles supplying the vertebral bodies also plays an important role and conditions such as arthrosclerosis, thrombosis, sickle cell anemia etc. severely hamper disc nutrition [35]. The charges on the molecules also plays an important role and uncharged dyes diffuse easier than their charged counterparts [35]. Similarly negatively charged antibiotics such as penicillin and cefuroxamine penetrate less efficiently than positively charged macrolides such as gentamicin and aminoglycosides whereas neutral antibiotics show intermediate diffusion [35, 38, 39]. The size of molecules also play an important role in diffusion and even glucose with MW 180 Da is partially restricted [35]. The transport of
solutes and metabolites also depends on diurnal loading and unloading of the disc due to physical activities and movement which helps drive in the nutrients from the capillary fluid derived from the vertebral capillary network at the endplate level [40, 41].

![Diagram of nutrient supply to intervertebral disc](https://www.chirogeek.com).

**Figure 5: Nutritional Supply of IVD**: Schematic diagram showing nutritional supply of intervertebral disc through endplates (in grey) and direct blood supply to outer 1/3 of annulus fibrosus [30]. (Reproduced with permission from [www.chirogeek.com](http://www.chirogeek.com)).

The indirect nutrient supply makes the disc hypoxic and more acidic compared to other tissues of the body [42]. The disc cells primarily derive their metabolic energy through glycolysis by conversion of glucose to lactic acid and making the disc pH
slightly more acidic [42]. Various metabolites such as glucose, O$_2$ and lactic acid (thus acidity), follow a gradient across the disc with glucose and O$_2$ having higher concentrations at the peripheral areas and lactic acid having highest concentration at the center of the disc [42] [Figure 6].

**Figure 6: Nutrient Gradients in IVD:** Schematics showing nutrient gradients for glucose, O$_2$ and lactic acid from endplate to endplate in IVD. (Reproduced from Grunhagen et al.,[42]).

Nutrients and metabolic products in the disc can follow convectional current or diffusion path. Disc loosens and regains 25% of its fluid content daily during diurnal
variation in activity and has been proven that the loss and increase in height daily is
due to loss and regaining of water [43] and creating convectional currents within the
disc. Smaller solutes have been shown to travel faster with diffusion than convection
[44, 45]. Also convection is outward in nature whereas diffusion is inwards.
Convections within the disc maybe be useful in extruding out waste metabolites and
for movement of larger molecules such as growth factors, proteases etc. which move
slower with diffusion. Convection can also have a role in distribution of newly
synthesized macromolecules within the IVD and loss of matrix degradation products
from the disc and subsequent loss of water retaining capacity of the disc leading to
collapsed disc and degeneration [26].

2.4 Spinal Column: The Phylogenetic Overview:
According to evolutionary embryology, *kingdom animalia* is divided into various
phylums. Among them *phylum chordata* is defined as organisms that have notochord
at some time during their development from embryo to full organism. Notochord is a
solid tube like structure derived from the endoderm of the embryonic germ layers
composed of endodermal stem cells. Notochord lies dorsal to the gut and gives rise to
the nervous system and its derivatives. It also provides a support structure in lower
animals during locomotion. Phylum *chordata* is divided in *subphylums* which are
further divided into *superclasses* and *classes* namely amphibia, reptilia, aves,
mammalian etc.
Human beings (*homo sapiens sapiens*) are classified as *kingdom animalia, phylum chordata, subphylum vertebrata, class mammalia*. Being part of *subphylum vertebrata* means that we have a solid back bone composed of bone. The backbone and its components in *subphylum vertebrata* are derived from mesodermal stem cells from mesoderm surrounding notochord which at different stages of development form different components of the backbone in addition to the nervous system being formed by the notochord. The various components of the backbone are the vertebral bodies, intervertebral discs, cartilage of facet joints, etc. The central portion of the intervertebral disc is formed by the notochordal cells whereas the peripheral region is formed by the mesenchymal cells. The mesodermal derivatives form a protective covering around the spinal cord and fuses on lateral aspects thus forming a hollow tube like structure around the spinal cord. The vertebral column thus protects the spinal cord from compressions and also provides a solid support structure for locomotion.

2.5 Embryological development of human intervertebral disc (IVD): the notochordal cells:

The IVD is a mesenchymal derivative arising from mesodermal germ layer. The disc originates from sclerotomes of the mesodermal somite [Figure 7 (A)]. There are two tubular structures; the dorsal hollow tube called neural tube and the ventral solid tube called notochord [Figure 7 (B)]. For some time the cells from the somites are seen as a thick band and they start to migrate and transverse to surround the neural tube. The mesenchymal cells from the somite arrange themselves into a cranial and a caudal
less condensed region sandwiching a dense region called perichordal disc [Figure 7 (C)]. The caudal less condensed region from one somite and cranial condensed region from the adjacent somite fuse to form the vertebral body, transverse process, neural arch and spinous process. The perichordal disc still has notochord in its center [Figure 7 (D)]. The perichordal disc forms the AF and gives rise to AF cells and the notochord forms and gives rise to NP cells. The origin of EP cells is still unclear and definite origins of these cells have to be investigated with modern genotyping tools available [29]. Some authors though believe the EP cells to arise from less condensed part of the somite giving rise to the vertebral body and EP cartilage would develop in the same way as a joint with bone and adjacent cartilage without the development of synovium and other joint encapsulating structures [26]. Other studies have suggested the endplate cells to have scleretomal origin [46, 47].
Figure 7: Embryonic Development of IVD: (A-E). Illustrations showing development of human IVD from mesodermal derivatives. (Figure courtesy Dr. Ovidiu Ciobanu, modified with permission).

Notochordal cells are embryonic in origin and have the capability of retaining embryonal features. Human IVD retain notochordal cells till birth and the number of these cells starts to decline and by age 10, which is controversial and not supported by molecular studies. On the contrary, recent studies suggest preservation of notochordal cells in adult human discs [48, 49]. Mouse lineage tracking studies also support this notion [50, 51]. Human IVD reaches maturity mature to have fibroblast like cells in AF and chondrocyte like cells in NP [26]. Some species such as rabbits,
mice, rats and some breeds of dogs such as non-chondrostrophoid breeds retain the notochord cells throughout their life [52, 53].

Notochordal cells have been shown to have a protective effect on NP cells [54], alter functioning of NP cells promoting them to produce proteoglycan [55] and direct stem cells towards NP like cells differentiation [56, 57]. In light of above facts, the choice of animal model both in vivo and in vitro thus should take presence or absence of notochordal cells in adult discs of that species into account as results coming out of these models cannot be extrapolated to human IVDs and no concrete assumption of efficiency of, for example a growth factor otherwise working in an animal model, be made in humans.

2.6 Cells of Intervertebral Disc:

IVD in fact is the most hypocellular tissue in the human body having the lowest cell density per gram tissue. Adult human IVD has three distinct cell populations, fibroblast like AF cells, chondrocyte like NP cells and cells found in EP. Few studies indicate that there is another phenotype of cells that are non-chondrocyte like and don’t produce matrix molecules generally produced by IVD cells such as collagen I &II, keratan sulphate (KS) and chondroitin sulphate (CS) [58]. AF cells are elongated and aligned parallel to the collagen fibers and adhere to them and have the ability to stretch along the axis of the collagen fibers when the collagen fibers stretch and coil.
along its triple helix to accommodate and redistribute the load applied on the disc. NP cells on the other hand are round, disorganised, sometimes found in clusters in many species such as [59] [Figure 8]. To date there are no specific markers that demarcate NP and AF cells. Few suggested markers to confirm differentiation of stem cells into NP and AF cells respectively is SOX 9, aggrecan and collagen I expression [60, 61]. Many genes have been studied to search for a marker for NP and AF cells and till date there is no markers that are “on or off” for identifying disc cells [61]. A total of 63 genes were studied by employing microarray analysis of which 7 genes were further analyzed with RT-PCR for some genes namely annexin A3, glypican 3 (gpc3), keratin 19 (k19), pleiotrophin (ptn), vimentin (vim), cartilage oligomeric matrix protein (comp) and matrix gla protein (mgp) in rat IVD but no fair conclusion could be drawn [61]. Recent works though clearly show that the expression of HIF-α at tissue level and its normoxic stabilization distinguishes NP from AF [62, 63] and can serve as a marker to distinguish between the two cells types.
**Figure 8: Cells of IVD:** Figure showing two major cell populations found in IVD, NP and AF cells. EP cell are found in cartilaginous endplates on both side of the disc.

(Reproduced from Mwale et al [64].)

In another study with canine IVD cells, 5 genes were selected that showed a high signal ratio in the NP versus AF microarray comparison: α-2-macroglobulin (A2M), annexin A4 (ANXA4), desmocollin-2 (DSC2), cytokeratin 18 (CK18), and neural cell adhesion molecule 1 (NCAM1, CD56 140-kDa isoform). In addition, 5 genes, that had been found differently expressed in NP versus articular chondrocytes in the rat, were evaluated, namely cartilage oligomeric matrix protein (COMP), glypican 3 (GPC3), matrix Gla protein (MGP), pleiotrophin (PTN), and vimentin (VIM). Still no conclusive markers for NP or AF cells could be proposed [65].
Other and additional markers have been proposed for disc cells such as HIF-1a and GLUT-1, CD-44 and cytokeratin-8 but their use as definitive phenotype markers for disc cells is still not validated [53].

In addition, “ghost cells” are also found in IVD which are non nucleated disc cells which have undergone apoptosis and may constitute up to 10% of total cell population in discs from older individuals [66].

A major issue with conducting studies with isolated disc cells is their unstable phenotype. When cultured in monolayers, disc cells lose their phenotype and when trypsinized and cultured in 3D scaffolds, they regain their phenotype thus indicating the importance of 3D micro-matrix around in disc cells in vivo and its influence in regulation disc cell metabolism [53]. The disc cells also lose/ change their phenotype after certain passages, thus common view in the research community is to use primary cells whenever possible. Unfortunately, harvested tissues are often very less in quantity and limits use of primary cells as they have to be expanded in monolayers to have ample starting material for conducting experiments.
3. Biochemical composition and matrix metabolism in IVD:

IVD has the same macro molecules like other fibrocartilages such as articular cartilage. The major components are proteoglycans and collagen whereas the minor non collagenous components are represented by glycoproteins, catabolic enzymes, their inhibitors, elastin and lipids. Proteoglycans constitute 50% of ECM in NP and 10-20% of ECM in AF in a healthy adult disc. The percentage in NP decreases with progressing age and grade of degeneration [67-70]. Collagen content in NP is around 15-20% of dry weight whereas in AF is close to 60-70% of dry tissue weight. Additionally water constitutes 90% of NP wet weight in young healthy discs whereas in older degenerated discs, 70% of water by wet weight tissue is reported. In AF, the water content is around 60-70% and remains fairly constant with age and degeneration [68, 71]. Around 20-45% of dry weight of NP and 5-20% of dry weight of AF is constituted by non-collagenous proteins such as decorin, lubricin, cartilage oligomeric matrix protein COMP etc. [72]. 0.5-2% of mature disc is comprised of lipids such as cholesterol, phospholipids, triglycerides etc.[72, 73]. IVD has a continuous turnover of matrix in states of health where there is constant degradation of already laid down matrix and production of new matrix to maintain matrix homeostasis. With increasing age, the number of cells in the disc decrease and the overall matrix turnover equilibrium is disturbed and shifts towards catabolism leading to degradation of matrix not accompanied by replacement and over time, there is loss
of disc matrix and the disc loses its structure and function and reaches a level of degeneration where repair, restoration and regeneration by disc physiology itself is not possible and the disc becomes pathological and gives rise to degenerative disc disease (DDD), symptomatic or asymptomatic.[Figure 9].

Figure 9: Matrix of IVD: Schematic showing simplified depiction of IVD matrix layout. Also to be noted are the IVD matrix homeostatic processes in play maintaining balance between anabolism and catabolism. (Figure courtesy Dr. Ovidiu Ciobanu, modified with permission).
3.1 Aggrecan:

Proteoglycans (PGs) of the disc are classified as aggregating and non-aggregating type. The aggregating PGs have more water retention capacity when compared to non-aggregating PGs due to the higher concentration of glycosaminoglycan (GAG) chains because of their close proximity. The non-aggregating PGs comprise of small leucine rich repeat proteoglycans such as biglycans, fibromodulin, lumican etc. [74, 75]. But still the major non aggregating PGs in the IVD are the degradation products of huge aggregating PG which accumulate in the disc due to the avascular nature of the disc that prevents the escape of these degradation products [67, 76].

The most common, most abundant and most important aggregating PG found in IVD is aggrecan. As the name suggests, the term aggrecan is derived from the word “aggregates” which in itself describes the structure of aggrecan. Aggrecan has a test tube cleaning brush like structure due to attachment of chondroitin sulphate (CS) and keratan sulphate (KS) side chains on the core protein [67].

Aggrecan aggregate molecule consists of hyaluronic acid (HA) backbone on which core protein of aggrecan are attached in the region of G1 domain of the core protein. The core protein has the KS and CS chains attached to it which are the ‘hairs’ of the cleaning brush. [Figure10].
Figure 10: Structure of Aggrecan: (A) Showing electron microscope image of aggrecan. (B) Showing the schematics of structure of aggrecan. (Reproduced from Fox et al 2012 [77].

Aggrecan core protein has 3 globular domains and one interglobular (IGD) domain lying between G1 and G2 domains. Core protein has 3 globular domains named G1, G2 and G3 respectively from the N terminal of the protein. These globular domains are formed due to folding of the protein and formation of disulfide bonds [67, 78].

The G1 domain in itself has 3 loops or modules. The G1 domain interacts with HA at 5 specific tandem repeat disaccharide on the COOH side. The loop on the NH₂ side interacts with G1 domain of Link protein. The IGD between G1 and G2 has various sites for cleavage by MMPs, aggrecanases and a disintegrin and metalloproteinase
with thrombospondin motifs (ADAMTs). This region is speckled with O-linked oligosaccharides.

The function of G2 domain is unknown. The region between G2 and G3 has KS chains (20-40 in number) in KS domain and CS chains (approx 100 in numbers) in CS region. CS region is divided into two sub domains, CS1 and CS2 domain with CS1 having shorter GAG chains compared to CS2 chains. This region between G2 and G3 is heavily GAG chained with on an average 1 GAG chain per 7 amino acids. This region also has N-linked oligosaccharides speckled between the CS and KS chains [67, 78].

The G3 domain also has 3 loops/modules. This domain is shown to have an effect on GAG chain attachment to the core protein and release of PG from the cytoplasm post processing [78, 79].

3.2 Collagen:

Collagen is fibrillar molecule similar to DNA in structure in many aspects. It is a left sided triplex helix comprising of 3 α chains arranged in polyproline type II helix. The special feature of collagen molecule is that every 3\textsuperscript{rd} amino acid on the polypeptide chain is glycine which is essential for the stability of the helix. Collagen triple helix gives the molecule high tensile strength by virtue of its coiling on along its axis and
accommodating large amounts of stretch forces by reducing the size of the major grooves of the molecule. There are 28 known types of collagens of which 9 are found in healthy IVD namely collagen I, II, III, V, VI, IX, XI, XII and XIV [67, 80, 81].

Collagen type I and II constitute 80% of the collagens found in IVD. Collagen type I is predominantly found in oAF region (30-40% of total collagen in the disc) whereas collagen type II is predominantly found in iAF and NP regions (30-40% of total collagen in the disc). Collagen type I and II follow reverse pattern of abundance in AF and NP respectively. The iAF or the transition zone is an interesting area of research and discussion as it has features of both AF and NP is composed of both collagen types I and II. Collagen is highly organized in oAF region and becomes less organized as one moves from oAF to NP. NP has strands of collagen type I intervened by proteoglycans in a mesh work like pattern which prevents excessive swelling of the proteoglycan molecules due to imbibition of water due to negative charges carried by the molecule.

Collagen IX of the IVD is different from the one found in hyaline cartilage as it doesn’t have the NC4 domain which is the site of interaction collagen type IX with other matrix component. This absence of NC4 domain is thought to be beneficial for the disc and contribute to providing fluid consistency to the NP [26].
Mature disc also has collagen type X which is found in iAF and NP and is generally associated with endochondral ossification of the growth plates and in young individuals is confined to the cartilage of the vertebral endplate [26].

### 3.3 Link Proteins:

Link protein was first identified in cartilage and was subsequently found in IVD matrix and characterized [82]. Link proteins are the glycoproteins found in association with proteoglycan aggregates and stabilizes the interaction between the G1 domain of core protein of aggrecan and HA protein backbone by neutralizing the negative charges on the two binding sites. In absence of Link protein, the association of aggrecan core protein and HA is very unstable and the aggrecan monomer dissociates spontaneously at pH 5 or below. Link protein is found in 1:1 ratio with G1 domain of aggrecan core protein and is around 100 per HA molecule. Link proteins also bind to collagen I and III in and the ratio of abundance with collagens is 13:1. Link protein has three globular loops named as loop A, B and B’ from N terminal [83, 84]. The G1 of core protein and loop A of Link protein is in close association and it is these domains that interact to stabilize the aggrecan interaction with HA. [Figure11]. The remaining two loops are similar to each other. Link protein in cartilage amounts to 0.05% of the total weight and represents 10μM conc. Link protein has different forms based on MW LP1, LP2 and LP3 with LP1 with the largest molecular weight [82]. The difference in forms is also species dependent [85]. This difference in forms is attributed to the degree of glycosylation. Most
interestingly, LP2 has a lower MW due to cleavage of a fragment from N terminal. In human, LP1 is predominantly found in neonatal IVD, LP2 in older (50-70 yrs) IVD and LP3 in young IVDs [82]. LP is tightly bound to HA and this binding is dependent upon the conc. of Zn$^{2+}$, Mg$^{2+}$ and Co$^{2+}$ ions. Link protein can be reversibly dissociated from HA by treating with 4M guanidinium hydrochloride [82, 84].

Figure 11: Interactions between Hyaluronic acid and Link protein: Diagram showing relative positions of G1 domain of core protein and loop A of Link protein and their site of interaction. Also showing the interaction of loop B and B’ of the Link protein. (Figure courtesy Dr. Ovidiu Ciobanu, modified with permission).
3.4 IVD extracellular Matrix Metabolism (ECM):

IVD contains metalloproteinases family of enzymes which cleaves various components of IVD matrix. These enzymes are required for normal turnover of IVD matrix which requires remodeling before laying down of newly synthesized matrix. Thus these enzymes also have role in homeostasis and not only catabolism. These enzymes also have role in cell proliferation, migration and apoptosis [86]. The metalloproteinases family of enzymes is divided into two subfamilies namely, (i) MMPs-metalloproteinases and (ii) ADAMTSs- the disintegrin-like and metalloproteases (reprolysin-type) with thrombospondin type-1 motif [67]. Some MMPs are named after their specific actions such as MMP-1, 3, 18 which are also called collagenases. Similarly, ADAMTS 4&5 are called aggrecanases 1&2 respectively. MMPs and ADAMTSs are secreted by disc cells or by cells associated with vascular system when there is vascular invasion of the disc in advanced grades of degeneration. MMPs are secreted in an inactive form and are converted from zymogen to the active form by cleavage of proenzyme part in the matrix. ADAMTs on the other hand are secreted by the cells in active form and their proenzyme part is cleaved within the cell. These enzymes cleave the matrix components at specific sites and specific site of action of enzymes on these macromolecules sometimes determine the degree of degeneration [67]. [Figure12] For example, MMP-3 or stromoeolysin 1 cleaves the aggrecan core protein in the IGD region close to G1 domain of aggrecan core protein and thus causes maximum damage as the major part of the molecule is
lost. Whereas ADAMTS 4&5 cleave aggrecan at a different site and create smaller fragment of aggrecan. These enzymes also cleave Link protein which is susceptible to MMPs but not ADAMTSs, thus creating fragmentation of aggrecan [87, 88]. Studies have shown that MMPs are less efficient in cleaving aggrecan compared to ADAMTSs probably due to their delayed activation in the matrix [89].

Figure 12: Enzymatic Cleavage sites in Aggrecan: Figure showing various enzymes and their cleavage sites on the aggrecan molecule. (Reproduced from Nagase et al, 2003 [90]. Figure courtesy Dr. Ovidiu Ciobanu, modified with permission).
3.5 Link-N Peptide:

Link-N peptide (DHLSDNYTLDHDRAIH) is the 16 amino acid peptide generated from proteolytic cleavage of Link protein from N terminal. The proteinases mainly MMPs that cleave and degrade aggrecan core protein also cleave Link proteins mainly MMP 3 and 13. There are other cleavage sites for other MMPs on Link protein which generate other fragments of Link protein but by large Link-N is the predominant form from generated as a result of proteolytic degradation of the IVD and cartilage matrix [90]. [Figure13] Link protein is resistant to degradation by ADAMTSs due to non-availability of cleavage sites. Current hypothesis for generation of this fragment is that due to increased proteolytic activity leading to degradation of matrix, this peptide is generated and in turn acts as a growth factor signalling the disc cells to produce new matrix and attempt repair of the disc. The already upregulated MMPs and ADAMTSs help in remodelling of this newly laid down matrix. This peptide has shown to have stimulatory effect on proteoglycan and collagen synthesis by disc cells and chondrocytes in pellet cultures and rabbit model [91-93]. Link-N also has effect on regulation of proteinases [94, 95], both MMPs and ADAMTSs thus making it a potent agent for biological repair of disc which not only stimulates matrix synthesis but also regulates its remodelling.
3.6 State of ECM health and disease:

Healthy IVDs have clearly demarcated AF and NP regions. NP is glistening and jelly like and highly hydrated. The AF has clear collagen rings which can be easily counted and distinguished. The transition zone between AF and NP is limited to innermost 2-3 collagen rings and can be easily separated from NP. IVD matrix in healthy discs as discussed already is composed of clearly concentrically arranged
collagen I fibers which number around 25-35 up til iAF region. Thereon the iAF has both collagen I and II with aggregan inlaying within the collagen fibres. The NP region has aggregating and non-aggregating PGs. The non-aggregating PGs amounting to 10% in adult NP [26], even though not aggregated to HA, are still functional in retaining water molecules and maintaining hydration of the NP and its gelatinous consistency.

In DDD, the matrix of the disc loses its gross morphology and the AF and NP in severely degenerated IVDs cannot be demarcated from each other. The NP is heavily fibrosed and transition zone cannot be demarcated. The glistening sheen of the NP is lost due to loss of proteoglycans. The matrix becomes brownish in color due to accumulation of lipids and carbohydrate derived adducts [26] which is age related and gradual time dependent accumulation. The accumulated products are called choromophores and have the ability to fluoresce [96]. The collagen of AF becomes fragmented and percentage of aggregan rises in an attempt to maintain the functionality of the disc. The PGs in NP become severely cleaved and fragmented to an extent that it is not able to retain water and the disc collapses [97]. The fragmentation products are not able to escape from the disc due to the avascular nature of the disc [26] [Figure 14]. The proteins of the disc also start to cleave such as aggregan core protein, Link protein and HA. The collagen in NP gets replaced by collagen type II to some extent with fair degree of cross linkage. The cell population of NP and AF is severely depleted and the endplates are heavily calcified with
obscured pores in the calcified part of the cartilage leading to severely impaired nutrition and oxygen to already hypoxic and “malnourished” IVD [27, 28, 98].

Figure 14: Change in IVD matrix composition with age: Figure depicting composition of IVD NP varying with age with mature adult degenerate matrix showing severe fragmentation and loss of functionality of PGs. Also collagen and proteins are cleaved with increasing grade of degeneration [26].
3.7 Effect of nutrition on IVD matrix metabolism:

Nutrition has a deep impact on the health and metabolic state of IVD. Nutritional state is of particular importance in IVD as it is the largest avascular organ in the human body. The nutritional state is also important owing to the accumulation of metabolic by products which are not able to diffuse out due to impaired diffusion through calcified endplate. This accumulates free radicals that have been sequestered owing to glycolytic cycle disc cells utilize for metabolising glucose [42, 99]. Synthetic rates are highest at 5% oxygen and become 1/5 of this rate at 1% oxygen tension. Some other studies have shown no effect of low oxygen tension at 1% when compared to 5% and 21% oxygen tension and sustained GAG synthesis at low oxygen tensions [42, 100].

Accumulation of lactic acid and other metabolites such as pyruvate due to glycolytic cycle arising due to hypoxia leads to drop in ECM pH which is less than the pH of blood, serum and capillary fluid (pH 7.2). Synthesis of ECM drops at pH lower than 7.2 and shows a considerable drop even at pH 7.0. The MMP production is less sensitive to pH changes and they show similar expression levels at pH 6.4 compared to pH 7.0. Thus lower pH in disc has a two pronged effect on matrix degradation and drives the process faster in absence of stimulated GAG synthesis and increased MMP production [42].
Another interesting effect of nutrition state is appearance and production of keratan sulphate (KS) by NP cells. Fetal aggrecan doesn’t have KS as notochordal cells do not produce KS. At birth, KS starts to appear due to replacement of notochordal cells by mesenchymal cells which produce the KS for KS region of aggrecan as described before [26]. The degree and amount of KS in aggrecan increases with age and an adult mature healthy disc has the highest KS/CS ratio [26]. KS production is seen in mature discs owing to the diminished oxygen supply to the center of the disc as vascularity and thus oxygen supply decrease with age and oxidation is required for the formation of glucouronic acid which is required to produce CS [26]. On the other hand KS production is stimulated as it needs galactose for its synthesis. This is seen as a beneficial event as it is an attempt by the disc cells to maintain the high negative charge density necessary for osmotic functioning of the disc [26].

In summary, nutrition plays a very important role in IVD matrix metabolism and any attempts made to regenerate IVD should factor into attempts made to better the nutritional rate of the disc, if any desired effect is to be seen.

4. IVD culture models:

IVD physiology, biomechanics, biology, metabolism and tissue engineering studies have been studied in variety of models and systems which range from cells in monolayers, in 3D cultures [95, 100] to whole organ culture system using IVDs from higher animals such as bovine [101, 102], ovine [103], rabbits [104, 105] and porcine [106, 107], with or without the utilization of custom built bioreactors for culture.
Some organ culture models evaluate discs with bony endplates (BEP), no endplates (NEP), and cartilaginous endplates (CEP) or with retention of part of vertebral bone on both sides of the IVDs. *In vivo* models generally utilize use of rabbits [91], rats, monkeys [108, 109] and dogs [110].

Various evaluations done with the above used models investigating the potential of growth factors on ECM synthesis, phenotypic evaluation of disc cells, effect of cyclic and static loading regimes, effect of oxygen tension on disc cell metabolism, genotypic evaluation of disc cells, suppression of MMP expression, GAG loss over a period of culture, co-culture studies, effect of nitrogen rich culture surfaces on ECM production, needle injury models, endplate injury models etc. [33].

Though many models are available and are being developed to study IVD biology and pathophysiology of human IVD in DDD, the problem occurs while extrapolating the results obtained from animal models to human discs. None of the animal or culture models available at the moment is parallel to the complexity of occurrence of human DDD. Many animal models induce degeneration by enzymatic approach or by needle stick injury but human disc degeneration is a continuous ongoing process which takes decades to reach the levels of degeneration needing repair and/or regeneration of IVD. Cellular studies and animal models provide good platforms for screening bioactive agents and evaluating their therapeutic response, but ultimate validation will need the candidates to be tested in human subjects or organ culture models before taking the drugs or therapies to clinical trial phase.
Current review of literature shows no report of any intact human IVD culture system or model in use or development, apart from our research group and enabling long term evaluation of lead candidates for treating DDD.

5. Regenerative Medicine approaches in regeneration and repair of degenerated IVD:

IVD is a complex tissue and is destined to degenerate and some people refer this to “programmed degeneration” owing to avascular nature of IVD, loss of notochordal cells at birth and age related depletion of IVD cells from NP and AF. Current therapies for low back pain are limited to symptomatic treatments including physiotherapy and alternative medicine. Advanced grades of degeneration of IVD leading to neural or neuromuscular sequelae are surgical emergencies and require immediate decompression through surgical intervention. Various surgical treatments for DDD are nucleotomies, nucleoplasty, annular repair, discectomy with implant insertion, motion segment fusion. The major problems with spinal surgeries are associated post-operative high morbidity, infections, implant failure, adjacent level disc disease. [111-116] Adjacent level disc disease often follows implant insertion and leads to accelerated degeneration of the adjacent level discs due to altered biomechanics [117, 118]. In view of these problems and advances and use of regenerative medicine therapies in other tissues, it is impossible to delay the attempt to test these therapies in IVD and regenerate this otherwise very well designed tissue.
5.1 Cell based therapies:

Many approaches have been adopted to repair degenerated IVDs with cell based therapies. The sources of cells varies from study to study and most commonly proposed cell source for injection in IVD are bone marrow, synovium or adipose derived mesenchymal stem cells (MSCs) [119, 120] from allogenic and autologous sources, NP and AF cells from donors from same species (allogenic source) [121] and chondrocytes [119, 122, 123].

The problem and concern with using cell based therapies varies with the proposed cell types to be used. For MSCs, the concern is differentiation of these injected cells into cells other than disc cells when injected and formation of ectopic tissue not native to the disc compounding the problem. This problem can be circumvented by using already differentiated or priming the MSCs with growth factor and injecting them with the growth factor laced vector to ensure downhill differentiation towards desired path [119, 124-126].

Due to avascular and hence non-immunocompetent nature of IVD, injection of allogenic disc cells would not be a problem but the real problem lies with number of cells harvested during the harvest. To have a measurable and beneficial regenerative response, adequate number of cells has to be injected. This could be a limiting factor as both allogenic and autologous disc cell source have the problem of having less number of cells. These harvested cells can be expanded before transplantation but
care and caution has to be taken to make sure that these cells have not lost their phenotype and are still capable of showing adequate response [53].

5.2 Use of growth factors in IVD:

Growth factors have a very important role to play in tissue engineering and regenerative therapies targeting IVDs [3]. Various growth factors have been evaluated for tissue engineering and regenerative medicine purposes in IVDs with variable success. The major growth factors evaluated are Insulin like growth factor -1 (IGF-1) [127], Bone Morphogenic Proteins (BMP) 2, 4, 6, 7 [128-132], transforming growth factor (TGF-β) [133-135], fibroblast growth factor (FGF)[136] and growth and differentiation factor-5 (GDF-5) [137, 138]. The limiting factor with these growth factors is their production and their cost as doses needed to see a significant response costs a significant amount of money and makes their use economically unfeasible. Another concern could be regarding their safety profile. Despite the potential high cost of production, growth factors are still way cheaper than spine surgeries and related morbidities.

The need for a cheaper alternative makes Link-N peptide a cost effective growth factor like bioactive agent as its production cost is 100 times less than commonly evaluated growth factors. Moreover being native to the disc, theoretically has less potential of stimulating the cells to produce ectopic matrix or induce calcification. Link-N has been shown to stimulate proteoglycan synthesis and suppress MMPs in 3D cell cultures and rabbit in vivo needle injury model [92, 95, 124, 139]. Link-N has
also shown a dose dependent increase in human IVD cells and intact organ culture model for human IVDs [140]. All these studies have established Link-N as a cost effective bioactive agent needing further development and evaluation to be taken to clinical trials for the treatment of DDD.

5.3 Other modern approaches:
New emerging technologies are being increasingly employed in the treatment of DDD namely gene therapy [141-144], hydrogel injections [145-149], biomodulation of catabolic enzymes etc. These therapies have not been fully validated and are under evaluation and shows promise to be used in the treatment of DDD in near future. Another interesting area of application that has recently attracted the attention of spine research community is the application of siRNA therapy and its use in DDD [150].

6. Conclusions:
In light of the above literature review, following points could be highlighted.

The evaluation of growth factor like synthetic peptides such as Link-N is warranted as it offers a cheaper and currently unknown alternative for the treatment of DDD compared to spinal surgeries. Link-N has so far shown promise in stimulating proteoglycan synthesis by disc cells in culture and in rabbit needle injury model.
Establishing its ability to stimulate PG synthesis in human IVDs will provide researchers and healthcare providers another tool to tackle DDD and treating LBP.

The effect of Link-N or similar peptides and growth factors has to be evaluated in a system whether be an animal model or organ culture system before proceeding forward with clinical trials with human subjects. Since the findings and evaluations in animal models and animal IVD cultures cannot be directly translated to the complexity of degeneration in human IVDs, there is an immediate need to develop and validate an organ culture model for human discs which ensures prolonged culture periods at physiological conditions making the findings and results as relevant as possible before clinical trials.

Thus, the current knowledge base in the field completely justifies the rationale and objects of this doctoral thesis.
Chapter 2:

(Methods and Materials)
1. Source of Materials:

1.1. Materials:

Link-N (DHLSDNYTLDHDRAIH), 5-TAMRA conjugated Link-N, Reverse Link-N (HIARDHDLTYNSLHD), Scrambled Link-N, (DLNRAHLHIDYHTDSD), Link-N peptide 1\textsuperscript{st} 8 peptide residues [Link-N (1-8) (DHLSDNYT)], and 2\textsuperscript{nd} 8 peptide residues [Link-N (9-16) (LDHDRAIH)] were synthesized by CanPeptide, (Pointe Claire, QC, Canada). 5-TAMRA (5-carboxytetramethylrhodamine) was from Ana Spec Inc., (Fremont, CA, USA). Pronase was from Calbiochem (Darmstadt, Germany). Collagenase 1A, GlutaMAX, NaCl, Sodium citrate, HEPES and EDTA were purchased from Sigma (St. Louis, MO, USA). Low viscosity alginate (Keltone LV), was obtained from Kelco Chemical Co. (San Diego, CA, USA). Penicillin/streptomycin, gentamicin sulphate, amphotericin B, Dulbecco’s modified Eagle’s medium (DMEM), Hank’s balanced salt solution (HBSS), Trypan Blue and fetal calf serum (FCS) were obtained from Gibco (Burlington, ON, Canada). 20G 1\textsuperscript{1/2} inch needles to make alginate beads and U-100 insulin syringes with 25G needles to inject intact discs were obtained from BD syringes, (Concord, ON, Canada). RNA Easy™ kit for RNA isolation and Omniscript™ Reverse Transcription kit were purchased from Qiagen, (Toronto, ON, Canada). RT-PCR kit, Taqman reagent and Taqman primers and probes were from Applied Biosystems (Foster City, CA, USA). Gas permeable sterile bags were obtained from VWR International (Ville Mount Royal, QC, Canada). HPLC grade Acetonitrile was purchased from Rathburn (Walkerburn, Scotland). Trypsin Gold mass spectrophotometry grade was purchased from Promega (Madison, WI, USA). TopSert, TPX-Short Thread-Vial, 32x11.6mm
with integrated 0.2ml Glass-Micro-Insert, 15mm top were purchased from Skandinaviska GeneTec AB (Västra Frölunda, Sweden). Vydac UltraMicro Spin® Silica C18 300Å columns were purchased from The Nest Group (Southborough, MA, USA). Sequencing Grade Chymotrypsin was purchased from Roche Diagnostics GmbH (Mannheim, Germany). EasyTag™ EXPRESS\(^{35}\)S Protein Labeling Mix, \(^{35}\)S-, 2mCi (74MBq), Stabilized Aqueous Solution was ordered from Perkin Elmer (Montreal, Quebec, Canada). NUNC 6 well culture plates were purchased from Corning Inc. (Edmonton, Alberta, Canada). Surgical round end-boring bit on a high-speed drill were purchased from Racine (WI, USA). Polymethylpentene containers for culture were purchased from Nalgene (Rochester, New York). Live/Dead® assay and 4% and 12% Novex gels were bought from Invitrogen (Burlington, Canada). Complete protease inhibitor cocktail tablet and Insulin-Transferrin- Selenium (ITS) were bought from Roche (Mississauga, Canada). Keratanase II and chondroitinase ABC were bought from Seikagaku (Tokyo, Japan). 6-mm and 4-mm biopsy punches were purchased from AcuPunch (Toronto, Canada).

1.2. Antibodies:

Anti-G1 antibody against G1 domain of aggrecan core protein was a generous gift from Dr. Peter Roughley, Shriners Hospital for Children, Montreal, Quebec, Canada. Anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidise was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).
1.3. Instruments and Softwares:

The secondary antibody bound to anti-G1 antibody in SDS-PAGE was visualized by chemiluminescence (GE Healthcare, Waukesha, WI). Cell counting and quantification for Live/Dead® assay was done with CellC software (MATLAB, USA). Cells in confocal microscopy were visualized using an inverted confocal laser-scanning microscope (Zeiss LSM510) and image export was done with LSM Image Browser® software. Stacking of confocal images was done with Adobe Photoshop® CS1 software. Fluorescence detection for 5-TAMRA and 5-TAMRA conjugated Link-N was done using an Infinite® M1000 Pro instrument (Tecan, Austria GmBH). Statistical analysis was performed using Microsoft Excel® and Prism® GraphPad softwares.

2. Sources of Tissues:

2.1. Bovine Intervertebral Discs (IVD):

Bovine tails were obtained from local abattoir (Abattoir Poirer, St. Louis des Gognaz, Quebec). The levels obtained were coccygeal (Co) levels Co1-Co7. The steers were 18-27 months of age. The discs used for these studies were apparently healthy and degenerated segments were excluded from the study. The samples were procured within 6 hours of sacrifice and the samples were transported to the laboratory on ice. All procedures with animal tissue were approved by the local institutional review board and proper ethics approval was obtained.
2.2. Human Lumbar Intervertebral Discs (IVD) Retrieval:

Human lumbar spines were obtained through organ donation program in coordination with Transplant Quebec. The spines were retrieved within 3-4 hours of cross clamping of carotids (official declaration of brain death). The donor’s next of kin filled out a questionnaire including low back pain assessment [see appendix] to point out the degenerated discs with or without symptoms. The exclusion criteria were: no cancer, age <65 years, no spine deformity and no major metabolic disease such as liver cirrhosis and kidney failure leading to altered bone mineral density of the vertebral bodies causing abnormal loading of IVDs.

The lumbar spines were retrieved by fracturing transverse processes of the levels being retrieved, fracturing the body of the vertebrae of the last disc to be retrieved from cephalic end, fracturing the sacro-iliac joints on caudal end and fracturing the vertebral body of S1. All these fractures when continuous, release the anterior segment of the spine and the posterior segment of the spine is left behind to stabilize the body [Figure 15 (A) and (B)].

Till the date of submission of this manuscript thesis, 111 human lumbar spine segments were retrieved by me and out of these segments, 30 spines having the inclusion criteria for the study were chosen and designated for cell isolations and preparation of IVDs for organ culture.
Figure 15: Retrieval of Lumbar Spine: (A). Figure depicting retrieval of lumbar spine. Thunderbolt arrows indicate the site of transverse process fractures and bold straight red lines indicating sites of vertebral body fractures. (B). The fractures are made continuous and communicating with each other to facilitate release from body (red arrows). (Figure courtesy of Dr. Ovidiu Ciobanu and modified with permission).

The IVDs used in the study are tabulated below [Table 1].

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Cause of Death</th>
<th>Disc Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>M</td>
<td>Cardiac Arrest</td>
<td>T10–T11</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>M</td>
<td>MI</td>
<td>T11–T12, T12-L1, L1–L2, L2–L3, L3–L4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>M</td>
<td>MVA</td>
<td>T11–T12, T12-L1, L1–L2, L2–L3, L3–L4, L4–L5, L5-S1</td>
</tr>
<tr>
<td>4</td>
<td>49</td>
<td>F</td>
<td>ICH</td>
<td>T12-L1, L1–L2, L2–L3, L3–L4, L4–L5, L5-S1</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>M</td>
<td>MI</td>
<td>L1–L2, L4–L5, L5-S1</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>M</td>
<td>Testicular Cancer</td>
<td>T10–T11, T11–T12, T12-L1, L1–L2, L2–L3, L3–L4</td>
</tr>
<tr>
<td>7</td>
<td>49</td>
<td>F</td>
<td>CVA</td>
<td>T11–T12, T12-L1, L1–L2, L2–L3, L3–L4, L4–L5, L5-S1</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>M</td>
<td>ICH</td>
<td>T12-L1, L1–L2, L2–L3, L3–L4, L4–L5, L5-S1</td>
</tr>
<tr>
<td>9</td>
<td>72</td>
<td>F</td>
<td>ICH</td>
<td>T12-L1, L1–L2, L2–L3, L3–L4, L4–L5, L5-S1</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>F</td>
<td>MVA</td>
<td>T10–T11, T11–T12, T12-L1, L1–L2, L2–L3, L3–L4, L4–L5, L5-S1</td>
</tr>
<tr>
<td>11</td>
<td>66</td>
<td>M</td>
<td>ICH</td>
<td>T12-L1</td>
</tr>
<tr>
<td>13</td>
<td>66</td>
<td>F</td>
<td>ICH</td>
<td>L1–L2, L2–L3, L3–L4, L4–L5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>14</td>
<td>72</td>
<td>F</td>
<td>ICH</td>
<td>L4-L5</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>M</td>
<td>Gunshot</td>
<td>L4-L5</td>
</tr>
<tr>
<td>16</td>
<td>29</td>
<td>M</td>
<td>ICH</td>
<td>L1-L2, L2-L3, L3-L4, L4-L5</td>
</tr>
<tr>
<td>17</td>
<td>59</td>
<td>M</td>
<td>ICH</td>
<td>T12-L1, L1-L2, L2-L3, L3-L4, L4-L5</td>
</tr>
<tr>
<td>18</td>
<td>42</td>
<td>F</td>
<td>ICH</td>
<td>L2-L3, L3-L4, L4-L5, L5-S1</td>
</tr>
<tr>
<td>19</td>
<td>53</td>
<td>F</td>
<td>ICH</td>
<td>L1-L2, L2-L3, L3-L4, L4-L5, L5-S1</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>M</td>
<td>MVA</td>
<td>L1-L2, L2-L3, L3-L4, L4-L5, L5-S1</td>
</tr>
<tr>
<td>21</td>
<td>26</td>
<td>M</td>
<td>Electrocution</td>
<td>L1-L2, L2-L3, L3-L4, L4-L5, L5-S1</td>
</tr>
<tr>
<td>22</td>
<td>61</td>
<td>F</td>
<td>ICH</td>
<td>L1-L2, L2-L3, L3-L4, L4-L5</td>
</tr>
<tr>
<td>23</td>
<td>21</td>
<td>F</td>
<td>Cocaine Overdose</td>
<td>T9-T10, T10-T11, T11-T12, T12-L1, L1-L2, L2-L3, L5-S1</td>
</tr>
<tr>
<td>24</td>
<td>20</td>
<td>F</td>
<td>MVA</td>
<td>T9-T10, T10-T11, T11-T12, T12-L1, L1-L2, L2-L3, L3-L4, L5-S1</td>
</tr>
<tr>
<td>25</td>
<td>16</td>
<td>F</td>
<td>Cardiac Arrest</td>
<td>T9-T10, T10-T11, T11-T12, T12-L1, L1-L2, L5-S1</td>
</tr>
<tr>
<td>No</td>
<td>Age</td>
<td>Sex</td>
<td>Cause of Death</td>
<td>IVD Levels</td>
</tr>
<tr>
<td>----</td>
<td>-----</td>
<td>-----</td>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td>26</td>
<td>36</td>
<td>F</td>
<td>MVA</td>
<td>T12-L1, L3-L4, L5-S1</td>
</tr>
<tr>
<td>27</td>
<td>25</td>
<td>M</td>
<td>Suicide</td>
<td>T12-L1, L1-L2, L5-S1</td>
</tr>
<tr>
<td>28</td>
<td>47</td>
<td>F</td>
<td>CVA</td>
<td>T12-L1, L3-L4, L5-S1</td>
</tr>
<tr>
<td>29</td>
<td>42</td>
<td>F</td>
<td>ICH</td>
<td>T12-L1, L5-S1</td>
</tr>
<tr>
<td>30</td>
<td>4 months</td>
<td>F</td>
<td>MVA</td>
<td>L1-L2, L2-L3</td>
</tr>
</tbody>
</table>

ICH = Intracranial Hemorrhage, MVA = Motor Vehicle Accident, CVA = Cardiovascular Accident (Stroke), MI = Myocardial Infarction.

Table 1: Tabulation of IVD donors with age, sex, cause of death and IVD levels used in the study.

All the human spine retrievals were performed after proper signed consent by the next of kin of the donors and the procedures were approved by local institutional review board. All documentations were procured and catalogued pre and post retrievals.

3. Radiology and grade of degeneration assessment on procured human lumbar spines:

3.1. X-ray of procured human lumbar spines:

Before starting experiments, the retrieved spines were X-rayed to roughly assess the grade of degeneration and spines not meeting the inclusion criteria were excluded based on the criteria outlined above. The spines were X-rayed in the emergency radiology room and four views; antero-posterior (AP), Postero-anterior (PA), left
lateral (Lt. Lat.) and right lateral (Rt. Lat.) were procured. The technique used for X-ray was, voltage 53kV and tube current at 6.2mA with cassette in the bucky of the table. This modified X-ray method was optimized by trial and error to obtain the best quality images as the surrounding tissue normally found in human body has been stripped. The views obtained with this technique are good enough to demarcate and highlight intradiscal, peri-discal and peri-vertebral landmarks to categorize the spines into various grades of degeneration [Figure 16 (A-D)].
Figure 16: Radiology of Harvested Lumbar Spine: Figure showing radiographs of retrieved spines. The four views procured were (A) antero-posterior, (B) postero-anterior, (C) right lateral and (D) right lateral.

3.2. Grade of degeneration assessment of retrieved spines:
To assess the grade of degeneration, the harvested spines were X-rayed. This was done due to ready availability of the X-ray machine and has an advantage over MRI based grading [151, 152] in visualizing disc space, endplate mineralization, mineral inclusions in the discs and osteophytes. The X-rayed spines were graded for the degree of degeneration using a grading scale developed for this study. It takes into account various radiological features such as osteophytes, intradiscal calcification, lipping of vertebral bodies, loss of disc height, loss of disc space and fusion of adjacent vertebral bodies. The grades were set from 1 to 5 with grade 1 being healthy with no signs of degeneration and grade 5 being severely degenerated. The discs used in this study were from grade 1-3 with no or minimal radiological signs of degeneration [Figure 17 (A-C)]. Spines with higher grades of degeneration were not used for the study.
**Figure 17: Grades of Degeneration of Harvested Spine:** Figure with X-rays from three different grades of degeneration. Arrows in red indicate radiological features of degeneration such as intradiscal calcification, osteophyte formation, lipping of vertebral bodies, loss of disc height in X-rays labelled mild and severe degeneration.

### 4. Definition of mediums and wash buffers used:

For washing the tissue post isolation, two buffer solutions were used, 100μg/mL gentamicin, 200ug/mL penicillin, 200U streptomycin and 0.5μg/mL fungizone supplemented double strength (2X) HBBS (HBBS-S) and PBS (PBS-S). The culture
medium used was Dulbecco’s modified Eagle’s medium supplemented with 25 mmol/L HEPES, 50μg/mL of gentamicin sulphate, 0.25μg/mL fungizone, 50μg/mL L-ascorbate, and 2mmol/L GlutaMAX. Modifications of this medium were used varying in conc. of glucose and FCS. For cell isolation and culture DMEM with either 4.5g/L glucose with no FCS (medium HN), 4.5g/L glucose with10% FCS (medium HC) or 4.5g/L glucose with 0.0006g/L selenium, insulin 1g/L and 0.55g/L transferrin (medium H-ITS) was used. For organ culture, DMEM with 4.5g/L glucose with 5%FCS (medium HH), 4.5g/L glucose with 1%FCS (medium HL), 1g/L with 5% FCS (medium LH) or 1g/L with 1% FCS (medium LL) was used respectively.

5. Preparation of Link-N solution:

For use in cell cultures, human sequence of Link-N (DHLSDNYTLDHDRAIH), Reverse Link-N (HIARDHDLTYNDLH), Scrambled Link-N, (DLNRAHLHIDYHTDSD), Link-N 1st 8 peptide residues [Link-N (1-8) (DHLSDNYT)], and 2nd 8 peptide residues [Link-N (9-16) (LDHDRAIH)] were ordered and were manufactured by CanPeptide. Received batches had >97% purity on mass spectrophotometry. The scrambled 16 amino acid peptide sequence was designed using a bioinformatics tool from Institut Pasteur, Paris, France (http://mobyle.pasteur.fr). The sequence was selected to mimic the overall properties, such as isoelectric point and solubility of the original peptide. The scrambled sequence was made and validated with the proteomic tool so as to break focal areas of charge densities due to individual amino acids to avoid charge based interactions, if any, by Link-N. 1mg/mL solution of Link-N peptide was made in medium HN. The
peptide owing to its PI value makes the solution very acidic. The 1mg/mL stock solution made was pH balanced with 10N NaOH and final pH set at 7.4. The pH was confirmed using pH measuring strips. This pH balanced stock solution was used to make serial dilutions in 1/10th decrements to make a series of solutions with a range of concentrations.

For injection in intact the IVDs, Link-N solution was prepared in 10mg/mL concentration in medium HN and pH balanced to 7.4. Link-N solution was also prepared in conc. of 20/mg/mL and pH balanced to 7.4. 50µCi/mL solution of radioactive $^{35}$SO$_4$ (1mCi/mL stock solution) was prepared in medium LL. Prepared Link-N solution was mixed with prepared radioactivity supplemented medium LL in 1:1 ratio. This way the concentration of Link-N fell to 10mg/ml and of $^{35}$SO$_4$ to 25µCi/mL.

For injection in cartilaginous endplate model (CEP) IVDs, 5-TAMRA conjugated Link-N was also prepared. The Link-N solution used for injecting IVDs (10mg/mL) is 0.502µM in molar concentration. Same molar concentration of 5-TAMRA-Link-N conjugate was prepared to have equimolar injections of Link-N in 5-TAMRA-Link-N conjugate injection experiments. Unconjugated 5-TAMRA solution was made in similar fashion. 0.113µM solution was made which is equimolar to 5-TAMRA Link-N conjugate (0.502µM) solution. The solutions were made in medium HN.
6. Isolation and culture of bovine and human intervertebral disc (IVD) cells in alginate microspheres:

6.1. Preparation of alginate solution:

1.2% alginate was used to embed the cells in microspheres as previously described [153]. The alginate preparation protocol was slightly modified to prepare a sterile solution to minimize risk of contaminations during culture. Low molecular weight alginate powder was dissolved in a buffer prepared in miliQ water containing 0.15M NaCl and 0.2M HEPES. The solution was put on a heating plate with a stir bar and alginate powder was added slowly. The solution was stirred until it was clear and all of the weighed alginate powder went into solution. Special care was taken not to overheat the solution to prevent caking and denaturation of alginate in solution. Prepared alginate solution was autoclaved using liquid cycle at 121°C and 1 atmosphere pressure for 30 mins.

6.2. Dissection of bovine and human intervertebral disc (IVD):

Procured bovine tails and human spines were generously sprayed with 70% ethanol and debris cleaned out. The dissection table was covered with sterile surgical drapes and paraspinal muscles and soft tissue around spinal segment was removed in an aseptic manner. The IVD was cut out from in between the vertebral bodies with a surgical blade. The IVDs were transferred to a sterile petridish and taken into biological safety cabinet. For bovine tails, the IVD was divided into NP and AF regions. Demarcation between the two regions was done by naked eye examination of
annular fibres and cutting and separating NP tissue from the innermost annular fibre. Due to young age of the disc, it was almost impossible to demarcate between iAF and oAF regions. For human spines, IVDs were separated and divided into NP, iAF and oAF regions. The tissue from iAF region was identified and taken as 5-6 rings starting from innermost ring next to NP region [154]. The tissue from human oAF was not included in this study. The separated NP and AF/iAF tissue was then diced and minced into very small pieces with surgical blades and transferred to 50mL conical tubes.

6.3. Isolation of bovine and human intervertebral disc (IVD) cells:
A previously described method [76] was used and modified to facilitate maximum cell release and high cell viability. The minced tissue was weighed and washed twice with HBBS-S and once with PBS-S. Each wash was 2-3 mins. The PBS-S solution was taken off and the tissue was weighed again to get the wet weight. The enzymes needed were calculated based on wet post wash tissue weight. The tissue was digested with 0.2% pronase prepared in medium HN first for 1 hour at 37°C, then spun down in a centrifuge for 5 min at 500Xg and pronase was then taken off. The tissue was washed twice with PBS-S to remove residual enzyme. The tissue was then digested with collagenase type IA at 0.01% for NP and 0.04% for AF/iAF tissue prepared in medium HN at 37°C. The collagenase IA treatment was done for 3-4 hours depending on the age of tissue and complete disappearance of NP was taken as the end point of treatment for both NP and AF/iAF tissues. 2.5mL of pronase and 5mL of collagenase type1A per gram of tissue with concentration mentioned above.
was used. Amount of tissue taken in each 50mL conical was 6gms to maximize enzymatic digestion by proper rotation in enzyme concoction.

The digest was then passed through 100μm cell strainers and NP and AF/iAF tissues in different tubes were pooled respectively and spun down in centrifuge for 5 min at 500Xg. The cell pellets were visualized and resuspended in medium HN and spun down again. This was done for a total of three times to wash the cell pellets of any matrix debris. After final wash, the pellet was resuspended in medium HN and 100μl aliquot taken and mixed with equal volume of Trypan Blue® solution to assess cell count and viability using Neuber’s haemocytometer. The cells were spun down again in centrifuge, medium taken off and the pellet was recovered and mixed with alginate solution for beading the IVD cells in alginate 3D microspheres.

For bovine tissue, 1million cells/gram of NP and AF tissue was consistently isolated. For human tissue, the cell harvest was variable depending on age and the grade of degeneration. On an average, 1million cells/gram NP and 750,000 cells/gram iAF tissues was obtained.

6.4. Embedding of disc cells in 1.2% alginate and culture in 3D microspheres:
Prior to making cell seeded alginate microspheres, the alginate solution was warmed to 37°C. The IVD cells from NP and AF/iAF regions were seeded at a density of 2million cells/mL of alginate. The cell pellets recovered as described above were mixed with required amounts of 1.2% prepared alginate solution. The cell pellets
were suspended gently without agitation with a 5mL pipette tip. The alginate was added slowly, to achieve a uniformly distributed cell suspension in alginate. 50mL of 102mM CaCl$_2$ solution was poured in sterile urine cups for polymerization of alginate cell suspension solution. Alginate cell suspension was taken up in a 10mL sterile syringe attached with 20G 1½ inch needle. The cell suspension was dropped drop by drop in aliquoted 102mM CaCl$_2$ solution. The beads were retained in CaCl$_2$ solution for 3 min to mature the beads. After formation of beads, the CaCl$_2$ solution was sucked off and the beads were washed twice with PBS-S solution. The beads were then transferred to 6 well culture plates. IVD cells in alginate beads were cultured at 37º C, 5% CO$_2$ in medium HC. The beads were stabilized for 7 days and cell viability was assessed by Live/Dead® assay prior to further treatment. The beads formed with the gauge of needle used were 3.5mm in diameter and 1mL of alginate cell suspension gave 65 beads. Approximately 29,000 cells per bead were embedded by this method.

The beads were checked under light microscope for uniform suspension and Live/Dead® assay was performed before starting experiments using a fluorescent microscope (Olympus). Fluorescent microscopy showed >95% cell viability for majority of bovine and human IVD cell batches. As a standard procedure, microspheres construct batch showing <85% cell viability was not used for experiments. Five regions across each bead from periphery to center were visualized for assessing cell viability for each cell batch.
6.5. Exposure of bovine and human intervertebral disc (IVD) cells to Link-N:

On the day of the experiment, alginate beads were transferred to 48 well culture plates with 5 beads per well (~150,000 cells per well). The wells were designated for controls and experiments in triplicates.

To determine dose of maximal response to Link-N by bovine and human IVD cells, alginate beads with cells in designated wells were exposed to increasing concentrations of Link-N (0.01μg/mL, 0.1μg/mL, 1μg/mL and 10μg/mL) in 0.5 mL of radioactive supplemented (25μCi/mL final concentration) medium HC.

Specificity of response to Link-N sequence was evaluated by exposing alginate beads containing IVD cells to 1μg/mL of human sequence, reversed sequence and scrambled sequence of Link-N in 0.5 mL of radioactive supplemented medium HC. Efficiency of Link-N to stimulate proteoglycan synthesis in an inflammatory environment was evaluated by exposing alginate beads to IL-1β (10ng/mL), Link-N (1μg/mL) or a combination of Link-N (1μg/mL) and IL-1β (10ng/mL) [155].

Biological activity of Link-N peptide fragment was assessed using equimolar amounts of all these peptides. The peptides were made in medium HC and pH balanced to 7.4. IVD cells were also exposed to IL-1β (10ng/mL), Link-N (1μg/mL), Link-N (1-8), Link-N (9-16) or a combination of Link-N, Link-N (1-8), Link-N (9-16) and IL-1β [155].
The exposure times to the peptides was 48 hours and at the end of the culture period, the medium was collected and frozen for further analysis at -20°C.

IVD cells isolated from NP and iAF regions from human IVDs from 3 different donors were also seeded in monolayer in a 6 well culture plate separately at a density of 250,000 cells/well. IVD cells were cultured in medium HC. After 24 hours, the cells were assessed and >98% cells adhered to the bottom of the well. The medium was taken off and cell layer washed with PBS twice to remove residual FCS. 3mL of Link-N solution (1µg/mL) in medium H- ITS was added to each well. Separate wells with no cell layer at the bottom of the wells with only Link-N solution were also set up to evaluate the stability of Link-N peptide in solution.

The reason for not using 3D alginate cultures for this set of experiment was that alginate interferes with mass spectrometric analysis.

200µL of media sample from all three conditions, NP cell layer, AF cell layer and no cell layer, were taken at time points 0, 6, 12, 24, 36 and 48 hours and frozen immediately at -20°C for further analysis using mass spectrophotometry.

7. Analysis of gene expression by disc cells exposed to Link-N:

7.1 Exposure of intervertebral disc (IVD) cells to Link-N:
The human and bovine IVD cells isolated from NP and AF/iAF regions were beaded in 1.2% alginate and cultured in medium HC. The experimental groups were set in
triplicates and each group was assigned 3 wells. On the day of the experiment, 17 beads (approx 0.5 million cells) were taken in each well and washed with medium HN three times for 5 min each to remove any residual serum from the previous medium. The alginate embedded cells were cultured in medium H-ITS to minimize any effect coming from growth factors in FCS. The beads were exposed to medium H-ITS alone or supplemented with, Link-N (1μg/mL), IL-1β (10ng/mL) or both Link-N (1μg/mL) and IL-1β (10ng/mL). The exposure time was 24 hours and at the end of this period, the medium was taken off and the beads harvested and transferred to RNAase, DNAase, and pyrogen free 2mL eppendorf tubes.

7.2 Isolation and preparation of RNA from beads harvested from Link-N and Link-N+ IL-1β experiments:

The alginate beads harvested from the control and experimental groups containing cells were dissolved using sterile PBS supplemented with 150mM NaCl, 55mM sodium citrate and 28mM EDTA. 1mL of the above supplemented PBS was used per 17 beads. The eppendorf tubes were gently flickered until the beads were completely dissolved. Cell pellets were collected after spinning at 500Xg for 5 mins. The cell pellets were washed by gently resuspending them using 1mL of sterile autoclaved RNAase, DNAase free PBS and spun down again at 500Xg for 5 mins. This was repeated 3 times to wash off any residual alginate and get a clean RNA preparation. 1mL of cell lysis buffer from RNA Easy™ kit was added to the cell pellet after final wash and RNA prepared following the manufacturer’s instructions. RNA
concentrations and purity were determined by measuring $A_{260}$ and by calculating the $A_{260}/A_{280}$ ratio, respectively.

7.3 Reverse transcription and real time PCR (RT-PCR) analysis of prepared RNA:

Total RNA isolated from the cells from each group was digested with DNAase I and used for reverse transcription using an Omniscript™ Reverse Transcription kit. Total RNA was mixed with required amounts of 1X RT buffer, 0.5mM dNTP mix, 2µM random hexamers, 10 units/ 20µL reaction volume of RNAase inhibitor and 4 units/20µL reaction volume of Omniscript® to prepare cDNA as per vendor’s instructions. cDNA from 1µg RNA equivalent (2µL of cDNA solution) was mixed with 10µL Taqman mastermix®, 1µL random primers and RNAase free water to a final volume of 20µL and used per reaction well of RT-PCR reactions using Taqman® chemistry run with 7500 Fast Real Time System (Applied Biosystems). Genes analysed were aggrecan, MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5, with 18S ribosomal RNA as a housekeeping gene [Table 2]. Gene expression was calculated using the $\Delta\Delta Ct$ method [156-158].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Catalogue Number</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 18S</td>
<td>Hs99999901_s1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>2. Aggrecan</td>
<td>Hs00202971_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td></td>
<td>Bt03212186_m1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gene</td>
<td>Transcript ID</td>
</tr>
<tr>
<td>---</td>
<td>---------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>3.</td>
<td>MMP-3</td>
<td>Hs00968305_m1 Bt04259490_m1</td>
</tr>
<tr>
<td>4.</td>
<td>MMP-13</td>
<td>Hs00233992_m1 Bt03214050_m1</td>
</tr>
<tr>
<td>5.</td>
<td>ADAMTS-4</td>
<td>Hs00192708_m1 Bt03224693_m1</td>
</tr>
<tr>
<td>6.</td>
<td>ADAMTS-5</td>
<td>Hs00199841_m1 Bt04230785_m1</td>
</tr>
</tbody>
</table>

Table 2: List of genes evaluated post Link-N exposure

8. Preparation of human and bovine intervertebral discs (IVD) for no endplate (NEP), cartilaginous endplate (CEP) and bony endplate (BEP) culture models:

To prepare IVDs with no endplates (NEP), bovine tails and human spines were processed aseptically as described in previous section. The IVDs were visualized by the stripping of muscles and soft tissues [Figure 19 (B)]. The IVDs were dissected from adjacent vertebral bodies with a no. 23 surgical blade [Figure 18 (A)].

To prepare IVDs with bony endplates (BEP), human spines were dissected and IVDs visualized by the stripping of muscles and soft tissues [Figure 19 (B)]. A hack saw was used to saw through the adjacent vertebral bones keeping 2-3 mm of vertebral bone on both sides [Figure 19 (C) and (D) and Figure 18 (B)].
Prior to preparation of IVDs with cartilaginous endplates (CEP) and other methods [Figure 18 (C)], bovine tails and human spines were X-rayed [Figure 19 (A)], dissected and IVDs visualized by the stripping muscles and soft tissue [Figure 19 (B)]. A hack saw was used to cut adjacent vertebral bodies as close to the IVDs [Figure 19 (C)] as possible so as to have 1-2 mm of vertebral bone on both sides [Figure 19 (D)]. The IVDs were then further processed so that the bone and the adjacent calcified part of the cartilage end plates were removed using a surgical round end-boring bit on a high-speed drill [Figure 19 (E)]. The stopping point for drilling was when the IVD had a “hard boiled egg” like consistency. At this point, all of bony endplate and some part of cartilaginous endplate are removed and the remaining cartilaginous endplate is enough to maintain the structural integrity of the IVD.

**Figure 18: Different IVD Culture Models:** No endplate (NEP) culture model. (B) Bony endplate (BEP) culture model. (C) Cartilaginous endplate (CEP) culture model. (Reproduced and modified with permission [159]).
Figure 19: Preparation of Different IVD Culture Models: (A) X-raying of harvested spine. (B) Dissection of harvested spines. (C) Parallel cuts on vertebral bodies using hack saw. (D) IVDs with thin layer of vertebral bone on both sides. (E) Removal of vertebral bone, bony endplate, and majority part of cartilaginous endplate with high speed burr. (F) Prepared CEP IVDs, pre-weighed and ready for culture. (G) Prepared NEP, BEP and CEP IVDs in culture with standard tissue culture techniques.

The isolated IVDs were weighed and washed twice with HBBS-S and once with PBS-S. The IVDS prepared by the BEP method were put in for additional three 3 min washes and extensively rinsed in PBS-S containing 20mmol/L sodium citrate, pH = 7.4 to remove bone debris and to chelate calcium to open the bony endplates. The PBS-S was removed and IVDs were transferred to sterile polymethylpentene containers. Designated culture medium (HH, HL, LH or LL) was added to the isolated IVDs. The amount of culture medium added was 3.5X the initial weight of
the IVD. The lid of the culture cups was loosened and put in the incubator with standard tissue culture technique environment at 37°C and 5% CO₂.

9. Culture of bovine and human intervertebral discs:
To assess the swelling potential of the IVDs cultured with NEP and CEP methods, IVDs were cultured in medium HH and after initial weighing at time point 0 hrs, weights were recorded after 15 minutes, 30 minutes, 1, 2, 4, 8, 21, 28, and 66 hours. At each time point, the IVD was taken out from the culture pot inside a biological safety cabinet, wiped with sterile surgical gauze to wipe off excess medium, weighed aseptically and put back in culture in the same culture pot. Photographs were also taken at each time point and values were plotted using GraphPad Prism software.

To assess the long term cell viability in NEP, BEP and CEP models, the IVDs were harvested at days 3, 7, 14, 21 and 28.
To assess the cell viability in CEP model IVDs were cultured for 1 and 4 months with varying nutritional states. The CEP IVDs were prepared as described before and designated to be cultured in four different culture mediums; HH, HL, LH or LL The IVDs were cultured without an external load for a period of 1 month. Similar experiment was repeated and IVDs cultured for 4 months to assess the sustainability of IVD cell viability by CEP model over a prolonged culture period. The medium was changed every 3rd day for the entire culture period.
A modified method for culturing human CEP IVDs was used by culturing them in gas permeable bags to reduce the amount of radioactive supplemented medium.
surrounding each disc. At the same time, high enough concentration of $^{35}$SO$_4$ was maintained in culture for the IVD cells to incorporate it into newly synthesized PGs. CEP discs were placed in the middle of the culture bag and 20ml of medium was added to the bag. Air bubbles were carefully removed and the bag sealed with common domestic food bag sealer and placed in a petridish and cultured in an incubator under standard tissue culture technique at 37°C and 5% CO$_2$ for 7 days. At the end of the culture period, the IVDs were sampled in NP, iAF and oAF regions with a 4mm skin biopsy punch. The punches were processed for Live/ Dead® assay using confocal microscopy to assess cell viability in different culture models at these time points.

10. Live/Dead® analysis:
Calcein- AM and ethidinium homodimer are provided in the commercially available kit as 4mM and 2mM stock conc. respectively. The working strength was 1µM for both the solutions.

10.1 Live/Dead® analysis of alginate beads:
Alginate beads with embedded IVD cells were assessed for cell viability by incubating 2 beads each from NP and AF/iAF groups at the working strength of Calcein-AM and ethidinium homodimer in medium HN for 30 mins in an incubator at 37°C. The beads were then transferred on a glass slide and observed under a fluorescent microscope with required filters.
10.2. Live/Dead® analysis of punches from CEP intervertebral discs (IVDs):

The samples were taken on the designated days of harvest from NP, iAF and oAF regions using a 4mm skin biopsy punch. The samples were taken in the same coronal plane from one half of the IVD. The samples were full thickness and spanned the complete height of the IVD. 10% glycerol supplemented medium HN was prepared for incubation with working strengths of Calcein- AM and ethidinium homodimer. 

2mL of 10% glycerol supplemented medium HN with Calcein- AM and ethidinium homodimer for each sample plug was used. Two cores from each region were assessed to evaluate cell viability.

The sample plug were transferred to a 12 well or a 24 well cell culture plate with one sample plug per well. 2mL of prepared dye solution was put in each well. The cell culture plate was incubated wrapped in aluminium foil at room temperature on a shaker for 1hr-11/2 hr. 

The dye solution was taken off from the sample plugs after incubation with a pipette and 2mL of 10% of glycerol solution in PBS was added and incubated for 10-15 min at room temperature on a shaker to remove unbound dye and avoid having a non-specific reddish hue in confocal or fluorescent microscope acquired images. 10% glycerol in PBS solution was removed after the incubation period and the culture plate was stored wrapped in aluminium foil in -80C for microscopy in future if the confocal microscope was not readily available.
10.3 Preparation of slices from punches for confocal microscopy:

On day of microscopy, cylindrical sample plug obtained [Figure 20 (A)] were cut in the middle having a thickness of 1mm [Figure 20 (B)] with a custom made cutting tool utilizing two microtome blades set 1mm apart so that the confocal laser is able to penetrate the samples [Figure 20 (C&D)]. Cutting the sample in the middle of the core removes the outer surface of the plug which is important as the cells on the this surface of the plug die while taking the sample and evaluating them gives a false result as these cells are not dead due to experimental conditions but get killed by the sampling procedure.

The cutting procedure was done on dry ice to keep the punches frozen and in shape. While assessing them under the microscope, remaining samples were stored on dry ice as the samples thaw and Calcein-AM dye leaks out of the cells resulting in loss of signal and difficult acquisition of images.
Figure 20: Preparation of IVD cores for confocal microscopy: Cylindrical plugs taken with skin biopsy punch. (B) 1mm slice cut in the middle of the punch with custom made cutting tool. (C) Orientation of slice post cutting. (D) Slice laid flat and probed with scanning laser of the confocal microscope.

10.4 Confocal microscopy of the tissue slices prepared from intervertebral disc (IVD) punches:

The cells were then visualized using an inverted confocal laser-scanning microscope (Zeiss LSM510). The cells were initially visualized with mercury lamp 10x/0.3 plan neofluar lens. After visualization, rapid x-y scan was run with 543nm and 488nm lasers. Pinhole was set at 1000µm for both red and green channels. Stack size was 1.27µm X 1.27µm X 5.95µm (x-axis, y-axis, z-axis). 20 consecutive 6µm sections were imaged from each disc and area. Confocal laser-scanning microscope stacks were split into single images. The first image to be analyzed was chosen.
approximately 5 images into the stack to further prevent artifacts from the cut surface and then extending another 4 images. The selected 5 images were merged, saved as single color JPEG files, and the labeled live (green) and dead (red).

The cell quantification was separately done using the CellIC software (MATLAB). The live-to-dead cell ratio was then calculated and the average viability was combined from all discs in each respective area.

11. Injection of Link-N in CEP human intervertebral discs (IVDs) in culture:

The human IVDs were processed and isolated with CEP method and cultured for 5-7 days in medium LL media to stabilize the IVDs before injection. The IVDs were brought out of the culture pots inside the biological safety hood and placed in a sterile petridish and wiped with a sterile surgical gauge. The IVDs were marked in the NP region with a Chinese grease pencil to mark the site of injection.

U-100 insulin syringes with 25G needles were used to inject Link-N in the IVDs. 100µL of prepared 10mg/mL Link-N solution with radioactivity supplementation was taken up in U-100 syringes. A small bubble was introduced in the syringe behind the liquid in the barrel so as to deposit the solution completely inside the IVD and minimize dead space losses in the syringe. During injection, the needle was introduced slowly inside the IVD and made to go past the marked area a bit to create a pocket so as to avoid the problem of backflow of the injected solution. After
injection, the needle was kept in for 2-3 minutes in the same position and gradually brought out with slow rotatory movement to avoid backflow and the injected solution seeping out along the needle track. *A total of 1mg of Link-N was injected per IVD with this method.* Control IVDs were injected with medium alone in similar fashion as Link-N injection. The IVDs were sealed in gas permeable bags with 20ml of medium LL, sealed and cultured in an incubator under standard tissue culture technique at 37°C and 5% CO₂ for 2 days.

At the end of the culture period, the human IVDs were brought out of the bag safely without spilling the radioactivity contained in the culture bag. The IVDs were sampled at the site of injection with an 8mm skin biopsy punch to cover the area of injection as much as possible. Circumferential sample plugs were taken with a 4mm skin biopsy punch 1cm from the site of injection, total 4 in number, to see the effect of the Link-N injection in surrounding areas.

The cell viability was determined in the IVDs injected with Link-N and compared to the viability in their controls to evaluate whether the dose of Link-N chosen for injection was detrimental or not. Furthermore, the effect of needle track on focal cell health was also evaluated. For this purpose, 4mm sample plugs were taken from NP, iAF and oAF regions in the Link-N injected and control discs, processed and evaluated with Live/Dead® assay.
To assess the diffusion pattern of radioactive $^{35}$SO$_4$, another set of human grade 2 and 3 IVDs were prepared with CEP method and injected with 25$\mu$Ci/mL solution of radioactive $^{35}$SO$_4$ (1mCi/mL stock solution) in medium LL. A total of 100$\mu$L of this solution was injected in the center of the IVD and the same injection methodology was followed as described in previous sections. The injected IVDs were put in culture pots and 3.5 X initial weights of the IVD equivalent medium LL was added and pots were transferred to the incubator. 200$\mu$L of medium samples were taken from the media surrounding the IVD at time points 0, 15 min, 30 min, and 1,2,3,4,5,6,7,8,9,10,11,12,13,14 hours. The initial readings were taken within short intervals and thereon spaced every hour to evaluate whether the out flux of $^{35}$SO$_4$ occurs rapidly post injection due to its small size or follows a time dependent diffusion pattern. The endpoint was determined when 5 consecutive readings showed no change and plateaued out. The collected samples were transferred to the scintillation vials and 5ml of scintillation fluid was added and read on a beta scintillation counter. The CPM reads were plotted using Microsoft Excel. Reverse experiment was also performed with $^{35}$SO$_4$ supplemented medium LL surrounding the disc and no radioactivity injected within the IVD. The IVDs were cultured till 16 hours, same as the injection experiment above and sampled at the end of experiment using 8mm skin biopsy punch.
12. Analysis of $^{35}$SO$_4$ incorporation in newly synthesized proteoglycans (PGs) under the effect of Link-N:

The sample plugs obtained from the injected IVDs were weighed and digested with 1mg/mL Proteinase K solution. 1mL per 100mg wet weight tissue was put in and digested overnight at 56°C. The digest was heat inactivated at 95°C for 5 min at the end of experiment to kill residual Proteinase K activity [76, 160]. The digest was cooled down and 0.5mL of it transferred to the sample wells of the micro dialyzer.

The radioactivity supplemented medium collected from Link-N exposure studies stored at -20°C was thawed and spun down with a burst for 10 sec. The samples were dialyzed to remove unincorporated radioactivity using a micro dialyzer (Spectr/Por 3, 3500 kDa molecular weight cut-off dialysis membrane). The collected media and radioactive digests were charged in sample wells and exhaustively dialysed against miliQ water at 18.2Ω purity. The end point for dialysis of samples was determined by intermittently taking samples from draining sludge and when the sludge reading was 0, the dialysis was stopped. This was followed by a cold chase with 1M MgSO$_4$ for two hours to remove any remaining unincorporated $^{35}$SO$_4$. The dialyzed medium was transferred to the scintillation vials and 5mL of scintillation fluid was added to them.

Residual radioactivity was measured by counting counts per minutes (CPM) on a beta scintillation counter. The CPM obtained were plotted using Microsoft® Excel with counts from unexposed beads taken as control for dose response experiments.
13. Diffusion and distribution of Link-N injected in intervertebral discs (IVDs)

prepared with CEP method:

To evaluate the diffusion of Link-N injected in CEP IVDs, fluorescent 5-TAMRA was injected in the CEP IVDs. To check for contribution of fluorescence by the medium and other solutions used and to choose best dissolving solution, a series of solutions were tested for inherent fluorescence at the excitation/emission of 5-TAMRA. The solutions tested were DMEM with 1g/L glucose with and without phenol red indicator without FCS, 1% glucose with and without phenol red indicator with FCS, PBS, Proteinase K solution and distilled water. The fluorescence values for all the tested solutions were same as blank well with only air and thus these solutions were not inherently fluorescence at the chosen wavelengths.

To rule out interference of the IVD matrix with fluorescent readings, Proteinase K disc digests were evaluated for autofluorescence from the IVD matrix which might contribute to fluorescence reads from 5-TAMRA. 5 different donor IVDs from different age groups ranging from 22-72 years of age were taken and punched in NP region and digested with Proteinase K. IVD matrix is known to have autofluorescence due to choromophores accumulated over time due to degradation of cholesterol products. 100µL of the digest was put in the fluorescence plate reader (Infinite M1000 pro, Tecan) and a diagnostic scan was run. The autofluorescence detected was in UV range for the IVDs from different age groups having different amounts of choromophores and didn’t overlap with emission spectra of 5-TAMRA [Figure 21].
Figure 21: Autofluorescence of IVD: Figure showing fluorescence emission ranges of the IVD matrix digested with Proteinase K and of 5-TAMRA. Digested IVD matrix autofluoresced in UV range (double arrows) and distinctly had no contribution to fluorescence by 5-TAMRA (single arrow). The intensity of autofluorescence varied with the age of the donor with intensity being higher with the older IVDs as expected [161].

A serial conc. curve was run on the fluorescence plate reader (Infinite M1000 Pro, Tecan) from 10mg/mL – 1pg/mL to gauge the detection limit of the reader and to evaluate when the detector is saturated due to excess fluorescence. The conc. injected by us fell in the detection range of the machine and did not saturate the detector. The 5-TAMRA Link-N conjugate solution was serially read at 2, 4, 6, 8, 12 hours being not exposed and exposed to light to evaluate spontaneous decay and light exposure
induced decay in fluorescence intensity of 5-TAMRA. Non light exposed 5-TAMRA Link-N solution retained fluorescence whereas the light exposed group showed decay in signal intensity after 12 hours.

To evaluate diffusion pattern of Link-N, bovine IVDs prepared with CEP method were injected with unconjugated 5-TAMRA and 5-TAMRA conjugated Link-N. Human IVDs were prepared with CEP method and were injected with 5-TAMRA conjugated Link-N solution only and analyzed as to save hard to come by and procure human IVDs. The injected IVDs were then put in medium LL with 3.5X initial weight of the IVD equivalent medium and cultured in an incubator under standard tissue culture technique at 37°C and 5% CO₂. 100µL samples were taken from surrounding media at different time points to evaluate the appearance of fluorescence in the media from inside of the IVD and to see when the system equilibrates. The time points for taking samples were 0, 15 min, 30 min, 1, 4, 6, 8, 11, 15, 19, 22, 25, 27, 31, 33, 36, 38, 40, 44, 48, 50, 52 hours. The detected fluorescence was plotted and the experiment was ended when 5 consecutive readings showed no change and the curve plateaued out.

To evaluate residual Link-N binding to the IVD matrix, IVDs prepared with CEP method were injected with unconjugated 5-TAMRA and 5-TAMRA conjugated Link-N and put in culture. 100µL samples were taken from surrounding medium at 0, 12, 24, 48, 96, 116, 168 hours until no further increase in fluorescence was detected. After taking the last sample, medium surrounding the IVDs was then changed at
regular intervals at 24, 48, 72, 96, 120 and 144 hours to actively drive out unconjugated 5-TAMRA and 5-TAMRA conjugated Link-N. The media changes were stopped and no further samples were collected when no fluorescence was detected for 3 consecutive reads.

The reverse experiment was also done with 5-TAMRA conjugated Link-N where the solution instead of being injected, was put in the surrounding medium and cultured till the noted time of equilibrium from previous experiments and the IVDs harvested at the end of the culture period.

The IVDs were sampled at the site of injection and 1cm circumferentially as described in previous sections. Extra sample plugs were also taken in oAF region of the IVDs. The sample plugs were digested with Proteinase K overnight with aluminium foil to prevent light decay of 5-TAMRA fluorescence signal intensity.

100µL IVD punches digests were read with a plate reader. Media collected at the time of equilibrium and at different time points was diluted to 1/10th dilution and read on the plate reader as Proteinase K digestion diluted the fluorescence of the IVD sample plug to 1/10th due to 1:10 addition of Proteinase K solution per 100mg wet wt. tissue as per protocol. The reads from sites of injection, circumferential punches, punches from oAF, surrounding media at equilibrium and media from different time points was plotted on Excel software and quantified.

After taking sample plugs from various region of the IVD post equilibrium and leaching with plain medium, full width slice was taken. The slice was 0.5mm in
thickness and obtained with an in house custom built cutting tool using two microtome blades set 0.5mm apart. The slices were washed with PBS for 3 hours with PBS change every ½ hour and then evaluated using LASS400 ImageQuant imaging system with epi green filter with detection range 475-495nm wavelength, capable of detecting fluorescence emitted by 5-TAMRA to see residual fluorescence even when naked eye was not able to see any fluorescent marks.

14. DMMB assay for GAG content estimation:
GAG content of the IVDs was done with DMMB assay. 8mm punch was taken in NP region of every IVD and digested overnight at 56ºC with 1mg/mL Proteinase K solution. 1mL of Proteinase K/100µg wet weight tissue was used [162]. 100µL of IVD extract was used for sulfated GAGs were quantified by a modified dimethylmethylene blue dye-binding assay [163]. Shark fin chondroitin sulphate was used to generate standard curve and some samples were diluted to fall in the middle of the linear range of the standard curve.

15. Western blot analysis to probe for G1 domain in CEP intervertebral discs:
Proteins and proteoglycans were extracted at 4°C under continuous shaking for 48 hours on a 15 volumes per wet weight volume basis using extraction buffer (4M guanidinium chloride, 50mmol/L sodium acetate, pH =5.8, 10mmol/L EDTA, Complete® protease inhibitor cocktail tablet). The extracts were centrifuged at 16,000g for 30 minutes. Proteins and proteoglycans in 5µL aliquots of disc extracts were precipitated in 95% ethanol (1:9) at 4°C overnight, and recovered by
centrifugation. The pellets were washed twice in 75% ethanol and lyophilized. The samples were redissolved in 50μL 50mmol/L Na Acetate, pH=6.0, and then digested with Keratanase II at 0.1mU per 5μL extract for 6 hours. The solution was then adjusted to 100mmol/L Tris, 100mmol/L sodium acetate, pH=7.3, and digested overnight with chondroitinase ABC at 1mU per 5μL extract. Finally, the solution was mixed with SDS/PAGE sample buffer and boiled for 5 mins. The samples were separated by SDS-PAGE (4%–12% NOVEX gels). The separated proteins were transferred on to nitrocellulose membranes, and western blotting was performed using antibodies recognizing G1 domain of aggrecan. The membranes were blocked with 3% skim milk and then incubated with the anti-G1 antibody at a 1:1000 dilution in blocking buffer containing 3% bovine serum albumin, followed by an anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (1:1000 dilution) in blocking buffer containing 1% dried skim milk. The bound antibody was visualized by chemiluminescence.

16. Mass spectrophotometry:
25μL medium samples, collected from the monolayer cultures described above, were enriched on C18 columns [164]. Peptides were eluted in 20μL, 2% Acetonitrile (ACN) and 0.2% Formic acid (FA). 1μl of samples was injected in light chromatography column of LC MS/MS with quadruple time of flight machine [165]. The results were analysed using multiple reactions monitoring (MRM) using Protein Lynx 2.1 and nano-lockspray calibration using the peak at 1922 Da as calibrant mass, the mass of intact non glycosylated Link-N peptide. The signal intensities for
calibrant mass were plotted to evaluate the stability of Link-N peptide over the collected time points.

1μL samples were loaded on customized plate for MALDI-TOF and air dried. The samples were analyzed using Bruker Scout 384 Reflex III matrix-assisted laser desorption/ionization time-of-flight as described [164, 166, 167]. A mass peak was detected consistently with samples collected from iAF cell layer other than the mass of Link-N (1922Da).

The identified fragment was analysed using the ExPASY peptfind bioinformatics tool from the Swiss Institute of Bioinformatics (SIB) (http://web.expasy.org/findpept/) and two possible sequences within the Link-N peptide were identified. MALDI-TOF MS was used to determine the sequence of the fragment present. Of the two, definitive sequence was identified based on presence/absence of cleavage products from the C-terminal end of the parent 16 amino acid peptide (Link-N). The identified fragment was named Link-N (1-8) and the remaining part was named Link-N (9-16).

17. Statistical analysis:

Results from experiments evaluating incorporation of radioactive sulphate were analysed by expressing the results normalized to controls. Each dose of radioactivity incorporation in every donor set was done in triplicates and means and standard errors of mean were calculated for each experiment. The means were normalized to control and expressed as a relative ratio with synthesis by chosen control groups set at
1. Standard error of means were converted to relative standard deviation (RSD) and plotted. For statistical significance, two tailed paired student t-test was performed with $p \leq 0.05$ taken as significant.

For gene expression analysis, the relative gene expression for each evaluated gene with 18S as control was calculated using $\Delta \Delta Ct$ method. The relative gene expression was pooled from three donors and mean calculated. The data was plotted as relative ratio with expression by chosen control group set at 1. Relative standard deviation were thus calculated and plotted. For statistical significance, two tailed paired student t-test was performed with $p \leq 0.05$ taken as significant.

The CPM counts from Link-N injected IVDs were normalized to CPM from their own individual, grade matched, adjacent level control IVDs. Data was again expressed as a relative ratio with synthesis of new PG by controls set at 1. For statistical significance, two tailed paired student t-test was performed with $p \leq 0.05$ taken as significant.

Five different IVDs from bovine and human donors were put in culture after preparation with NEP or CEP method and weight increase was calculated for each IVD for each isolation method at each time point. Mean and standard of mean was calculated for each time point and date pooled in on to a single curve and plotted with their standard error of mean.
Cell counts from Live/Dead® assay were pooled and mean and standard error of means calculated for each isolation method and each culture condition. The data was tabulated to compare effect on cell viability under different conditions.

Data plotting and statistical analysis was done by using Microsoft Excel® and GraphPad Prism® softwares.
Chapter 3

(Results)
1. Effect of Link-N on proteoglycan synthesis and protease production by bovine and human IVD cells cultured in non inflammatory and inflammatory environment:

1.1 Effect of Link-N on proteoglycan synthesis by bovine and human IVD cells:

1.1.1 Dose response curve for proteoglycan synthesis by bovine and human IVD cells:

Link-N has been previously demonstrated to have growth factor like properties and has an anabolic effect on IVD cells. Like any other growth factor, Link-N also has an optimal dose. To assess the effect and optimal dose of Link-N for maximal proteoglycan synthesis by bovine and human IVD cells, cells isolated from NP and AF/iAF regions of the IVD beaded in 1.2% alginate were exposed to radioactivity supplemented medium HC with increasing doses of Link-N. Control beads were exposed to medium alone. The exposure was for 48 hours and incorporation of $^{35}$SO$_4$ was used to assess neo proteoglycan synthesis in response to Link-N exposure. The data was normalized to $^{35}$SO$_4$ incorporation by controls set at 1 and expressed as a relative ratio.

In bovine IVDs (n=4), both NP and AF cells showed a dose dependent increase in $^{35}$SO$_4$ incorporation with the dose of maximal response being at 1μg/mL for both NP and AF cells [Figure 22 (A)]. The incorporation of $^{35}$SO$_4$ was significant at all doses (*); 0.01μg/mL (NP p=0.006, AF p=0.027), 0.1μg/mL (NP p=0.009, AF p=0.007),
1μg/mL (NP p=0.003, AF p=0.004) and 10μg/mL (NP p=0.009, AF p=0.004) respectively.

In humans IVDs (n=4), both NP and iAF cells showed a similar trend as bovine IVD cells and there was a dose dependent increase in $^{35}$SO$_4$ incorporation with the dose of maximal response being at 1μg/ml for both NP and iAF [Figure 22 (B)]. The incorporation of $^{35}$SO$_4$ was significant at all doses (*); 0.01μg/ml (NP p=0.003, AF p=0.02), 0.1μg/ml (NP p=0.002, AF p=0.004), 1μg/ml (NP p=0.03, AF p=0.009) and 10μg/ml (NP p=0.01, AF p=0.04) respectively.

The cells from oAF were not used as regions targeted to be stimulated by Link-N in intact human IVD were NP and iAF.
Figure 22: Proteoglycan synthesis dose response curve in bovine and human IVD cells: (A) Proteoglycan synthesis increased in a dose dependent manner in bovine NP and AF cells (n=4) exposed to Link-N with dose of maximal response at 1μg/ml. (B) Proteoglycan synthesis increased in a dose dependent manner in human
NP and iAF cells (n=4) exposed to Link-N with dose of maximal response at 1μg/mL. Data is represented as relative ratio normalized to incorporation by controls (dotted line) set at 1. For both response by NP and AF/iAF cells at different doses (*), values of p≤ 0.05 were taken as significant.

1.1.2 Variation in response to Link-N exposure in human IVD cells from different donors:

Human donors show a high degree of variability in physiological response to therapeutic agents and make establishment of a compound as a therapeutic agent difficult. To evaluate the degree of variation, IVD cells from 7 donors from NP and iAF regions beaded in alginate were exposed to the dose of maximal response of Link-N (1μg/mL). Control beads were exposed to radioactivity supplemented medium HC alone. NP and iAF cells from all the donors showed a significant increase in $^{35}$SO$_4$ incorporation in response to Link-N exposure (*). The response to Link-N in different donors showed as expected variability but invariably, a significant response was seen in all the donors, indicating that Link-N therapy will have benefits but to different magnitudes in different donors making patient selection very important. [Figure 23].
Figure 23: Variation in response to Link-N in IVD cells from different human donors: IVD cells from different donors (n=7, age range 25-53 yrs.) showed an increase in proteoglycan synthesis at the dose of maximal response of Link-N (1μg/mL). The response was variable but consistently a response was seen in all the donors. Data is represented as relative ratio normalized to incorporation by controls (dotted line) set at 1. Values of p≤ 0.05 were taken as significant.

1.1.3 Evaluation of response to Link-N in human IVD cells from a very young donor:

IVD cells vary in their metabolic states depending upon the age of the subject. IVD cells from young individuals are already in a high state of metabolic activity as compared to cells from older individuals where cell senescence is common. To assess whether Link-N can stimulate an increase in proteoglycan synthesis in IVD cells from
very young donors that have not undergone degeneration, IVD cells from NP region only from a 4 month old donor beaded in alginate were exposed to increasing concentrations of Link-N, 0.01μg/mL, 0.1μg/mL, 1μg/mL and 10μg/mL respectively in radioactivity supplemented medium HC. AF tissue was restricted to 1-2 annular rings and not fully developed, and thus was not used. Control beads were exposed to radioactivity supplemented medium HC alone. Only NP cells were isolated and used as at this young age, AF is not fully developed and is restricted to mere 2-3 annular rings and the majority of the IVD tissue is gelatinous grade 0 NP tissue. Link-N was only able to significantly stimulate $^{35}$SO$_4$ incorporation in newly synthesized proteoglycan at 1μg/mL dose (p=0.012) indicating cells from non-degenerate young donors behave differently compared to adult donors and can only be stimulated with high dose due to already active IVD cells [Figure 24].

Figure 24: Evaluation of proteoglycan synthesis in human IVD cells from young donors: Proteoglycan synthesis in human NP cells from a young donor (4 months
old) exposed to Link-N was only increased at the previously determined dose of maximal response to Link-N (1μg/mL). Data is represented as relative ratio normalized to incorporation by controls (dotted line) set at 1. Values of $p \leq 0.05$ were taken as significant.

1.1.4 Evaluation of specificity of Link-N sequence in stimulating proteoglycan synthesis in bovine and human IVD cells:

Link-N is a peptide with positively and negatively charged amino acids, giving it focal charge densities. The receptor interaction with Link-N could be charge based and not sequence specific. To evaluate the sequence specificity of Link-N in stimulating proteoglycan synthesis, bovine (n=3) and human (n=3) IVD cells from NP and AF/iAF regions embedded in alginate were exposed to human sequence of Link-N, reverse sequence of Link-N and scrambled sequence of Link-N at the dose of maximal response to Link-N (1μg/mL) in radioactivity supplemented medium HC. Control beads were exposed to radioactivity supplemented medium HC alone.

Bovine IVD cells from both NP and AF regions showed $^{35}$SO$_4$ incorporation in newly synthesized proteoglycan only with the native human sequence. The increase by both NP and AF ($p=0.03$ and $p=0.03$ respectively) [Figure 25(A)].

Human IVD cells from both NP and iAF regions also showed $^{35}$SO$_4$ incorporation in newly synthesized proteoglycan only with the native human sequence. The increase by both NP and iAF ($p=0.008$ and $p=0.02$ respectively) was statistically significant.
The scrambled and reverse sequences failed to stimulate any response in either bovine or human IVD cells and incorporation levels were comparable to controls.

Figure 25: Evaluation of Link-N sequence on neo proteoglycan synthesis by bovine and human IVD cells: (A) Bovine and (B) human IVD cells showed
significant increase in proteoglycan synthesis only with exposure to human sequence of Link-N (1μg/mL conc.). Reverse and scrambled sequences had no significant effect on proteoglycan synthesis when comparable to controls (dotted line) set at 1. Values of p≤ 0.05 were taken as significant.

1.2 Effect of Link-N on gene expression of aggrecan and proteases by bovine and human IVD cells cultured under non inflammatory and inflammatory environment:

For use as a bioactive agent in vivo, any growth factor or growth factor like agent should have the ability to work in physiological conditions present in the tissue at the time of administration. DDD is associated with a pro-inflammatory cytokine rich environment. For this IL-1β exposure was chosen as the model as it is one of the most commonly implicated cytokines involved in DDD [155, 168]. Effect of Link-N on gene expression of aggrecan and proteases by bovine and human IVD cells was evaluated in non-inflammatory conditions and inflammatory environment to determine the differential effect of Link-N under these conditions.

1.2.1 Effect of Link-N on aggrecan and proteases gene expression by bovine and human IVD cells under non inflammatory environment:

To evaluate the effect of Link-N on gene expression of aggrecan and proteases in a non-inflammatory environment, bovine and human IVD cells embedded in alginate from NP and AF/iAF regions were exposed to 1μg/L conc. of Link-N for 24 hours in medium H-ITS as described in previous sections. Control alginate beads were
exposed to medium H-ITS alone. Genes analysed were aggrecan, MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 normalized to 18S as endogenous control. GAPDH was also tested as an endogenous control but 18S was found to give stable Ct values for the IVD cells used. The evaluation results for GAPDH corroborated with personal communications with other groups conducting research in the field of IVD pathology. Results were expressed as relative gene expression using ΔΔCt method compared to controls set at 1.

In both bovine NP and AF cells (n=3), aggrecan gene expression was upregulated in response to Link-N exposure (*) (NP p=0.012, AF p=0.001). MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 expressions were downregulated by Link-N in both NP (*) (p=0.03, p=0.026, p=0.012, p=0.005), and AF cells (*) (p=0.0009, p=0.001, p=0.0035, p=.0008) [Figure 26(A)].

In both human NP and iAF cells (n=3), aggrecan gene expression was upregulated in response to Link-N exposure (*) (p=0.021 and p=0.003). MMP-3 expression was upregulated by Link-N in both NP and iAF cells (*) (p=0.009 and p=0.001). MMP-13, ADAMTS-4 and ADAMTS-5 expression was downregulated by Link-N in NP cells (*) (p=0.0003, p=0.003, p=0.005), whereas their expression was upregulated in iAF cells (*) (p=0.002, p=0.002, p=.003) [Figure 25 (B)].
Figure 26: Gene expression analysis in bovine IVD cells exposed to Link-N in non-inflammatory environment: Changes in gene expression of aggrecan, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 by (A) bovine NP and AF cells (n=3) and (B) human NP and iAF cells (n=3) embedded in 1.2% alginate, 24 hrs after Link-N (1μg/mL) exposure. Data is expressed as a ratio relative to gene expression by control.
cells exposed to medium alone set at 1 (dotted line). Values of \( p \leq 0.05 \) were taken as significant.

1.2.2 Effect of Link-N on gene expression by bovine IVD cells cultured under inflammatory environment:

DDD is associated with elevated levels of cytokines and for a bioactive agent to have a beneficial effect; it should be able to function in such a pro-inflammatory environment. To evaluate the effect of Link-N on gene expression of aggrecan and proteases in an inflammatory environment, bovine and human IVD cells embedded in alginate from NP and AF/iAF regions were simultaneously exposed to Link-N (1μg/mL) and IL-1β (10ng/mL) for 24 hours in medium H-ITS as described in previous sections. Control alginate beads were exposed to IL-1β in medium H-ITS alone. Genes analysed were aggrecan, MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5. Results were expressed as relative gene expression using \( \Delta\Delta Ct \) method compared to controls set at 1.

In bovine NP and AF cells (n=3), aggrecan gene expression was significantly upregulated in response to Link-N exposure (*) (NP \( p=0.022 \), AF \( p=0.006 \)). MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 expressions were significantly upregulated by Link-N in both NP (*) \( (p=0.03, p=0.026, p=0.012, p=0.039) \), and AF cells (*) \( (p=0.009, p=0.005, p=0.02, p=0.012) \) [Figure 27 (A)].
In human NP and iAF cells (n=3), aggrecan gene expression was significantly upregulated in response to Link-N exposure (*) (p=0.0031 and p=0.002). MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 expression was significantly downregulated by Link-N in NP cells (*) (p=0.003, p=0.002, p=0.002, p=0.0003). Whereas in iAF cells, MMP-3 and ADAMTS-5 was significantly upregulated (p=0.002, p=0.0003) and MMP-13 and ADAMTS-4 significantly downregulated (*) (p=0.0001, p=0.0002) Figure 27 (B)].
Figure 27: Gene expression analysis in bovine IVD cells exposed to Link-N in an inflammatory environment: Changes in gene expression of aggrecan, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 by (A) bovine NP and AF cells (n=3) and (B) human NP and iAF cells (n=3) embedded in 1.2% alginate, 24 hrs after Link-N (1μg/mL) and IL-1β (10ng/mL) exposure. Data is expressed as a ratio relative to gene expression by cells exposed to IL-1β alone set at 1(dotted line). Values of $p \leq 0.05$ were taken as significant.

The differential response to Link-N by bovine and human IVD cells in the levels of gene expression of MMPs and ADAMTSs in the presence or absence of IL-1β may be species dependent.

1.2.3 Effect of Link-N on proteoglycan synthesis by bovine and human IVD cells cultured in an inflammatory environment:

In addition to upregulating gene expression for aggrecan, the synthesis and release of post translational modified end products in an inflammatory environment is critical for any bioactive agent to be successfully established as a therapeutic agent. To evaluate the potential of Link-N on stimulation of proteoglycan synthesis in an inflammatory environment, bovine and human IVD cells from NP and AF/iAF regions were simultaneously exposed to Link-N (1μg/mL) and IL-1β (10ng/mL) and to Link-N (1μg/mL) alone for 48 hours in medium H-ITS as described in previous sections. Control alginate beads were exposed to IL-1β in medium H-ITS alone. The incorporation of $^{35}$SO$_4$ was used to assess proteoglycan synthesis in response to Link-
N exposure. The data was normalized to $^{35}$SO$_4$ incorporation by controls set at 1 and expressed as a relative ratio.

Bovine NP and AF cells (n=3) showed a significant increase in proteoglycan synthesis when co-exposed to Link-N and IL-1β (*) (NP p=0.002, AF p=0.002). Though the response to Link-N alone was better than Link-N and IL-1β co-exposure, Link-N still was able to induce proteoglycan production significantly in the presence of cytokines [Figure 28 (A)].

Human NP and AF cells (n=3) also showed a significant increase in proteoglycan synthesis when co-exposed to Link-N and IL-1β (NP p=0.003, AF p=0.004). Though the response to Link-N alone was better than Link-N and IL-1β co-exposure, Link-N still was able to induce proteoglycan production significantly in the presence of cytokines [Figure 28 (B)].
Figure 28: Evaluation of Link-N sequence on neo proteoglycan synthesis by bovine and human IVD cells: (A) Bovine and (B) human IVD cells showed
significant increase in proteoglycan synthesis with co exposure to Link-N (1μg/mL conc.) and IL-1β (10ng/mL). Data was expressed as a relative ratio to controls (dotted line) set at 1. Values of p≤ 0.05 were taken as significant.

2. Development of a whole organ culture model for long term culturing of IVD:

2.1 Evaluation of the swelling potential of bovine and human IVD prepared with NEP and CEP isolation techniques:

IVDs in culture without external load swell due to the presence of PGs which draw water from the medium into the disc. The amount and rate of such swelling is critical for the survival and adaptation of the IVD cells in organ culture [35, 169-171]. Thus, very rapid or excessive swelling will lead to failure of the culture system due to rapid cell death.

Bovine and human IVDs prepared with NEP and CEP as described in previous sections (Section 6.3) were evaluated for the rate of swelling. The discs prepared with NEP and CEP method were weighed to determine the pre culture weights and put in culture in medium HH. Bovine NEP IVDs (n=5) swelled rapidly and gained 52% of their initial weight within the first 6 hours in culture. The culture system stabilized between 24-28 hours in culture with swelling to a maximum of 55% of the initial weight and with no further increase in swelling. The IVDs prepared with the CEP method (n=5) swelled and gained 20% of the initial weight within the first 9 hours in culture and the system stabilized at 21 hours into culture with a maximum swelling of
22% of initial weight and with no further increase in swelling [Figure 29 (A3)]. During the culture period, the NEP IVDs lost their morphology and bent out of shape and formed a “hat like” structure [Figure 29 (A1)]. CEP IVDs on the other hand, retained the shape and morphology throughout the culture period [Figure 29 (A2)].

Human IVDs prepared with the NEP showed a similar trends in swelling and the discs prepared with NEPs (n=5) swelled to 40% of their initial weight within 4 hours in culture and the system stabilized at 21 hours in culture with 42% maximum swelling from initial weight and no further swelling. The IVDs prepared with the CEPs swelled to a maximum of 20% of their initial weight within the first 8 hours in culture and the system stabilized between 19-21 hours in culture with 21% maximum swelling and no further increase in swelling [Figure 29 (B3)]. The NEP discs again bent out of shape [Figure 29 (B1)] whereas the CEP discs retained their shape, morphology and structural intactness [Figure 29 (B2)].

Schmorl’s nodes are swellings found to arise from the IVD cartilage and protrude within the vertebral bodies. These nodes are considered as hallmarks of early stage DDD. There are two prevalent theories about Schmorl’s nodes; some authorities believe that it is a lytic, immune/inflammatory reaction that follows protrusion of NP into marrow cavity of vertebral bone through endplate microfractures, while some think that this immune reaction occurs due to an infection in the endplate region. The majority of the Schmorl’s nodes are found in the caudal end of the IVD [172-175].
The direction of swelling in our experiments was always towards the cephalic end of the IVD in all the samples contrary to the literature on the subject.

Figure 29: Evaluation of swelling potential in NEP and CEP IVDs: Swelling potential and morphology of bovine (A1-3) and human (B1-3) IVDs prepared with NEP (without endplate) and CEP (with endplate) methods. CEP IVDs maintained their shape and morphology (A2, B2) whereas NEP IVDs failed to retain it (A1, B1). The swelling in CEP discs was to a maximum of 22% of initial weight. (Figure 29(B) Adapted and modified with permission from [159]).
2.2 Evaluation of effect of surface tension on direction of swelling in NEP IVDs:

Schmorl’s nodes can be present on cephalic or caudal end of the IVD but the majority incidence is on caudal end according to published literature (68%) [172]. Thus the protrusion in event of compromised endplates occurs mainly on the caudal end. In our experiments, we found the direction of “hat like coning” to be towards cephalic end, away from the gravity. Swellings tend to be directed towards least surface tension which in this case is air medium interface. To rule out such an effect of surface tension on the direction of swelling in NEP model, bovine IVDs (n=3) were marked on cephalic end and suspended sideways on medium HH to obliterate the effect of surface tension on cephalic end and direction of swelling assessed [Figure 30 (A &B)]. After the IVDs were cultured for a total of 60 hours, they showed the swelling to be in the cephalic direction even after negating the effects of surface tension on the cephalic end [Figure 30 (C)].

The experiments were done only with bovine IVDs. With the similar trends observed in bovine and human IVDs, the results could be extrapolated to human tissue.
Figure 30: Evaluation of direction of swelling in bovine IVDs: Bovine IVDs were suspended sideways to obliterate effect of surface tension (A&B) and marked with a suture on cephalic end (white arrow) showed the direction of swelling to be in
cephalic direction (black arrow) irrespective of whether the discs were cultured upright or sideways.

2.3 Evaluation of effect of IVD proteoglycan on swelling potential of CEP model:
The sponge like properties of the IVD is due to the water content of the matrix. The water in IVD matrix is drawn in from the surrounding capillary fluid and retained due to the presence of negatively charged PGs. Human IVDs prepared with CEPs (n=70) showed an increase in weight due to swelling in culture ranging from 5%-25.5%. The evaluated IVDs were divided into groups based on the degree of swelling into low (L), medium (M) and high (H) swelling groups. Half of the discs were found to increase in weight between 15% and 23% (M), about one quarter of the discs increased in weight more than 23% (H), and 22% of the discs increased in weight less that 15% (L). Measurement of the glycosaminoglycan content by the DMMB assay in the discs with the high (H) and low (L) swelling groups revealed that the swelling of the tissue was directly related to its proteoglycan content thus indicating that the swelling potential not only depends on the isolation method selected but also on the individual properties of the disc such as matrix composition [Figure 31].
Figure 31: Correlation of proteoglycan content to swelling potentials of human IVDs: Human IVDs cultured with CEP method and divided into low (L) and high (H) swelling groups (n= 20) showed a direct correlation between the amount of proteoglycan present in them and to the degree to which they swelled. (Adapted and modified with permission from [159]).

2.4 Evaluation of effect of isolation method on cell viability of human IVDs in short term culture:

For employing organ culture models in studying IVD physiology and pathophysiology, it is important that high cell viability be maintained during the
culture period. The effect on cell viability of 3 IVD isolation and culture methods; NEP, BEP and CEP as described in previous sections (Section 6.3), was evaluated for 30 days. Human IVDs were prepared with the 3 methods and put in culture in medium HH without external load. Discs were harvested at selected time points and analyzed for cell viability using Live/Dead® assay.

Human discs isolated with NEP method (n=12) were harvested after 3 and 7 days in culture, cores of NP, iAF and oAF tissue was analysed for cell viability. After 3 days in culture, the IVDs showed a drop in cell viability across all regions; NP, iAF and oAF (41%, 35% and 77%) which further dropped to (34%, 11% and 25%) respectively at day 7 [Figure 32(A)]. After 14 days in culture, signs of tissue death such as no change in media color and putrid smell were noted. The cell viability dropped to <10% across all regions and the cultures were terminated.

Human discs isolated with BEP method (n=9) were harvested at 7 and 14 days in culture, cored in NP, iAF and oAF and analysed for cell viability. Day 3 was not evaluated due to restricted amount of tissue obtained at that given time. At day 7 in culture, the IVDs showed a drop in cell viability across all regions; NP, iAF and oAF (4%, 14% and 14%) [Figure 32(B)]. At day 14, no viable cells were detected in the whole disc. Starting at day 3, signs of tissue death such as minimal change in media color and putrid smell were noted. The vertebral bone in the BEP model showed punctate black areas of necrosis and dissolution of bone. The cultures were
terminated and were not continued to time point 30 days in light of tissue necrosis and massive cell death.

Human discs isolated with the CEP method (n=12) were harvested at designated time points 3,7,14 and 30 days in culture, punched in NP, iAF and oAF and analysed for cell viability. At days 3 and 7 in culture, in contrast to NEP and BEP models, the CEP IVDs showed >96% cell viability across all regions; NP, iAF and oAF (98%, 96.8% and 97%) and (96%, 98% and 97%) respectively [Figure 32(C)]. Cultures were continued to days 14 and 30 and cell viability was still maintained at >96%.
**Figure 32:** Evaluation of cell viability with different isolation methods for IVD whole organ culture: Cell viability in human IVDs prepared with (A) NEP (B) BEP.
and (C) CEP isolation methods. NEP model showed a drastic drop in cell viability from day 3 to 7. BEP model had the worst cell viability even at day 7. CEP model for the same time period showed that the cell viability was >96%. Live cells are represented by green dots whereas dead cells are represented by red dots. Scale bar represents 100μm. (Adapted and modified with permission from [159]).

2.5 Effect of different nutritional levels on cell viability in IVDs prepared with CEP method:

IVDs in vivo are in an hypoxic environment [176] and have a very low nutrient supply owing to its avascular nature and nutrients reaching the center of the IVD through diffusion. Glucose levels in human blood (serum) range from 1-1.2 g/L and estimated glucose concentrations at the center of the IVD is around 0.6-0.7 g/L [177, 178]. In order to extrapolate the results of in vitro studies to in vivo conditions, it is essential to culture the IVDs near the physiological state. To evaluate the effect of varying nutritional levels on cell viability in human IVDs in culture, IVDs prepared with CEP method were cultured in mediums HH (n=9), HL (n=9), LH (n=9) and LL (n=10). The levels of nutrients were gradually made to reach to near physiological levels. The IVDs were maintained in culture without external loads for a period of 4 weeks. At the end of the culture period, cell viability was assessed across all the regions of the IVD; NP, iAF and oAF, and was comparable in all nutritional states and was maintained at >96% [Table 3], [Figure 33].
Figure 33: Evaluation of cell viability in human IVDs CEP model cultured in different conditions for 4 weeks: Cell viability in all culture conditions across all regions of the IVD was maintained at >96%. Live cells are represented by green dots whereas dead cells are represented by red dots. Scale bar represents 100μm. (Adapted and modified with permission from [159]).
<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>NP (% Viability)</th>
<th>iAF (% Viability)</th>
<th>oAF(% Viability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Glucose + High FBS (HH)</td>
<td>98.44 ± 1.6</td>
<td>97.25 ± 2.75</td>
<td>98.06 ± 3.78</td>
</tr>
<tr>
<td>High Glucose + Low FBS (HL)</td>
<td>97.61 ± 1.58</td>
<td>97.37 ± 0.93</td>
<td>96.05 ± 2.75</td>
</tr>
<tr>
<td>Low Glucose + High FBS (LH)</td>
<td>97.18 ± 1.22</td>
<td>97.81 ± 0.68</td>
<td>96.23 ± 2.51</td>
</tr>
<tr>
<td>Low Glucose + Low FBS (LL)</td>
<td>96.38 ± 2.48</td>
<td>97.10 ± 1.85</td>
<td>97.19 ± 1.95</td>
</tr>
</tbody>
</table>

Table 3: Table showing cell viability in IVDs cultured in different culture conditions for 4 weeks.

2.6 Effect of culture conditions on metabolic state of human IVD prepared with CEP method:

During the progression of DDD, the endplates can be calcified and nutrient supply and metabolic waste removal is impaired in the IVDs. This can lead to a decrease in the pH of the disc matrix and an upregulation of proteases. Even though cell viability
was not affected by the different glucose and FCS concentrations, altered nutritional status could have the potential to adversely affect protease production and matrix breakdown. Since aggrecan is the major proteoglycan in IVD tissue and is very susceptible to degradation by both metalloproteinases and aggrecanases, it was chosen to address this question. G1 domain of aggrecan generated by aggrecanases and MMPs was evaluated by using western blot analysis to determine differences in G1 band intensity due to altered nutritional states. The G1 fragments are normally found in the IVD matrix due to ongoing degenerative process spanning decades. After 4 weeks in culture, the harvested IVDs were sampled across different regions of IVD. The G1 bands produced by aggrecanases and MMPs activity were found in all the samples across all the regions of IVDs cultured in different nutritional states but while fragments of aggrecan were present in the disc extracts, there was no evidence for increased accumulation of degradation products due to any particular culture condition [Figure 34] indicating that the low nutritional states had no added adverse affects to the ongoing metabolism of the IVD matrix.
Figure 34: Evaluation of the effect of varying nutritional states on the metabolism of disc matrix in human IVDs in culture for 4 weeks: The human IVDs cultured for 4 weeks in different culture conditions showed no changes in G1 band intensity for both aggrecanases and MMPs in all the culture conditions tested across all regions. (Adapted and modified with permission from [159]).

2.7 Effect of prolonged culture periods under different nutritional states on cell viability in IVDs prepared with CEP method:

In order to induce a substantial regenerative response, the administered growth factors or bioactive peptides have to act for a longer duration of time. To be able to evaluate the beneficial effect of such compounds in organ culture models, there is a need for a culture system, which is able to sustain cell viability for longer durations of time. To evaluate the effect of prolonged culture periods under different nutritional states on cell viability in human IVDs in culture, IVDs prepared with CEP method
were cultured in mediums HH (n=10), HL (n=10), LH (n=11) and LL (n=10) with nutrients gradually reaching near physiological levels. The discs were maintained in culture without external loads for a period of 4 months. At the end of the culture period, the cell viability was assessed in all the culture conditions across all the regions of the IVD; NP, iAF and oAF, and was comparable in all nutritional states and was maintained at >96% [Table 4], [Figure 35].

Figure 35: Evaluation of cell viability in human intervertebral discs (IVDs) CEP model cultured in different culture conditions for 4 months: Cell viability in human IVDs prepared with CEP isolation method cultured in varying culture conditions for 4 months. Cell viability in all culture conditions across all regions of
the IVD was maintained at >96%. Live cells are represented by green dots whereas dead cells are represented by red dots. Scale bar represents 100μm. (Adapted and modified with permission from [159]).

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>NP (% Viability)</th>
<th>iAF (% Viability)</th>
<th>oAF (% Viability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Glucose + High FBS</td>
<td>97.88 ± 0.96</td>
<td>98.25 ± 1.75</td>
<td>98.16 ± 1.04</td>
</tr>
<tr>
<td>High Glucose + Low FBS</td>
<td>96.51 ± 1.85</td>
<td>98.28 ± 1.63</td>
<td>96.88 ± 0.95</td>
</tr>
<tr>
<td>Low Glucose + High FBS</td>
<td>98.76 ± 1.45</td>
<td>97.81 ± 1.58</td>
<td>97.23 ± 1.34</td>
</tr>
<tr>
<td>Low Glucose + Low FBS</td>
<td>96.65 ± 1.38</td>
<td>97.91 ± 2.05</td>
<td>97.49 ± 1.25</td>
</tr>
</tbody>
</table>

Table 4: Table showing cell viability in IVDs cultured in different culture conditions for 4 months.

3. Effect of Link-N injection in CEP model human IVDs in culture:

3.1 Evaluation of $^{35}$SO$_4$ diffusion and equilibrium post injection in human IVDs prepared with CEP method:

In DDD, the CEPs are calcified and thus hamper transport of nutrients and other solutes across the IVDs. Human IVDs prepared with CEP method is a semi open
culture system with nutrients, medium and solutes entering and leaving the IVD passing through CEPs which are opened during isolation process. For IVD cells to incorporate radioactive sulphate into newly synthesized PGs, it is important to maintain considerable levels of available radioactivity during the period of exposure. The IVDs prepared with CEP method might have an accelerated diffusion of injected radioactivity thus a reduced amount of radioactivity available for the IVD cells to incorporate. Thus to assess the diffusion and equilibrium of radioactivity injected in the IVD prepared with CEP method, the IVDs were injected with 100μL of 25μCi/mL $^{35}$SO$_4$ alone and put in culture with 20 mL of medium LL surrounding the disc. IVDs from grade 2 and grade 3 donors were injected and 100μL samples were taken off from the surrounding medium at designated time points. The injected radioactivity started to appear in surrounding medium as early as 15 min post injection and gradually increased and reached equilibrium between 10-12 hours post injection. The diffusion from grade 3 discs followed a similar pattern as grade 2 discs but the amount of radioactivity and the rate of it coming out was slightly more than that of grade 2 discs, probably due to pre-existing NP or AF fissures. At equilibrium, the discs were cored at the site of injection and assessed for CPM. Counts detected inside the IVDs were more than those detected in surrounding medium and were in the range of ~ 0.5-0.7 million CPM and thus even post equilibrium, there was high enough level of radioactivity available to IVD cells to incorporate in newly synthesized PGs under the effect of Link-N injection [Figure36].
Figure 36: Evaluation of $^{35}$SO$_4$ diffusion and equilibrium injected in human IVDs prepared with CEP method: Radioactivity injected in human IVDs prepared with CEP method showed equilibrium with surrounding medium between 10-12 hours post injection. Grade 3 IVDs had more radioactivity diffusing out at a faster rate compared to grade 2 IVDs. (n=2)

3.2 Effect of modified culture technique on cell viability in human IVDs prepared with CEP method:
In order to minimize the amount of radioactivity in the culture medium around the human IVDs prepared with CEP method, IVDs (n=3) were cultured in gas permeable bags and 20 mL of medium LL was added and the bags were sealed. At the same time, high enough available radioactivity was maintained in the culture medium for
the cells to incorporate it into newly synthesized PGs. The discs were cultured in these bags for a period of 2 days, the intended duration for future experiments. At the end of culture period, the discs were harvested and sampled in NP, iAF and oAF regions for Live/Dead® assay. The cell viability was maintained at >97% across all regions (NP 97.5%, iAF 98%, oAF 97.1%) at the end of culture period indicating that the modified culture method was not detrimental to the health of IVDs in culture [Figure 37].

![Image](image_url)

**Figure 37:** Evaluation of effect of culturing human IVDs prepared with CEP method in gas permeable bags on cell viability: Cell viability in human IVDs prepared with CEP method cultured in gas permeable bags for 2 days. Cell viability across all regions of the IVD was maintained at >97%. Live cells are represented by green dots whereas dead cells are represented by red dots. Scale bar represents 100µm.
3.3 Effect of Link-N injection on cell viability in human IVDs prepared with CEP method:

The injection dose of Link-N was chosen after a series of preliminary experiments evaluating 1mg/L, 5mg/L and 10mg/L as injection doses. 10mg/mL showed maximum stimulation of proteoglycan synthesis in injected discs. This chosen dose was 20X more than the dose used in 3D cultures of isolated IVD cells. Effect of this high dose on viability of IVD cells in the organ culture system was evaluated to rule out any toxicity due to proximity to such high dose of Link-N. Human IVDs prepared with CEP method were injected (n=2) with 100μL of 10mg/L Link-N solution depositing 1mg/disc of Link-N. The control discs (n=2) were injected with 100μl medium alone. Injected discs were cultured in gas permeable bags in medium LL for 3 days. At the end of the culture period, the discs were harvested and sampled in NP, iAF and oAF regions for Live/Dead® assay. The cell viability was comparable in control (NP 97.8%, iAF 99%, oAF 98.1%) and Link-N injected discs (NP 97.5%, iAF 99%, oAF 97.5%) and maintained at >97% across all regions at the end of culture period indicating that the chosen dose of Link-N was not detrimental to the health of IVDs in culture [Figure 38].
**3.4 Effect of Link-N injection on proteoglycan (PG) synthesis in human IVDs prepared with CEP method:**

IVD degeneration is associated with loss of PGs mainly aggrecan and any attempt at regenerating and repairing degenerated IVDs should aim at increasing the PG content of the IVDs. Link-N has shown to stimulate PG synthesis in IVD cells in culture and in order to take Link-N to clinical trials; its potential has to be tested in animal...
models or organ culture systems. Since there is no ideal animal model to study human IVDs *in vitro*, the potential of Link-N to stimulate PG synthesis was evaluated in CEP culture model for human IVDs, the closest we have to human *in vivo* IVD conditions. The human IVDs prepared with CEP method were graded and grouped according to similar grades of degenerations. The IVDs injected with 1mg/disc of Link-N in radioactivity supplemented medium LL, showed a significant increase in neo proteoglycan synthesis compared to their adjacent level, grade matched control discs was set at 1. Each IVD injected with Link-N had its own individual control; adjacent level and matched to grade of degeneration. The control IVDs were injected with radioactivity supplemented medium LL alone. The response to Link-N was variable in different donors but every disc injected with Link-N showed a significant increase in PG synthesis [Figure39].

![Figure 39](image)

**Figure 39: Evaluation of stimulation of proteoglycan synthesis in human CEP IVDs injected with Link-N**: Human IVDs prepared with CEP method injected with 130
1mg/disc of Link-N showed an increase in proteoglycan synthesis compared to adjacent level grade matched control disc set at 1 (dotted line). Each injected disc had its own individual control injected with radioactivity supplemented medium LL alone. Grades of degeneration (2+, 3, and 3+) of individual discs and their grade matched control are indicated. Values of p≤ 0.05 were taken as significant.

In order to evaluate interspinal variation in response to Link-N injection, injected IVDs from different donors from the same levels (L2-L3) were compared. The IVDs from different donors showed a variation in response but invariably a response was seen in every disc. [Figure 40(A)].

In order to evaluate intraspinal variation in response to Link-N injection, IVDs from different levels from the same donors were compared. Each IVD level had its own adjacent level grade matched control. The response to Link-N in IVDs from the same donor was variable but a response was present in every injected IVD indicating that IVDs from the same donor behave as individual IVDs and intraspinal variation in response to Link-N injection is to be taken into account while choosing candidates and IVDs for Link-N injection therapy. [Figure 40(B)].
Figure 40: Evaluation of interspinal and intraspinal variation in stimulation of proteoglycan synthesis in human CEP IVDs injected with Link-N: Human IVDs prepared with CEP method injected with 1mg/disc of Link-N showed variation in proteoglycan synthesis compared to adjacent level grade matched control disc set at 1 (dotted line). (A) IVDs from same level (L2-L3) from different donors showed...
interspinal variation in response to Link-N injection. (B) IVDs from different levels from the same donor showed intraspinal variation in response to Link-N injection. Each injected disc had its own individual control injected with radioactivity supplemented medium LL alone. Grades of degeneration (2+, 3, and 3+) of individual discs and their grade matched control are indicated. Values of p≤ 0.05 were taken as significant.

3.5 Evaluation of stimulation of proteoglycan synthesis in areas adjacent to Link-N injection site:

Strategies for restoration of function in degenerated IVDs will involve increase in PG content all across the disc. This might be achieved with a single injection of a bioactive agent if the agent diffuses from the site of injection to the adjacent areas or else a need for multiple injections at multiple sites may arise. To evaluate the effect of Link-N injection on the areas adjacent to the injection site, samples were taken circumferentially 1cm. away from the site of Link-N injection. The sampled sites showed a significant increase in proteoglycan synthesis in the Link-N injected discs compared to adjacent level grade matched control cored at similar location. The CPM detected in peripheral sites was nearly 1/5th compared to the CPM detected at the site of injection in both Link-N injected and control IVDs. Thus, Link-N has maximal stimulation of PG synthesis at the site of injection but is also able to stimulate PG synthesis at sites away from the site of injection to some extent, demonstrating that the need for multiple site multiple injection therapy with Link-N may be circumvented. [Figure41].
Figure 41: Evaluation of stimulation of proteoglycan injection at sites adjacent to the site of Link-N injection: (A) Schematics showing the sites of circumferential sampling (1-4) around the site of Link-N injection (denoted by X). (B) Proteoglycan synthesis at circumferential sampling sites (1-4) normalized to corresponding sampling sites in control discs injected with radioactivity supplemented medium alone (dotted line). Each Link-N injected disc had its own individual adjacent level grade matched control. Values of $p \leq 0.05$ were taken as significant.
3.6 Evaluation of diffusion of Link-N injected in human (IVDs) prepared with CEP method:

3.6.1 Diffusion of injected Link-N through endplates of IVDs prepared with CEP method:

Whether an injectable therapy would be single administration or multiple administrations depends upon the clearance rate of the injected therapeutic agent from the target site, both metabolic and clearance through diffusion. To assess the rate of diffusion of Link-N injected in human IVDs prepared with CEP method, 5-TAMRA conjugated Link-N was injected in the IVD and 100µL samples were collected at designated time points. Injected fluorescent Link-N was detectable in surrounding medium by 8 hours post injection and increased gradually until 38 hours post injection and equilibrated thereafter with no further detectable increase in fluorescence in the surrounding medium [Figure 42]. The diffusion of Link-N out of the CEP discs was through endplates and not laterally through AF fibres as during diffusion, the pink fluorescence mark started to appear on the endplates and gradually increased in size at equilibrium Figure 43(B)].
Figure 42: Evaluation of diffusion of Link-N injected in human IVDs: 5-TAMRA conjugated Link-N injected in human IVDs prepared with CEP method started to diffuse out post injection and appeared in the surrounding medium at 8 hours post injection. The diffusion gradually increased until 38 hours post injection and equilibrated thereafter with no further increase in fluorescence detected in surrounding medium.

3.6.2 Evaluation of binding of injected Link-N to the matrix of human IVDs:

Sustained effect of a therapeutic agent could be due to its binding to substrates in the target site. Since fluorescent Link-N showed a slow diffusion release out of the IVD through endplates [Figure 43(B)], the amount of Link-N retained inside the IVD and its binding to matrix was evaluated. The IVDs injected with 5-TAMRA conjugated Link-N were taken out of culture post diffusion equilibrium and were placed in fresh culture pots with fresh medium LL. The medium was changed at designated time
points and 100µL sample collected for fluorescence detection. The medium was changed to actively draw out the injected fluorescent Link-N and binding of Link-N to the IVD matrix was analyzed. The medium changes were stopped when no fluorescence was detected in the surrounding medium. At equilibrium, the pink mark was seen at the endplates and was restricted by iAF fibres and didn’t extend into oAF [Figure 43(B)]. Sample cores were taken at the site of injection and 1 cm circumferentially. The residual injected fluorescent Link-N was detected mainly at the site of injection and in surrounding iAF region but not in oAF regions [Figure 43(D)]. Link-N thus diffused out through the endplates and diffused within the IVD until iAF and was retained and bound to the IVD matrix creating a local depot of supply for IVD cells.

After drawing out fluorescence actively from the injected IVDs, a 0.5mm slice in coronal plane was taken and washed three times with PBS for half hour each to assess the binding of Link-N to the matrix. After the washes, the slices still showed residual fluorescence in the NP and iAF regions under epi green filter, generally not visible to the naked eye, indicating the binding is not due to ionic interaction [Figure 44(A&B)].
Figure 43: Evaluation of binding of injected Link-N to IVD matrix: 5-TAMRA linked Link-N was injected in NP region of intact IVDs (A) and was observed at the endplates at equilibrium (B). Punches were taken at equilibrium across various regions of the injected disc (C). Link-N was mostly detected at the site of injection (NP) and in the surrounding iAF region (D). No Link-N was detected in the medium or outer AF at the time of equilibrium.
Figure 44. Residual binding of Link-N to IVD matrix: (A) Naked eye view of slice from 5-TAMRA injected human IVD post active drawing out of fluorescence. The pink fluorescent stain was restricted to the site of injection (NP) and to iAF and was not seen in oAF (indicated in black arrows). (B) Fluorescence detection visualized with CCD image system with epi green filter. The fluorescence was restricted to site of injection (NP) and to iAF and was not seen in oAF (indicated in black arrows).

3.6.3 Evaluation of specific binding of 5-TAMRA conjugated Link-N in bovine IVDs through the Link-N moiety:

The injected 5-TAMRA conjugated Link-N can theoretically bind to IVD matrix through the 5-TAMRA moiety or the Link-N part. To rule out binding of 5-TAMRA conjugated Link-N through 5-TAMRA moiety, unconjugated 5-TAMRA was
injected in bovine IVDs prepared with CEP method. Bovine IVDs were used to save difficult to procure human tissue. The injected unconjugated 5-TAMRA rapidly diffused out of the IVD in to the surrounding medium with maximum loss occurring within first 48 hours post injection. The medium was changed every 24 hours and by 120 hours post injection, no fluorescence was detected in the surrounding medium. At the end of the experiment, the site of injection showed no fluorescence and the majority of the fluorescence diffused into the surrounding medium collected with first 96 hours post injection [Figure 45(A)]. The fluorescent pink mark seen at the endplate during diffusion also disappeared from the cephalic and caudal end of the IVD [Figure 45 (B&C)]. The cross section of the IVD showed no fluorescence at the site of injection [Figure 45 (D&E)].

![Figure 45](image)

**Figure 45: Evaluation of specific binding through Link-N moiety in 5-TAMRA**

**Link-N conjugate injected in IVDs prepared with CEP method:** (A) The majority of the injected unconjugated 5-TAMRA diffused out of the IVD within the first 48 hours post injection. No residual 5-TAMRA was detected at the site of injection.
Disappearance of pink fluorescent mark which was seen during diffusion at cephalic end (B) and caudal end (C). (D&E) No detection of 5-TAMRA was observed at the site of injection in the slice taken from the injected discs 160 hours post injection.

3.7 Evaluation of sustained stimulation of proteoglycan synthesis in human IVD injected with Link-N:

For any repair to be significant in DDD, the effect of any bioactive peptide has to be sustained, in order to be able to accumulate newly synthesized PGs and to regain the structure and function of the IVD. Since Link-N is retained in the IVDs, it has the potential to have a sustained effect on PG synthesis. To assess whether the stimulatory effect on PG synthesis by Link-N injection is sustained to prevent the need for repeated injections, human IVDs prepared with CEP method were injected with 1mg of Link-N and cultured for 7 days with radioactivity injected on day 8 and the IVDs harvested on day 9. The Link-N injected IVDs showed a significant sustained stimulation of PG synthesis even 9 days post Link-N injection. The response was variable but the injected IVDs invariably showed a response. The control IVDs were adjacent level grade matched and injected with medium LL alone during 7 day culture period. [Figure46].
Figure 46: Evaluation of sustained effect of Link-N injection on proteoglycan synthesis in human IVDs: Sustained effect of Link-N on proteoglycan synthesis in intact human discs (n=2). Intact human IVDs were injected with $^{35}\text{SO}_4$, 7 days post Link-N (1 mg/disc) injection. Discs were harvested and proteoglycan synthesis was evaluated by estimating $^{35}\text{SO}_4$ incorporation in intact human IVDs matched to their adjacent level controls in grade of degeneration. Data are expressed as a ratio relative to control discs injected with medium alone set at 1 (dotted line). Values of $p \leq 0.05$ were taken as significant (*).
4. Metabolism of Link-N by IVD cells and evaluation of biological effect of generated metabolic products:

4.1 Stability of Link-N peptide in culture and its modulation by nucleus pulposus (NP) and annulus fibrosus (AF) cells:

Stability of any bioactive agent in vivo or in vitro is crucial for maintaining its function and ability to exert its effect on the target tissue. A stable agent has less clearance from the target tissue site and the need for repeated administrations may be circumvented. Link-N peptide was cultured without cells (control) and with human NP and AF cells in monolayer for 48 hours and its stability assessed with mass spectrophotometry. Link-N cultured without cells as control was stable throughout the 48 hour culture period and equivalent amounts of the peptide was detected in culture medium (medium H-ITS) at designated time points [Figure 47 (A)]. The peptide was also stable with human NP cells in culture and again equivalent amounts of the peptide were detected at designated time points [Figure 47(B)]. Link-N peptide when cultured with human AF cells showed a drop in the detected amount within the first 6 hours of culture and the amount drastically fell by 24 hours in culture. By 36 hours in culture, the amount of Link-N was barely detected [Figure 47(C)]. Thus Link-N is modulated by human AF cells but not by NP cells.
**Figure 47: Evaluation of stability of Link-N cultured with and without human IVDs:**

(A) Stability of Link-N cultured without human IVD cells. The peptide remained stable throughout the culture period. (B) Stability of Link-N cultured with human NP cells. The peptide remained stable throughout the culture period. (C) Stability of Link-N cultured with human AF cells. The peptide was modulated by AF cells and the amount started to fall within first 6 hours of culture and was barely detectable 36 hours in culture. (n=3).

### 4.2 Detection of generated cleaved by products of Link-N modulation by human annulus fibrosus cells:

The Link-N peptide was modulated by human AF cells and the parent 16 amino acid peptide was barely detectable by the end of the 48 hour culture period. It was not clear whether the parent peptide was completely cleaved into individual amino acids or broken down into shorter peptides or fragments. The collected medium from NP and AF cultures was analyzed using IonTrap and MALDI-TOF and a peptide with molecular weight of 964.4Da was identified in the medium from AF cultures. This newly generated peptide was not found in medium from NP cultures [Figure 48A]. The sequence corresponding to this molecular weight was not known and a proteomic tool was used to identify the possible sequences with this molecular weight, which can be generated from the parent peptide. Two possible sequences were identified with the corresponding molecular weight, peptide with amino acid sequence 1-8 from N-terminal of the parent peptide (Link-N 1-8) and peptide with amino acid sequence
3-10 from N-terminal of the parent peptide (Link-N 3-10). Cleavage products from C-terminal of the parent peptide were identified and corresponded with the Link-N (1-8) sequence and thus the cleavage product of Link-N modulation by AF cells was identified [Figure 48 B]. It was not clear though whether the cleavage occurred sequentially starting from C-terminal of the parent peptide until amino acid position 8 from N-terminal or the parent peptide was cleaved at the 8-9 amino acid site and the fragment with amino acid sequence 9-16 (Link-N 9-16) was later completely degraded to individual amino acids.
Figure 48: Identification of sequence of cleaved peptide generated by modulation of Link-N by human AF cells: (A) Mass spectrum of peptides detected in medium
from human NP (black) and AF (red) cells. Fragmented Link-N with a mass of 964.4 Da is indicated in the graph. The 964.4 Da peptide eluted from the column in 2 different regions, with retention times of around 23 and 32 min. (B) Schematic illustration of the two possible Link-N fragments of 964.4 Da, Link-N 1-8 (highlighted in blue) and Link-N 4-11 (highlighted in green). (C) The amino acid sequence of the generated 964.4 Da fragment was identified by tandem MS. The sequence was confirmed by evaluating the generated fragmentation products of the peptide. Major detected peaks are A [(845.3Da) DHLSDNY (+1)], B [(682.28Da) DHLSDN (+1)] and C [(568.2Da) DHLSD (+1)], masses that can only be generated by the 1-8 sequence.

4.3 Evaluation of biological activity of Link-N (1-8) peptide by assessing stimulation of proteoglycan production in human and bovine IVD cells exposed to the sequence in a non-inflammatory environment:

For many therapeutic and bioactive agents, the main mechanism of action to exert their effect is through metabolism into more or equally potent by products which retain their activity [179, 180]. Otherwise, bioactive agents could be normally metabolized in the target tissue but still continue to exert their action through by products which might retain the activity of the parent compound [179, 181]. To assess the effect of Link-N (1-8) and any possible effect of Link-N (9-16), if generated has no interference on proteoglycan synthesis by bovine and human IVD cells, cells isolated from NP and AF/iAF regions of the IVD beaded in 1.2% alginate were exposed to 0.5µg/mL of Link-N (1-8), 0.5µg/mL of Link-N (9-16) and 1µg/mL of Link-N with control beads exposed to the medium alone. To rule out interference
by Link-N (9-16), if generated, on the activity of Link-N (1-8), the cells were also co-
exposed to 0.5µg/mL of Link-N (1-8) and 0.5µg/mL of Link-N (9-16). The potential
to stimulate proteoglycan synthesis was also compared to that of the parent 16 amino
acid Link-N. The exposure was for 48 hours and incorporation of $^{35}$SO$_4$ was used to
assess proteoglycan synthesis in response to the peptide exposures. The data was
normalized to $^{35}$SO$_4$ incorporation by controls set at 1 and expressed as a relative
ratio.

In bovine IVDs (n=3), both NP and AF cells showed an increase in $^{35}$SO$_4$
incorporation into newly synthesized PGs in response to Link-N (1-8) exposure (NP
p=0.014, AF p=0.014) compared to control exposed to medium alone. The
stimulation of PG synthesis was comparable to the parent 16 amino acid Link-N
peptide. There was no stimulation with exposure to Link-N (9-16) peptide and
incorporation levels were at baseline levels [Figure 49(A)].

In humans IVDs (n=3), both NP and iAF cells showed an increase in $^{35}$SO$_4$
incorporation into newly synthesized PGs in response to Link-N (1-8) exposure (NP
p=0.009, AF p=0.02) compared to control exposed to medium alone. The stimulation
of PG synthesis was comparable to the parent 16 amino acid Link-N peptide. There
was no stimulation with exposure to Link-N (9-16) peptide and incorporation levels
were at baseline levels. [Figure 49(B)].
Bovine and human IVD cells co-exposed to Link-N (1-8) and Link-N (9-16) showed sulphate incorporation comparable to Link-N (1-8) exposure alone, thus demonstrating that Link-N (9-16), if generated, has no interference or additional effect on activity of Link-N (1-8).

Figure 49: Evaluation of the effect of Link-N (1-8) on proteoglycan synthesis in bovine and human IV) cells in a non-inflammatory environment: (A) Bovine IVD cells exposed to Link-N (1-8) showed significant stimulation of PG synthesis
compared to controls exposed to medium alone set at 1 (dotted line). (B) Human IVD cells exposed to Link-N (1-8) showed significant stimulation of PG synthesis compared to control exposed to medium alone set at 1 (dotted line). The response was comparable to the exposure to 16 amino acid parent Link-N peptide. There was no stimulation with exposure to Link-N (9-16) peptide and incorporation levels were at baseline levels. Values of $p \leq 0.05$ were taken as significant.

4.4 Evaluation of biological activity of Link-N (1-8) peptide by assessing stimulation of proteoglycan production in human and bovine IVD cells exposed to the sequence in an inflammatory environment:

DDD is associated with elevated levels of cytokines and for a bioactive agent to have a beneficial effect; it should be able to trigger a response in cells exposed to such pro-inflammatory mediators such as cytokines. IL-1β is one of the most commonly implicated cytokines in DDD. To assess the effect of Link-N (1-8) and any possible effect of Link-N (9-16), if generated, on proteoglycan synthesis by bovine and human IVD cells in an inflammatory environment, cells isolated from NP and AF/iAF regions of the IVD beaded in 1.2% alginate were exposed to 0.5µg/mL of Link-N (1-8), 0.5µg/mL of Link-N (9-16) and 1µg/mL of Link-N with IL-1β (10ng/mL). Control beads exposed to IL-1β supplemented medium alone. To rule out interference by Link-N (9-16), if generated, on the activity of Link-N (1-8), the cells were also co-exposed to 0.5µg/mL of Link-N (1-8) and 0.5µg/mL of Link-N (9-16) in the presence of 10ng/mL IL-1β. The potential to stimulate proteoglycan synthesis was also
compared to that of parent 16 amino acid Link-N. The exposure was for 48 hours and incorporation of $^{35}$SO$_4$ was used to assess proteoglycan synthesis in response to Link-N exposure. The data was normalized to $^{35}$SO$_4$ incorporation by controls set at 1 and expressed as a relative ratio.

In bovine IVDs (n=3), both NP and AF cells showed an increase in $^{35}$SO$_4$ incorporation into newly synthesized PGs in response to Link-N (1-8) exposure (NP p=0.014, AF p=0.013) compared to control exposed to medium alone. The stimulation of PG synthesis was comparable to the parent 16 amino acid Link-N peptide exposure. There was no stimulation with exposure to Link-N (9-16) peptide and incorporation levels were at baseline levels [Figure 50(A)].

In humans IVDs (n=3), both NP and iAF cells showed an increase in $^{35}$SO$_4$ incorporation into newly synthesized PGs in response to Link-N (1-8) exposure (NP p=0.006, AF p=0.004) compared to control exposed to medium alone. The stimulation of PG synthesis was comparable to the parent 16 amino acid Link-N peptide. There was no stimulation with exposure to Link-N (9-16) peptide and incorporation levels were at baseline levels [Figure 50(B)].

Bovine and human IVD cells co-exposed to Link-N (1-8) and Link-N (9-16) showed sulphate incorporation comparable to Link-N (1-8) exposure alone, thus demonstrating that Link-N (9-16), if generated, has no interference or additional effect on the activity of Link-N (1-8).
Figure 50: Evaluation of effect of Link-N (1-8) on proteoglycan synthesis in bovine and human IVD cells in an inflammatory environment: (A) Bovine IVD cells exposed to Link-N (1-8) showed significant stimulation of PG synthesis compared to control exposed to medium alone set at 1 (dotted line). (B) Human IVD cells exposed to Link-N (1-8) showed significant stimulation of PG synthesis compared to controls exposed to IL-1β supplemented medium alone set at 1 (dotted line). The response was comparable to the exposure to 16 amino acid parent Link-N peptide. There was no stimulation with exposure to Link-N (9-16) peptide and
incorporation levels were at baseline levels. Values of $p \leq 0.05$ were taken as significant.
Chapter 4:
(Discussion)
The current study investigates the role of Link-N in regenerating or repairing IVD degeneration by stimulating proteoglycan synthesis in IVD cells and in an organ culture system. The described model for culturing human IVDs was developed to test the potential of Link-N in stimulating proteoglycan synthesis, which is the closest system we have so far to human in vivo conditions. The data presented in the result section has provided substantial information regarding the potential of Link-N to stimulate proteoglycan synthesis in an inflammatory and non-inflammatory environment both in cell cultures and in intact human IVDs. The presented data also warrants the need for discussion in the following six areas:-

- The advantages of the developed CEP model for human IVD culture over other organ culture systems and its limitations.

- Link-N as a therapeutic agent for regenerating IVD degeneration.

- Difference in response to Link-N by IVD cells reported by recent works and difference in response by NP and AF cells to Link-N exposure.

- Degradation of Link-N by human AF cells and discovery of minimum sequence which is still bioactive.

- The variation in response to Link-N by different human donors and selection of candidate cohort for Link-N therapy.

- The mode of delivery of Link-N to human IVDs in vivo and its clinical relevance.
1. Advantages of cartilaginous endplate (CEP) model of human IVD culture:

Whole organ IVD culture has long been an area of intense curiosity and research. The main issue with long term culture and maintenance of a viable cell population is providing nutrition to the center of disc. The density of IVD cells depends on the blood supply of the adjacent vertebral bone providing nutrition to the IVD through capillary network. The nutrition to the center of the IVD also depends on the height of the IVD with the center being the most nutrient deprived and hypoxic. The cell density does not vary much with the height of IVD but varies largely with the amount of nutrient supply. The larger IVDs have more vascularity supplying nutrients with more pores present in the bony endplates [99, 182, 183]. Different whole organ culture models have been in development adopting different approaches namely, without endplates [184], with retention of bony endplates [103, 120, 185-191] and with retention of cartilaginous endplates [101, 159, 192, 193]. The common denominator for all the organ culture models with retention of bony endplates has been the loss of IVD cell viability over short culture periods, even with heparinization of the animal before sacrifice to maintain patent unclotted vasculature of the vertebral body and the bony endplate. The culture model without endplates using an alginate culture system showed maintenance of high cell viability up to one month in culture but rapid release of produced ECM components into the surrounding medium [184]. Thus, no good culture system for IVDs, small or large, capable of sustaining high cell viability over prolonged culture periods was available.
The objective of this study was to develop an organ culture system using intact human IVDs, utilizing IVDs with different degrees of degeneration, which would be ideal for studying the potential of various bioactive agents to induce biologic repair. Such a system will also truly evaluate regeneration and repair in human IVD discs where the degeneration present is physiological, natural and occurs over a period of decades in comparison to one that is enzymatically induced [102] or induced by a stab injury with a needle [91, 191, 194-196] creating a non-physiological degeneration of IVD. The problem again with using such large human IVDs (average lateral diameter 7 cm, average antero-posterior diameter 4 cm) is adequate nutrient supply during culture to maintain high cell viability. The role of cartilaginous endplates in providing a selective barrier, whereby allowing free transport of nutrients but limiting the excessive swelling of IVD matrix exposed to medium was thus utilized.

Recently it has been shown that heparinization of the animals prior to sacrifice partly overcomes the problem of coagulation of vasculature and maintains a healthy IVD cell population for short periods of IVD culture with bony endplates [103] provided that the concentration of glucose in surrounding medium is more than 2.5g/L. The IVDs used in this thesis work were harvested from heparin treated donors but BEP method of isolating IVDs for culture failed to maintain cell viability past 7 days in culture even at 4.5g/L glucose. The BEP model would be ideal for studying IVDs under loads due to the presence of a flat loading surface but its use becomes restricted
to biomechanical or short term studies due to the failure to maintain a viable cell population.

For any repair response to be significant in IVD degeneration, the effect of a therapeutic agent has to be studied for prolonged periods as accumulation of the newly synthesized matrix components is essential to see any noticeable improvement of hallmarks of IVD degeneration such as increase in disc height. In comparison to the BEP method, the CEP model is able to maintain a high cell viability for periods of culture ranging from 7 days to 4 months primarily due to enhanced nutrition and limited swelling. The open capillary entrance ports are sometimes visible on cartilaginous endplates during drilling away of the bony endplates, confirming the interplay of the isolation technique with now patent routes of nutrition to the IVD.

Apart from maintaining high cell viability up, another advantage the CEP isolation method has over other isolation methods is the maintenance of such high cell viability even at near physiological nutritional states. The standard culture conditions used for IVD whole organ culture is 3.5-4.5g/L of glucose and 5% FCS. In human in vivo conditions, according to World Health Organization (WHO) definition of standard reference male, the blood glucose level should range from 0.8-1.2g/L. In terms of nutrition to IVDs through diffusion by capillary fluid via cartilaginous endplates, the center of the IVD has around 0.6-0.75g/L of glucose. The serum growth factors or regulatory proteins constitute <1% of the total serum proteins [197-199]. The serum growth factors should follow similar trends as glucose and thus the standard IVD
whole organ culture techniques are not physiological or near physiological and thus the response in the culture systems in use is not a true reflection of otherwise expected response in \textit{in vivo} conditions. \textit{In vitro} experiments performed using isolated IVD cells verify that they adapted very well to low glucose concentrations and that isolated IVD cells proliferate to a higher extent when cultured under very low glucose conditions (no glucose added to the culture medium, 0.07–0.15 g/L was calculated to be provided as part of the glucose in FCS) if the media was supplemented with FCS. When omitting FCS, either in the presence or absence of glucose, no cell proliferation was observed, and with absolutely no glucose and no FCS present the cells started to die within a few days in culture [200]. The nutritional conditions were thus gradually brought down to a near physiological state to 1g/L of glucose and 1\% FCS (medium LL), with an assumption that this culture condition will mimic human \textit{in vivo} condition. Any effect evaluated in this culture model under LL culture condition, such as response to injectable growth factor, will be more clinically relevant and results translated to human \textit{in vivo} conditions before undertaking clinical trials. The IVDs isolated with CEP method maintained high cell viability under LL culture conditions for a period of at least 4 months in culture, thus providing an ideal tool to study IVD physiology and pathophysiology.

Another major factor at play in IVDs isolated with CEP method is the potential of swelling. In evaluating NEP model against CEP model, the NEP system swelled to twice as much as CEP system in a very short time span. The CEP system on the other
hand swelled and equilibrated gradually and swelled to a maximum to 25% in some IVDs compared to up to 44% in NEP system. This gradual and lesser swelling also contributes to high cell viability seen in the CEP model due to more time for the IVD cells to get “acclimatized” to new osmolar conditions due to greater amounts of glucose available in culture. The gradual swelling also prevents sudden traction on the IVD cells anchored to other matrix components and leading to their rupture. The IVDs in vivo experience up to 25% increase in disc weight due to increased water content diurnally and height in an average human being increases by about 1.25 cm every night [201-203]. It was initially thought to occur due to relaxation of paraspinal muscles but has been demonstrated to happen due to imbibition of water by the IVDs due to unloading of the spinal column while sleeping. The same has been confirmed by anthropometry, CT-scan and MRI studies [204-207]. Thus the amount of maximum swelling experienced by the IVD cells in CEP system is what they experience diurnally in vivo and thus this amount of swelling should not have a detrimental effect on adaptation of cells in IVD in culture post retrieval from the body.

Another advantage of the CEP system over NEP system is the retention of shape and structure in culture even post swelling. Although some cells survive in NEP system, the IVDs undergo severe deformation which makes them unsuitable for studying repair. Some previous studies using NEP isolated discs have shown increased cell survival if the IVDs are compressed to limit swelling [184]. However, for this a
customized loading device or bioreactor is needed and the influence of load introduces another variable to the experiment. In addition, the porous platens of the loading device will have a direct contact with the gelatinous NP, which might lead the pores of the loading platen to become blocked with time. This method has been used for shorter culture periods, up to 1 week, which is sufficient for metabolic studies, but is not sufficient for significant changes in the ECM to take place in a repair model. Moreover evaluation of injectable therapeutic agents becomes almost impossible due to seepage from the site of injection into the surrounding medium even with applied loads. Only advantage perceived for the NEP system of culturing IVDs is conducting release studies where release of different cleaved ECM components in response to co culture with cytokines is undertaken as release of such degraded matrix components in a similar experiment with CEP culture system might not show a good release profile.

The IVDs isolated with CEP method can either be maintained in “limited swelling” without any external load or be maintained under external applied loads, both static and dynamic [101]. This increases the versatility of use of this organ culture system enabling to study and evaluate the effect of load under near physiological culture conditions.
Despite all the cited advantages CEP culture system has over other whole organ culture systems in use or development, this culture system has some limitations of its own, mainly technical. The foremost limitation is the procurement of human IVD tissue, which the majority of the spinal research groups have trouble with. All the major experiments were thus conducted in bovine IVDs too and were replicable to facilitate use of this method by other research groups where procurement of bovine tissue is the easiest and the most viable option. The bovine IVDs cultured with the CEP method failed to maintain high cell viability when cultured with 1g/L of glucose due to larger total cell number/disc and probably higher rate of metabolism (unpublished data). Another major limitation is the isolation method itself, which requires a fair bit of expertise and experience. Drilling to the right depth and extent is critical as too less drilling will lead to retention of bony endplates and closed cartilaginous endplate pores, thus compromising nutrition to the IVDs in culture. On the other hand, far too much drilling will lead to the cracking of the cartilaginous endplate and loss of the NP material from the crack site due to excessive swelling as in NEP discs. Also too much drilling will lead to cracking of IVDs in culture while they are swelling in an otherwise macroscopically normal pre cultured CEP IVD. Special care also has to be taken while drilling IVDs with higher grades of degeneration due to the presence of intradiscal osteophytes which pose additional problems while drilling and sometimes lead to over drilling in an attempt to get rid of calcification. The CEP model sometimes does not have a flat surface as BEP model for loading in a bioreactor for biomechanical studies and customized loading platens
have to be used in some situations. Nonetheless, high cell viability surpasses the flat surface advantage of BEP model in metabolic studies with bioreactor.

Thus, in summary, this novel method of isolation increases nutrient supply to the IVDs, thus allowing them to be cultured in near physiological states for prolonged periods.

2. Link-N as a therapeutic agent for treating IVD degeneration:
Link-N has been previously shown to act as a growth factor and stimulate the synthesis of proteoglycans and collagens in articular cartilage [208], bovine IVD cells in vitro [92, 93], as well as in a rabbit model of disc degeneration [91]. Link-N has recently been shown to interact with bone morphogenetic protein (BMP) type II receptor. The interaction results in the expression of BMP-4 and BMP-7 which through a cell-autonomous loop intensified Smad1/5 signaling though a feed-forward circuit involving BMP-RI [209].

In this thesis work, the potential of Link-N to stimulate PG synthesis was evaluated in 3D cultures of IVD cells and in the developed intact whole organ culture model for culturing human IVDs. Various studies have been done with IVD cells in monolayer and IVD cells expanded in monolayers and embedded in 3-D scaffolds thereafter [94, 95]. In this study, only primary (P0) IVD cells isolated from freshly harvested tissue were used. The isolated primary cells were then beaded in alginate without expansion in monolayers. This methodology preserves the phenotype of the isolated cells as the IVD cells cultured in monolayers lose their phenotype [53]. Moreover using IVD
cells past P₃₋₄ has the similar disadvantage of losing the phenotype and functionality of the IVD cells. Thus, evaluations and studies in primary IVD cells in 3D scaffolds are more physiologically relevant and translatable to clinical outcomes. A major limitation to this approach is the ready availability of human tissue in ample amounts to forgo the need for expanding them in monolayers to increase cell number.

The reason for conducting experiments in bovine and then in human IVD cells is that bovine IVDs are a good agreed upon replacement for human IVDs [210, 211] [188] due to similar size and morphology and absence of notochordal cells at and near skeletal maturity. IVDs from skeletally mature 18-24 months old steers correspond to 21-25 years of age in human years. The screening experiments were done in bovine to save on precious human tissue and for other groups with limited access to human tissue, to be able to replicate the experiments and further advance the studies. Previous work with Link- N in bovine IVD cells showed dose of maximal stimulation of PG synthesis to be at 0.1µg/L [93]. The dose response curve was run for bovine and human IVD cells from NP and AF/iAF embedded in alginate and the dose of maximal response for stimulation of PG synthesis was found to at 1µg/L. The possible reason for a 10 fold shift in dose of maximal response for both bovine and human IVDs cells could be that the previous study evaluated the response to Link-N in bovine IVD cells in pellet cultures which is a different culture system compared to alginate culture system. Also doses greater than 0.1µg/L were not evaluated.
To restore function in a degenerated disc, restoration of PG content is the main strategy. The principal site of PG production in a young developing IVD is the NP. With progressing age and falling cell number in NP, the PG production is partly shifted to AF mainly the iAF. The matrix composition in NP, iAF and oAF regions begin to change seen with changing GAG/hydroxyproline ratio with age [68]. Thus, if a therapeutic agent can stimulate both NP and AF cells, it will lead to an increased production of PG all across the IVD and restoration of IVD function. Though, the structure of the IVD might not be restored but that is of less importance if the newly synthesized matrix is biochemically stable and the IVD retains/regains its biomechanical stability. Link-N has shown to stimulate the synthesis of other ECM components namely collagens [91, 92, 212] in NP and AF cells and its ability to stimulate PG production in these cell types makes it an ideal therapeutic agent warranting further investigation into its function in more complex culture systems such as whole organ culture systems for IVDs.

Aggrecan is the major PG responsible for maintaining disc height due to its water retention capacity owing to the presence of a high density of negatively charged sulphate groups. Thus restoration of PGs especially aggrecan will restore disc height and possibly reverse disc degeneration. Stimulation of production of new proteoglycans under the influence of Link-N was studied by evaluating $^{35}$SO$_4$ incorporation. The radioactive sulphate from the culture medium or injection concoction can be incorporated into other sulphated PGs other than aggrecan,
including biglycan, decorin and versican. Such PGs may be present in similar (biglycan and decorin) or lower (versican) molar concentrations when compared to aggrecan. However, aggrecan has many more sulphated GAG chains than other PGs and hence is the major site for sulphate incorporation. Thus, the increase in incorporation of radioactive sulphate under the influence of Link-N is due the utilization of the same in newly synthesized aggrecan.

IVD degeneration in vivo is a process that results from years of increased catabolism of the IVD matrix involving various cytokines which upregulate proteases [213-216]. If a bioactive agent is to reverse and/or retard the degenerative process in the IVD, it should be able to exert its anabolic effect in this cytokine rich inflammatory milieu. Link-N stimulated proteoglycan synthesis in the presence of inflammatory cytokines, thus showing the potential for regeneration in ongoing IVD matrix degeneration. Link-N, at the dose of maximal response induced an upregulation of aggrecan in both NP and AF cells. In NP cells, Link-N increased gene expression for aggrecan and a downregulation of the expression levels of the proteases namely MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 in the presence of IL-1β. In AF cells, Link-N showed upregulation of aggrecan along with MMP-3 and ADAMTS-5 gene expression in the absence or presence of IL-1β. It is commonly believed that remodelling of the newly laid down connective tissue matrix such as healing fractured bones, scar tissue in skin etc. is crucial to generating a functional matrix. The same should hold true for newly laid down IVD matrix. This remodelling involves upregulation of various proteases.
It will only be clear with time whether the upregulation of MMP-3 and ADAMTS-5 observed here is due to remodelling.

Degenerative disc disease is associated with calcification of endplates and compromised nutrition and circulation and injecting growth factors or growth factor like agents such as Link-N would be an easy delivery modality. The dose of Link-N to be injected in the whole organ culture disc was taken 20 times more than the dose of maximal response determined in 3D cultures of disc cells. The average weight of the disc was around 22 grams and 20 mL of media was used around the disc to minimize culture volume. The injected 1 mg/disc of Link-N thus distributed in 42 mL media on an average. An assumption was made and 5% of the injected was thought to be available to disc cells owing to the tissue MMPs and other proteases present in the intact disc. If these assumptions are correct, the dose available to disc cells in intact disc organ culture was calculated to be approximately 1μg/mL. Intact discs also were injected with 0.1 and 0.5 mg/disc dosage of Link-N but 1 mg/disc was found to be the dose with best response. This dose might not be the dose of maximal response for whole organ system, but was not detrimental to the cells as the cell viability in both control and Link-N injected discs was maintained at >96%.

Fluorescently tagged Link-N was injected in the human CEP IVDs to evaluate its diffusion out of the IVD and its distribution within the IVD. Rapid outward diffusion of the injected Link-N would lead to Link-N being injected repeatedly to maintain optimal dosage, which might not be a feasible option in an outpatient clinical setting.
owing to the invasive nature of the injection in living subjects. A low clearance rate out of the injection site is one way that multiple injections can be avoided. Link-N diffuses out slowly in the injected CEP IVDs through the endplates but this in an *in vivo* setting should not pose a problem as the IVDs in a living human being is not as open a system as CEP IVDs. This will lead to even slower diffusion out, if any, and the IVD cells will be exposed to injected Link-N peptide and it can exert its effect for a longer duration of time. The only issue could be the seepage through the injection tract but that can be overcome by using sealing techniques used in nucleoplasty with hydrogel insertion in NP. The tagged Link-N distributed within the IVD reaching the iAF/oAF junction and was restricted further by oAF fibres providing the PG synthesizing regions of the IVD access to the injected Link-N. The retention of Link-N within the IVD possibly due to interaction with the ECM, causing it to be released slowly to the cells is one probable explanation for the sustained effect of Link-N on stimulation of PG synthesis in the injected IVDs. Another possibility is that Link-N may have a long half-life within the tissue. Nevertheless, it provides a clinical advantage of not requiring repeated injections of Link-N *in vivo*.

Previous studies have shown many recombinant growth factors such as GDF-5, OP-1, and IGF-1 to increase proteoglycan synthesis by rabbit and bovine disc cells [217]. OP-1 was injected in rabbit discs after inducing degeneration with chondroitinase ABC and showed restoration of disc height [218]. Similarly Link-N injection in needle stick induced degeneration showed increase in disc height on MRI within 3 months post Link-N injection [91]. In another study, TGF-β was shown to rescue the
decrease in proteoglycan production by IL-1β in cartilage explants, but interestingly co-injection of TGF-β and IL-1β was not able to show a rescue effect [219]. Similarly induction of proteoglycan synthesis under the influence of TGF-β was shown in intact disc culture model of IVD [101]. In these studies, tissues from younger animals were used and degeneration induced either enzymatically or mechanically with a needle injury. The present study is first of its kind to show that Link-N can induce proteoglycan synthesis in human disc cells in an inflammatory milieu. This establishes the potential of Link-N to be active when injected into human discs in vivo.

3. Differences in response to Link-N by IVD cells reported by other recent studies and difference in response by NP and AF cells:

The current work draws the need to discuss recent publications evaluating the potential of Link-N to stimulate PG synthesis by IVD cells. The recent work on the potential of Link-N include works by Abbot et al [220] and Wang et al [94]. In the present study human IVD cells in their 3D microenvironment, under the influence of Link-N, showed an increase in aggrecan synthesis and downregulation of proteases by NP cells. In contrast the recent work by Abbott et al [220] evaluating the effect of Link-N, CTGF and TGF-β + Dex on human IVD cells in alginate, did not show much upregulation of aggrecan by IVD cells exposed to Link-N compared to other groups. Furthermore, MMP-3 was upregulated whereas in this work, there was a downregulation of the MMP-3. This difference could be attributed to the source of tissue, which in their case was from moderate to severely degenerated surgically removed discs where it is difficult to separate the different regions of discs due to loss
of gross morphology. This leads to isolation of a mixed population of IVD cells that is then cultured. Also, in contrast to our study, Abbott et al expanded the cells to increase cell numbers before embedding them in 3-D scaffolds, which could influence the phenotype of cells [53]. Importantly, the dose of Link-N used was 10 times less than the dose of maximal response reported by us for human IVD cells. Another recent study by Wang et al [94] evaluated the effects of Link-N on human NP cells from surgical donors in producing ECM components at the gene expression and protein translation levels and showed increased production of aggrecan and collagen type II but no effect on protease production. This study again used expanded IVD cells and the dose of maximal response was 5 times less than the one reported by us. There was a trend towards increase in ECM protein production with increasing doses, but doses greater than 200ng/mL were neither evaluated nor used, which might explain protease production remaining unaffected by Link-N contrary to results shown by us. In this study, even though the increase in aggrecan gene expression and proteoglycan synthesis may be small in magnitude, but shows the pro-anabolic potential of Link-N and the PG accumulated over an extended time following Link-N administration, may restore the function of the IVD.

In our work, even though high PG content in the disc is commonly associated with NP, AF cells responded better than NP cells to Link-N at all the tested doses in promoting PG synthesis. Other studies have previously reported the ability of AF cells to respond more strongly than NP cells in stimulating PG synthesis with other growth factors [221]. In earlier work with bovine IVD cells, it was reported that AF
cells were capable of producing more PG than NP cells when stimulated with TGF-β, another commonly investigated growth factor in IVD metabolism [222]. However, other studies with bovine cells reported that NP and AF cells were capable of responding in a similar manner when cultured on 3D polyester mesh [223]. In young human discs, NP is the main source of PG, but with age and degeneration there is an increased PG content in the AF [224] and much of the PG needed to maintain disc function is presumably synthesized by the AF cells. Thus, it is not surprising that PG synthesis can be strongly stimulated in the AF cells from adults. This can lead to an altered therapeutic strategy for repairing degenerating IVDs by inducing annular repair.

4. Degradation of Link-N by human AF cells and discovery of minimum sequence which is still bioactive:

The present work demonstrates that AF cells have the ability to proteolytically process the Link-N peptide resulting in a fragment spanning 1-8 amino acid residues. The generated peptide is biologically and is able to increase PG synthesis in both NP and AF cells in both a non-inflamatory and an inflammatory milieu.

In a recent study by Wang et al, the stimulatory effect of Link-N was lost when they evaluated a number of shorter truncated Link-N peptides [94]. In that study, one of the evaluated peptides truncated at residues 1-12, was also found to be inactive. In
contrast, our study demonstrated that residues 1-8 of Link-N were active. The loss of effect in their system could be due to the different concentrations and different time frames that were evaluated. Wang et al used 200ng/mL of the different peptides independent of size and exposed their cultures for a period of 21 days. In this work, 1μg/mL of Link-N 1-16 and 0.5μg/mL of the shorter peptides [(1-8) and (9-16)] were evaluated in order to have equimolar concentrations and treated our cultures for a period of 48 hours. As pointed out in earlier sections, the cell source used by Wang et al was from degenerated disc tissue extracted during surgery for spinal fusion, and the IVD cells were isolated and expanded in monolayer cultures before embedding them into 3D scaffolds, a procedure that may have altered the phenotype and therefore the response by the IVD cells. The IVD cells used in this thesis work, as mentioned before, were isolated from IVD donors with only mild degeneration, and to preserve the phenotype, the cells were not expanded in monolayer and used as freshly isolated P₀ cells embedded directly in alginate.

Disc degeneration in vivo is strongly associated with increased catabolism in the disc matrix. This is a process lasting over decades, involving an upregulation of various cytokines and proteases [225, 226]. Current thesis work indicates that although Link-N is processed by AF cells, a minimal active sequence remains intact. The current data suggest that Link-N is being degraded by a protease that cleaves within the C-terminal half of the peptide to leave Link-N 1-8 as an end product. At present it is not clear whether such cleavage occurs in a sequential manner from the C-terminus, as
might be expected for a peptidyl dipeptidase such as cathepsin B, or in a more random manner. The most probable protease involved in this could be cathepsins as this family of proteases is unregulated in DDD [85, 227, 228]. MMPs seem to be unlikely culprit as there is no cleavage site on Link-N for MMP to act [85, 229]. Out of the Cathepsin family, namely cathepsin L, H, B and X, Cathepsin B could be the cleaving enzyme in question as out of all the cathepsins, cathepsin B has the binding site to cleave from the C-terminal, as suggested by our data. But Cathepsin B was ruled out as the enzyme in question as 50ng/mL and 100ng/mL of Cathepsin B cultured with Link-N failed to cleave it and generate the discovered sequence in a preliminary experiment. Thus the hunt for the enzyme generating the sequence continues and will form one of the main directives for the work in future.

Nonetheless, Link-N and the generated fragment were still able to stimulate PG synthesis in the presence of inflammatory cytokines, thereby demonstrating the potential to regenerate the disc tissue during ongoing matrix degeneration. Taken together, this makes Link-N (1-8) a promising bioactive substance for the treatment of degenerative disc disease. This could also explain the sustained effect of Link-N in stimulating PG synthesis in CEP IVDs, and when in future administered in humans in vivo, as even after being metabolized, Link-N continues to exert its action through its daughter fragment, Link-N (1-8).
5. Variation in response to Link-N by different human donors and selection of candidate cohort for Link-N therapy:

Current research and advances in the field of spinal research are aimed at finding clinical solutions for DDD. The evaluation of various bioactive agents, development of therapies, therapeutic intervention analysis etc are all conducted in cells derived from animals and in animal models to keep the experimental setup simple and avoid high variations in experimental outcomes. This approach is very common and ideal for screening and discovery of novel therapeutic agents. After success in animal models, the ultimate goal of every research is to have a clinical translation.

In this work, a fair degree of variability was observed in stimulation of proteoglycan by Link-N in human tissue which was not only dependent on the cell type, but also on individual donors and on the disc level within the same donor. It is not clear whether the observation of variability in proteoglycan stimulation by Link-N is a universal phenomenon with all growth factors. Various factors can influence the ability of Link-N to stimulate matrix repair namely differences in age and gender, as well as nutritional status, size, cell density and grade of degeneration, which vary within among donors and even the same individual. These factors may influence patient selection for Link-N therapy for degenerative disc disease.

The hypothesis behind Link-N therapy is that during the degenerative process, there is up regulation of proteases mainly MMPs and ADAMTSs and these proteases, in
addition to cleaving the PGs and other ECM components, also cleave Link protein which generates the 16 amino acid N terminal peptide called Link-N. This peptide then gets accumulated in the degenerating discs and starts to exert its stimulatory effect on IVD cells to increase PG content of the IVD and rescue the IVD from further degeneration. Thus, Link-N peptide might work through a feedback loop mechanism with degeneration producing Link-N and after a critical conc., Link-N retarding or reversing the degenerative process. This probable mechanism surely needs a viable cell population to be able to exert its effect and therefore IVDs with higher grades of degeneration are not suited for Link-N therapy due to depleted cell population.

In this work, we observed variation in response to Link-N by human IVD cells from different donors but invariably a response was seen with every donor. In this study, IVDs having grade 2 and 3 degeneration were used as in clinical practice, this would be the target grade of degeneration where repair or regeneration or at least retardation of degenerative process can be attempted due to still viable cell population. Thus, higher grades of degeneration would not be suitable for Link-N therapy. To investigate whether IVDs with no degeneration can still be stimulated by Link-N, IVD cells from a very young donor aged 4 months were exposed to Link-N and the response was only seen at the dose of maximal response to Link-N, whereas other doses failed to trigger any response. Although the response was statistically significant, but compared to other donors, it was still on the lower side. This can be possibly explained that the IVD cells from a young donor are in an already high ECM
turnover state and the effect of Link-N gets masked. Also the IVD cells from a young donor are not exposed to inflammatory cytokines and degradation products and may not follow the proposed pathway of Link-N stimulation. Thus, cells from a young donor behave differently from cells from degenerating IVDs and makes target age and degeneration grade critical for Link-N therapy.

The IVDs prepared with CEP method showed a variation in response to Link-N injection, in the IVDs from different donors (interspinal variation) and in the IVDs from the same donor (intraspinal variation). While preparing discs for organ cultures, varying degrees of both degeneration and calcification within discs from the same individual was observed. This could be attributed to various systemic factors implicated in disc degeneration. Intraspinal variation indicates that every disc behaves as a separate entity and thus disc selection within the same individual is also crucial in having desired optimal effect following Link-N treatment. Clinically, this translates to that not every donor and not every IVD within the same donor will respond to Link-N therapy to the same extent.

The problem then becomes which patients to choose for Link-N therapy and how to predict among the selected patients, who will respond to Link-N therapy and to what extent. Patients with higher grades of degeneration and neuromuscular sequelae should definitely be ruled out. The grade 2 and 3 patients with painful IVDs should be the target cohort.
Link-N can also play a significant role in higher grades of degeneration where it can be used post-surgery to delay adjacent level disc disease. Link-N might still be useful in individuals with these higher grades of degeneration, where it could be used to delay adjacent level disc disease after fusion [117, 118, 230, 231]. For higher grades of degeneration, Link-N might be injected along with stem cells to attempt regeneration.

6. Possible modes of delivery of Link-N to human IVDs in vivo and its clinical relevance:

One of the major areas of discussion at this stage is the mode of delivery of Link-N to target IVDs in vivo. The commonest route of delivery for any therapeutic agent if the chemical properties permit, is par oral or vial oral route in form of tablets, capsules or syrups, in single or divided doses. This route provides best patient compliance, lesser side effects and toxicity. This route of administration for Link-N might not be feasible as even after absorption through the lining of the gastrointestinal tract, the delivery to the target tissue will be a cause of concern due to the avascular nature of IVDs and presence of calcified endplates associated with DDD, which further limits the access to the degenerated IVD. Moreover, even if it gets absorbed, the systemic side effects of Link-N are not fully understood and thus safety spectrum of the peptide will be a concern with regulatory authorities.
To bypass systemic administration, targeted delivery methods are the next best option. Injecting Link-N directly into the IVD through postero-lateral approach via fine spinal puncture needles is one such option. The needle tract will be narrow with this approach and the needle tract can be sealed with tissue glue used in nucleoplasty with hydrogel insertion. The NP region can be injected and the delivered solution will distribute itself up to iAF region stimulating both the regions. The need for repeated injections can be bypassed if a sustained release or depot preparation of Link-N can be formulated with the help of medicinal chemists. The concern with this method of delivery will be firstly, repeated injections of Link-N to see significant benefit and secondly, accidental seepage of Link-N into cerebrospinal fluid surrounding the spinal cord. Repeated injections of Link-N to maintain optimal conc. of Link-N at target site will reduce patient compliance and may increase the problem due to masking of the beneficial effect of Link-N injection due to upregulation of proteases induced by the needle stab itself [191].

Another targeted delivery approach could be using intravenous contrast dyes used in discography such as gadolinium [232-236] which have an affinity for tissues like IVD and cartilage. Injecting gadolinium conjugated Link-N will direct Link-N to IVD in a targeted manner. The problem with this approach is that gadolinium also has affinity for cartilage; hence the amount of Link-N injected will have to be more than the calculated amount to compensate for losses to cartilage tissue. Moreover gadolinium injection has its own safety issues and is known to have systemic side effects including headache, nausea, vomiting and more fatally nephrogenic systemic fibrosis.
This would restrict the amount of gadolinium that can be injected and also narrow down the patient selection base as many people past age 60, the commonest age group for DDD, have associated compromised renal function.

Another suggestion that has come up during conversations and discussions with spine surgeons if the possibility of injecting Link-N in the vertebral bone close to the endplate and have few small pores with a micro drill in the cartilaginous endplate through vertebral approach to facilitate delivery of Link-N through calcified endplates. This approach though requires a high level of training and is highly invasive.

In view of the discovery of biologically active cleavage product of Link-N, newer approaches for drug development and delivery can now be explored. This shorter fragment can be dimerized and when administered, can provide twice the amount of peptide that can be designed to be a sustained release preparation. Another drug delivery method with high potential is dendrimer technology [237-239] which can be used to formulate a sustained release or a controlled release preparation with a gadolinium moiety to have a targeted delivery with a sustained release. Since the Link-N fragment is not open access and is in the process of intellectual property protection, it has the potential to attract collaborations from pharmaceutical industry for further development.
In conclusion, delivery of Link-N to the IVDs is an area which needs further development and once a safe delivery platform has been devised, it will make Link-N one of the major medical therapies to treat DDD and significantly cut down healthcare costs.

7. Conclusions:
In conclusion, Link-N is a promising agent for biological repair of degenerated human IVDs but its success in repairing IVD degeneration will largely depend upon the patient selection. In the future, an alternative therapeutic delivery system for Link-N which would avoid the need for injection would greatly enhance its acceptance as a valid therapeutic option. With the potential of Link-N in stimulating matrix synthesis and development of an alternate delivery system, this peptide could be taken to the stage of clinical human trials and be used as a therapeutic agent for treating DDD. Furthermore, Link-N has a significant cost advantage over other tested recombinant growth factors. Based on prior in vivo studies in the rabbit, Link-N is over 100 times less expensive than recombinant growth factors that have a similar repair response.
Chapter 5:

(Contributions to Knowledge)
The experiments presented in this thesis describe the stimulatory effect of Link-N peptide on bovine and human IVD cells in producing PGs, restoration of which might be able to regenerate degenerated IVDs in DDD. The specificity of response to Link-N by IVD cells was demonstrated along with its effect on IVD cells cultured in an inflammatory environment. A whole organ culture model was developed to culture human IVDs for prolonged durations of time. The potential of Link-N to stimulate synthesis of PGs in this whole organ culture was also demonstrated. Furthermore, the cleavage of Link-N by human AF cells was evaluated and production of a new bioactive peptide was reported.

**The major original findings of the study are:-**

- Dose of optimal response to Link-N in IVD cells cultured in 3D culture system.

- Sequence specific response to Link-N.

- Stimulation of PG synthesis in response to Link-N exposure in IVD cells cultured in an inflammatory environment.

- Developed and validated a long-term organ culture system for intact human intervertebral discs, in which the potential for biologic repair of disc degeneration can be studied.
• Stimulation of PG production in response to Link-N injection in human whole organ IVD culture system.

• Interaction of injected Link-N with IVD matrix and sustained PG synthesis in response to Link-N injection.

• Cleavage of Link-N by human iAF cells producing a new peptide as a cleavage by product.

• Demonstration of ability of generated new peptide to stimulate PG synthesis in both a non-inflammatory and inflammatory environment.

This study is the first of its kind developing an whole organ culture model for human IVDs, which is the closest we have till date to the human in vivo situation. The model allows for prolonged culture periods of up to 4 months, a feat that has never been achieved before. The study also tests the potential of Link-N peptide in the developed model. This brings us one step closer to taking Link-N peptide to clinical trials. Finally, the discovery of a biologically active product of Link-N generated by human iAF cells will pave way in future for drug discovery and efficient drug delivery techniques.

**Suggestions for future work include:-**

• Evaluation of potential of Link-N injection in human IVDs cultured under load using a bioreactor.
• Identification of the enzyme(s) causing cleavage of Link-N to generate a bioactive peptide Link-N (1-8).

• Evaluation of the potential to stimulate PG synthesis with Link-N (1-8) injection in whole organ culture model for human IVDs.

• Identification of receptor(s) for Link-N and deciphering the molecular pathway of action of Link-N in stimulating PG synthesis.

• Development of delivery vehicles for Link-N circumventing the need for injections. The discovery of shorter bioactive product of Link-N can potentially develop and utilize dendrimer technology for its delivery.

List of publications, intellectual property rights and presentations:

A. Publications:


2. Gawri, R; Antoniou, J; Ouellet, J; Awwad, W; Steffen, T; Roughley, PJ; Haglund, L; Mwale, F. Best Paper NASS 2013: Link-N can stimulate proteoglycan synthesis in the degenerated human intervertebral discs. (Accepted, eCM Journal, in press, August 2013).

185
3. **Gawri, R; Önnerfjord, P; Antoniou, J; Ouellet, J; Roughley, PJ; Heinegard, D; Mwale, F; Haglund, L.** Link-N is cleaved by annulus fibrosus cells generating a product with retained biological activity. (Manuscript submitted, eCM Journal, August 2013).

**B. Intellectual property:**

Report of invention (ROI) filed with McGill University titled (as equal co-inventor with 20% inventorship share)

“IVT-2: a Novel Therapeutic Agent for Cartilage and Intervertebral Disc Repair”

Co-inventors: Rahul Gawri, Fackson Mwale, John Antoniou, Peter Roughley, Lisbet Haglund.

**C. Presentations at scientific conferences:**


Possible. 46th Annual Meeting of Canadian Orthopaedic Association (COA) and Canadian Orthopaedics Research Society (CORS), Ottawa, Canada, 2012.


18. R Gawri, F Mwale, J Ouellet, T Steffen, P Roughley, J Antoniou, L Haglund. Long Term Human Intervertebral Disc Organ Culture: From Impossible to I’m Possible. 45th Annual Meeting of Canadian Orthopaedic Association


191

Long Term Human Intervertebral Disc Organ Culture: From Myth to Reality.
7th Combined Meetings of Orthopaedics Research Societies (CORS), Kyoto, Japan, 2010,


Development of a Whole Disc Organ Culture System to Study Human Intervertebral Disc Metabolism. 2nd World Forum for Spine Research (WFSR), Montreal, Canada, 2010.


Whole IVD organ culture systems to study the potential of biological repair.


A Whole Organ Culture System for Human Intervertebral Disc and the Effect
of Link-N on Disc Repair. 16th Canadian Connective Tissue Conference (CCTC), Toronto, Canada, 2010.

Reference List: -


225. Millward-Sadler, S.J., et al., *Regulation of catabolic gene expression in normal and degenerate human intervertebral disc cells: implications for the


Appendix

(Containing permission from copyright holders of figures used in the thesis, ethics approval from local review board, questionnaires and forms used).
Appendix 1(A): Questionnaire to evaluate whether the harvested spines had associated pain symptoms (English version).
Appendix 1(B): Questionnaire to evaluate whether the harvested spines had associated pain symptoms (French version).
Appendix 2: Harvest form citing inclusion criteria for the harvested spine

***ATTENTION HARVESTER ***PLEASE FILL OUT THIS FORM

Transplant Quebec Donor #: ______________________________

Inclusion Criteria
18-65 years of age □ Male □ Female □

Exclusion Criteria
HIV or Hepatitis □
Malignant Tumors □
Spinal Deformity □
History of moderate to severe chronic back pain □

Deceased DOB: / / DOD: / / Ht: Wt: 

Known medical history/C.O.D:
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Date of Harvest: __________________ Location: ______________
Time of Harvest: __________________
Time of Storage: __________________ fridge □ Freezer □

AFFIX TISSUE SAMPLE IDENTIFIER HERE AND ON TISSUE SAMPLE PACKAGE
See labels package in Manual
Appendix 3: Permission from copyright holder of figures used in the thesis

This is a License Agreement between Rahul Gawri ("You") and Wolters Kluwer Health ("Wolters Kluwer Health") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Wolters Kluwer Health, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Terms and Conditions

1. A credit line will be prominently placed and include: for books - the author(s), title of book, editor, copyright holder, year of publication; For journals - the author(s), title of article, title of journal, volume number, issue number and inclusive pages.
2. The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or authors of the material, or to Wolters Kluwer.
3. Permission is granted for a one-time use only within 12 months from the date of this invoice. Rights herein do not apply to future reproductions, editions, revisions, or other derivative works. Once the 12-month term has expired, permission to renew must be submitted in

https://r100.copyright.com/RpsDispatchServlet

1/3
writing.

4. Permission granted is non-exclusive, and is valid throughout the world in the English language and the languages specified in your original request.

5. Wolters Kluwer cannot supply the requestor with the original artwork or a "clean copy."

6. The requestor agrees to secure written permission from the author (for book material only).


8. If you opt not to use the material requested above, please notify Rightslink within 90 days of the original invoice date.

9. Please note that articles in the ahead-of-print stage of publication can be cited and the content may be re-used by including the date of access and the unique DOI number. Any final changes in manuscripts will be made at the time of print publication and will be reflected in the final electronic version of the issue.

Disclaimer: Articles appearing in the Published Ahead-of-Print section have been peer-reviewed and accepted for publication in the relevant journal and posted online before print publication. Articles appearing as publish ahead-of-print may contain statements, opinions, and information that have errors in facts, figures, or interpretation. Accordingly, Lippincott Williams & Wilkins, the editors and authors and their respective employees are not responsible or liable for the use of any such inaccurate or misleading data, opinion or information contained in the articles in this section.

10. This permission does not apply to images that are credited to publications other than Wolters Kluwer journals. For images credited to non-Wolters Kluwer journal publications, you will need to obtain permission from the journal referenced in the figure or table legend or credit line before making any use of the image(s) or table(s).

11. The following statement needs to be added when reprinting the material in Open Access publications: "promotional and commercial use of the material in print, digital or mobile device format is prohibited without the permission from the publisher Lippincott Williams & Wilkins. Please contact journalpermissions@lww.com for further information.

12. In cases of Disease Colon Rectum, Plastic Reconstructive Surgery, The Green Journal, Critical Care Medicine, Pediatric Critical Care Medicine, the American Heart Publications, the American Academy of Neurology the following guideline applies: no drug brand trade name or logo can be included in the same page as the material re-used.

13. When requesting a permission to translate a full text article, Wolters Kluwer Lippincott Williams & Wilkins requests to receive the pdf of the translated document.

14. Other Terms and Conditions:

v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RUN:031025:201. Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 021
P.O. Box 8433006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support:
https://s100.copyright.com/DisPatchServlet

229