Developing an *In vitro* Model of the OAF-CEP Interface: Towards Tissue Engineering the Intervertebral Disc

by

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Institute of Biomaterials and Biomedical Engineering
In the University of Toronto

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Masters of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto
2015

**Abstract**

Bioengineering a biologically relevant interface is an essential element toward developing an intervertebral disc (IVD) replacement for the treatment of low back pain. To this end, this study further characterized the native outer annulus fibrosus (OAF)/cartilage endplate (CEP) interface of adolescent bovine and fetal human disc by immunohistochemical and mechanical methods. Secondly, an *in vitro* model of the interface was developed between *in vitro*-formed cartilage tissue and a trilamellar AF tissue formed using OAF cells and polycarbonate urethane (PU) scaffolds. The native bovine OAF-CEP interface showed mechanical stability up to peak forces of $2.5 \pm 0.8$ MPa. The *in vitro* generated tissues integrated themselves to form an AF-cartilage interface with some characteristics that mimic native fetal and adolescent bovine interface. These data convey that tissue engineered disc replacements have the potential to integrate following implantation and this model is anticipated to be valuable to investigate conditions promoting *in vivo* integration.
Acknowledgements

I would like to thank my supervisors Dr. Rita Kandel and Professor Paul Santerre who have prepared me to excel as a young investigator and clinician. My committee members Prof. Mark Grynpas, Prof. Eli Sone and assistant Prof. Tom Willet have been invaluable and I thank them for their generous guidance.

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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ADO</td>
<td>anionic dihydroxyl oligomer</td>
</tr>
<tr>
<td>AF</td>
<td>annulus fibrosus</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ALPa</td>
<td>alkaline phosphatase activity</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARD</td>
<td>artificial disc replacement</td>
</tr>
<tr>
<td>β-GP</td>
<td>beta-glycerophosphate</td>
</tr>
<tr>
<td>BEP</td>
<td>boney endplate</td>
</tr>
<tr>
<td>CEP</td>
<td>cartilage endplate</td>
</tr>
<tr>
<td>COL I</td>
<td>type I collagen</td>
</tr>
<tr>
<td>COL II</td>
<td>type II collagen</td>
</tr>
<tr>
<td>COL III</td>
<td>type III collagen</td>
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<tr>
<td>COL IV</td>
<td>type IV collagen</td>
</tr>
<tr>
<td>COL V</td>
<td>type V collagen</td>
</tr>
<tr>
<td>COL VI</td>
<td>type VI collagen</td>
</tr>
<tr>
<td>COL IX</td>
<td>type IX collagen</td>
</tr>
<tr>
<td>COL X</td>
<td>type X collagen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DDD</td>
<td>degenerative disc disease</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fn</td>
<td>fibronectin</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>IAF</td>
<td>inner annulus fibrosus</td>
</tr>
<tr>
<td>IVD</td>
<td>intervertebral disc</td>
</tr>
<tr>
<td>LBP</td>
<td>low back pain</td>
</tr>
<tr>
<td>NP</td>
<td>nucleus pulposus</td>
</tr>
<tr>
<td>NP-CEP</td>
<td>nucleus pulposus cartilage endplate</td>
</tr>
<tr>
<td>OAF</td>
<td>outer annulus fibrosus</td>
</tr>
<tr>
<td>OAF-CEP</td>
<td>outer annulus fibrosus- cartilage endplate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature formulation</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PU</td>
<td>polycarbonate urethane</td>
</tr>
<tr>
<td>RNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SAED</td>
<td>selected area electron diffraction</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>VB</td>
<td>vertebral body</td>
</tr>
</tbody>
</table>
Chapter 1: Literature Review

1.0 Intervertebral Disc Degeneration and Treatment

1.1.1 Intervertebral Disc and Back Pain

Low back pain (LBP) is an important health concern. Direct and indirect healthcare costs are estimated at over 118 billion dollars each year in the US (Katz et al., 2006). It has an estimated prevalence of 80% and can be associated with degeneration of the intervertebral disc (IVD; Walker et al., 2000; Freemont et al., 2009). IVD degeneration or degenerative disc disease (DDD), affects 45% of the population by 40 years of age, rising to 96% by the age of 50 (Cheung et al., 2009). Disc-related LBP has a chronic and persistent natural history, with pain and disability not improving over time in most patients (Peng et al., 2012).

It appears DDD is an irreversible process, in part associated with aging, characterized as the structural failure of the IVD including annular fissures, loss and disorganization of nucleus pulposus tissue and microfracture of the cartilage endplate (Guiot and Fessler 2000; Adams and Roughley 2006; Rutges et al., 2013;Wang et al., 2014). Patients with moderate levels of disc degeneration are also at an increased risk for recurrent disc herniations and protrusion of the nucleus pulposus into the outer layers of the annulus fibrosus, following discectomy (Kim et al., 2014). While the cause(s) of DDD has not been fully elucidated, genetics (Battie et al., 2006), loss of notochordal cells (Roughley et al., 2004), mechanical stress (Stefanakis et al., 2014) and calcification of the cartilage endplate (Roberts 1996; Kang et al., 2014) have all been implicated in the pathogenesis.
1.1.2 Current Treatments

A multitude of interventions (pharmacological, manual therapy and surgical) are used to manage chronic LBP (Peng et al., 2012). Increased utilization rates for interventional therapies, such as epidural steroid injection, facet joint injections and surgeries, have not been associated with improved health status among patients with LBP (Chou et al., 2009a). Furthermore, none of the treatments currently available restore the structure or biomechanical function of the IVD. There is evidence demonstrating favorable effectiveness of non-steroidal anti-inflammatories (NSAIDs) in the treatment of chronic LBP, with topical NSAIDs associated with less risk for gastrointestinal, cardiovascular and renal toxicity (Rainsford et al., 2008; White et al., 2011; Klinge and Swayer 2013). For chronic LBP patients who do not improve with pharmacologic therapy alone, intensive interdisciplinary rehabilitation including spinal manipulation, acupuncture, massage therapy and exercise is recommended (Chou et al., 2007).

When unresponsive to conservative care, disc-related LBP can be managed by local injections of steroids or anesthetics. However, a systematic review of mainly fluoroscopically-guided epidural injection, found good evidence for radiculitis secondary to disc herniation with local anesthetics and steroids but only fair for radiculitis secondary to spinal stenosis or axial pain without disc herniation (Benyamin et al., 2012). Percutaneous treatments that alter the internal mechanics or innervation of the IVD (intradiscal electrothermal annuloplasty) demonstrate a limited effect with positive outcomes observed only in a small number of select subjects (Pauza et al., 2004; Freeman et al., 2005). In one study, less than 40% of the patients achieved greater than 50% relief of their
pain, with the majority of patients experiencing no appreciable benefit following five treatments (Pauza et al., 2004).

Literature supports fusion of affected segments as a viable treatment option for reducing pain and improving function of patients with discogenic chronic LBP unresponsive to conservative care (Phillips et al., 2013). Surgical fusion of the affected segments has been the current gold standard treatment for discogenic LBP, however, outcomes vary widely across different studies (Mirza and Deyo 2007). Fusion can be associated with recurrent episodes of pain and complications including failure of segment fusion (Lee et al., 1995; Fritzell et al., 2001; Chou et al., 2009b; Glassman et al., 2009). While still under debate, some studies have shown that spinal fusion increases the stress levels in adjacent segments, accelerates rates of degeneration and may necessitate additional surgery (Lopez-Espina et al., 2006; Harrop et al., 2008; Maldonado et al., 2011; Harrod et al., 2012; Kasliwal et al., 2012; Sakaura et al, 2013; Choi et al., 2014).

Prosthetic disc implants have the potential to preserve spinal motion and prevent degeneration of adjacent segments (van den Eerenbeemt et al., 2010). However, studies have shown prosthetic discs are associated with heterotrophic ossification and often fail to restore IVD motion (Putzier et al., 2006; Anderson et al., 2012; Brenke et al., 2012; Chen et al., 2011 and 2012; Yang et al., 2012; Wang et al., 2012a). One study reported 60% of prosthetics spontaneously fused after a mean follow up of 17 years (Putzier et al., 2006). Complication rates have been reported as high as 40% (van den Eerenbeemt et al., 2010). Between 2% and 19% of complications following prosthetic disc implantation were related to the surgical approach (e.g. vascular injury, nerve root
damage and retrograde ejaculation), between 2% and 39% were related to the prosthesis (e.g. subsidence, migration, implant displacement, implant failure and endplate fracture) and between 2% and 62% were related to the treatment (e.g. wound, pain, neuromusculoskeletal). While total disc replacement did restore IVD height, mean pain and physical function scores did not differ between subjects in disc replacement and surgical fusion groups (Berg et al., 2011). Thus, a recent systematic review cautioned the widespread use of prosthetic discs until long term outcomes and potential complications are known (Jacobs et al., 2013).

There continues to be a great need for biological alternatives that both alleviate pain and create tissues that restore the structure and biomechanics of the IVD. Replacing a degenerate IVD with a bioengineered tissue equivalent is one way to circumvent the limitations of current therapeutic options (O’Halloran et al., 2007; Kandel et al., 2008; Nerurkar et al., 2010a; Costi et al., 2011; Smith et al., 2011; Hudson et al., 2013).

1.2 Intervertebral Disc: Development to Maturity

Tissue engineering strategies aim to regenerate the mature IVD. It is an immunologically privileged, poorly vascularized and innervated organ separating the 24 articulating vertebral bodies (VB) in the spine (Olmarker et al., 1995; Roughley 2004; Wang et al., 2007). It is made up of three biochemically and structurally unique tissues that merge through complex interfaces: the annulus fibrosus (AF), nucleus pulposus (NP) and the endplate which anchors the IVD to the VB (Figure 1.1.; Roughley et al., 2004; Raj 2008).
1.2.1 The Mature Intervertebral Disc

The mature IVD is composed of water, collagens and proteoglycans. Water and proteoglycan content are highest in the NP and decrease towards the AF while collagen content increases from the NP to the AF (Roberts 1989). The extracellular matrix (ECM) of the IVD contains several types of collagens (Reviewed in Table 1.1). Fibrillar collagens, type I (Col I) and type II (Col II)
make up 80% of the collagenous content of the IVD while aggrecan is the most abundant IVD proteoglycan (Roberts et al., 1991a and 1994).

<table>
<thead>
<tr>
<th>Type</th>
<th>Collagen</th>
<th>Gene</th>
<th>Function</th>
<th>Distribution</th>
<th>Amount</th>
<th>References</th>
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<tbody>
<tr>
<td>Fibrillar</td>
<td>I</td>
<td>Col 1A1, Col 1A2</td>
<td>Tensile Strength</td>
<td>Gradient: high to low OAF to IAF</td>
<td>70% dry wt in AF</td>
<td>Eyer and Mar, 1976; Beard et al., 1991; Antoniou et al., 1996; Nerlich et al., 1997; Schollmeier et al., 2000; Meissner et al., 2006; Nosikova et al., 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weakly associates with SLRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Col 2A1</td>
<td>Resist Compression</td>
<td>OAF, IAF, NP, CEP</td>
<td>20% dry wt in NP</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weakly associates with SLRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Col 5A1</td>
<td>Col fibril Assembly, matrix-repair molecule, Associate with Col I and II</td>
<td>Gradient: high to low IAF to OAF</td>
<td>20% dry wt in NP</td>
<td>Feisthauer et al., 1993; Roberts et al., 1991a; Beerd et al., 1991; Nerlich et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased in DDD</td>
<td>Loose fibrillar network: NP, AF</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CEP: Pericellular and Intertorial Matrix</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>Col 5A1</td>
<td>Associated with Col II</td>
<td>Pericellular Matrix</td>
<td>3% total collagen</td>
<td>Roberts et al., 1991; Nerlich et al., 1998; Meissner et al., 2006; Clouter et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Col 5A2</td>
<td>Increased in DDD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>Col 6A1-A3</td>
<td>Associated with proteoglycans</td>
<td>AF, NP, CEP: pericellular and Intertorial matrix</td>
<td>5% dry wt</td>
<td>Wu et al., 1987; Roberts et al., 1991a; Meissner et al., 2008; Wu et al., 1992; Nerlich et al., 1993</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Increased in DDD</td>
<td>Transformellar cross bridges</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FACIT</td>
<td>IX</td>
<td>Col 9A1-5</td>
<td>Associated with Col II-regulate fibril size</td>
<td>IAF, NP, CEP: pericellular</td>
<td>1-2% collagen</td>
<td>Roberts et al., 1991; Roughley et al. 2004; Wu and Eyre, 1993; Watson et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased in DDD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XII</td>
<td>Col 12A1</td>
<td>Co-distributes with Col X and Col II</td>
<td>NP</td>
<td>3% total collagen</td>
<td>Roughley, 2004; Eyre and Matsumoto, 2002</td>
</tr>
<tr>
<td>Other</td>
<td>X</td>
<td>Col 10A1</td>
<td>Candidate repair, remodeling and mineralization molecule, Associates with Col II</td>
<td>IAF, NP, CEP, Pericellular</td>
<td></td>
<td>Roberts et al., 1993; Lomm et al., 1998</td>
</tr>
</tbody>
</table>

Table 1.1 Collagen Types of the Mature Intervertebral Disc. Fibril Associated Collagens with Interrupted Triple Helices (FACIT), Cartilage Endplate (CEP), Inner Annulus Fibrosus (IAF), Outer Annulus Fibrosus (OAF), Nucleus Pulposus, Weight (wt).
The NP is made up of 77% water, 14% proteoglycans and 4% collagen by wet weight (Raj 2008). The NP is largely composed of Col II, aggregan and minor amounts of elastin making the tissue suited to resist compressive loads (Roberts et al., 1991a and 1994; Nerlich et al., 1998; Yu et al., 2002; Iu et al., 2014). Notochordal cells and NP cells are two cell types found in the NP. Notochordal cells are highly vacuolated cells between 25-85 µm in diameter that are lost with age in humans even though they are retained by other animal species (Peacock 1951a and 1951b; Hunter et al., 2003 and 2004; Cappello et al., 2006). However, it has been shown that notochordal cells are retained in humans but their morphology changes (Weiler et al., 2010). The NP tissue also has a low density of NP cells (4x10⁶ cells/cm³) characterized by similar proteoglycan synthesis and significantly less pro-collagen type II mRNA expression in comparison to articular chondrocytes (Poiraudreau et al., 1999).

The NP is circumferentially constrained by the AF. Different ECM composition and cell populations divide the AF into outer (OAF), inner (IAF) and interlamellar regions (Bruehlmann et al., 2002). Water forms the bulk of the tissue weight of the AF comprising 65–75% of the IAF and 55–65% of the OAF (Urban 1996). Collagens comprise 40–60% of the dry weight of the OAF and 25–40% of the IAF with the most prevalent forms, Col I and Col II, forming opposing gradients (Eyre and Muir 1976; Urban 1996). Col I is most abundant in the OAF while the IAF contains mostly Col II (Eyre and Muir 1976). Proteoglycans, principally aggregan, form an increasing gradient from the OAF to the NP and comprise 5–8% of the dry weight of the OAF and 11–20% of the IAF (Eyre 1979; Urban 1996).
In humans, the AF is composed of discontinuous lamellae whose number and thickness vary by anatomic location and age. The AF contains between 15-25 lamellae varying between 0.14 mm in thickness near the periphery to 0.2 mm near the centre of young discs. This corresponds to values of 0.33 mm at the periphery and 0.52 mm in the centre of older IVDs (Marchand and Ahmed 1990). In the AF, each lamella is made up of bundles of collagen fibrils oriented 25° to 45° from the transverse plane and alternate between successive lamellae to create a multilayered radial-ply structure (Cassidy et al., 1989; Marchand and Ahmed 1990). Aligned parallel to collagen fibres in the OAF lamellae are fusiform shaped AF cells (density of 4x10⁶ cells/cm³) with elongated processes that may play a role in mechanical sensing and cell-cell communications (Errington et al., 1998; Bruehlmann et al., 2002).

Recovery of the multilaminate AF structure following deformation is aided by elastin networks found between lamellae and traversing several non-consecutive lamellae (Pezowicz et al., 2006; Smith and Fazzalari 2009; Schollum et al., 2009). Elastin fibres within the OAF are densely concentrated at the interlamellar space or interface between consecutive lamellae (Yu et al., 2002 and 2007; Smith and Fazzalari 2009). Cells in the interlamellar space are spherical with branching cell processes (Bruehlmann et al., 2002). Translamellar cross-bridges are radial networks which include elastin fibres forming connections between non-consecutive lamellae that also assist the recovery of the OAF. These cross-bridges also contain aggrecan, versican, Col I and collagen type IV (Yu et al., 2002 and 2007; Melrose et al., 2008; Schollum et al., 2009). Understanding how the AF and other tissues of the IVD develop may help guide efforts to bioengineer a replacement tissue (Smith et al., 2011; Chan et al., 2014).
1.2.2 Development of the Intervertebral Disc

Development of the vertebral column is a multistep process whereby embryonic tissues are organized and remodeled (Christ et al., 1992; Sivakamasundari and Lufkin 2012). This is facilitated by the neural tube which secretes molecules that in turn trigger the differentiation of surrounding somites into dermis/muscle and the axial skeleton (Adams et al., 1990; Pourquie et al., 1993; Stemple 2005). Several molecules have been described in the development of the axial skeleton, including the hedgehog family and transforming growth factor beta superfamily (DiPaola et al., 2005). Further consideration of the signaling pathways involved in IVD development may allow us to determine which factors may be necessary for the formation of a stable AF-CEP interface in vitro.

Signaling Molecules: The hedgehog genes encode a family of secreted signaling peptides that direct a variety of developmental events such as patterning tissue formation, cell proliferation and cell survival (reviewed in Robbins et al., 2012). Studies in pre- and postnatal mice have shown that Indian Hedgehog (Ihh) mRNA is expressed by condensing chondrocytes of the VB and is later become confined to the endplate (DiPaola et al., 2005). Conditional knockdown of Ihh from chondrocytes in postnatal mice resulted in loss of the annulus fibrosus and enlargement of the nucleus pulposus (Choi and Harfe 2011). Sonic hedgehog (Shh) mRNA expression has also been shown in the NP of pre- and postnatal mice (DiPaola et al., 2005). Early defects in axial patterning were observed in Shh-null mice following failure to maintain the newly formed notochord and absence of the spinal column (Chiang et al., 1996). Furthermore, Shh expression in the notochord
is sufficient to pattern the vertebral column and IVD (Choi and Harfe 2012). These data suggest that Shh and Ihh play a role in IVD development.

Shh may also interact with Pax-1, a transcription factor, thought to play an early role in patterning the sclerotome and later during IVD development (Dietrich et al., 1993; Wallin et al., 1994; Peters et al., 1998; DiPaola et al., 2005). In Pax-1 deficient mice lack VB and IVDs (Wallin et al., 1994). With age, Pax-1 expression is found in disc anlagen and lining the skeletal structures and may have a role in the maintenance of the boundaries between skeletal elements (Wallin 1994).

C-Fos, a member of the mitogen-activated protein kinase (MAPK) pathway, may play a role in ECM homeostasis in the IVD and cartilage. C-Fos protein expression was observed in rat (3-11 week) AF and NP cells and the notochordal cells of fetal mice (Yokoyama et al., 2013). Overexpression of c-Fos in rat NP cells directly inhibited the promoter activities and gene expression of aggrecan and Col II (Yokoyama et al., 2013). Similarly, in ATDC5 cartilage cell line, overexpression of c-Fos was found to down-regulate Col II and PTH/PTHR expression and inhibit chondrocyte differentiation (Thomas et al., 2000).

TGF-β regulates many aspects of axial skeletal development and postnatal maintenance of IVD cells (Sanford et al., 1997; Matsunaga et al., 2003; Jin et al., 2011). TGF-β signaling plays a critical axial spine development as suggested by the overlapping expression patterns of TGF- β isoforms (Pelton et al., 1990; Millan et al., 1991; Schmid et al., 1991). TGF-β signaling also plays a role in
the postnatal IVD and is required for normal CEP growth (Jin et al., 2011). During postnatal development, deletion of TGF-β2 receptor in sclerotome cells expressing Col type IIA resulted in alterations of posterior spinal elements while the IVDs were either missing or incomplete (Baffi et al., 2004). Furthermore, alterations in the boundary between the future vertebrae and future IVD could be detected in the axial spine suggesting that TGF-β2 influences the maintenance of boundaries in the sclerotome (Baffi et al., 2006). TGF-β2 mRNA was also expressed in the adult mouse AF and decreased expression is correlated with aging and IVD degeneration (Matsunaga et al., 2003).

**Patterning the Axial Skeleton:** Signaling from the notochord patterns the surrounding paraxial mesoderm will give rise to the sclerotome and subsequently the AF and CEP of the IVD. (Sivakamasundari and Lufkin 2012).

**Vertebral Body Formation:** Endochondral ossification of the VB involves proliferation and maturation of chondrocytes to an enlarged or hypertrophic state (Kronenberg et al., 2003). Hypertrophic chondrocytes synthesize type X collagen (Col X), mineralize their ECM and trigger the differentiation of perichondrial cells into osteoblasts (Noonan et al., 1998). Osteoblast and osteoclasts then remodel the mineralized cartilage matrix into cortical and trabecular bone (Kronenberg et al., 2003; Lui et al., 2014).
Increased VB width is facilitated by periosteal bone apposition. VB height is facilitated by the growth plate located at the cranial and caudal ends of the VB. The growth plate is continuous with the apophyseal ring, a secondary ossification centre that surrounds the peripheral margins of the VB (Bick and Copel 1951; Grignon et al., 2000; Piaetta et al., 2013). The apophyseal ring begins to ossify at age 9 and fuses to the VB by 13 years of age in humans (Bick and Copel 1951).

The cranial and caudal ends of the postnatal mouse VB contain thick CEPs that can be divided into an inner and outer region. The inner region lies adjacent to the VB and the outer region of the CEP interfaces with the IVD (Higuchi et al., 1982). One week after birth, the inner cartilage zone contains parallel columns of hypertrophic chondrocytes embedded in a rich proteoglycans matrix. The outer zone of the CEP is further distinguished into superficial and deep layers. The superficial layer contains a columnar arrangement of chondrocytes surrounded by a proteoglycan rich ECM. The deep layer stains non-uniformly for proteoglycans and exhibits calcification (Higuchi et al., 1982). Calcification of the CEP has also been reported at the interface 8 weeks after birth in humans (Paietta et al., 2013). Bone remodeling cells (osteoblasts, osteoclasts) and blood vessels are seen in the deep layer of the inner zone of postnatal mice (Higuchi et al., 1982).

**Intervertebral Disc Formation:** IVD development occurs concomitantly with ossification of the VB. Despite previous controversy, recent studies suggest the NP is formed by notochordal cells (Risbud and Shapiro 2011; Maier and Harfe 2011; McCann et al., 2012). In a mouse model of development, organization and differentiation of the AF is thought to be triggered by bulging of the notochord (Peacock 1951a and 1951b; Walmsley 1953; Rufai et al., 1995). This occurs at
embryonic day 55 in the human (Peacock, 1951a) and between embryonic days 15 and 16 in the rat (Rufai et al., 1995). Cellular organization, spreading and proliferation is mediated by actin stress fibres and fibronectin (Fn), a multifunctional glycoprotein (Miyamoto et al., 1998; Hayes et al., 1999). Fn has also been identified in the VB and endplate where it may play a role in regulating collagen fibre orientation (Hayes et al., 2001). Following alignment, AF cells then deposit a highly ordered ECM essential to the mechanical properties of the OAF (Eyre and Muir 1976 and 1977; Nerlich et al., 1998; Elliott and Setton 2001; Hayes et al., 2001 and 2011).

ECM deposition in the IVD begins with collagen type III (E.16; Col III) which is replaced by Col I in the OAF and Col II in the IAF by E21 (Hayes et al., 2001). Col III has been shown to form heterotypic fibrils with Col I and is essential for fibrillogenesis in developing tissues where it has been suggested to modulate fibre size (Fleischmajer et al., 1983).

Versican, the first proteoglycan to appear in the OAF, is also present in the cartilaginous VB which stains strongly for aggrecan (Hayes et al., 2001). Like aggrecan, versican can bind to hyaluronan and form highly hydrated aggregates that contribute to the swelling pressure of the IVD (Hardingham and Muir 1974; Roughley and Lee 1994). Versican may also play a role in collagen fibrillogenesis (Kalamajski and Oldberg 2010). In the fetal disc, versican is present in the AF and has been shown to co-localize with elastin where it may aid in generating tensional forces to direct the assembly of the AF (Melrose et al., 2001 and 2008; Hayes et al., 2001; Smith et al., 2009). Versican may also facilitate joint morphogenesis, regulate cell adhesion and proliferation as well
as interact with signaling proteins to modulate cellular responses (Yang et al., 1999 and 2003; Hirose et al., 2001; Choocheep et al., 2012; Nagchowdhuri et al., 2012).

Small leucine rich proteoglycans (SLRP) biglycan and decorin in sheep IVD, function as key regulators of ECM assembly and tissue growth in oriented fibrous connective tissues (Banos et al., 2008; Kalamaijski and Oldberg 2010). SLRPs have also been identified in the developing IVD of human, mouse and sheep while their function remains unknown (Melrose et al., 2001; Smith et al., 2009; Hayes et al., 2011). In mice, biglycan is detected in the OAF and endplate and decorin is weakly detectable within the developing VB at E15 and OAF by E21 (Hayes et al., 2001). It is possible decorin also plays a role in collagen alignment in the IVD. Decorin knock-out mice show skin fragility and abnormal collagen fibril morphology, supporting a role for decorin in the lateral assembly of collagen fibrils during tissue assembly and wound healing (Danielson et al., 1997; Jarvelainen et al., 2006). Decorin and biglycan also bind a diverse range of cytokines and growth factors, such as TGF-β, and may modulate tissue formation by regulating levels of these factors or affecting presentation of these molecules to their receptors (Hildebrand et al., 1994; Merline et al., 2009).

As the IVD matures, tensional and compressive forces increase in the IVD causing blood vessels to recede to create the translamellar cross-bridges that are observed in the mature AF (Smith and Elliott 2011).
1.3 The Endplate

The endplate has two thinly demarcated parts: the bony endplate (BEP) adjacent to the VB and cartilage endplate (CEP) adjacent to the IVD. The endplate can be single-layered (Silva et al., 1994; Edwards et al., 2001; Zhao et al., 2009; Rodrigues et al., 2012; Nosikova et al., 2012; Wang et al., 2012b; Moon et al., 2013; Paietta et al., 2013) or double layered (Vernon-Roberts et al., 1977; Edwards et al., 2001; Zhao et al., 2009; Rodrigues et al., 2012; Fields et al., 2012). The BEP of single layered endplates was twice the thickness (0.37 ± 0.18 vs 0.62 ± 0.34 mm) of the BEP in double-layered samples (Fields et al., 2012). Despite the differences in thickness, micro CT-based finite element analysis predicts single and double layered endplates have similar tensile strains (Fields et al., 2012). However, double-layered endplates may influence IVD health as the mean ratio of proteoglycan/cell of NP tissue was significantly higher adjacent to double-layered endplates compared to single endplate (0.018 ± 0.007 vs. 0.008 ± 0.007 μg/cell) (Fields et al., 2012; Rodriguez et al., 2012).

Figure 1.2: Intervertebral Disc Interface with the Cartilage Endplate. (A) Coronal section of (6-9 month) bovine caudal intervertebral disc and outer annulus fibrosus interface (box) with the cartilage endplate. (B) Outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope following haematoxylin and eosin staining and (C) toluidine blue (purple) and von Kossa (black) staining. Outer Annulus Fibrosus (OAF); Nucleus Pulposus (NP); Vertebral Body (VB).
The endplate withstands and redistributes repetitive mechanical forces which may otherwise initiate DDD through inhibition of cellular metabolism and acceleration of ECM degradation (Adams et al., 1993; Ishihara et al., 1996; Handa et al., 1997; Fields et al., 2012). The endplate also facilitates the exchange of nutrients and lactic acid with the capillary network in the underlying subchondral bone (Roberts et al., 1996; Urban et al., 2004). Decreased nutrient availability, as a result of BEP sclerosis or CEP disruption, may disrupt IVD homeostasis (Nachemson et al., 1970; Roberts et al., 1996; Rajasekaran et al., 2004; Benneker et al., 2005).

The CEP is not uniform in thickness, ranging between 0.1 - 1.6mm in humans with a mean thickness reported at 0.62 ±0.29 in humans that is generally thinnest in the region of the NP (Roberts et al., 1989; Moon et al., 2013; Silva et al., 1994; Paietta et al., 2013). The CEP contains 55% water, 8% proteoglycans and 25% collagen by wet weight (Raj 2008). There is considerable variation of composition through the CEP vertically and horizontally. Proteoglycan and water content decrease while collagen content increases from the disc to the bone. Across the CEP sagittally, the endplate adjacent to the AF has a higher collagen but lower proteoglycan and water content than adjacent to the NP (Roberts et al., 1989). It has been suggested the ECM turns over in three phases. Phase I was characterized by synthesis of Col II pro-collagen, aggrecan and denaturation of Col II, a maturation phase distinguished by a decline in ECM synthesis and denaturation of Col II and a final catabolic phase where there is an increase in Col II denaturation and Col I pro-collagen synthesis (Antoniou et al., 1996).

Collagen content of the CEP is the highest in the 5- to 15-year age group (about 10% of tissue dry weight) and decreases with age, falling to the lowest (6-7%) between the ages of 60 and 80
Fields et al. reported that proteoglycan content of the CEP also decreases with age (ages 40–60 years: 356 ± 140 μg/mg dry wt; ages 60–80 years: 188 ± 117 μg/mg dry wt), which is similar to the reports by Antoniou et al. (8-415 μg/mg dry wt and within the lower range (~70–900 μg/mg dry wt) reported by Benneker et al. (Antoniou et al., 1996; Benneker et al., 2005; Fields et al., 2012).

The ECM proteins of the mature CEP have been characterized in cow (Roberts et al., 1991a; Nosikova et al., 2012), human (Roberts et al., 1991a; Paietta et al., 2013) and sheep (Melrose et al., 2001). The CEP contains a layer of calcifying cartilage. The transition from high mineral volume fractions (35–50%) in the calcified cartilage to the hyaline-like cartilage (0%) occurs abruptly over 3–15 microns (Paietta et al., 2013). With age the CEP decreases in diameter to cover only the NP and IAF (Roughley 2004; Nosikova et al., 2012; Moon et al., 2013; Paietta et al., 2013). The mature CEP also contains elastin fibres, collagens type II, III, IV, V, VI, IX, Col X and proteoglycans aggrecan, hyaluronan, perlecan, decorin and biglycan (Roberts et al., 1991a,b; Smith and Fazzalari 2009; Boos et al., 1997; Gotz et al., 1997; Aigner et al., 1998; Nerlich et al., 1998; Melrose 2002; Yu 2002; Nosikova et al., 2012). Collagen type IX was present in the endplate of bovine and rat but not human (Roberts et al., 1991a). However, the specific distribution of these ECM molecules along in the CEP (vertically: inner to outer region of the CEP; horizontally: OAF to NP) remains unclear. This knowledge will enhance our ability to assess the integration site of a tissue engineered IVD following implantation.
1.3.1 **Nucleus Pulposus-Cartilage Endplate Interface**

The NP interface with the CEP (NP-CEP) contains closely packed fibrils largely oriented parallel to the BEP. Collagen fibres extending from the NP are locked in place by transverse fibres from the NP and multidirectional fibrils from the CEP. The nucleus-endplate junctions was able to support, on average, 20 N before tensile failure occurred. (Wade et al., 2011 and 2012). These microanatomical features may contribute to the tensile properties of the NP-CEP interface. The organization of the NP-CEP matrix has yet to be characterized.

1.3.2 **Outer Annulus Fibrosus-Cartilage Endplate Interface**

The OAF-CEP interface is a potential region of stress concentration in that connects the compliant AF (20 MPa in tension) to relatively stiff bone (20 GPa in compression) through a soft cartilage endplate (compressive modulus of 0.4 MPa; Reilly and Burstein 1975; Setton et al., 1993; Acaroglu et al., 1995; Ferguson et al., 2003; Donnelly et al., 2006). The presence of mineral and branched morphology of inserting collagen fibres at the OAF-CEP interface is thought to help anchor the OAF to the calcified cartilage (Nosikova et al., 2012; Rodrigues et al., 2012; Rodriguez et al., 2012; Paietta et al., 2013). Aligned Col I fibres of the OAF lamella form a dual attachment with the VB (Hashizume 1980; Inoue 1981; Nosikova et al., 2012). The outermost fibres of the OAF merge with the periosteum lining the VB. The remaining fibres enter a proteoglycan-rich ECM containing horizontally aligned collagen fibres to subdivide into a multidimensional leaflet structure (Nosikova et al., 2012; Rodrigues et al., 2012; Paietta et al., 2013). Collagen fibres from penetrating into the interface in the posterior OAF, appear to possess a more consistent orientation (10°- 20° insertion angle) as compared to anterior regions (10° to 50° degree insertion angle;
Paietta et al., 2013). Collagen fibres ultimately anchor into the hypermineralized CEP and in areas to be in direct continuity with the marrow cavity of the VB (Inoue 1981; Nosikova et al., 2012; Rodrigues et al., 2012; Paietta et al., 2013). The thickness of the hypermineralized region remained constant with age in cow (cow 6-24 months: 120-152 ± 46 μm) and humans (humans 22-45 years old; 40-42 ± 26 μm) OAF-CEP interface (Nosikova et al., 2012).

The OAF-CEP interface contains a mixed population of AF cells and chondrocytes. Alkaline phosphatase activity (ALPa) was detected around cells in the interface and OAF tissue above the interface. Enzymatic activity in these regions also appeared to increase with age (Nosikova et al., 2012). Col X was absent from OAF tissue adjacent to the interface.

1.4 Tissue Mineralization

The CEP is a mineralizing tissue whereby hydroxyapatite is deposited into the ECM through the process of biomineralization (Christoffersen et al., 1991). Mineralization begins inside matrix vesicles, membrane invested particles (50-200nm diameter) that are derived from the membrane of chondrocytes, osteoblasts and odontoblasts (Boskey 1998; Anderson 2003). When calcium ions and inorganic phosphate (Pi) concentrations exceed the solubility of calcium phosphate (CaPO₄), a critical nucleus is formed inside the vesicle (Anderson 2003 and 2005). These crystals propagate and form clusters around the matrix vesicle and in spatially distinct nucleation sites along collagen fibres if calcium ions and phosphate (Pi) concentrations in the ECM are adequate (Glimcher 1987; Landis et al., 1993; Mann et al., 1993).
Several enzymes influence ECM mineralization by regulating the ratio of available Pi and inorganic pyrophosphate (PPi). ANKH (a homolog of the mouse progressive ankylosis [ANK] gene product) is localized to the membranes of hypertrophic chondrocytes and forms PPi, an inhibitor of hydroxyapatite formation (Addison et al., 2007). Local Pi concentrations are raised by phosphohydrolases such as alkaline phosphatase (ALP), a membrane-bound ectoenzyme that hydrolyzes PPi into Pi to promote the formation of hydroxyapatite crystals (Ali et al., 1970; Hoshi et al., 1997; Roach et al., 1999). In humans, ALP is classified into 4 types: tissue non-specific, intestinal, placenta and germ cell. Tissue nonspecific ALP is expressed by many tissues including liver, bone, cartilage and kidney (Orimo 2010).

Both ALPa and Col X are involved in endochondral ossification of the growth plate and have been reported in the healthy mature CEP of cow and human (Aigner et al., 1998; Miao and Scutt 2002; Boos et al., 1997; Nosikova et al., 2012). Col X and ALP mRNA gene expression have also been detected in the CEP of beagle dogs and mice (Lammi et al., 1998; Liang et al., 2011). ALPa has also been demonstrated in the OAF adjacent to the interface and AF cells express ALPa and mineralize their matrix in 2-D culture (Nosikova et al., 2012 and 2013). Extension of calcification and Col X beyond the CEP has been associated with IVD degeneration (Boos et al., 1997; Aigner et al., 1998; Resnick, 1985; He et al., 2004; Urban et al., 2004; Melrose et al., 2009; Madiraju et al., 2013). The presence of Col X and von Kossa stained deposits in the AF, suggestive of calcification, corresponds with the Thomas grade of IVD degeneration (Rutges et al., 2010). Human IVDs from patients with DDD or adolescent idiopathic scoliosis also have higher levels of ALPa, calcium ions and Pi concentrations than healthy controls (Hristova et al., 2011). Recently, a study found parathyroid hormone can potentially retard DDD by stimulating ECM synthesis and
decreasing ALP, calcium ion, Pi and Col X expression in degenerated disc cells via both MAPK and protein kinase A signaling pathways (Madiraju et al., 2013).

1.5 Mechanics of the Intervertebral Disc

The IVD absorbs compressive, shear, torsion and tensile loads and redistributes those forces to the VB and surrounding musculature (Shirazi-Adl et al., 1984). Under compression, the NP radially expands to generate circumferential tensile stresses in the AF causing crimped collagen fibres to straighten and slide relative to one another in the direction of loading (Cassidy et al., 1989 Bruehlmann et al., 2004). Maximum shear strains in the OAF never exceed 10%/mm ranging between 4%/mm when the IVD is compressed and 6%/mm when flexed or extended (Costi et al., 2007). Without external loads residual stresses from swelling of the NP range from 230 ± 22 kPa of compression at the IAF to 54 ± 0.2 kPa of tension at the OAF (Michalek et al., 2012).

Ex-vivo studies of the healthy mature IVD have shown the tensile behaviour of single and multilayer AF samples vary with specimen orientation, region of the IVD and testing conditions (Ebara et al., 1996; Elliott and Setton 2001; Walsh and Lotz 2004; Guerin and Elliott 2007). Multilamellar OAF tissue harvested from the anterior IVD has higher tensile moduli and failure stress than the posterolateral OAF; the OAF has higher elastic moduli and failure stress than the IAF (Skaggs et al., 1994; Ebara et al., 1996). Collagen fibre orientation also influences the tensile properties of multilamellar AF samples. Stiffness along the principal collagen fibre direction (ranging between 5- 30 MPa) was several orders of magnitude higher than the transverse and radial direction (ranging between 0.2- 2 MPa; Setton and Chen 2004). The stiffness of a single OAF
lamellar specimen, under uniaxial tension perpendicular to fibre direction was $1.44 \pm 1.26$ MPa (Holzapfel et al., 2005).

Finite element models suggest AF cells, in a collagen fibre-reinforced lamella, are subject to a 5% tensile stretch (Stokes et al., 1987; Baer et al., 2000 and 2003; Setton and Chen 2004). Micropipette aspiration studies estimate that healthy OAF and IAF cells have an average shear modulus of 0.09 kPa which is an order of magnitude lower than NP cells (0.55 kPa; Guilak et al., 1999).

1.6 Tissue Engineering an Intervertebral Disc

There is a great need to create cell-generated tissues that restore the structure and biomechanics of the IVD while retaining a capacity for tissue remodeling. Many different approaches and biomaterials have been developed to create isolated or multi-component IVD tissues (Mizuno et al., 2004 and 2006; Hamilton et al., 2006 and 2010; Wilda and Gough 2006; Gruber et al., 2009; Chou and Nicoll 2009; Calderon et al., 2010; Gebhard et al., 2010; Nerurkar et al., 2010b; Bowles et al., 2010 and 2012; Ramakrishnan et al., 2011; See et al., 2011; Park et al., 2012; Bhattacharjee et al., 2014; Grunert et al., 2014; Iu et al., 2014; Martin et al., 2014).

While composite IVD tissues provide proof of principle that the IVD can be bioengineered, these constructs often accumulate limited tissue and fail to model the native AF architecture and ECM composition (Mizuno et al., 2004 and 2006; Nesti et al., 2008; Lazebnik et al., 2011; Bowles et al., 2012; Grunert et al., 2014). An AF-NP composite was formed by seeding AF cells on fibrous
polyglycolic acid and polylactic acid scaffolds followed by the injection of an alginate suspension containing NP cells into the centre of the AF tissue. While a cohesive tissue was formed with Col I localized to the AF and Col II in the NP, however, AF tissue lacked hierarchical structure and failed to reach the level of collagen found in the native IVD (Mizuno et al., 2004). At 16 weeks, DNA, proteoglycan and collagen content in the AF reached 50 % of native tissue levels, while collagen levels in the NP were 15% of native values (Mizuno et al., 2006). Other groups have attempted to model the multilaminate AF microstructure in using electrospun polycaprolactone scaffold (seeded with chondrocytes), wrapped around an NP-agarose disc, to develop an AF-NP composite tissue (Lazebnik et al., 2011). Cells in the model remained viable at 24hr and the construct displayed aspects of cellular alignment and increased elastic moduli compared with acellular hydrogels. However, the long term viability of the IVD-like construct is yet unknown. Mescenchymal stem cells cultured in the presence of TGF-β were used to generate a biphasic construct that consisted of a nanofibrous scaffold (NFS) enveloping a hyaluronic acid hydrogel centre (Nesti et al., 2008). While the construct did accumulate matrix during 28 days of culture, AF tissue failed to exhibit lamellar organization and the construct failed to exhibit the differential Col I and Col II distribution observed in native tissue. Thus, more work is needed to develop composite IVD-like tissues reflective of the complex architecture of the native IVD.

1.6.1 Polycarbonate Urethane Scaffold and Bioengineering a Multilayer Annulus Fibrosus

Efforts to bioengineer the AF have focused on identifying suitable scaffolds that mimic the native AF structure and serve as 3-D structures to guide cellular alignment and tissue formation including natural (collagen, silk and alginate/chitosan hybrid) and synthetic fibres (Vasita and Katti, 2006;
Electrospinning is one method to create scaffolds for tissue engineering highly ordered collagenous tissues. Nanofibrous scaffolds can be spun into parallel sub-micron fibres that emulate the alignment and diameter of collagen fibres and have been used to regenerate the AF (Bhattarai et al., 2004; Riboldi et al., 2005; Nerurkar et al., 2007; Nesti et al., 2008; Gruber et al., 2009; Yeganegi et al., 2010). These scaffolds can be microporous and have a high surface area to volume ratio which favours cell adhesion, proliferation, migration and differentiation (Vasita and Katti 2006).

Because of their reproducibility, several synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA) and polyurethane have been used to generate electrospun scaffolds to regenerate the AF. While PGA and PLA scaffolds can support the growth of AF cells, collagen and proteoglycan retention remains low (Gruber et al., 2009). Furthermore, studies have shown that these scaffolds generate acid by-products during biodegradation which can incite inflammatory reactions and are potentially cytotoxic (Taylor et al., 1994; Gunatillake and Adhikari, 2003; Li and Chang 2005). These findings suggest these materials may not be suitable for IVD engineering.

The biocompatible and biodegradable properties of polyurethanes have made them desirable materials for the fabrication of implant components that directly contact biological systems, such as pacemakers, vascular grafts and soft tissues (Grad et al., 2003; Xue and Greisler 2003; Santerre et al., 2005; Riboldi et al., 2005; McBane et al., 2011). Polyurethanes are segmented polymers composed of repeating urethane linkages and alternating crystalline and amorphous segments. The
ratios of hard and soft segments can be tailored to create biomaterials of differing mechanical properties and degradation products (Premraj et al., 2005; Guelcher et al., 2008).

Nanofibrous polycarbonate urethane (PU) scaffolds are an ideal material to bioengineer the AF because it is a biodegradable, non-toxic elastomer that has been shown to promote cellular attachment and alignment. Tensile strength of the aligned PU scaffold is within the same order of magnitude as the native AF lamella (6 MPa) and withstands strains of 40% or greater before failure (Yeganegi et al., 2010). PU-ADO scaffolds are biodegradable as shown by their sensitivity to cholesterol esterase (7wt% mass loss/week) which generates non-toxic by-products and ultimately degrades into alcohol and carbon dioxide (Premraj et al., 2005; Yeganegi et al., 2010). Cellular attachment can be improved by introducing an anionic dihydroxyoligomer (ADO) to the PU increasing the surface energy and protein content of the PU (Yang et al., 2008). Similarly, coating PU scaffold with Fn positively influences the spreading and alignment of AF cells (Attia et al., 2011). Nanofibrous PU scaffolds also enabled the maintenance of most of the characteristic features of the IAF cell phenotype, maintaining relatively higher levels of Col II, aggrecan and versican gene expression and relatively lower levels of Col I gene expression when compared to OAF cells after 10 days of culture (Iu et al., 2014). While tissue from both cell types accumulated similar amounts of aggrecan, tissue formed by IAF cells accumulated higher amounts of versican (Iu et al., 2014). These findings suggest PU-ADO scaffolds are an appropriate biomaterial to support AF tissue formation and model the various regions of the AF in vitro.
1.6.2 Mechanical Stimulation and Bioengineering

The IVD develops in an environment rich with mechanical stimuli. However, little is known about the influence of loading on CEP formation *in vivo* and its potential role in bioengineering an OAF-CEP interface *in vitro*. Studies indicate that mechanical cues can influence patterning and growth during fetal joint development as well as the formation of tendon-to-bone attachments (Slack et al., 1984; Mikic et al., 2000 and 2004; Moukoko et al., 2004; Kirkos et al., 2005; Thomopoulous et al., 2007; Schwartz et al., 2013). For example, neonatal brachial plexus palsy results in a number of shoulder pathologies including defects in bone and tendon (Kim et al., 2009 and 2010). Similarly, in botulinum toxin (BtxA)-induced paralysis of mouse rotator cuff muscles, collagen fibre alignment and crystal morphology are altered at the tendon-to-bone attachment site (Schwartz et al., 2013).

Ex-vivo studies of the mature IVD demonstrate that the pattern, duration, magnitude and frequency of load can independently and synergistically influence the biological response and that this response can differ dependent on the type of disc cell (Chen and Setton 2004). In the IVD, static and dynamic compression patterns may induce different biological responses. Static (0.5 and 1 MPa) compression of whole organ rabbit IVD/endplate explants suppressed gene expression of Col I, Col II and aggrecan in the AF and Col I and II expression at the endplate (Wang et al., 2007). In contrast, dynamic compression of IVD explants (0.5-1MPa; 1Hz; 6 h) increased Col I, Col II and aggrecan expression in the AF and endplate. The duration of the loading pattern can also induce changes in collagen, proteoglycan, protease gene expression and cell viability (Ohshima et al., 1995; Hutton et al., 1998; Lotz et al., 1998 and 2000; Iatridis et al., 1999; Ariga et al., 2003; Hsieh et al., 2003; MacLean et al., 2003). Short (1–2 hours) and long (3 hours daily for 7 days)
intervals of dynamic compression have been shown to differentially alter the cell-specific expression of ECM genes (Col I, II, aggrecan) and catabolic genes (MMP-3, MMP-13, ADAMTs-4, and COMP; Walsh et al., 2004).

Cell death, mediated by the mitochondrial and MAPK pathways, has been reported in vivo and in vitro following static and dynamic compression (0.4 MPa, 0.01 Hz, 24 h; Ariga et al., 2003; Rannou et al., 2004). Cells of the IVD have also been shown to respond differently to the same mechanical forces. In bovine explants, short-term of static compression (5–10 ± 0.2–0.4 MPa) increased proteoglycan and collagen synthesis in NP and IAF cells relative to controls while only proteoglycan synthesis increased in the OAF (Ohshima et al., 1995).

The application of mechanical loads to in vitro-formed tissues has different effects on cell shape, proliferation and ECM synthesis (Benallaoua et al., 2006; Deschner et al., 2006; Nerurkar et al., 2007; Sowa and Agarwal 2008; See et al., 2011; Turner et al., 2014). Changes in the strain and elastic modulus of a single layer of PU-ADO scaffold increased AF cell proliferation, collagen synthesis and gene expression of Col I and TGFβ-1 compared to controls (Turner et al., 2014). Radial compression (5-25% axial compression, 0.25Hz, 15 min) of AF tissue on silk scaffold for four weeks increased Col II synthesis and decreased Col I gene expression and protein synthesis (See et al., 2011).
Similarly, ECM deposition of *in vitro*-formed cartilage has also been shown to be influenced by a single dose or prolonged period of mechanical stimulation (Mauck et al., 2000 and 2003; Chowdhury et al., 2003; Waldman et al., 2003a,b and 2006). The type of mechanical force applied also influences the composition and mechanical properties of a tissue. *In vitro* cartilage exposed to shearing forces accumulated larger amounts of collagen and proteoglycans than compression-stimulated constructs and the tissue demonstrated a 3-5 fold increase in equilibrium stress and modulus compared to controls (Waldman et al., 2003b).

### 1.7 Summary and Rationale

Successful treatment of chronic LBP associated with DDD involves alleviation of pain and restoration of normal IVD function. Tissue engineering aims to solve these challenges (O’Halloran and Pandit 2007; Kandel et al. 2008; Costi et al., 2011; Smith et al., 2011; Hudson et al., 2013). Previous work by the Kandel and Santerre labs have begun to outline protocols for the independent culture of IVD-like tissues *in vitro* (Sun et al., 2001; Seguin 2004; Kandel et al., 2007; Yang et al., 2008; Hamilton et al., 2010; Attia et al., 2011; Turner et al., 2014; Iu et al., 2014). Preliminary efforts to bioengineer a bilayer AF tissue on Fn coated PU-ADO scaffolds produced distinct layers of tissue that, in regions of the construct, were comparable in thickness to the native lamella (Turner 2011). Furthermore, when grown in contact co-culture with NP tissue, formed on a calcium-phosphate bone substitute, the multilayered AF tissue combines with the NP tissue to form a cohesive composite IVD-like tissue (Li, Unpublished).
The functionality of a bioengineered IVD tissue is, in part, dependent upon its ability to form a stable interface with the host VB following implantation to redistribute physiological loads (Nerurkar et al., 2010a). Several studies have begun to explore how composite AF/NP IVD-like tissue may integrate a host VB upon implantation (Gebhard et al., 2010; Bowles et al., 2012; Grunert et al., 2014). A bioengineered AF/NP tissue, with circumferentially aligned collagen fibrils, maintained their disc height and accumulated proteoglycans and collagens when implanted in the rat spine for up to 6 months (Gebhard et al., 2010). Histological evaluation demonstrated no separation between the bioengineered AF-NP tissue and the host VB. Similar results were shown when AF (collagen)/NP (alginate) composite IVD-like tissue was placed in the L4-5 disc space of athymic mice. IVD construct maintained disc space in 3 of 5 animals and formed a continuous interface between host and bioengineered tissue containing Col II (Bowles et al., 2012). These studies suggest bioengineered IVD-like tissues can fuse with host tissue following implantation. However, the studies fail to characterize composition and spatial organization of collagens and proteoglycans in the de-novo interface which, studies in tendon/ligament-to-bone suggest, can influence the mechanical properties of the interface between native and bioengineered tissues. For example, it is unknown if these integration sites between bioengineered and host tissues contained Col X or mineral, both known to be present in the native interface between the IVD and VB (Lammi et al., 1998; Nosikova et al., 2012). Thus, the functionality of these in vivo-formed interfaces is yet unknown.

Currently, little is known about the effect(s) of co-culture between articular chondrocytes and cells in the IVD. Co-culture of different cells or tissues have also been shown to influence cell metabolism. For example, proteoglycan and collagen accumulation by passaged chondrocytes is
enhanced by contact-free co-culture with primary chondrocytes (Taylor et al., 2010). Contact-free co-culture of chondrocytes and NP cells has also been shown to increase aggregan and collagen gene expression at 2 weeks in NP tissue compared to NP tissue grown alone (Arana et al., 2010). Substituting the CEP with in vitro-formed cartilage had a similar positive effect, suggesting bovine articular chondrocytes are an appropriate cell source to bioengineer the CEP in vitro (Arana et al., 2010). These findings support the use of NP cells in 3-D contact co-culture with articular chondrocytes to develop a model of NP-CEP interface in vitro (Hamilton et al., 2006). At 8 weeks the tissues integrated and cells retained their phenotype. Furthermore, NP-cartilage constructs attached more securely to the bone substitute than NP tissues grown alone demonstrating increased peak loads during shear testing. This may be due to the anabolic effect chondrocytes have on NP cells (Arana et al., 2010). Furthermore, these data suggest that incorporation of a CEP-like layer into a multi-component IVD construct may improve the bone substitute-to-IVD interface characteristics.

The goal of this thesis was to bioengineer a model of the OAF-CEP interface which has yet to be successfully regenerated in vitro. Preliminary efforts to co-culture a single layer AF tissue on PU-ADO scaffold with chondrocytes resulted in a fused tissue but lacked the hierarchical microstructure of the native interface (Nosikova 2011). Further characterization of the mechanical properties, cellular and ECM microstructure of the developing and mature native interface, which have yet to be fully characterized, is necessary to guide bioengineering efforts and evaluate the appropriateness of the model interface. The adolescent cow was chosen as a suitable experimental animal model, since it was previously shown to have similar disc dimensions to human IVD and experiences similar magnitudes of compressive forces of the human IVD in prone position.
(Oshima et al., 1993; Ishihara et al., 1996; Alini et al., 2008). In addition, the biochemical composition of the bovine IVD has also been reported to be similar to that of a healthy young (less than 40 years old) human lumbar IVD (Roberts et al., 1991; Oshima et al., 1993; Demers et al., 2004).

The first aim of the study was to further characterize the adolescent bovine OAF-CEP interface to gain insight into it microstructure. The second aim was to further characterize the human fetal interface to gain further insight into its resemblance to the in vitro model developed. Finally, an AF cell and chondrocyte contact co-culture system was developed and serve as a starting point to understand how IVD tissues integrate in vitro.

1.8 Hypothesis and Specific Aims

Hypothesis: An AF-cartilage construct can be generated in vitro that will yield an integrated tissue that models aspects of the histological appearance and biochemical composition of the native OAF-CEP interface.

Specific Aims:

1. Further characterize the adolescent bovine OAF-CEP interface.
2. Further characterize the human fetal OAF-CEP interface.
3. Develop and characterize an in vitro-formed AF-cartilage interface model and
compare it to the human fetal and native bovine OAF-CEP interface where appropriate.

4. Investigate the effect(s) of compression on the histological appearance and biochemical composition of the *in vitro* AF-cartilage interface model.
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Chapter 2 Manuscript
TITLE: Characterization of the *In vivo* Bovine Annulus Fibrosus-Cartilage Endplate Interface

**Abstract**

**SUMMARY:** The outer annulus fibrosus interface with the cartilage endplate (OAF-CEP) efficiently transfers multiaxial loads from the intervertebral disc (IVD) to the vertebral body (VB). Obtaining a detailed understanding of the native OAF-CEP is a necessary step towards creating a stable interface between an IVD tissue equivalent and host tissue upon implantation. The objective of this study was to characterize the physical strength and composition of the bovine OAF-CEP interface, an animal model currently used in the development of potential IVD therapies.

**METHODS:** IVDs were harvested from bovine (6-9 months) caudal discs and characterized biochemically as well as immunohistochemically using antibodies reactive with type I, II, III or X collagens, aggrecan, versican, biglycan or decorin. To determine the tensile strength, VB-AF-VB samples were harvested from the anterior and posterior regions of the IVD, embedded in polymethacrylate, and tested to failure in uniaxial tension at a rate of 0.02 mm/sec. The fracture site was assessed histologically.

**RESULTS:** The OAF-CEP interface measured between $450 \pm 31 \, \mu m$ and $600 \pm 19 \, \mu m$ in width. Aggrecan and collagen type II was present throughout the interface while collagen type I, III, versican, biglycan and decorin were observed surrounding cells. A mineralized zone, composed
of hydroxyapatite crystals and collagen type X, was localized to the inferior region of the interface continuous with the boney endplate or marrow of the VB. The OAF-CEP interface contained significantly more proteoglycans (198.6 ± 28 µg/mg dry wt) than the OAF tissue (164.2 ± 14 ug/mg dry wt; p=0.041). Cells in the interface, as well as the annulus fibrosus cells immediately adjacent to the interface, express alkaline phosphatase activity (17.6 ± 2.9 µM PNP/hr per µg DNA; n=9). Mechanical failure did not occur through this interface in any samples upon uniaxial tensile loading. VB-AF-VB samples containing 18-20 lamellae failed through the proliferative zone of the growth plate (2.5 ± 0.8 MPa; n=20) while failure occurred within the OAF tissue for samples containing 6-10 lamellae (1.4 ± 0.4 MPa; n=9).

CONCLUSION: The OAF-CEP interface is an organized calcifying tissue. Characterizing the complexity of the bovine OAF-CEP interface provides important information for the design of a bioengineered IVD replacement tissue for the treatment of low back pain.
2.1 Introduction

The poorly vascularized and innervated intervertebral disc (IVD) stabilizes the spine in six axes of motion. It is composed of an annulus fibrosus, nucleus pulposus (NP) and endplate that anchors the IVD to the vertebral body (VB; Adams et al., 2006; Raj 2008). The endplate is a specialized interface that withstands repetitive mechanical loads while permitting nutrient exchange between the IVD and capillaries in the VB (Roberts et al., 1989 and 1996 and Rodrigues et al., 2012). The importance of the endplate is highlighted by its association with IVD degeneration in addition to the improved mechanical stability and health of IVDs adjacent to double-layered endplates (Roberts et al., 1996; Benneker et al., 2005; Fields et al., 2012; Kang et al., 2014).

IVD degeneration, including radial AF fissures, posterior NP herniation, endplate defects and IVD reinnervation, is common and associated with chronic low back pain (LBP; Guiot and Fessler 2000; Roughley 2004; Videman et al., 2004; Adams and Roughley 2006; Rutges et al., 2010; DePalma et al., 2011; Lavelle et al., 2011; Peng et al., 2012; Scheele et al., 2012; Fields et al., 2014). Specifically, failure of the CEP is commonly associated with vertebral rim avulsions, Schomorl’s nodes, IVD herniation and degeneration (Hilton and Ball 1984; Hellstrom et al., 1990; Walters et al., 1991; Yang et al., 1994; Sward et al., 1990a,b; Martinez-Lage et al., 1998; Wang et al., 2012b; Lotz et al., 2013; Rajasekaran et al., 2013; Wu et al., 2013). In vivo, 65% of lumbar disc herniations are the result of lower endplate junction failure as evidenced radiologically by defects in the VB corner or rim (Rajasekaran et al., 2013). This mechanisms of disc herniation is supported by IVD explant studies where repetitive multiaxial loads results in the migration of nuclear material through the posterior outer annulus fibrosus, an area of weak interlaminar
cohesion, and eventual rupture through the thinner inferior endplate (Gordon et al., 1991; Grant et al., 2001; Zhao et al., 2009).

Current treatment options include physical rehabilitation, medication and surgery. However, these therapies are associated with recurrent episodes of pain, do not regenerate the tissue to restore normal spinal movement and with the exception of total disc replacement, fail to restore disc height (Javendan and Dickman 1999; Okuda et al., 2004; Putzier et al., 2005; Mirza and Deyo 2007; van den Eerenbeemt et al., 2010; Berg et al., 2011; Costi et al., 2011). Replacing a degenerated IVD with a bioengineered tissue equivalent may be one way to restore the biological and biomechanical functions of the IVD (O’Halloran and Pandit, 2007; Kandel et al., 2008; Bowles et al., 2011; Smith et al., 2011; Hudson et al., 2013).

The importance of regenerating a functional interface between bioengineered and host tissues is highlighted by the complication rates associated with total joint arthroplasty and total disc replacements as well as surgically re-attached tendons and ligaments (van den Eerenbeemt et al., 2010; Sadoghi et al., 2013; Smith et al., 2012). For example, nearly 40% of all complications following prosthetic disc replacements result from lateral migration of the prosthetic IVD, subsidence into the VB and endplate fracture (van den Eerenbeemt et al., 2010). The surgical repair of tendon/ligament attachments are also associated with high re-injury rates (Smith et al., 2012). Failure at the surgically attached site is attributed to an inability to regenerate the zonal variations in cell morphology, extracellular matrix (ECM) composition and organization of the native fibrocartilaginous interface (Rodeo et al., 1993; Galatz et al., 2004; Silva et al., 2006; Newsham-West et al., 2007). The gradual change in ECM composition, and associated changes in tissue
mechanical properties, is hypothesized to facilitate the transfer of load from the flexible tendon/ligament to the stiff bone and thus minimize potentially damaging stress concentrations at the interface (Thomopoulus et al., 2002 and 2003a and 2006; Genin et al., 2009; Liu et al., 2013). These findings suggest the success of regenerative strategies is dependent, in part, on the stable integration of a bioengineered IVD tissue replacement to the VB following surgical implantation. Thus, a detailed understanding of the interface between the IVD and VB is necessary to inform future repair and regenerative strategies.

The human endplate (17-35 years of age) varies in thickness across the IVD (0.1 - 1.6 mm); in general, it is thickest adjacent to the OAF (Roberts et al., 1989; Silva et al., 1994; Moon et al., 2013; Paietta et al., 2013). It contains cartilaginous and boney regions; the cartilaginous endplate (CEP) interdigitates directly with the bone or marrow cavities of the underlying VB (Roberts et al., 1989; Benneker et al., 2005; Nosikova et al., 2012; Wang et al., 2012a; Paietta et al., 2013). The biochemical composition varies horizontally and vertically through the CEP. Collagen content of the CEP is highest near the OAF while proteoglycan and water content is highest adjacent to the NP (Roberts et al., 1989). In all regions of the CEP, collagen content is highest and proteoglycan content is the lowest adjacent to the BEP.

Studies have described the complex microstructure of the mature outer annulus fibrosus (OAF)-cartilage endplate (CEP) interface (OAF-CEP; Buckwalter et al., 1989; Roberts et al., 1991a,b and 1994; Carlson et al., 1993; Gotz et al., 1997; Melrose et al., 2001; Fields et al., 2012 and 2014; Nosikova et al., 2012; Rodrigues et al., 2012; Rodriguez et al., 2012; Lotz et al., 2013; Paietta et
al., 2013). The aligned collagen type I (Col I) fibres of the OAF form a dual attachment with the VB. The outermost fibres of the OAF merge with the periosteum lining the VB (Hashizume 1980; Inoue 1981; Nosikova et al., 2012). The remaining fibres pass through a type II collagen (Col II) and proteoglycan-rich ECM, subdivide into a multidimensional leaflet structure, and insert into the hypermineralized region of the CEP (Nosikova et al., 2012; Rodrigues et al., 2012; Paietta et al., 2013).

The OAF-CEP interface contains a mixed population of AF cells and chondrocytes throughout the ECM. Alkaline phosphatase activity (ALPa) is seen in the mineralizing matrix surrounding OAF cells and chondrocytes and is also present in OAF tissue above the interface. With age, enzymatic activity appears to increase in the IAF and OAF (Nosikova et al., 2012). Aggrecan, hyaluronan, perlecan, decorin and biglycan staining have been detected in the CEP of humans, sheep and monkeys (Carlson et al., 1993; Roberts et al., 1994; Gotz et al., 1997; Melrose et al., 2002). The interface contains Col II fibres aligned parallel to the BEP and collagens type III (Col III), type V, type VI (Col VI) and type IX (Roberts et al., 1991a,b; Boos et al., 1997; Gotz et al., 1997; Nerlich et al., 1998; Nosikova et al., 2012). Col X is present in the mineralized matrix around chondrocytes in the adolescent and mature cow and human OAF-CEP interface and is absent from the OAF (Aigner et al., 1998; Boos et al., 1997; Nosikova et al., 2012). With age, the OAF-CEP undergoes mineralization and the CEP decreases in diameter so that in adults it underlies the inner annulus fibrosus and NP (Roughley 2004; Nosikova et al., 2012; Moon et al., 2013).
Microstructural features of the CEP, such as mineralization, branched collagen fibre orientation and branched insertion, are suggested to stabilize the CEP and transmit forces from the IVD to the VB (Rodrigues et al., 2012; Paietta et al., 2013). Despite the importance of this interface, ex-vivo studies documenting the mechanical properties of the OAF-CEP are limited (Lundin et al., 1998 and 2000; Kasra et al., 2004; Baranto et al., 2005a,b). One study examining the effect of strain rate on the AF in a healthy sheep model, documented failure of the interface loaded under uniaxial tension (0.02 mm/sec 1000 N load cell). However, without histological evaluation, it is unclear where fracture occurred in the CEP (Kasra et al., 2004). Coupled bending-compression forces (flexion-compression, extension-compression) of immature spinal units, following experimentally induced degenerated IVD, most commonly resulted in failure at the growth plate. Few samples failed at the margins of the endplate (Baranto et al., 2005a). In an immature porcine model, compression of healthy spinal units (VB-IVD-VB) resulted in rupture of the NP through the CEP, displacing the AF with a boney fragment at the point of insertion of the vertebra (Lundin et al., 1998).

The field of bioengineering is currently limited in part by its inability to replicate the organization and thus the mechanical function of the OAF-CEP interface in vitro. This may be due to the fact that the ECM of the interface has yet to be fully characterized. The use of cow coccygeal discs provides the opportunity to obtain sufficient numbers of healthy IVD in contrast to the limited number of healthy human IVD specimens available. While postural variations are likely to result in different IVD loading patterns in humans and cows, sufficient similarities exist to suggest that the cow IVD may be a good animal model to study OAF-CEP interface (Demers et al., 2004; Alini et al., 2008; Beckstein et al., 2008; Nosikova et al., 2012). Coccygeal IVDs of young cows have
similar disc dimensions to the human IVD and experiences similar magnitudes of compressive forces as the human IVD in the prone position (Oshima et al., 1993; Ishihara et al., 1996; Alini et al., 2008). Also, the IVD of cows contain similar amounts of water and proteoglycans as well as similar distributions of collagen and aggrecan as the adult human IVD (Oshima et al., 1993; Roberts et al., 1991a; Demers et al., 2004). Specifically at the OAF-CEP interface, the characteristic dual OAF insertion, layer of mineral and ECM components (type II, III and X collagens) observed at the human interface are also present in the cow model (Roberts et al., 1991a, b and 1998; Nosikova et al., 2012).

The current study characterizes the biomechanical, biochemical content and composition of the bovine OAF-CEP interface with the goal to define specific design parameters that may aid in the bioengineering a successful OAF-CEP interface in vitro and ultimately in vivo.

2.2 Methods

2.2.1 Tensile Strength of the Native Bovine OAF-CEP Interface

The strength of the native OAF-CEP interface of the immature (6-9 months) bovine IVD was evaluated in uniaxial tension. Within 4 hours (hrs) of death, the soft tissues were removed from the cephalad motion segments (C3-C5) of a refrigerated (4°C) bovine caudal spine. The spine was wrapped in gauze and stored at -20°C until mechanical testing. VB-OAF-VB samples were cut in anterior and posterior regions of the IVD with a continuously irrigated slow speed diamond saw. This method has been used by other groups to assess the mechanical properties of the IVD tissues
(Kasra et al., 2004; Baranto et al., 2005a; Changoor 2010). The length and width of each sample were measured with an electronic calliper from three different locations along the interface and then averaged for each specimen. The number of lamellae inserting into the OAF-VB interface was counted using a dissecting microscope.

To determine the tensile strength of the OAF-CEP interface, VB ends were embedded in polymethacrylate, to reduce sample slippage (Figure 2.1). Samples were then placed in 2M PBS solution to reduce leaching of proteoglycans which can influence the tissues’ mechanical properties (Han et al., 2012). For example, in the OAF, the tensile modulus decreased by over 70% with 0.15 M PBS treatment but was unchanged with 2 M PBS treatment. Samples were mounted between plinths of an Instron 1850 testing machine (1,000N load cell) and preconditioned with 10 cycles of 1.5N of uniaxial tension at a rate of 0.08 mm/sec and then tested to failure at a rate of 0.02 mm/sec. Preconditioning was used to provide samples with a common loading history in order to allow for comparisons between different experimental groups (Miller et al., 2012). Strain values were not calculated as changes in interface length could not be accurately ascertained and applied forces were not only distributed within the OAF-CEP interface. Ultimate failure was determined at the point in which there was a decrease in the slope of the linear region of the stress vs strain curve indicative of a plastic and non-recoverable region. Tensile strength values were derived from force displacement curves. Selected tested samples were examined histologically as described below.
Figure 2.1: Schematic of Uniaxial Tensile Testing of Bovine Outer Annulus Fibrosus Cartilage Endplate Interface. (A) Anterior and posterior regions of motion segments from which (B) vertebral body–annulus fibrosus–vertebral body sections were harvested using an irrigated diamond saw. (C) VB ends of AF-VB-AF samples (55-133mm² in cross-sectional area) were embedded and (D) placed in an Instron 1850 testing machine (1,000N load cell). Arrow indicates direction of applied uniaxial tension. Annulus Fibrosus (AF); Intervertebral Disc (IVD); Vertebral Body (VB).
2.2.2 Histological Characterization

Native bovine (6-9 months) IVD were isolated within 4 hours (hr) of death and fixed in 10% buffered formalin for 24 hrs as previously described (Nosikova et al., 2012). Select IVD samples were decalcified in 0.5mM ethylenediaminetetraacetic acid (EDTA pH 7.4; Sigma Chemical, St Louis, MO, USA) at 4 °C for 2-3 weeks and embedded in OCT (Tissue-Tek, VWR). Eight-micron-thick sections of decalcified and undecalcified specimens (3 sections/animal) were cut and stained with haematoxylin and eosin (H&E) to visualize cellular organization at the interface. Toluidine blue and von Kossa were used to assess the presence of sulfated proteoglycans and mineral. The thickness of the OAF-CEP interface was measured from the H&E images at three different locations using ruler measurement tools (mm) in Adobe Photoshop CS5 (Adobe Systems Incorporated) and averaged (N=3 animals).

2.2.3 Location of Alkaline Phosphatase Activity

Azo dye histochemical staining (Sigma Chemical) was used to determine the location of ALP activity in the undecalcified bovine interface as previously described (Allan et al., 2007). Briefly, sections were incubated in Azo dye solution [3 mg naphthol AS-MX phosphate, dissolved in 20 mL dimethyl sulfoxide and 10 mg of Fast Blue BB salt and brought to a final volume of 10 mL with 0.2 M Tris buffer (pH 9.2) for 15 minutes at 37°C. Conditions were optimized experimentally by testing various time points (between 5 and 15 min) at room temperature (RT) and 37°C. The tissues were counterstained with eosin to visualize cellular organization, coverslipped and examined by light microscopy. Bovine articular cartilage was used as a positive control.
2.2.4 Quantification of Alkaline Phosphatase Activity

As previously described, the ALP\(\alpha\) of freshly isolated cells of the OAF-CEP interface and the deep zone of articular cartilage, as a control, was measured (Nosikova et al., 2012). The cells were re-suspended into Buffer A (0.1% Triton X, 0.2 M Tris HCl pH7.4, and 45.7 mM NaCl) and freeze/thawed 3 times to lyse the cells. The solution was clarified by centrifugation at 2800 rpm for 20 minutes at 4 °C and stored at -20°C until used. Aliquots of the extracts were mixed with 0.02 M solution of p-nitrophenol phosphate (Sigma Chemical Co.) in 0.2 M Tris buffer at pH 9.3 for 1 hr at 37 °C. The reaction was stopped by the addition of 50 µL of 1.5 N NaOH and then assayed spectrophotometrically (wavelength of 405 nm; Titertek Multiskan). A standard curve was generated from p-nitrophenol and results were normalized to DNA content. OAF cells were used as a negative control.

2.2.5 Immunohistochemistry

To further characterize the matrix components of the native bovine (6-9 months) interface, representative sections were cut from decalcified samples. To detect Col X and Col II, sections were digested with pepsin (2.5mg/mL of phosphate-buffered saline (PBS) at pH 2; Sigma Aldrich, P7012-1G; ≥2,500 units/mg solid) for 30 minutes at RT. When co-staining for collagen type II (Col II) with either collagen types I (Col I) or type III (Col III) the tissues were sequentially digested with 2.5mg/mL pepsin (2.5 mg/mL PBS lowered to pH 2 with HCl; ≥2,500 units activity/mg solid) for 10 minutes at RT, then trypsin (2.5 mg/mL of Tris-buffered saline [TBS] Sigma Aldrich T7409; 1,000-2,000 units activity/mg solid) for 30 minutes at RT and finally hyaluronidase (25mg/mL in PBS; Sigma Aldrich H3506; 400–1,000 units) for 30 minutes at 37°C.
Sections were rinsed 3 times with PBS (10 minutes each) between digestions. Sections to be stained for Col I, Col II, Col III and Col X were then blocked with 20% goat serum (Invitrogen 10000C) for 1 hr at RT. Co-staining sections for Col I and aggrecan or Col II and versican, decorin and biglycan, followed the sequential digestion protocol outlined above with a final 30 minute digestion of chondroitinase ABC (in 0.25uM in 0.1M Tris pH 8, 0.05M sodium acetate; Sigma Aldrich, C2905-10UN) at 37°C. These sections were blocked with 1% BSA (v/v) in PBS for 1 hr at RT. Antibodies reactive with Col I (1 : 500 dilution; polyclonal T59103R; Meridian Life Sciences), Col II (1 : 500; monoclonal, clone 6B3; Thermo Scientific, Labvision, Freemont, CA, USA; TBS with1% Triton X-100), Col III (1:1000; Abcam AB7778), Col X (1:2000; Sigma Aldrich 7974), aggrecan (1:200; monoclonal 969D4D11; Invitrogen), versican (0.66μg/mL; polyclonal; 16770002; Novus Biologicals), biglycan (1:500; Dr. P J Roughly, Genetics Unit, Shriners Hospital for Children, McGill University, Montreal, Canada), or decorin (4μg/mL; sc-22753; Santa Cruz) were incubated overnight at 4°C and then washed five times in PBS. To detect Col II reactivity, samples were incubated with Alexa Fluor® 594 conjugated goat anti-mouse and Col I and III reactivity was detected with Alexa Fluor® 488 conjugated goat anti-rabbit IgG (both used a 1:1000 dilution; Invitrogen, Eugene, OR, USA) in darkness for 1hr at RT. Col X reactivity was detected after a 1 hr incubation with Alexa Fluor® 594 conjugated goat anti-mouse IgM (1:1000 dilution; Invitrogen, Eugene, OR, USA). Versican, decorin and biglycan reactivity were detected by incubation with Alexa Fluor® 488 conjugated goat anti-rabbit IgG antibody (1:250 dilution; Invitrogen, Eugene, OR, USA) for 1 hr at RT and then washed three times in PBS (10 min each). To visualize nuclei, the sections were terminally stained with 4’,6-diamidino-2-phenylindole (DAPI; 4μgmL; Pierce Biotechnology, Rockford, IL, USA) for 5 min at RT and then washed three times in PBS (10 min each). Sections were coverslipped and images of stained
sections were visualized and captured with a Leica Microsystems (type 090-135.002) microscope (Leica Microsystems, Buffalo Grove, IL) and a Hamamatsu digital CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). For negative controls, the primary antibody was replaced with either IgG or IgM as appropriate. Bovine articular cartilage and tendon served as positive controls.

2.2.6 Transmission Electron Microscopy and Electron Diffraction

Mineral at the native bovine OAF-CEP interface was identified as previously reported (Allan et al., 2007). Briefly, samples of the OAF-CEP interface were fixed in 2% glutaraldehyde in 0.1 M Sorenson phosphate buffer. After fixation, samples were rinsed in buffer, post-fixed in 0.1% osmium tetroxide and embedded in Spurr epoxy resin. Sections were cut (RMC MT6000 ultramicrotome, Leica, Toronto, ON) and viewed using a transmission electron microscope (FEI Tecnai 20, Portland, OR) at 100 kV. The mineral at the OAF-CEP interface was compared to calcified articular cartilage using selected area electron diffraction (SAED). SAED patterns were generated using a 200 mm selected-area aperture and a camera length of 890 mm. The patterns generated by the OAF-CEP interface and calcified cartilage were calibrated by comparison with a gold diffraction standard under the same conditions. Ring diameters were measured and d-spacings calculated.
2.2.7 Biochemical Characterization

**DNA ASSAY:** Tissues were digested with papain (40 mg/mL; Sigma Chemical Co.) in a digestion buffer (20mM ammonium acetate, 1mM ethylenediaminetetraacetic acid [EDTA] and 2mM dithiothreitol) for 48 hrs at 65°C as previously described (St. Pierre et al., 2012). Papain digests were then stored at - 20°C until use. DNA content was determined in PBS-diluted papain digest aliquots using Hoechst 33258 dye binding assay (Polysciences) and fluorometry (excitation wavelength 365nm, emission wavelength 458nm). A DNA standard curve was produced using thymus DNA (Sigma Chemical Co).

**GLYCOSAMINOGLYCAN QUANTIFICATION:** Proteoglycan content was determined by quantifying the amount of sulfated glycosaminoglycans (GAG) in the papain digests using the dimethylmethylen blue dye binding assay and spectrophotometry (wavelength 525 nm) as previously described (St. Pierre et al., 2012). A standard curve was generated using bovine chondroitin sulfate (Sigma Chemical Co.) and the amount of GAG was expressed per microgram DNA.

**HYDROXY-PROLINE QUANTIFICATION:** To determine collagen content, aliquots of the papain digest were hydrolyzed in 6 N HCl for 18 hrs at 110°C (St. Pierre et al., 2012). The hydrolysate was neutralized (pH 7) with 5.7 N NaOH and hydroxyproline content was determined by chloramine-T/Ehrlich’s reagent assay and spectrophotometry (wavelength 560nm). The standard curve was generated using cis-hydroxy-L-proline (Sigma Chemical Co.).
2.2.8 Statistical Analysis

All test conditions were carried out in triplicate and each independent experiment was repeated at least three times from the combined tissues of 3 animals (n=3). The results were combined and analyzed using a Student’s T-test with the assumption that the data were normally distributed and of equal variance. Significance was assigned at p-values < 0.05.

2.3 Results

2.3.1 Vertebrae-Annulus-Vertebrae Samples Did Not Fail at Interface

The OAF-CEP interface did not fail in any test specimens (n=30) with a mean peak tensile strength of $193 \pm 51$ N (range 129-259 N; n=30). Failure of the VB-OAF-VB samples (18-20 lamellae) occurred at $1.4 \pm 0.8$ MPa and the region of failure typically was through the proliferative zone of the growth plate (n=20) under uniaxial tension (Figure 2.2). In VB-OAF-VB samples containing between 6-10 lamellae (n=9), failure occurred in the OAF tissue adjacent to the interface at $1.2 \pm 0.5$ MPa. A single sample failed during preparation. No differences in strength were detected between samples harvested from the anterior and posterior regions of the IVD (Appendix A: Supplementary Table 2.1).
Figure 2.2: Tensile Strength of Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Tensile strength of vertebral body-outer annulus fibrosus-vertebral body samples based on location of fracture (n=30). (B,C) Haematoxylin and eosin staining of decalcified coronal sections to visualize (B) the intact outer annulus fibrosus interface (*) with the cartilage endplate. (C) Samples with 18-20 lamella failed along the columnar zone of the growth plate (n=20). (D) Demonstration of intact interface in samples containing fewer lamella (6-10 lamella). Fracture site in the OAF is not visible in the image (n=9). Cartilage Endplate (CEP); Columnar Zone (CZ); Growth Plate (GP); Outer Annulus Fibrosus (OAF); Outer Annulus Fibrosus-Cartilage Endplate (OAF-CEP); Resting Zone (RZ); Vertebral Body (VB). Images are representative of one experiment done in triplicate and repeated three times.
2.3.2 Interface Contained More Proteoglycans than Outer Annulus Fibrosus

The OAF-CEP interface contained significantly more proteoglycans ($198.6 \pm 28$ ug/mg dry wt) than the OAF ($164.2 \pm 14$ ug/mg dry wt; $n=3$ experiments). No significant differences were detected between the proteoglycan and collagen content of the OAF-CEP interface and articular cartilage (Figure 2.3).

![Graphs showing OH-Pro, GAG content, and ALP/DNA for OAF, DZ, and OAF-CEP interfaces.](image)

**Figure 2.3:** Biochemical Characterization of Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) hydroxyproline content, (B) s- glycosaminoglycans content and (C) alkaline phosphatase activity of the outer annulus fibrosus- cartilage endplate compared with outer annulus fibrosus and deep zone articular cartilage. A significant difference (*) in alkaline phosphatase activity (Pair wise t-test; $p < 0.05$). Alkaline phosphatase activity (ALP); Deep Zone (DZ); S- Glycosaminoglycans (GAG); Hydroxyproline (OH-Pro); Outer Annulus Fibrosus (OAF); Outer Annulus Fibrosus- Cartilage Endplate Interface (OAF-CEP).

2.3.3 Organization of the Outer Annulus Fibrosus-Cartilage Endplate Interface

The CEP measured on average $526 \pm 51$ μm in thickness. Calcium deposits were detected in the CEP and VB by toluidine blue/von Kossa staining (Figure 2.4). The mineral in the CEP was determined to be hydroxyapatite by SAED.
Alkaline phosphatase activity (ALPa) was seen around cells immediately adjacent to and in the interface (Figure 2.4). Cells harvested from the native bovine (6-9 months) OAF-CEP interface expressed levels of ALPa comparable to OAF (OAF-CEP: 17.6 ± 2.9 µM PNP/hr per µg DNA vs OAF: 16.9 ± 5.7 µM PNP/hr per µg DNA; n=3 experiments; Figure 2.3) but were significantly lower than chondrocytes harvested from the deep zone of articular cartilage (224 ± 20 µM PNP/hr per µg DNA; n=3).

Col II was present throughout the interface and absent from the OAF clearly defining a boundary between the two tissues (Figure 2.5). Col I and Col III were present throughout the OAF lamella and seen around cells in the Col II rich interface tissue (Figure 2.6). Col X was seen around cells adjacent to the boney endplate (Figure 2.6). Enlarged images of collagen distribution at the OAF-CEP interface is available in Supplementary Figure 2.1-2.3 (Appendix B).

Aggrecan, versican, biglycan and decorin were present in the OAF-CEP interface but differed with respect to distribution. Aggrecan co-localized with Col I in the OAF and stained strongly throughout the CEP interface (Figure 2.7). Versican was present in the ECM and interlamellar space of the OAF and around cells in the interface. Biglycan and decorin were present in the OAF and around cells in the Col II-rich interface (Figure 2.7). Enlarged images of proteoglycan distribution at the OAF-CEP interface is available in Supplementary Figure 2.4-2.7 (Appendix B).
Figure 2.4: Characterization of Bovine Annulus Fibrosus Interface with the Cartilage Endplate. (A) Annulus fibrosus cells (arrow) and chondrocytes (open arrow head) as seen by light microscopy following haematoxylin and eosin staining at the outer annulus fibrosus interface (*) with the cartilage endplate. (B) Toluidine blue (blue) and von Kossa (black) staining shows proteoglycan and mineral (circle) at the interface. (C) Alkaline phosphatase activity visualized by azo dye (blue) and eosin counter stain. Circle indicates sites of alkaline phosphatase activity. (D) Electron diffraction pattern obtained from the crystals present in the interface. Scale bars represent 100 µm. Inner Annulus Fibrosus (IAF), Outer Annulus Fibrosus (OAF); Outer Annulus Fibrosus-Cartilage Endplate (OAF-CEP); Vertebral Body (VB). Images are representative of one experiment done in triplicate and repeated three times.
Figure 2.5: Regional Distribution of Collagens and Proteoglycans in the Bovine Outer Annulus Fibrosus Interface with the Cartilaginous Endplate. (A) Haematoxylin and eosin stained interface (*) as seen by light microscopy or (B-H) immunostained for collagen type II (red) with (B) collagen type I (green), (C) collagen type III, (D) collagen type X, (E) versican, (F) biglycan and (G) decorin. Co-staining of (H) Col I (red) and aggrecan (green). Negative controls seen in inset (20x magnification). Section counterstained with DAPI (blue) to visualize nuclei. Collagen Type I (Col I); Collagen Type II (Col II); Collagen Type III (Col III); (D) Collagen Type X (Col X); Inner Annulus Fibrosus (IAF), Outer Annulus Fibrosus (OAF), Vertebral Body (VB). Scale bars represent 250 μm. Images are representative of one experiment done in triplicate and repeated three times.
Figure 2.6: Detailed Collagen Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained interface (*) as seen by light microscopy or immunostaining of the (B-D) outer annulus fibrosus, (E-G) cartilage endplate and (H-J) calcifying region of the interface (box). Co-staining of (B-J) collagen type II (red) with (B, E, H) collagen type I (green), (C, F, I) collagen type III, (D, G, J) collagen type X. Negative controls seen in inset (40x magnification). Section counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type I (Col I); Collagen Type II (Col II); Collagen Type III (Col III); Collagen Type X (Col X); Outer Annulus Fibrosus (OAF); Vertebral Body (VB). Scale bars represent 100 μm. Images are representative of one experiment done in triplicate and repeated three times.
Figure 2.7: Detailed Proteoglycan Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained interface (*) as seen by light microscopy or (B-M) immunostaining of the (B-E) outer annulus fibrosus, (F-I) cartilage endplate and (J-M) calcifying region of the interface (box). Co-staining of (B-I) collagen type II (red) with (B,F,J) versican (green), (C,G,K) biglycan or (D,H,L) decorin (green). Co-staining of (E,I,M) collagen type I (red) and aggrecan (green). Interlamellar space (arrow). Negative controls seen in inset (40x magnification). Section counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type I (Col I); Collagen Type II (Col II); Outer Annulus Fibrosus (OAF); Vertebral body (VB). Scale bars represent 100 μm. Images are representative of one experiment done in triplicate and repeated three times.
2.4 Discussion

These results indicate the heterogeneous but organized native OAF-CEP interface was stronger in uniaxial tension than the OAF and VB growth plate. The interface contained significantly more proteoglycans compared to the OAF. Cells throughout the interface were surrounded by Col I, II, III, versican, decorin and biglycan. Col II, aggrecan and ALPa were present throughout the interface while Col X and hydroxyapatite were only seen in the CEP adjacent to the bone. This is in keeping with other studies that have demonstrated mineral and mineral associated proteins at the interface (Higuchi et al., 1982; Eurell and Kazarian 1986; Roberts et al., 1989 and 1998; Carlson et al., 1993; Lammi et al., 1998; Liang et al., 2011; Nosikova et al., 2012; Paietta et al., 2013). Consistent with previous studies in the mature IVD, the OAF contained Col I, III, versican and minor amounts of biglycan and decorin (Roberts et al., 1991a,b; Antoniou et al., 1996; Gotz et al., 1997; Nosikova et al., 2012).

In this study, the differential distribution of Col II in the OAF and CEP (absent in the OAF and present in the CEP) was used to determine if ECM components such as Col I, Col III, versican, decorin and biglycan were present in the OAF-CEP interface. While previous studies suggested that Col III, biglycan and decorin were present at the interface, the CEP is often considered as a whole and it is unclear if regional variations exist throughout the tissue (Beard et al., 1981; Roberts et al., 1991a,b; Carlson et al., 1993; Gotz et al., 1997; Schollmeier et al., 2000). For example, studies describing the presence of Col III at the interface often use low power images of the IVD which fail to clearly define the boundary between the IVD and CEP or use high power images
without indicating the region of the CEP shown (Beard et al., 1981; Roberts et al., 1991a,b; Nerlich et al., 1998; Schollmeier et al., 2000).

Cells of the OAF-CEP interface were surrounded by a complex matrix. In cartilage, the pericellular matrix has been shown to play an important role in regulating the biomechanical, biophysical and biochemical interactions between the cells and ECM by buffering mechanical loads, forming protein networks to anchor cells, binding growth factors and ultimately regulating cellular gene expression (Adams and Watt 1993; Boudreau et al., 1995; Loeser 1997; Guilak et al., 2006).

This study was novel in reporting Col I around cells at the immature bovine OAF-CEP interface. Col I gene expression has been detected in the immature CEP of humans and both Col I gene and protein expression have been detected in the fibrocartilaginous region of tendon-to-bone insertions (Kumagai et al., 1994; Antoniou et al., 1996; Waggett et al., 1998; Thomopoulos et al., 2003b). However, Col I has not been previously shown by immunohistochemistry in the healthy adolescent CEP (Beard et al., 1981; Roberts et al., 1991a; Nerlich et al., 1998; Schollmeier et al., 2000; Nosikova et al., 2012). This is likely due to differences in tissue processing. Previous studies used a single enzymatic digestion prior to staining which may have been insufficient to penetrate the ordered network of Col II fibres, elastin fibres and proteoglycans at the CEP (Roberts et al., 1989 and Yu 2002). This density of the ECM may physiologically limit the access of antibodies to their epitopes at the interface. In support of this hypothesis, other groups have found exposure of carbohydrate and protein epitopes in the IVD and at tendon-to-bone attachments by employing various enzymatic digestions. (Wu et al., 1987; Kumagai et al., 1994; Roberts et al., 1994). For
example, intact collagen type VI (Col VI) could only be extracted from the bovine IVD by serially digesting disc tissue with chondroitin ABC and hyaluronidase (Wu et al., 1987). Similarly, increased pericellular staining of proteoglycan epitopes following pre-digestion of disc tissue with trypsin rather than chondroitinase or hyaluronidase alone was attributed to unmasking of epitopes hidden in the proteoglycan tertiary complex (Roberts et al., 1994). Interestingly, the distribution of Col I remains inconsistent with previous studies using backscatter scanning electron microscopy (SEM) and polarized light microscopy techniques that show fibres from the OAF, composed primarily of Col I, anchor into the calcifying region of the CEP (Eurell and Kazarian, 1986; Nosikova et al., 2012; Paietta et al., 2013). It is possible that other collagens present in the OAF and tendon/ligament-to-bone interfaces, such as collagen type V, VI or IX may be the collagens responsible for anchoring the lamellae into the layer of calcifying cartilage (Wu et al., 1987; Roberts et al., 1991a; Sagarriga and Visconti et al., 1996; Nerlich et al., 1998; Thomopoulos et al., 2003a). Further work is needed to clarify the distribution and role of Col I at the interface.

The finding of versican in the bovine interface was unexpected as it has not been previously described in the mature CEP, cartilage or fibrocartilaginous interfaces (Poole, 1997; Waggett et al., 1998; Claudepierre et al., 2005). Versican associates with the elastic networks in various connective tissues and can form highly hydrated aggregates (LeBaron et al., 1992; Zimmermann et al., 1994; Bode-Lesniewska et al., 1996). As such, versican has been proposed to facilitate the gliding and attachment of adjacent lamellae in the OAF (Melrose, 2001 and Melrose, 2008). An extensive elastic fibre network has also been demonstrated in the CEP and versican may similarly aid in stabilizing inserting lamellae at the interface during weight bearing (Yu, 2002; Smith et al., 2009). However, the presence of versican around cells at the OAF-CEP interface may suggest
versican also mediate cell-ECM interactions. In articular cartilage, versican is thought to help maintain cell-ECM stability through its interactions with hyaluronan (Chen et al., 2003). In vitro studies further demonstrate that versican can influence cell proliferation and adhesion of cells to ECM components such as Col I and fibronectin in vitro (Yamagata et al., 1989; Yang et al., 1999 and 2003). Versican has also been shown to modulate cellular responses by binding of signaling molecules through their glycosaminoglycan side chains (Hirose et al., 2001). Thus, the distribution of versican in the CEP suggests novel functions of versican in the IVD which require further investigation.

Decorin and biglycan were also detected around cells in the OAF-CEP interface. These findings are consistent with previous studies identifying decorin and biglycan proteins in the mature CEP of humans and monkeys (Carlson et al., 1993; Johnstone et al., 1993; Gotz et al., 1997). While the function of decorin and biglycan at the OAF-CEP interface is unknown, literature suggests they may play a role in regulating ECM organization and mineralization as well as cell-ECM interactions (Waddington et al., 2003; Kalamajski and Oldberg 2010). For example, decorin and biglycan can interact with soluble growth factors, bind to cell surface receptors and as such have the potential to influence cell-cycle progression and gene expression (Iozzo and Murdoch, 1996; Iozzo 1997). Decorin also regulates fibre assembly and has been shown to protect Col I and Col II fibres from degradation by collagenases (Vogel et al., 1984; Zhang et al., 2006; Orgel et al., 2009; Geng et al., 2006). In articular cartilage, decorin has also been shown to interact with Col VI which is also a component of the pericellular matrix in the CEP (Roberts et al., 1991a; Nerlich et al., 1998; Bidanset et al., 1992). In articular cartilage, Col VI is suggested to form a protein network
that helps to anchor chondrocytes to the surrounding ECM and transduce biomechanical signals (Marcelino and McDevitt 1995; Buckwalter and Mankin 1998; Alexopoulos et al., 2009).

In addition to aiding matrix assembly, biglycan and decorin may play a role in mineral formation at the OAF-CEP which has been shown to calcify with age (Roughley 2004; Nosikova et al., 2012; Paietta et al., 2013). Biglycan and decorin have been extracted from the mineralized matrices of bone and have the capacity to bind calcium and interact with hydroxyapatite (Waddington and Embery 1991; Fujisawa et al., 1991; Embery et al., 1998; Sugars et al., 2003). In primary bone cell cultures biglycan was expressed during cell proliferation, ceased at early matrix deposition and then was re-expressed at the onset of mineralization while decorin was only expressed during stages of mineralization (Waddington et al., 2003). More work is needed to clarify the role of these proteoglycans at the interface.

Given the differing elastic moduli of the OAF and CEP, the interface between these tissues is a potential region of high stress concentration and is subject to failure at slow strain rates (Green et al., 1993; Ebara et al., 1996; Kasra et al., 2004). However, no VB-AF-VB samples failed through the CEP which may be attributed to specimen age, preparation or type of applied mechanical loads. For example, in Kasra et al. the growth plate may have been developmentally more mature or fully embedded in the polymethylmethacrylate (PMMA). However, in this study, it was not possible to fully embed the growth plate of bovine samples in PMMA without also compromising the CEP. Furthermore, an immature IVD was used in this study to better approximate the tensile properties of a partially mineralized interface. The CEP may also be more vulnerable to multiaxial loads.
Disc herniations, commonly associated with CEP failure, have been documented in disc explants following repetitive loading (1.5 Hz) in a combination of flexion, rotation and compression (Gordon et al., 1991; Rajasekaran et al., 2013). Thus, future studies should investigate the strength of the interface in compression, bending and torsional moments which are also experienced at the IVD *in vivo*.

Failure of IVD samples occurred most commonly through the growth plate in uniaxial tension at comparable strength (2.97 ± 0.80 MPa) and fracture pattern as observed in the bovine tibial growth plate (Cohen et al., 1992). These results support clinical observations, finite elemental modeling and biomechanical studies that have demonstrated the growth plate is weaker than the CEP in shear stress and bending motions (Ikata et al., 1996; Sairyo et al., 1998 and 2001 and 2004; Kajiura 2001; Konz et al., 2001; Baranto et al., 2005a,b). Imaging studies of pediatric patients with isthmic spondylolisthesis, or anterior translation of one VB relative to the adjacent segment, demonstrate most vertebral slippages developed or progressed in the cartilaginous or apophyseal stage of the lumbar skeletal development and ceased with maturity (Ikata et al., 1996; Sairyo et al., 2001). In a spondylolisthesis rat model, forward slippage of the immature VB occurred only in spines following growth plate injury with no evidence of abnormalities in the AF or IVD degeneration (Sakamaki et al., 2003; Sairyo et al., 2004). Ex vivo compression studies further suggest the growth plate is the weakest link in the adolescent spine (Brinckmann 1983; Kajiura et al., 2001; Lundin et al., 1998; Sairyo et al., 1998; Barranto et al., 2005a,b). Growth plate fracture was observed without IVD derangement when shear forces applied to immature calf spines (neonate to 24 months) resulted in failure at the growth plate in all age groups at 986.4 ±124.16 N in 2 month old calves (Kajiura 2001). These studies imply the growth plate may be weaker than the CEP as well as the
intact AF. This is further supported by this study where the majority IVD samples containing 6-10 lamellae failed in the OAF rather than the growth plate.

It is possible that structural differences between the OAF-CEP interface and VB growth plate may contribute to the observed differences in mechanical properties. For example, collagen fibre orientation at the tendon/bone insertion have been shown to influence stress concentrations at the interface (Thomopoulos et al., 2006). It has also been suggested that the decreased width and density of the collagen fibrils as well as the absence of transepiphyseal fibres in the proliferating zone of the growth plate make this region particular vulnerable to fracture (Bright and Elmor 1968; Speer 1982; Moen and Pelker 1984; Cohen et al., 1992). In contrast, the OAF-CEP interface is stabilized by a dense network of elastic fibres, Col II fibres aligned parallel to the subchondral bone and collagen fibres from the inserting lamellae (Roberts et al., 1989; Yu 2002; Nosikova et al., 2012; Paietta et al., 2013). Furthermore, the unique multidimensional leaflet structure of the collagen fibres in the OAF-CEP interface may contribute to the increased strength of the interface in comparison with the growth plate (Rodrigues et al., 2012).

The OAF-CEP has been shown to be unique from other soft tissue-to-bone attachments functionally and structurally (Claudepierre and Voisin, 2005; Fields et al., 2012; Nosikova et al., 2012). Functionally, the CEP balances opposing biomechanical and nutritional functions. It must withstand repetitive mechanical loading to uniformly redistribute IVD pressures which otherwise may initiate IVD degeneration (Shirazi-Adl et al., 1984; Adams et al., 1993; Handa et al., 1997; Ishihara et al., 1996; Lotz and Chin 2000; Walsh and Lotz 2004; Fields et al., 2012). The CEP also
facilitates the inflow of nutrients and outflow of lactic acid which if disrupted by CEP sclerosis or CEP disruption can compromise IVD health (Roberts et al., 1996; Rajasekaran et al., 2004; Benneker et al., 2005; Kang et al., 2014). In contrast, tendon/ligament-to-bone attachments do not play a role in nutrient exchange. Rather, these tissues interfaces primarily function to transmit force from the muscle/bone to the bone (Mackey et al., 2008).

Structurally, the OAF-CEP interface has been shown to be unique compared with fibrous, fibrocartilaginous, or cartilaginous soft tissue-to-bone attachments (Claudepierre and Voisin, 2005; Nosikova et al., 2012). It has been previously shown the OAF-CEP differs from fibrous (tendon or ligament) insertions, as collagen fibres from the OAF pass through a chondroid region before anchoring into a hypermineralized tissue (Benjamin et al., 2002; Claudepierre and Voisin, 2005; Benjamin and McGonagle 2009; Nosikova et al., 2012). This study demonstrated the OAF-CEP interface also differs from fibrocartilaginous insertions (Achilles tendon, supraspinatus tendon) in that cells were surrounded by versican and demonstrated ALPa (Rufai et al., 1996; Benjamin and Ralphps 1998; Claudepierre et al., 2005). ALPa has not been characterized in the mineralizing fibrocartilage of tendon/ligament-to-bone attachments. Rather, ALPa has been reported in bone, the intermediate and hypertrophic zones of articular cartilage and in the hypertrophic zone of the vertebral growth plate (Miao and Scutt et al., 2002; Wang et al., 2006). The OAF-CEP interface also differs from articular cartilage in ECM composition and collagen fibre alignment. While absent from healthy articular cartilage, this study detected versican and Col I around cells at the OAF-CEP interface (Poole et al., 1997; Guilak et al., 2006). It has also been previous shown that collagen fibres from the OAF insert into the calcifying cartilage at 120° angle whereas Col II fibres in the deep zone of articular cartilage are angled near perpendicular to
the layer of calcifying cartilage (Kaab et al., 1998; Rodrigues et al., 2012). Collectively, these findings suggest the OAF-CEP interface is highly specialized interfacethat differs from other tendon/ligament-to-bone attachments and may reflect the delicate balance between the mechanical and nutritional functions of the OAF-CEP interface.

The distinct composition and structure of the OAF-CEP may reflect the multidirectional tensile and compressive loads experienced by the interface (Shirazi-Adl 1984; McNally and Adams 1992; Gregory and Callaghan 2011). Changes within the mechanical environment may also explain differences in fibre alignment between the OAF-CEP interface and the NP interface with the CEP. In the OAF, region of high torsional, circumferential and axial tensions, collagen fibres passing through the OAF-CEP interface are aligned (Costi et al., 2007; Nosikova et al., 2012; Paietta et al., 2013). In contrast, collagen fibres are less aligned at the NP-CEP, a region that bears primarily compressive loads (Wade et al., 2011 and 2012). These and other structural differences may explain why the mean peak tensile strength of the OAF-CEP interface determined in this study was nearly 10x greater than that recently reported for the NP-CEP interface (193N vs 20N; Wade et al., 2011).

This study has several limitations. Despite the acceptance of the bovine IVD as an appropriate animal model, the composition and tensile properties of the OAF-CEP interface described herein should be confirmed in human samples. Also, during mechanical testing, samples were stored in a hypertonic saline solution and some studies have suggested that this may influence sample hydration and thus affect the viscoelastic properties of the tissue (Gu and Yao 2003). For example,
Reducing hydration levels of IVD explants by creep loading was associated with a decrease in compressive modulus at high rates of loading (3 MPa/sec) while no change was observed at slower rates of 0.3kPa/sec (Race et al., 2000). Estimation of the strength of the OAF-CEP interface was also limited in that tensile forces were distributed across all tissues of the VB-AF-VB sample (VB, growth plate, OAF-CEP interface and AF). Notwithstanding, the peak tensile strength of the interface reported in this study is consistent with previous works and provides a measure of the regional characteristic of the OAF-CEP interface (Kasra et al., 2003).

Despite these limitations, this study highlights several characteristics of the native bovine OAF-CEP interface which may be important in the development of a model of the OAF-CEP interface in vitro. Collagen and proteoglycan content of in vitro tissues are valuable in providing objective measures of the type of tissue formed. However, the collagen and proteoglycan content of in vitro constructs often fail to reach native values (Alini et al., Hudson et al., 2013). Thus, it may be more informative to establish a collagen to proteoglycan ratio similar to that of the native interface in early efforts to tissue engineer the interface. The mechanical stability of an in vitro interface may likewise be influenced by the ECM composition as well as the number of AF lamellae incorporated in the model interface. A mineral gradient may likewise influence the mechanical stability of an in vitro model. In osteochondral bioengineered tissues, the presence and location of mineral between the in vitro-formed cartilage and a bone substitute resulted in a 3.3-fold increase in the interfacial shear strength (St-Pierre et al., 2012). While factors beyond the scope of this study, such as the number and orientation of inserting collagen fibres, may also influence the tensile properties observed at the native bovine OAF-VB interface, these structural features characterized in this study will aid in the development of a stable model in vitro.

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In summary, this study provides detailed knowledge of the microstructure, biochemical composition and tensile properties of the bovine OAF-CEP interface. This will assist in bioengineering functional soft-tissue interfaces and facilitate clinical translation of biological disc repair.

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2.6 References


Chapter 3 Manuscript
TITLE: A Novel *In vitro* Model of the Annulus Fibrosus- Cartilage Endplate Interface

**PURPOSE:** Replacing the damaged intervertebral disc (IVD) with a tissue engineered equivalent, composed of annulus fibrosus and nucleus pulposus, is an alternative approach to the treatment of disc-related chronic low back pain. Success of this biological IVD replacement following implantation depends, in part, on stable integration with the host vertebral body to fix adajacent segments and redistribute physiological loads. **However, little is known about how or if bioengineered disc will integrate between the different tissues following implantation. Thus, the purpose of this study** was to generate and characterize an *in vitro* model of the outer annulus fibrosus (OAF)-cartilage endplate (CEP) interface so as to enable identifying factors that could influence implant integration.

**METHODS:** Deep zone chondrocytes were isolated from bovine articular cartilage and grown on membrane inserts to form cartilage tissue. Outer annulus fibrosus cells were isolated from bovine caudal intervertebral discs and seeded on multilayered nanofibrous polycarbonate urethane (PU-ADO) scaffolds. A 3-dimensional model of the OAF-CEP interface was then generated by contact co-culture of these tissues. The effect of a single application of cyclic compression for 30 minutes (1 kPa, 0.5 Hz, 1800 cycles) on the interface was determined. Tissues were analyzed histologically and biochemically and compared to human fetal OAF-CEP interface.
**RESULTS:** PU-ADO scaffold supported the growth of a multilayered AF tissue with evidence of cellular alignment and tissue ingrowth. When placed in co-culture with calcifying cartilage, the tissues fused to form an *in vitro* model of the OAF-CEP. Calcific deposits and alkaline phosphatase activity (ALPa) were observed in the chondroid region. The spatial organization of collagen type I, II, III, aggrecan, versican, biglycan and decorin in the interface model reflected aspects of the *in vivo* human fetal interface while mineral and Col II differed from tissues grown alone. Significantly more proteoglycans and collagen accumulated at the interface as compared to independently cultured AF tissues. Dynamic compression of the interface model significantly increased proteoglycan retention and altered ALPa distribution. Col X was also observed in the interface model following dynamic compression. Similar to unloaded models, cells in the stimulated interface were surrounded by Col I, Col II, Col III, versican, biglycan and decorin.

**CONCLUSION:** This study demonstrated that bioengineered AF tissue and cartilage tissues could fuse to form a model of the OAF-CEP interface. As the interface responded to mechanical load, this model may be suitable to begin investigating factors that will strengthen the integration of a biological disc replacement.
3.1 Introduction

To successfully replace the intervertebral disc (IVD), a multicomponent tissue found between adjacent vertebrae in the spine, functional and stable integration of the bioengineered IVD replacement tissue following surgical implantation is required to maintain tissue health and prevent future dislocation (O’Halloran and Pandit, 2007; Kandel et al. 2008; Costi et al., 2011; Sahoo et al., 2011; Nosikova et al., 2012; Hudson et al., 2013). In the native IVD, the annulus fibrosus anchors the IVD which functions to transmit forces across the interface from IVD to the adjacent vertebral bodies (VB) and permit the diffusion of nutrients into the IVD (Broberg 1983; Urban 2004; Roberts et al., 1989; Raj 2008).

The importance of regenerating a functional interface between implanted bioengineered and host tissues is highlighted by the high failure rates reported in prosthetic IVD replacements as well as surgically reattached tendons and ligaments (van den Eerenbeemt et al., 2010; Smith et al., 2012). For example, nearly 40% of all complications following prosthetic disc replacements result from lateral migration of the prosthetic IVD, subsidence into the VB and endplate fracture (van den Eerenbeemt et al., 2010). The surgical repair of tendon-to-bone attachments also report high reinjury rates at the site of graft fixation. Frequent failure rates have been attributed to the inability of surgical procedures to drive the regeneration of cell phenotype, extracellular matrix (ECM) composition and mineral formation at the native fibrocartilaginous interface (Rodeo et al., 1993; Galatz et al., 2004; Newsham-West et al., 2007; Genin et al., 2009). These gradients in compositional, structural and thus mechanical properties are hypothesized to minimize the concentration of compressive and shear forces at the interface between the compliant
ligament/tendon (200 MPa) and to relatively stiff bone (20 GPa; Benjamin and Ralphs 1998; Thomopoulos et al., 2002 and 2003; Ferguson et al., 2003). As more is learned about the composition, architecture, and function of the CEP, it may be possible to engineer tissues that can interface with the host VB. Thus, several groups have begun to characterize the regional heterogeneity of the CEP, such as the interface between the OAF and CEP (OAF-CEP), to guide future repair and regenerative strategies (Inoue 1981; Roberts et al., 1989; Johnstone et al., 1993; Antoniou et al., 1996; Gotz et al., 1997; Moore 2006; Wade et al., 2012; Nosikova et al., 2012; Moon et al., 2013; Paietta et al., 2013).

Aligned Col I fibres of the OAF form a dual attachment to the underlying VB as described below. The outermost fibres of the OAF merge with the periosteum of the VB while the remaining fibres branch into a multi-leaflet structure as they pass through the CEP and anchor into a hypermineralized matrix (152 ± 21 μm; Inoue 1981; Fields et al., 2012; Nosikova et al., 2012; Rodrigues et al., 2012; Paietta et al., 2013). In other areas, the OAF appears to be in direct continuity with the marrow cavity of the VB (Nosikova et al., 2012; Paietta et al., 2013; Chapter 2). It is hypothesized that the circumferential and flexion-induced tensile loads experienced by the OAF are resisted, in part, by the anchorage of fibres in the CEP which in turn creates maximal shear strains at the fibre-cartilage matrix interface (Broberg et al., 1983; Schmidt et al., 2007; Rodrigues et al., 2012). It is also possible that, similar to tendon/ligament-to-bone attachments, the unique microarchitecture of the OAF-CEP interface may be a functional adaptation to this complex mechanical environment (Benjamin and Ralphs 1998; Nosikova et al., 2012; Chapter 2).
In the adolescent cow, the OAF-CEP interface measures average $526 \pm 51$ μm in thickness and has a minimum tensile strength of $2.54 \pm 0.77$ MPa (Chapter 2; Paietta et al., 2013). The OAF-CEP stains diffusely for Col II and aggrecan while collagen type X (Col X) and mineral were concentrated along the subchondral bone of the VB (Boos et al., 1997; Nosikova et al., 2012; see Chapter 2). AF cells and chondrocytes at the interface are surrounded by Col I, II, III, aggrecan, versican, and small leucine rich proteoglycans (SLRPs) decorin and biglycan (Nosikova et al., 2012; see Chapter 2). While others have described collagen type IV, hyaluronan and perlecan throughout the CEP, the presence and spatial organization of these molecules in the OAF region of the CEP remains unclear (Nerlich et al., 1998; Melrose 2002; Smith et al., 2009). Cells in the interface and OAF cells immediately adjacent to the interface demonstrate alkaline phosphatase activity (ALPa; Nosikova et al., 2012; see Chapter 2). Alkaline phosphatase is a membrane-bound ectoenzyme that hydrolyzes pyrophosphate to promote hydroxyapatite crystal formation (Roach et al., 1999).

Many studies have explored the use of different biomaterials, culture conditions and mechanical loading parameters to bioengineer individual and multi-component IVD tissues, (Mizuno et al., 2004; Chou and Nicoll 2009; Wilda and Gough 2006; Gruber et al., 2009; Calderon et al., 2010; Nerurkar et al., 2010; Bowles et al., 2010, See et al., 2011 and 2012; Zhuang et al., 2011; Pan et al., 2012; Bhattacharjee et al., 2014; Chik et al., 2014; Feng et al., 2014; Lu et al., 2014; Martin et al., 2014; Turner et al., 2014). However, few studies have explored how bioengineered IVD replacement tissues may integrate with the host VB following implantation (Gebhard et al., 2010; Bowles et al., 2012; Grunert et al., 2014). A bioengineered AF/NP tissue, containing circumferentially aligned collagen fibres around an alginate NP core, was found to maintain disc
height (between 68% -74 % of healthy disc) 8 months after implantation in the rat spine (Gebhard et al., 2010; Grunert et al., 2014). The AF-NP accumulated matrix, had similar water content to control discs and histological evaluation demonstrated no separation between the bioengineered tissue and the host VB (Gebhard et al., 2010; Grunert et al., 2014). In another study, magnetic resonance imaging demonstrated that AF (collagen)/NP (alginate) maintained L4/5 disc space in 3 of 5 athymic mice and formed a Col II containing interface between host and bioengineered tissues after 16 weeks (Bowles et al., 2012). While these studies suggest that bioengineered IVD-like tissues can fuse with the host VB following implantation, the functionality of the de-novo interface is unknown as the latter studies only had limited characterization of the tissues’ composition or mechanical properties. For example, it is unknown if the integration sites between bioengineered IVD tissues and the host VB contains Col X or mineral, both of which are components of the native CEP (Lammi et al., 1998; Nosikova et al., 2012; see Chapter 2).

Tissue engineering of fibrous soft tissue-to-bone interfaceshave been studied more extensively in the repair of tendon/ligament-to-bone attachments rather than for the OAF-CEP interface (Spalazzi et al., 2006 and 2008; Lu and Spalazzi 2009; Yang and Temenoff 2009; Dickerson et al., 2013). Triphasic scaffolds have been used in an attempt to regenerate the multi-tissue transition (ligament, fibrocartilage, calcifying fibrocartilage, bone) of the anterior cruciate ligament-to-bone attachment (Cooper et al., 1970; Messner et al., 1997; Spalazzi et al., 2006 and 2008). Following an 8 week subcutaneous implantation in athymic rats, fibroblasts, chondrocytes and osteoblasts were tri-cultured on a stratified scaffold to form a phase-specific matrix with a distinct mineral and fibrocartilage-like region containing Col I, Col II and Col X (Spalazzi et al., 2008). In a sheep rotator cuff repair model, surgical implantation of a regionally demineralized cancellous bone
scaffold, seeded with human adult adipose stem cells after 16 weeks, was shown to form a calcifying fibrocartilaginous interface between the tendon and bone which was thicker than that observed in healthy controls but completely absent from sites of standard surgical fixation of the tendon by bone tunnel (Dickerson et al., 2013).

A stable in vitro model of the OAF-CEP interface has yet to be regenerated in vitro in part owing to the structural complexity of the OAF. We have previously shown that AF tissue can be formed on electrospun non-toxic, biodegradable nanofibrous polycarbonate urethane (PU) scaffolds that contain an anionic dihydroxyoligomer to promote protein attachment and subsequently cell attachment (Santerre et al., 2005; Yang et al. 2008; Yeganegi et al., 2010; Attia et al., 2011). PU-ADO is electrospun into nanofibrous sheets of scaffold with comparable tensile strength, alignment and fibre diameter to the native AF lamella (Fleischmajer et al., 1983; Marchand and Ahmed, 1990; Yang et al. 2008). Similar to that observed during IVD development, AF cells on PU-ADO scaffold orient parallel to aligned fibres and deposit a similarly aligned ECM that contains Col I (Wang et al., 2003; Attia et al., 2011). AF cells also respond to changes in PU-ADO scaffold tension by increasing rates of proliferation, collagen synthesis, Col I gene expression and TGFβ-1 gene expression (Turner et al., 2014). These findings suggest the PU-ADO scaffold is an appropriate biomaterial to regenerate AF tissue in vitro and can be used to model the multilamellar insertion into the CEP.

Thus, the objective of this study was to develop an in vitro model of the OAF-CEP interface in vitro, comparing it to the collagen and proteoglycan composition of both native human fetal tissue
and independently cultured *in vitro*-formed tissues, to further understand how these tissues integrate to form a stable interface.

### 3.2 Methods

#### 3.2.1 Multilayer Annulus Fibrosus Tissue

**POLYCARBONATE URETHANE SCAFFOLD FABRICATION:** Polycarbonate Urethane (PU) was electrospun into a nanofibrous scaffolds as described by Yang et al., (2008). Briefly, the base PU polymer was synthesized by reacting poly (1,6-hexyl 1,2-ethyl carbonate) diol, 1,6-hexane diisocyanate and 1,4-butanediol at a controlled temperature (60°C-70°C). Anionic dihydroxyl oligomer (ADO) was added to PU (0.15% to 0.5% by weight) to improve scaffold wettability and cellular attachment (Yang et al., 2009). ADO was synthesized by reacting polyteramethylene oxide, hydroxyethylmethacrylate and lysine diisocyanate to yield a polyurethane structure with terminal hydroxyls and pendant carboxylic acid groups. The PU-ADO polymer was then electrospun on a rotating mandrel to form a scaffold (106 ± 5 µm thickness, average fibre diameter of 273 nm; Yang et al., 2009). Scaffolds were placed in a vacuum oven (Isotemp Vacuum Oven Model 280A; Fisher Scientific, Pittsburgh, PA) overnight at 45°C, sterilized by gamma radiation (4 M Rad, 48 hrs) under conditions that were previously shown not to affect nanofibre alignment or influence tissue formation (Yang et al., 2009; Iu et al., 2014). Scaffolds were then stored at room temperature (RT) until use.

**MULTILAYER AF TISSUE:** AF tissue was formed by seeding multilayered PU-ADO scaffold with OAF cells from bovine caudal spines (6-9 months). IVDs were aseptically excised within 4
hrs of death and OAF tissue was digested with 0.5% protease (Sigma, St. Louis, MO) for 1 hr at 37°C, followed by 0.3% collagenase A (Roche Diagnostics, Laval, Quebec, Canada) overnight at 37°C to isolate OAF cells. The cell suspension was filtered using a sterilized mesh and washed 3 times with Dulbecco Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 5% fetal bovine serum (FBS; PAA, Etobicoke, ON) and 1% antibiotics (penicillin G, streptomycin sulphate and amphotericin B). Cells were re-suspended in DMEM with 10% FBS, seeded in monolayer at a density of 200 cells/cm² and cultured at 37°C, 95% relative humidity and 5% CO₂ until ~80% confluence. Cells were then harvested by incubating with 1% trypsin–EDTA for 5 min at 37°C, pelleted and re-suspended in DMEM with 20% FBS.

Pre-wet gamma irradiated PU-ADO scaffold was prepared for cell seeding by soaking overnight at 4°C in fibronectin (20μg/mL PBS 20 mg/mL; F0895; Sigma-Aldrich, St. Louis, MO), an integrin binding ECM glycoprotein previously shown to mediate cellular and ECM alignment in the fetal disc and bioengineered AF tissue (Hayes et al., 1999 and 2001; Attia et al., 2011). The scaffold was rinsed 3 times (5 min each) with phosphate buffered saline (PBS) and cut into rectangular strips (0.3 cm x 4 cm) with nanofibres aligned parallel to the shortest side of the strip. Under sterile conditions, scaffold was wrapped 3 times around a Teflon tube and pinned to form a 3-layered construct (0.35 mm outer diameter x 0.3 mm in height; Figure 3.1). Constructs were secured to the impeller of a bioreactor and dynamically seeded (35 revolutions per minute) with single passaged OAF cells (5 x 10⁶ cells/construct) resuspended in DMEM with 20% FBS. To remove all unattached cells, all the media was replaced after 24 hrs using fresh DMEM with 20% FBS and changed every 2 to 3 days and cultured for 2 and 4 weeks. Ascorbic acid (100mg/mL; Sigma Chemical Co.) was added to the culture media starting 72 hrs after the seeding of AF cells.
3.2.2 Calcifying Cartilage Tissue

Articular chondrocytes, previously shown to be an appropriate cell source to regenerate the CEP, were isolated from the deep zone of bovine (6-9 months) metacarpal-carpal joints as previously described (Arana et al., 2010). Within 4hrs of death, tissue was aseptically excised, placed in Ham’s F12 (F12; HyClone, Logan, UT) with 5% FBS and 1% antibiotics. Cells were isolated by sequential digestion in 0.5% protease for 1 hr followed by 0.1% collagenase A for 12 hrs at 37°C. Cells were filtered, pelleted and re-suspended in F-12 (1 x10^6 cells) and cultured on Col II-coated sterile membrane inserts for 24 hrs (0.5mg/mL ascorbic acid in 0.1N acetic acid; Sigma Chemical Co., St. Louis, MO; 60 mm²; Millicell-CM²; Millipore Corp., Bedford, MA). After seeding, cartilaginous tissue was cultured for 2 weeks and the media changed every 2 to 3 days. Calcification-inducing media was added to cartilaginous tissue on day 3 consisting of DMEM supplemented with 20% FBS, 10mM β-glycerophosphate (Sigma Chemical Co.) and 100µg/mL ascorbic acid.

3.2.3 Annulus Fibrosus-Cartilage Interface Model

A model of the OAF-CEP interface was formed by placing a 2 week old multilayer AF tissue on top of 3 day old cartilage such that the nanofibres were aligned perpendicular to the cartilage surface (Figure 3.1). Co-culture conditions were optimized by assessing how AF and cartilage tissue integration was influenced by nutrient availability as well as the duration of independent tissue culture (Appendix C; Supplementary Figure 3.1). The interface model was statically co-cultured in DMEM supplemented with 20% FBS, 10mM β-glycerophosphate and 100µg/mL...
ascorbic acid for 1 week and then subject to mechanical stimulation. AF-cartilage models were compared to independently cultured AF and cartilage tissues grown for 4 and 2 weeks respectively. For histological and biochemical evaluation, the AF-cartilage interface was defined as the tissue which formed along the PU-ADO scaffold (extending 0.15mm from cartilage surface) and tissue which formed on the membrane insert (extending 0.5 mm from the outermost edge of the AF scaffold).

In select experiments, chondrocytes were labelled with a green fluorescent dye, carboxyfluorescein diacetate succinimidyl ester (CFDA SE 2.5ug/mL in PBS; Molecular Probes, Eugene, OR, USA) according to the manufacturer’s protocol. Previous studies have shown that cartilage formation was not affected by cell labelling (Ahmed et al., 2009; Theodoropoulos et al., 2011). Chondrocytes were incubated in CFDA (2.5ug/mL in PBS) for 15 minutes at 37°C, washed twice in F-12 (30 minutes each), re-suspended in F-12 supplemented with 5% FBS and then seeded onto membrane inserts. To evaluate the efficiency of labelling, an aliquot of cells was examined by fluorescent microscopy and > 95% of the cells were fluorescent.
Figure 3.1: Schematic of Cell Harvest and 3D Co-Culture of Annulus Fibrosus and Cartilage Tissue. Annulus fibrosus (AF) cells are isolated from bovine caudal discs and dynamically cultured (35rpm) for 2 weeks on aligned polycarbonate urethane (PU-ADO) scaffold wrapped around a teflon tube to form a 3-layer AF tissue construct. Calcifying cartilage was formed by culturing deep zone (D2) chondrocytes on membrane inserts. Multilayer AF tissue was placed on top of 3 day old cartilage and statically co-cultured for 2 weeks under mineralizing conditions.
MECHANICAL STIMULATION OF AF-CARTILAGE MODEL: After 1 week of co-culture, 3% (vol/wt) agarose disc was placed in the centre of an AF-cartilage model (Figure 3.2). Cyclic uniaxial compression (1 kPa, 0.5 Hz, 1800 cycles; MACH-1, Biomomentum, Montreal, Canada) was applied directly to the 3% agarose disc and the model interface was assessed 1 week post-stimulation (Waldman et al., 2006). Loading conditions were selected by assessing tissue integration following compression at different frequencies (0.1 Hz, 0.5 Hz, 1.0 Hz) and forces (1 kPa, 2 kPa) that have previously been shown to have an anabolic effect (Waldman et al., 2003 and 2006; Wang et al., 2007a; See et al., 2011; Appendix C; Supplementary Figure 3.2). To form the agarose disc, molten agarose (3% wt/vol; Sigma-Aldrich) was poured into a polystyrene dish to a final height of 3 mm, cooled up to 4 hrs and cut with a biopsy punch (3 mm). A 3% agarose disc was chosen for mechanical loading of the interface model as it remained structurally undamaged during all testing conditions and had compressive moduli within the same order of magnitude as the native NP (Cloyd et al., 2007; Appendix C; Supplementary Figure 3.3). Confocal microscopy was used to confirm the increased diameter of the 3% agarose disc subject to 1 kPa compression (Appendix C; Supplementary Figure 3.3).
Figure 3.2: Schematic of Mechanical Stimulation of Interface Model. To generate an in vitro model of the OAF-CEP interface, a multilayer AF tissue was placed on top of 3 day old cartilage and statically co-cultured under mineralizing conditions. Following 1 week of co-culture, a single does of cyclic compression (1 kPa, 0.5 Hz, 1800 cycles) was applied to an agarose disc placed at the center of the construct. Tissue was histologically and biochemically assessed 1 week post stimulation.
3.2.4 Biochemical Characterization

**DNA ASSAY:** *In vitro* tissues were digested with papain (40 mg/mL; 20mM ammonium acetate, 1mM ethylenediaminetetraacetic acid [EDTA] and 2mM dithiothreitol; Sigma Chemical Co.) for 48 hrs at 65°C as previously described (St. Pierre et al., 2012). Papain digests were then stored at -20°C until further analysis. DNA content was determined using Hoechst 33258 dye binding assay (Polysciences) and fluorometry as previously described (excitation wavelength: 365nm, emission wavelength: 458nm). A DNA standard curve was produced using thymus DNA.

**GLYCOSAMINOGLYCAN QUANTIFICATION:** Proteoglycan content was determined by quantifying the amount of sulfated glycosaminoglycans (GAG) in the papain-digested tissue spectrophotometrically using the dimethylmethylene blue dye binding assay (wavelength 525 nm) as previously described (St. Pierre et al., 2012). The standard curve was generated using bovine chondroitin sulfate (Sigma Chemical Co.). As the method of PU scaffold fabrication can result in variable thickness, and thus weight, the amount of GAG synthesized was expressed per microgram DNA.

**HYDROXY-PROLINE QUANTIFICATION:** To determine collagen content, aliquots of the papain digest were hydrolyzed in 6 N HCl for 18 hrs at 110°C as previously described (St. Pierre et al., 2012). The hydrolysate was neutralized (pH 7) with 5.7 N NaOH and hydroxyproline content was determined spectrophotometrically (wavelength 560nm) using the chloramine-T/Ehrlich’s reagent assay. The standard curve was generated using cis-hydroxy-L-proline (Sigma Chemical
Co.). The amount of hydroxyproline synthesized was expressed per microgram DNA to account for any potential variation in PU scaffold thickness.

**ALKALINE PHOSPHATE ACTIVITY**: To measure alkaline phosphate (ALP) activity, freshly isolated cells from *in vitro*-formed tissues were re-suspended in Buffer A (0.1% Triton X, 0.2 M Tris HCl pH7.4, and 45.7 mM NaCl) and freeze/thawed 3 times to lyse the cells (Nosikova et al., 2011). The solution was clarified by centrifugation at 2800 rpm for 20 min at 4 °C and then stored at -20 °C until used. ALP activity was determined by mixing aliquots of the extracts with 0.02 M solution of p-nitrophenol phosphate (Sigma Chemical Co.) in 0.2 M Tris buffer (pH 9.3) for 1 hr at 37 °C. The reaction was stopped by addition of 1.5 N NaOH and the test solution assayed spectrophotometrically (wavelength of 405 nm; Titertek Multiskan). A standard curve was generated using p-nitrophenol and results were normalized to DNA content.

### 3.2.5 Transmission Electron Microscopy and Electron Diffraction

To analyze calcium deposits in the *in vitro*-formed tissues, samples were fixed in 2% glutaraldehyde in 0.1M Sorenson phosphate buffer as previously described (Allan et al., 2007). After fixation, samples were rinsed in buffer, post-fixed in 0.1% osmium tetroxide and embedded in a Spurr epoxy resin. Sections (100nm) were cut (RMC MT6000 ultramicrotome, Leica, Toronto, ON) and viewed using a transmission electron microscope (FEI Tecnai 20, Portland, OR) at 100 kV. Selected area electron diffraction (SAED) patterns were generated using a 200 mm selected-area aperture (camera length of 890 mm) and calibrated by comparison with a gold diffraction
standard under the same conditions. Ring diameters were measured and d-spacings of diffraction patterns calculated.

3.2.6 Histological Characterization

**HUMAN FETAL TISSUE:** The human fetal OAF-CEP interface, which has yet to be fully characterized, was selected as a comparison for immature *in vitro*-formed tissues. Human fetal spines (< 15 weeks gestation, n=3) were harvested at the termination of pregnancy with informed parent consent in accordance with Mount Sinai Hospital research ethics board. Soft tissues were removed and spines were fixed in 10% buffered formalin up to 3 days, were embedded in OCT (Tissue-Tek, VWR), frozen, and cryosectioned into eight-micron-thick sections and processed as described below.

**HISTOLOGY:** *In vitro*-formed tissues were fixed in 10% buffered formalin up to 1 day, embedded in OCT (Tissue-Tek, VWR), frozen and eight-micron-thick sections cut. Native and *in vitro*-formed tissues were then stained with haematoxylin and eosin (H&E) to visualize cellular organization or toluidine blue and von Kossa to assess the presence of sulfated proteoglycans and mineral respectively. To determine the uniformity of tissue integration between the *in vitro*-formed AF/PU and cartilage tissues, each AF-cartilage construct was divided into 3 regions and then 10 representative sections were cut from each area (2 potential attachment sites/section= 20 potential attachment sites/region of the construct) resulting in 60 potential sites of attachment between AF and cartilage tissue evaluated in each construct. The number of continuous tissue
interface between AF lamella and cartilaginous tissue in each construct was then counted using phase contrast microscopy.

**ALKALINE PHOSPHATASE ACTIVITY:** Azo dye histochemical staining (Sigma Chemical Co.) was used to determine the location of ALPa. Undecalcified sections were incubated at 37°C in Azo dye solution (3 mg naphthol AS-MX phosphate, dissolved in 20mL dimethyl sulfoxide and 10 mg of Fast Blue BB salt and brought to a final volume of 10 mL with 0.2 M Tris buffer [pH 9.2]) for 15 minutes at 37°C. Tissues were washed in distilled water and counterstained with eosin, coverslipped and examined by light microscopy.

### 3.2.7 Immunohistochemistry

The *in vitro*-formed AF-cartilage interface was evaluated for the presence of collagens (type I, II, III and X) and proteoglycans (aggrecan, versican, decorin and biglycan) as these molecules were either differentially distributed in the fetal IVD or suggested to play a role in mineral or collagen fibril formation in the IVD (Gotz et al., 1997; Nerlich et al., 1998; Melrose 2002; Nosikova et al., 2012) Tissues were embedded in OCT and stored at -20°C until sectioning. To detect Col X and Col II reactivity, sections were digested with pepsin (2.5mg/mL of phosphate-buffered saline (PBS) at pH 2; Sigma Aldrich P7012-1G; ≥2,500 units/mg solid) for 30 minutes at RT. For co-staining of Col II with Col I, Col III, samples were sequentially digested with pepsin (2.5mg/mL in phosphate-buffered saline (PBS) lowered to pH 2 with HCl; Sigma Aldrich P7012-1G; ≥2,500 units activity/mg solid) for 10 min at RT, then trypsin (2.5 mg/mL Tris-buffered saline [TBS] Sigma Aldrich T7409; 1,000-2,000 units activity/mg solid) for 30 min at RT and finally
hyaluronidase (25mg/mL PBS; Sigma Aldrich H3506; 400–1,000 units activity/mg solid) for 30 min at 37°C. Sections were rinsed 3 times with PBS (10 min each) between digestions. Sections for Col I, Col II, Col III and Col X were then blocked with 20% goat serum for 1 hr at RT (Invitrogen 10000C). Co-staining Col I and aggrecan or Col II and versican, decorin, and biglycan, followed the sequential digestion protocol outlined above with a final 30 minute digestion in 0.25μM of chondrotinase ABC (in 0.1M Tris pH 8, 0.05M sodium acetate) at 37°C. Sections were blocked with 1% BSA (v/v) in PBS (Sigma Life Sciences A7906-1004) for 1 hr at RT. Treated sections were incubated with antibodies reactive with Col I (1:500 dilution; polyclonal T59103R; Meridian Life Sciences) or Col II (1:500; monoclonal, clone 6B3; Thermo Scientific, Labvision, Freemont, CA, USA; TBS with 1% Triton X-100), Col III (1:1000; Abcam AB7778), Col X (1:2000; Sigma Aldrich 7974) or aggrecan (1:200; Invitrogen 969D4D11), versican (0.66μg/mL; Novus Biologicals 16770002), biglycan (1:500; Dr. P J Roughley, Genetics Unit, Shriners Hospital for Children, McGill University, Montreal, Canada), or decorin (4μg/mL; Santa Cruz sc-22753) overnight at 4°C and then rinsed 5 times in PBS. Primary antibodies have been previously shown to be reactive and have appropriate distributions in bovine and human IVD tissues (Nosikova et al., 2012; Iu et al., 2014; Chapter 2). To detect Col II reactivity, samples were incubated with Alexa Fluor® 594 conjugated goat anti-mouse and Col I and III reactivity was detected with Alexa Fluor® 488 conjugated-goat anti-rabbit IgG (both 1:1000; Invitrogen, Eugene, OR, USA) in darkness for 1 hr at RT. Col X reactivity was detected by 1 hr incubation with Alexa Fluor® 594 conjugated goat anti-mouse IgM (1:1000 dilution; Invitrogen, Eugene, OR, USA). Versican, decorin and biglycan reactivity were detected by incubation with Alexa Fluor® 488 conjugated-goat anti-rabbit IgG antibody (1:250 dilution; Invitrogen, Eugene, OR, USA) for 1 hr at RT. All samples were washed 3 times in PBS (10 min each) in PBS. To visualize nuclei, the sections were
terminally stained with 4’,6-diamidino-2-phenylindole (DAPI; 4µg/mL; Pierce Biotechnology, Rockford, IL, USA) for 5 min at RT and then washed three times in PBS (10 min each). Sections were coverslipped and images of stained sections were visualized and captured with a Leica Microsystems (type 090-135.002) For negative controls, primary antibody was replaced with either IgG or IgM as appropriate. Bovine articular cartilage or tendon served as a positive control.

3.2.8 Statistical Analysis

All test conditions, except mechanical testing, were done in triplicate where as the latter was done in duplicate. Each independent experiment was repeated at least three times. The results from these experiments were combined and expressed as a mean ± standard deviation and analyzed using Student’s t-test when comparing 2 conditions with the assumption that the data were normally distributed and of equal variance. Significance was assigned at p-values < 0.05.

3.3 Results

3.3.1 Independently Cultured Tissues Model Aspects of Native Annulus Fibrosus and Cartilage Endplate

A cohesive multilayered AF tissue was formed with cells oriented parallel to scaffold nanofibres (Figure 3.3). Col I, versican and biglycan were seen throughout the multilayered construct while Col III, aggrecan and decorin were present between scaffold layers. The AF tissue contained no ALPα, mineral or Col II (Figure 3.3).
Cartilaginous tissue was rich in proteoglycans and contained von Kossa positive deposits adjacent to the membrane insert, characterized by electron diffraction as a poorly crystalline apatite (Figure 3.4). ALPα was visible throughout the mineralized and non-mineralized regions of the tissue (Figure 3.4). Col II and aggrecan were diffusely present throughout the ECM while Col X and biglycan showed a localized distribution to the inferior aspect of the tissue (Figure 3.4). There was very little versican detected. Col I, Col III, versican and decorin were present around cells. These findings are consistent with previous studies in developing articular cartilage (Morrision et al., 1996; Yang et al., 2012)
Figure 3.3: Characterization of Multilayered Annulus Fibrosus Tissue. (A) Haematoxylin and eosin stained tissue formed on aligned polycarbonate urethane scaffolds (S) after four weeks. In vitro-formed AF tissue (box) stained for (B) proteoglycan (purple) and mineral (black) content seen by toluidine blue and von Kossa staining, (C) alkaline phosphatase activity (blue) using azo histochemical dye (eosin counterstain) or (D-K) immunostained (green) for (D) collagen type I, (E) collagen type II, (F) collagen type III, (G) collagen type X, (H) versican (I) biglycan (J) decorin or (K) aggrecan. Positive controls for (L) von Kossa staining in the IVD and (M) ALPAl (N) collagen type II (O) collagen type X in bovine articular cartilage. (P) Negative control irradiated Fn coated PU-ADO scaffold. Section counterstained with DAPI (blue) to visualize nuclei. Negative control for each stain seen in inset (20X magnification). Images are representative of one experiment done in triplicate and repeated three times. Scale bars represent 100 μm.
Figure 3.4: Characterization of Calcifying Cartilage. (A) Toulidine blue (purple) and von Kossa (black) staining of 2 week old tissue. *In vitro*-formed cartilage tissue stained for (B) alkaline phosphatase activity (blue) as seen by azo histochemical dye (eosin counterstain) or (C-J) immunostained for collagen type I, (D) collagen type II, (E) collagen type III, (F) collagen type X, (G) versican (H) biglycan (I) decorin or (J) aggrecan. Section counterstained with DAPI (blue) to visualize nuclei. (K) Electron diffraction pattern obtained from the crystals present in the *in vitro*-formed calcifying cartilage. Negative controls for each stain seen in the inset (20x magnification). Scale bars represent 100 μm.
3.3.2 AF-Cartilage Model Displays Cellular and Matrix Organization

The AF-cartilage interface model displayed early signs of cellular and ECM organization. AF cells were observed between scaffold layers (Figure 3.5). CFDA-labelled chondrocytes were present throughout the cartilage and were seen in the inferior aspect of the multilayered AF tissue (Figure 3.5). The cartilaginous tissue was rich in proteoglycans and von Kossa stained deposits throughout were present non-uniformly throughout the cartilaginous region. As such, the absence of mineral from certain images is an artifact of sampling. Electron diffraction identified the mineral as a poorly crystalline apatite (Figure 3.5). ALPa was only seen in the cartilaginous region of the interface model, however, not all ALPa expressing cells appeared to be associated with mineral deposits (Figure 3.5).

Immunohistochemistry revealed Col I, Col II, Col III, versican and decorin staining at the interface of AF and cartilage (Figure 3.6). Col II was seen at the inferior aspect of the scaffold. Diffuse Col II and aggrecan was present throughout the cartilaginous region whereas Col I, Col III and decorin were observed near cells. Punctate versican and biglycan staining were observed throughout the calcifying layer of cartilage. No Col X was observed in the model interface.
Figure 3.5: Characterization of Annulus Fibrosus-Cartilage Interface Model. (A) Schematic of interface model formed using aligned polycarbonate urethane scaffolds (S). (B) Cells (arrow) at interface visualized by haematoxylin and eosin staining and (C) proteoglycan and mineral content (arrow head) seen by toluidine blue (blue) and von Kossa staining (black). Rectangle identifies region of interface assessed by transmission electron microscopy and electron diffraction. (D) Alkaline phosphatase activity (blue) detected by azo histochemical dye staining (eosin counterstain). (E) Distribution of CFDA labelled chondrocytes (green) in collagen type II (red) immunolabelled tissue. DAPI staining (blue) of nuclei. (F) Electron diffraction pattern obtained from the crystals present in the interface model. Negative control seen in inset (20x magnification). Images are representative of one experiment done in triplicate and repeated three times. Scale bars represent 250 μm.
Figure 3.6: Extracellular Matrix of Anrulus Fibrosus-Cartilage Interface Model. (A) Haematoxylin and eosin stained interface formed using aligned nanofibrous polycarbonate urethane scaffolds (S) as seen by light microscopy. Immunostaining of interface (box). (B-G) Co-staining of collagen type II (red) with (B) collagen type I (green), (C) collagen type III (green), (D) collagen type X (green), (E) versican (green), (F) biglycan (green) or (G) decorin (green). (H) Collagen type I (green) and aggrecan (red). Negative control seen in inset (40x magnification) and (I) collagen type X staining in bovine articular cartilage as a positive control. Section counterstained with DAPI (blue) to visualize nuclei. Images are representative of one experiment done in triplicate and repeated three times. Scale bars represent 100 μm.
3.3.3 Fetal Interface Displays Cellular and Matrix Organization

By 14 weeks, AF cells were arranged into parallel layers that merged with the proteoglycan-rich cartilage to form a model of the OAF-CEP interface that contained both AF cells and chondrocytes (Figure 3.7). The OAF lamella stained diffusely for Col I, Col III and versican whereas biglycan and decorin were localized around AF cells. Col II was absent from the OAF but present in the aggrecan rich interface and VB. Cells at the interface were surrounded by Col I, Col III, versican, biglycan and decorinCol X, ALPa and mineral were absent from the developing interface (Figure 3.7 and Supplementary Figure 3.4). Enlarged images of collagens and proteoglycan distribution can be found in Appendix C (Supplementary Figures 3.5 to 3.10).
Figure 3.7: Collagen and Proteoglycan Distribution in the Human Fetal Outer Annulus Fibrosus Interface with the Cartilaginous Vertebral Body. (A) Haematoxylin and eosin staining of the interface (*) seen by light microscopy. (B-G) Co-staining of the interface (box) with collagen type II (red) with (B) collagen type I (green), (C) collagen type III (green), (D) collagen type X (green), (E) versican (green), (F) biglycan (green) or (G) decorin (green). (H) Collagen type I (green) and aggrecan (red). Negative controls seen in inset (40x magnification). (I) Positive control for collagen type X in bovine cartilage. Section counterstained with DAPI (blue) to visualize nuclei. Cellular orientation (arrow), lamellar organization (arrow head). Scale bars represent 100 μm. Images are representative of one experiment done in triplicate and repeated three times.
3.3.4 Mechanically Loaded *In vitro* Interface Shows Changes

A single dose of cyclic compression (1kPa, 0.5Hz, 1800 cycles) significantly increased the uniformity of tissue integration between the AF/PU and cartilage tissues within each construct when compared to unstimulated controls (Stimulated: 56.8 ± 3.49 vs Unstimulated 47.8 ± 6.7 sites of attachment/construct; N=3; n=2/experiment; p=0.041).

CFDA labelled chondrocytes were present in the inferior aspect of the scaffold of stimulated constructs (Figure 3.8). Col X was present in the cartilaginous region of the loaded interface model (Figure 3.9). Mechanically stimulated constructs contained significantly more proteoglycans than unstimulated constructs (loaded: 31.3 ± 2.8 vs unloaded: 16.9 ± 2.1 µg s-GAG/ µg DNA; N=3; n=2/experiment; p=0.0017; Figure 3.10). Interestingly, compared to unloaded AF tissue, mechanical stimulation significantly increased the amount of DNA (loaded: 12.7 ± 2.3 vs unloaded: 7.6 ± 0.1 µg DNA N=3; n=2/experiment; p=0.0001) and collagen (loaded: 7.9 ± 1.8 vs unloaded: 2.6 ± 1.0 µg OH-Pro/ µg DNA N=3; n=2/experiment; p=0.0041) present in the tissue (Figure 3.10).
Figure 3.8: Mechanically Stimulated Annulus Fibrosus-Cartilage Interface Model. (A) Schematic of a dynamically compressed model (1 kPa, 0.5 Hz, 1800 cycles) formed using aligned nanofibrous polycarbonate urethane scaffolds (S). (B) Cells (arrow) in model seen by haematoxylin and eosin staining. Separation of cartilage is a result of tissue processing. (C) Proteoglycan and mineral (arrowhead) content seen by toluidine blue (blue) and von Kossa (black) staining. Rectangle identifies region of interface assessed by transmission electron microscopy and electron diffraction. (D) Alkaline phosphatase activity (blue) detected by azo histochemical dye staining (eosin counterstain). (E) Distribution of CFDA labelled chondrocytes (green) in collagen type II (red) immunolabelled tissue. DAPI staining (blue) of nuclei. (F) Electron diffraction pattern obtained from the crystals present in the stimulated interface model. Negative control seen in inset (20x magnification). Images are representative of one experiment done in triplicate and repeated three times. Scale bars represent 250 μm.
Figure 3.9: Extracellular Matrix of Mechanically Stimulated Annulus Fibrosus-Cartilage Interface Model. (A) Haematoxylin and eosin stained interface formed using aligned nanofibrous polycarbonate urethane scaffold (S) following mechanical stimulation (1800 cycles, 1.0 kPa, 0.5Hz) as seen by light microscopy. Immunolabel of interface (box). (B-G) Co-staining of collagen type II (red) and (B) collagen type I (green), (C) collagen type III (green), (D) collagen type X (green), (E) versican (green), (F) biglycan (green) or (G) decorin (green). (H) Collagen type I (green) and aggrecan (red). Section counterstained with DAPI (blue) to visualize nuclei. Negative control seen at inset (40X magnification). Images are representative of one experiment done in triplicate and repeated three times. Scale bars represent 100 μm.
Figure 3.10: Biochemical Characterization of In Vitro-Formed Tissue Following Dynamic Compression. (A) Glycosaminoglycans (GAG) (B), hydroxyproline (OH-Pro), (C) DNA content and (D) alkaline phosphatase activity (ALP) of stimulated in vitro-formed tissue compared to unstimulated controls. A significant difference (*) in mechanically loaded compared to unloaded tissues (Pair wise t-test, p < 0.05). a* loaded AF contained significantly more GAG than unloaded AF; b* Loaded interface contains more GAG than unloaded interface; c* Loaded AF contained significantly more OH-Pro than unloaded AF; d* loaded AF contained significantly more DNA than unloaded AF. N=3; n=2/experiment.
3.4 Discussion

A model of the OAF-CEP interface was formed through static contact co-culture between in vitro-formed multilayered AF tissue and calcifying cartilage tissue. The appropriateness of the resulting AF-cartilage model was then assessed by comparing the histological characteristics of the in vitro-formed tissue with that of the human fetal OAF-CEP interface. The tissues achieved integration to form a fibrocartilage-like tissue that showed similar protein (Col I, Col II and Col III) and proteoglycan (aggrecan, versican, biglycan and decorin) composition as observed at the human fetal interface. Furthermore, the interface was responsive to mechanical stimulus suggesting the fibrocartilage-like tissue is an appropriate model to further explore how in vitro-formed IVD tissues may develop and remodel to achieve stable biological fixation. The ECM components reported here are consistent with previous reports of Col I, aggrecan and versican deposition by OAF cells cultured on PU-ADO scaffolds (Attia et al., 2011; Iu et al., 2014). Deep zone articular chondrocytes cultured on membrane inserts have also been shown to express mineralization markers such as Col X, osteopontin, ALP, in addition to aggrecan, Col I and Col II (Sun and Kandel 1999).

The ECM organization and composition of the fetal OAF-CEP interface resembled, but were not identical to that reported at the developing interface described in rats and humans (Bianco et al., 1990; Rufai et al., 1995; Aigner et al., 1998; Nerlich et al., 1998; Hayes et al., 2001 and 2011; Melrose 2001; Smith et al., 2009). Similar to previous studies, ALPa, Col X and mineral were not present in the prenatal interface (Bianco et al., 1990; Paietta et al., 2013). In this study, the differential distribution of Col II between the OAF and CEP was used to clarify if previously described ECM proteins (Col I, Col III, versican, decorin and biglycan) extended beyond the OAF.
into the OAF-CEP interface. For example, studies in prenatal rat demonstrated Col III and Col I in the OAF by E21. However without definition of the CEP, it remained unclear from the low magnification images provided if these molecules extended from the OAF into the OAF-CEP interface (Hayes et al., 2001). In the current study, co-localization of Col II with these proteins clearly demonstrated Col I, Col III, versican, decorin and biglycan were present, although each to varying extents, in the 14 week OAF-CEP interface. Furthermore, these molecules differed in distribution between the OAF and interface which demonstrates the enabling of differential gradients of ECM in the in vitro-formed tissues which may in turn lead to different functions. For example, studies suggest that biglycan may play a role in collagen fibrillogenesis in the AF while in cartilage tissues biglycan has been shown to modulate matrix mineralization (Xu et al., 1998; Furukawa et al., 2009; Wang et al., 2010). Thus, this study is the first step towards exploring the potential regional heterogeneity of the fetal CEP and serves as a benchmark to assess the suitability of the interface formed between in vitro-formed AF and cartilage tissues following 3-D contact co-culture.

All ECM components observed in the fetal OAF-CEP interface were present in the AF-cartilage interface suggesting that in vitro-formed IVD-like tissues have the potential to attach and form an organized and heterogenous tissues upon co-culture. The similarity in ECM composition was anticipated to contribute to the function of the AF-cartilage model. Specifically Col I, a major constituent of the OAF, may contribute to the tensile properties of the model (Burgeson and Nimni 1992; Elliott and Setton 2001; Hayes et al., 2001). Col I and Col III were also detected around cells in the fetal OAF-CEP interface and cartilaginous region of the model. While the role of Col I in this region is unclear, Col I mRNA expression has also been detected in the unmineralized
fibrocartilaginous zone in a rat model of developing tendon-to-bone insertion up to day 21 (Galatz et al., 2007). Col I mRNA has been extracted from the total RNA of epiphyseal cartilage and has been detected in chondrocytes during in vitro chondrogenesis (Vuorio et al., 1984; Elima et al., 1985; Kravis et al., 1985; Kosher et al., 1986) Other studies have reported pericellular localization Col I in the hypertropic zones of the articular cartilage as well as Col III in the subchondral plate (Morrison et al., 1996). Through its ability to form rapid intermolecular cross-links and mediate Col I fibre assembly, Col III may aid early tissue development in the bioengineered tissue (Fleischmajer et al., 1983; Burgeson and Nimni 1992; Rucklidge et al., 1992; Hayes et al., 2001).

The interface model also contained proteoglycans similar to those present in the fetal interface. It has been proposed that aggrecan plays a role in water retention and collagen fibrillogenesis in the developmental spinal tissues (Kobayakawa et al., 1985; Schmidt et al., 1990; Watanabe and Yamada 1997 and 2002; Smith et al., 2009). Age-associated spinal degeneration observed in mice producing a truncated aggrecan molecule was attributed to abnormal formation and packing of collagen fibrils (Watanabe and Yamada 1997). Similar distributions of versican and aggrecan are observed during rat development suggesting versican may also facilitate ECM organization and water binding capacity of the model interface (Hardingham and Muir 1974; Melrose et al., 2008 and 2001, Smith et al., 2009; Choocheep et al., 2012; Nagchowdhuri et al., 2012). Versican has also been shown to regulate cell adhesion and proliferation as well as interacting with signaling proteins to modulate cellular responses (Yang et al., 1999 and 2003; Hirose et al., 2001).
Previous studies suggest biglycan and decorin, present in the AF-cartilage model and fetal interface, may play a role in mineralization and the formation and maintenance of the collagenous matrix (Waddington et al., 2003; Geng et al., 2006; Mochida et al., 2009; Kalamajski and Oldberg 2010; Hayes et al., 2011). The most noted phenotype in a decorin knock-out mouse model is delayed wound healing, skin fragility and collagen fibre abnormalities (Danielson et al., 1997; Jarvelainen et al., 2006). Biglycan knock-out mice are characterized by an irregularity in collagen fibre size and shape and are associated with accelerated IVD degeneration (Furukawa et al., 2009).

Both biglycan and decorin have been extracted from the mineralized matrices of bone and have the capacity to bind calcium and interact with hydroxyapatite (Waddington and Embery 1991; Fujisawa et al., 1991; Embery et al., 1998; Sugars et al., 2003). Biglycan has also been shown to influence bone formation in mice and modulate osteoblast differentiation, matrix mineralization in vitro (Xu et al., 1998; Wallace et al., 2006; Wang et al., 2010). Thus, biglycan and decorin may facilitate the formation of mineral throughout the cartilaginous region of the interface model.

Based on observations in ligament/tendon-to-bone attachments and in vitro-formed cartilage, mineral is anticipated to increase the stability of the AF-cartilage interface during early stages of tissue deposition and organization (Thomopoulos et al., 2003; Genin et al., 2009; St-Pierre et al., 2012).

Contact co-culture of AF cells and chondrocytes produced changes in the interface that were different from AF and cartilage tissues grown alone. After co-culture, the scaffold immediately adjacent to the cartilage contained Col I, Col II and a mixed population of CFDA positive and negative cells. It is unclear from these data if Col II is deposited by chondrocytes or OAF cells present in this region. Previous studies have shown that OAF cells cultured on PU-ADO scaffold
express Col II mRNA and deep zone chondrocytes cultured in calcification-inducing media produce a Col II-rich calcifying matrix (Gan and Kandel, 2007; St. Pierre et al., 2012; Lu et al., 2014). This mixed Col I/Col II region may represent the formation of an early precursor to a potential fibrocartilaginous-like region between the in vitro-formed AF and cartilage tissues. Similarly, others have shown that tri-phasic scaffolds support the migration of chondrocytes and osteoblasts into an intervening region where their interaction resulted in a calcifying fibrocartilage-like matrix containing Col I and Col II (Spalazzi et al., 2008). In a tri-lineage co-culture system (osteoblasts–bone marrow stem cell (BMSc)-fibroblasts), BMSc that interacted with osteoblasts expressed osteoblasts gene markers Runx2 and osteonectin (He et al., 2012). In contrast, BMSC cells that interacted with fibroblasts expressed the transcription factor Sox9 and aggrecan (He et al., 2012).

Co-culture also appeared to disrupt the discrete layer of mineral observed in cartilage tissues grown alone. While little is known about the mechanisms regulating mineral patterning, both chondrocytes and AF cells have shown the capacity for mineral formation. In the mature IVD, Col X and ALPα are observed in the CEP while only ALPα is present in AF tissue adjacent to the CEP. Furthermore, subcutaneous implantation of AF tissue in nude mice increased Col II, Col X and osteocalcin expression of AF cells (Nosikova et al., 2012; Jin et al., 2014). OAF cells can also mineralize their ECM in 2-D culture and have been shown to express ALP, runt-related transcription factor 2, Runx2, osteopontin and BMP2 genes (Nosikova et al., 2012; Jin et al., 2014). Downstream targets of RUNT-2 include Col X and ALPα, both of which are known to be involved in the calcification process (Roach 1999; Pfander et al., 2001; Kamekura et al., 2006; Madiraju et al, 2013). This and other studies have shown that chondrocytes cultured on membrane inserts
express mineralization markers such as Col X, osteopontin and ALP (Sun and Kandel 1999). These results suggest that both AF cells and chondrocytes may, under appropriate stimuli, mineralize their ECM. Interestingly, it also appeared that AF cell-chondrocyte co-culture decreased the amount of von kossa stained deposits visible throughout the histological samples. Although these observations must be confirmed quantitatively, mineral attenuation has been demonstrated in cartilaginous tissue formed by deep zone chondrocytes following non contact co-culture with chondrocytes from the superficial zone of articular cartilage (Bromand et al., 2014). Similarly, differentiation of bone marrow-derived mesenchymal stem cells in media conditioned by a parallel culture of human articular chondrocytes suppressed alkaline phosphatase activity and reduced the expression of Col X and Col I relative to Col II (Jiang et al., 2008). In a 2-D osteoblast-fibroblast non-contact co-culture system, osteoblast-induced mineralization decreased while fibroblast-induced mineralization increased (Wang et al., 2007b).

Cell-cell interactions have been shown to influence the stability of osteochondral composite tissues and may likewise impact tissue integration between in vitro-formed AF and cartilage tissues (Schaefer et al., 2000; Gao et al., 2001). Superior tissue integration was observed between bovine articular chondrocytes seeded on polyglycolic acid meshes and periosteal cells on PLGA/polyethylene glycol foams when tissues were stitched together 1 week after seeding compared to 4 weeks. Likewise, integration between AF and cartilage tissues in the current study improved when AF/PU tissue was co-cultured with 3 day old in vitro-formed cartilage compared to AF tissue co-cultured with 2 week old cartilage.

While less is known about the role of mechanobiology during IVD development, there is evidence that mechanical forces induced by muscle contraction guide joint patterning and development.
Underdeveloped, brittle and misshapen bone as well as abnormal joint cavitation are observed in animal models where muscle contractions are altered using neuromuscular blocking agents (Drachman and Sokoloff et al., 1966; Persson 1983; Osborne et al., 2002; Nowlan et al., 2008), surgery (Drachman and Sokoloff et al., 1966), immobilization (Drachman and Sokoloff et al., 1966; Ruano-Gil et al., 1978; Persson 1983; Hogg and Hosseini 1992; Mikic et al., 2000). Muscle inactivation has also been shown to influence collagen fibre alignment and crystal morphology of tendon-to-bone attachment sites (Kim et al., 2009 and 2010; Schwartz et al., 2013).

The current study demonstrated that immature in vitro-formed AF and cartilage tissues were sensitive to mechanical stimuli and that small amounts of micromotion (0.8% strain) can influence ECM accumulation, organization and composition (Figure 3.9). Dynamic compression significantly increased proteoglycan content in the model interface. Others have shown that dynamic compression of whole organ rabbit disc/endplate IVD explants (0.5-1MPa; 1 Hz; 6 h) similarly resulted in anabolic changes with significant increase in gene expression for aggrecan, Col I and Col II in the AF and CEP (Wang et al., 2007a). However, interpretation of the data is limited given that the mechanical loading system used in this study was unable to quantify the type and magnitude of forces experienced at the interface between in vitro-formed AF and cartilage tissues. Work is ongoing to optimize loading conditions and to clarify the mechanisms governing proteoglycan accumulation. Others have shown that mechanically induced ECM accumulation in bioengineered cartilage was influenced by integrin-mediated cell spreading where inhibition of α5β1 integrin decreased matrix accumulation (Spiteri et al., 2010).
ALPa was observed in the inferior aspect of the non-mineralized multilayered AF tissue and Col X was observed in the cartilaginous region of the dynamically compressed AF-cartilage interface. While not present in the fetal OAF-CEP interface, Col X expression, mineral and ALPa have been previously described to be present in the healthy adolescent bovine interface (Nosikova et al., 2013; Chapter 2). Others have shown that shear stress increased in ALPa as well as the expression of odontogenic-related mRNAs amelogenin, bone sialoprotein and vimentin protein in bioengineered dental constructs (Honda et al., 2006). In tendon-to-bone attachments, Col X is expressed by hypertrophic chondrocytes during postnatal maturation, which coincides with bone mineralization and increased mechanical loads. (Fujioka et al., 1997; Galatz 2006). As mineral was not quantified in the present study, it is unclear if increased ALPa and Col X protein expression in the model interface was similarly associated with increased mineral formation and is an area for future investigation. Thus, future studies should aim to quantify the amount of mineral formed in the model and correlation mineral formation with Col X protein expression and ALPa. Interestingly, not all ALPa expressing cells in the in vitro-formed AF-cartilage interface and cartilage controls appeared to be associated with mineral deposits. These data suggest that ALP may play another, yet undefined role at the in vitro interface. These observations are supported by previous studies in the native IVD reporting ALPa activity in non-mineralized AF tissue adjacent to the OAF-CEP interface (Rutges et al., 2010; Melrose et al., 2009; Nosikova et al., 2012). Interestingly, ALP has also been shown to play a role in cell cycle progression in addition to cell adhesion and differentiation (Kim et al., 1991; Hui 1993; Anderson et al., 2004). Thus, the role of ALP at the OAF-CEP interface remains unclear and requires further study.
While this initial model has value, future strategies have been identified for improving it. Specifically, the 3-layer AF assembly does not have the full thickness (15-25 lamellae) or the cross-ply structure of the native AF which is believed to be essential for this tissue’s inherent mechanical properties (Elliott and Setton 2001). Thus, the current method of AF/scaffold assembly may have limited our ability to achieve the full extent of integration needed between in vitro-formed AF and cartilage tissues in order to form regenerated discs intended for implantation. It would also be of interest to characterize collagen fibre alignment of the in vitro model as the orientation and dual insertion of OAF fibres into the CEP and VB have also been highlighted as being a contributor to the mechanical properties of the native OAF-CEP interface (Paietta et al., 2013).

The importance of the mechanical stimulation is also highlighted in the literature for nutrient exchange and is a critical area for future study that has been identified by several groups and will become increasingly important as more tissues such as the IVD are assembled (Nerurkar et al., 2010; Bhattacharjee et al., 2014; Martin et al., 2014). Several studies have developed bioreactors that can mechanically stimulate and maintain IVD explants up to 4 months which may be adapted to the co-culture of the bioengineered composite IVD tissues (Haglund et al., 2011; Gawri et al., 2011; Paul et al., 2012; Chan et al., 2013).

Notwithstanding these limitations, this study developed a novel co-culture method to study the integration of IVD-like tissue in vitro. The resulting AF-cartilage interface is an appropriate model of the native OAF-CEP interface as it shows composition and organization that mimics some aspects of the native ECM and responds to mechanical loading. Thus, this model may be suitable
to investigate factors such as growth factors and culture conditions that will strengthen this interface to enhance the success of a biological disc replacement.

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Chapter Four: Summary & Future Directions
4.1 Summary

The aim of the present study was to develop an *in vitro* model of the OAF-CEP interface. First, the biochemical composition and ECM organization of the bovine and human fetal interfaces were characterized. The tensile properties of the bovine interface was also determined. Second, the growth of a 3-layered AF tissue on aligned PU-ADO scaffold was optimized in a spinner flask culture system at speeds shown to promote AF cell attachment and ECM accumulation (Turner 2011; Appendix E; Supplementary Figure 4.1). Third, a 3D co-culture system was developed to investigate if AF and cartilage tissues would integrate to create an *in vitro* model of the OAF-CEP interface. Finally, the responsiveness of the model interface to compression was assessed. The combined results demonstrated that cellular and ECM organization of the native interface occurred by 14 weeks in humans but interestingly, no mineral was present. Co-culture of AF cells and chondrocytes produced an ECM that shared some aspects of the human native interface and differed from independently cultured tissues. The AF-cartilage model was responsive to mechanical stimulus, as a single dose of cyclic compression increased Col X protein expression and proteoglycan content.

4.2 Discussion

4.2.1 Native Interface

The method of co-staining used in this study highlighted the complex cellular and ECM organization present in the bovine (Chapter 2) and human fetal (Chapter 3) interface. In the bovine interface, some ECM components were diffusely present throughout the entire interface (Col II, aggrecan), localized around cells (Col I, III, versican, biglycan, decorin) and others were concentrated to specific regions (mineral, Col X). The interface was stronger in uniaxial tension
than the multilamellar OAF and cartilage growth plate. As previously discussed (Chapter 2), structural features, such as the gradation of ECM components, may contribute to the mechanical properties of the interface as is observed in tendon/ligament-to-bone interfaces (Benjamin et al., 1998; Benjamin et al., 2002; Thomopoulos et al., 2002 and 2006; Genin et al., 2009). The characteristics described herein distinguish the OAF-CEP from other soft tissue-to-bone interfaces and is expected to contribute to the functionality of the interface in vivo.

The most notable difference between human fetal and adolescent bovine disc tissue samples was the absence of calcification markers at the prenatal interface (Supplementary Figure 3.4 and Figure 2.4 and 2.6). Calcification has been reported in the postnatal interface at 1 week in mice (Higuchi et al., 1982) and in 8 weeks postnatal in humans (Paietta et al., 2013). Mineral is an integral part of the healthy OAF-CEP interface but can be associated with degeneration when present in the AF and NP (Resnick, 1985; Roughley 2004; Melrose et al., 2009; Nosikova et al., 2012; Madiraju et al., 2013; Paietta et al., 2013; Chapter 2). Currently, little is known about regulation of mineralization at the OAF-CEP from development to maturation.

### 4.2.2 3-D Co-Culture of Annulus Fibrosus Cells and Chondrocytes

Currently, the mechanisms guiding tissue patterning during interface regeneration are unknown. In Chapter 3, an AF-chondrocyte 3-D co-culture system was developed to assess if integration between in vitro-formed AF and cartilage tissues would occur and if it would affect matrix organization. The appropriateness of the resulting AF-cartilage model was then assessed by comparing the biochemical and/or histological characteristics of the in vitro tissue with that of the human fetal (Chapter 3) and adolescent bovine (Chapter 2) OAF-CEP interfaces.
AF and cartilage tissues achieved a level of integration and formed a fibrocartilaginous-like tissue. Co-culture of AF cell-seeded PU scaffolds with chondrocytes produced a tissue that was different from when AF (Figure 3.3) and cartilage (Figure 3.4) tissues were grown alone. After co-culture, Col II was accumulated in AF tissue and Col X was absent from the cartilaginous region of the interface (Figure 3.6). Little is known about AF cell-chondrocyte interactions in vitro and in vivo. As previously discussed, cell-cell interactions may contribute to the formation of a calcifying chondroid interface under appropriate conditions (Chapter 3). While OAF cells cultured on PU-ADO scaffolds have been shown to express Col II mRNA, the current study confirms previous reports that Col II is not deposited on the scaffold (Atti et al., 2011; Iu et al., 2014; Figure 3.3). However, Col II is detected in AF tissue following 2 weeks of contact co-culture with chondrocytes (Figure 3.6). The presence of Col II may represent the initial formation of a region within the AF-cartilage interface model containing both Col I and Col II which appropriately models the composition of native fetal and adolescent interface. Alternatively, it may represent chondrocytes growing up the AF/scaffold as fluorescent labelled CFDA positive cells were shown to be present along the scaffold immediately adjacent to the cartilage tissue (Figure 3.5e and 3.8e). Other studies have shown that fluorescently-labelled chondrocytes can migrate from tissue-engineered cartilage (up to 1.5mm) into host articular cartilage 8 weeks after implantation (Theodoropoulos et al., 2011).

Similarly, AF cell-chondrocyte co-culture influenced tissue mineralization (Chapter 3). The discrete layer of mineral observed when cartilage tissues were grown alone was not observed following co-culture (Figure 3.4 and Figure 3.5). Changes in ECM composition and organization
following 3-D contact co-culture of AF cells and chondrocytes may be mediated by the secretion of growth factors, such as TGF-β, which have been shown to mediate tissue patterning during IVD development (Reviewed Chapter 1).

### 4.2.3 Mechanical Stimulation of Model Interface

The mechanical loading protocol described in Chapter 3 improved integration of AF and cartilage tissues and increased proteoglycan retention at the interface. As discussed in Chapter 3, dynamic compression of whole organ rabbit disc/endplate IVD explants (0.5-1MPa; 1 Hz; 6 h) similarly resulted in an increase in proteoglycan aggrecan, in addition to Col I and Col II, in the CEP (Wang et al., 2007). While the mechanism by which this occurs was not explored in this study, the cyclic loading may have assisted the transport of large soluble factors across the tissue in addition to applying direct and indirect stimulus to disc cells (Chan et al., 2011).

Dynamically compressed constructs also displayed ALPα (Figure 3.8) and Col X (Figure 3.9) which are structural features of the healthy adolescent bovine OAF-CEP interface (Figure 2.4 and 2.6). Col X is often associated with mineralization as a result of its spatiotemporal association with hypertrophic chondrocytes during osteochondral ossification and through its interaction with annexin V to mediate calcium influx into matrix vesicles (Gress and Jacenko 2000; Kirsch et al., 2000). However, Col X has also been proposed to function as a stabilizing molecule during skeletal development, through interactions with Col II and proteoglycans, as knock-out studies in mice only demonstrate subtle changes in mineralization of the growth plate (Rosati et al., 1994; Grskovic et al., 2012). While the role of Col X in the in vitro-formed interface is unclear, its presence is appropriate as Col X is a component of the healthy OAF-CEP interface (Lammi et al.,
1998; Nosikova et al., 2012). Furthermore, the expression of Col X following dynamic compression may suggest that mechanical stimulation is more reflective of the native IVD environment and is needed to generate a functional interface in vitro. Thus, these culture conditions represent a starting point for future studies that would enable further investigation of mechanical stimulation upon protein and proteoglycan expression and localization and mineral formation.

4.3 Study Limitations and Future Work

4.3.1 Limitations of Native Interface Characterization

This thesis work further characterized the ECM structure of a healthy OAF-CEP interface. Unfortunately, comparisons between fetal and adolescent interfaces was not possible as tissues were harvested from different species, human and bovine respectively. However, given the limited availability and ethical considerations associated with obtaining healthy human IVD or fetal bovine tissues, the bovine IVD was characterized as it has been proposed to be suitable as a model for the human disc under certain conditions (Demers et al., 2004; Alini et al., 2008). Nonetheless, the composition and organization of the adolescent interface described herein should be confirmed in adolescent human samples. It is also unclear if the composition and spatial organization of the interface varies with increasing age or degrees of degeneration as is observed in other regions of the IVD. For example, immunohistochemical studies in sheep show versican, decorin and biglycan throughout the developing AF and with age, versican is limited to the interlamellar space while decorin and biglycan decrease (Melrose et al., 2001). While this study investigates the spatial organization of many ECM components at the OAF-CEP interface, many other collagens
and proteoglycans have been described in the IVD. For example, perlecan and hyaluronan are thought to play a role in the terminal differentiation of chondrocytes at the growth plate and CEP in sheep (Melrose et al., 2002). Single-nucleotide polymorphisms in the hyaluronan gene are also associated with disc degeneration (Urano et al., 2011). Thus, the ECM composition characterized in this study, as well as other groups, should be characterized across a range of developmental stages and disease states to further our understanding.

The tensile strength of the adolescent bovine interface must be interpreted with caution. The current testing scheme failed to concentrate forces at the OAF-CEP interface. Rather tensile forces were distributed across the vertebrae, growth plate, OAF-CEP interface and multilaminate AF (Figure 2.1). A multi-component tissue was chosen because it was not possible to firmly secure smaller samples (containing only AF tissue and the OAF-CEP interface) to the testing machine during tensile testing. Thus, the current model was adopted to provide an estimate of the tensile strength of the OAF-CEP interface.

While the AF-VB interface did not fail in any sample under uniaxial tension, failure of the AF-VB interface has been described clinically (Hellstrom et al., 1990; Yang et al., 1994; Sward et al., 1990a,b; Figure 2.2). Uniaxial tension, as was applied in our study, does not reflect the range of loads (compressive, shear, torsion and tensile loads) experienced by the interface in vivo (Roughley 2004; Schmidt et al., 2007). Thus, it is possible that interface is more vulnerable to multiaxial loads. Future work should also investigate the strength of the interface in compression, bending and torsional moments, and/or combinations of these.
4.3.2 Limitations of Bioengineered Multilayered Annulus Fibrosus Tissue

In Chapter 3, a 3-layered AF tissue was formed from a single piece of PU-ADO scaffold wrapped around a modified tygon tube. Upon co-culture scaffold nanofibres were oriented approximately perpendicular to the cartilage surface. This assembly did not introduce the cross-ply structure of the OAF, a feature that is believed to be essential to the mechanical properties of the AF (Reviewed in Chapter 1; Elliott and Setton 2001). The stability of bioengineered AF tissue may be similarly influenced by this hierarchical structure and the current method of scaffold assembly may influence stable integration between AF and cartilage tissues in vitro. Several studies have demonstrated that it is possible to model the native multilaminate cross-ply architecture of the AF using aligned electrospun nanofibrous scaffold. Scaffold was cut into strips with fibres aligned at 30° relative to the strip long axis and then two strips with opposing fibre orientation were wrapped concentrically to form multilayered (4mm thick) AF region in a bioengineered IVD tissue replacement (Nerurkar et al., 2010; Martin et al., 2014). Thus, additional experiments are required to determine if AF organization will influence integration.

The heterogeneity of AF tissue formation observed within a single construct, between replicates and between successive experimental runs may have contributed to the large deviations observed in the rates of attachment between AF and cartilage tissue (Chapter 3). As bioengineering efforts aim to model the insertion of 20-25 layered AF tissue, improving AF tissue formation remains an important issue. Histological evaluation demonstrated that cells were present between all scaffold layers while tissue deposition and varied considerably in the radial and axial directions. In general, thicker tissue formed on the outermost surface of the construct at 35 rpm (Figure 3.3). However, minimal tissue was formed on the innermost layers of the PU-ADO scaffold. Culture media was
monitored regularly to ensure that nutrient depletion or waste accumulation did not limit tissue formation. These may be a consequence of culture practices suggesting optimal culture conditions have yet to be defined (Turner 2011). It has been suggested that superior surface-zone (or outer zone) tissue formation by AF cells and chondrocytes may limit nutrient diffusion to underlying tissue (Vunjak-Novakovic et al., 1998; Neves et al., 2005; Saini and Wick 2003; Turner 2011). Small molecules such as glucose and oxygen reach the IVD cells by establishing gradients between cellular demand and the rate of tissue transport (Grunhagen et al., 2006). When placed in a DMEM (25mM concentration of glucose) a multilayered PU-ADO scaffold construct (cross-sectional area of 11.19mm$^3$) has a glucose flow rate of less than 2x native tissue (1.87 nmol/h vs 46.49nmol/h; Chak unpublished; Jackson et al., 2008). This rate of diffusion may impact tissue formation in the innermost layers of the AF tissue during dynamic culture.

Further exploration of fluid flow and resulting rates of diffusion through multilayered cellularized bioengineered AF tissues may provide greater insight into how culture conditions may be further optimized to improve AF tissue formation. The importance of adequate nutrient diffusion and waste removal is highlighted by the vascular network established in the developing IVD at 35 weeks gestation (Nerlich et al., 2007; Smith and Elliott 2011). Developing a perfusion network that penetrates the multiple layers of scaffold/developing AF tissue may overcome diffusion barriers and facilitate nutrient diffusion and waste removal to the innermost layers of the AF and ultimately the developing NP tissue. To support this hypothesis, it has been shown that interspersing layers of rapidly degrading poly(ethylene oxide) throughout the angle-ply AF structure improved cellular infiltration and tissue formation in the bioengineered IVD construct (Martin et al., 2014).
It is possible that the dynamic culture practices adopted in Chapter 3 may have negatively impacted cellular attachment and tissue retention. Studies characterizing the turbulent and unsteady flow characteristics of a spinner flask bioreactor at 34 rpm have reported horizontal and vertical flow rates (0-1 cm/s and -0.2 to 0.5 cm/s respectively) and mean shear-stress field (1.1 dyne/cm²) for constructs in close proximity to the stir bar (Sucosky et al., 2004). External forces ranging between 3.24 and 50 dyn/cm² have also been shown to trigger cellular detachment: 20% of cells were found to detach from a laminin precoated polycarbonate membrane (Furakawa et al., 2003; Tang et al., 2012). Thus, current seeding and culture methods (Chapter 3) may contribute to the observed heterogeneity in AF tissue formation by impairing AF cell attachment and shearing off newly formed tissue. This hypothesis is supported by current and previous data demonstrating that slower rotational speeds (35-45 rpm) improve cellular attachment and tissue retention (Appendix E; Supplementary Figure 4.1; Turner 2011). Continued efforts to improve the formation of multilayered AF tissue should consider the influence of flow rates and shear forces during cell seeding and tissue culture and this alternative ways of culturing the constructs.

4.3.3 Limitations of AF-Cartilage Interface Model

Co-culture of AF and cartilage tissues altered the distribution of Col II and mineral compared to tissues grown alone. However, the mechanism by which this occurs remains unclear. TGF-β has been shown to influence tissue patterning in vivo and may similarly play a role during in vitro tissue formation. This hypothesis is supported by the finding that bovine AF cells cultured on PU-ADO scaffold express TGF-β1 and is associated with increased DNA content, cell proliferation and total collagen synthesis (Turner et al., 2014). Furthermore, several studies have exogenously
added TGF-β during in vitro culture of composite AF-NP tissues to improve tissue formation (Nesti et al., 2008; Nerurkar et al., 2010b; Park et al., 2012). These findings suggest TGF-β may also play a role during in vitro tissue formation. Thus, future studies in gene expression and protein analysis are required to explore the potential role of signaling molecules on the composition of tissues formed following 3-D contact co-culture between AF and cartilage tissues.

In Chapter 3, successful attachment between AF and cartilage tissues was defined as direct contact between the PU-ADO scaffold and cartilaginous tissue. However, this definition does not assess the functionality of the interface which is defined in the literature by its mechanical properties, permeability, composition and structure (Roberts et al., 1991 and 1994 and 1996; Adams and Roughly 2006). Furthermore, the approximation of the tissues does not ensure continuity of tissues between scaffold layers or between all regions of the AF and cartilage tissues. In vivo, ECM disorganization and discontinuity, such as annular tears or endplate changes, can accelerate degenerative changes in other regions of the IVD (Hu et al., 2009; Wang et al., 2012; Li et al., 2014). Likewise, disruptions in bioengineered interfaces may also influence degenerative changes in other regions of an IVD replacement. Thus, sufficient tissue integration and mechanical stability should be achieved in tissue engineered constructs prior to implantation. Furthermore, surgical approaches, such as external fixation systems recently developed in a rat model, may be used to prevent displacement of IVD implants and permit characterization of tissue integration during early effort to develop a tissue engineered composite IVD tissue (Martin et al., 2014).

Limited tissue formation at the interface may have also contributed to the inconsistency in attachment between AF and cartilage tissues observed between replicates and successive
experimental runs. As discussed in section 4.3.2, tissue formation at the interface may be limited by inadequate media perfusion through the scaffold and cartilage tissue. This hypothesis is supported by the observation that percent attachment increased by improving media circulation during static co-culture (Appendix E; Supplementary Figure 3.1). The influence of nutrient exchange upon tissue formation is a critical area for future study that has been identified by several groups assembling IVD tissues and will become increasingly important as more tissues of the IVD are assembled (Nerurkar et al., 2010; Bhattacharjee et al., 2014; Martin et al., 2014). Several studies have developed bioreactors that can mechanically stimulate and maintain IVD explants up to 4 months which may be adapted to the co-culture of bioengineered composite IVD tissues (Parolin et al., 2010; Haglund et al., 2011; Jim et al., 2011; Gawri et al., 2011; Paul et al., 2012; Chan et al., 2013). Thus, development of a bioreactor with physiological rates of nutrient diffusion, fluid flow and external forces may improve AF tissue formation and improve the stability of interfaces formed in a composite IVD tissue.

4.3.4 Limitations Associated with Method of Mechanical Stimulation

The current method of mechanical stimulation does not fully model the complex loading patterns observed in the IVD in vivo (Setton et al., 2004; Schmidt et al., 2007; Nerurkar et al., 2010a). Efforts to quantify the radial expansion of the 3% agarose disc under 1kPa compression is limited by potential batch to batch variability and plastic deformation of the agarose disc during repeated compression. While 1kPa of force was applied to the agarose disc during compression of interface model, it was not possible with the current apparatus to quantify the type and magnitude of forces being transmitted along the length of the AF tissue, cartilage and interface. Finite element analysis modeling has been used extensively to characterize the complex mechanical environment of the
native IVD and may be similarly used to model forces experienced along the tissue engineered interface (Shirazi-Adl et al., 1984; Baer et al., 2003).

Despite these limitations, Chapter 3 demonstrated an increase in proteoglycan accumulation at the interface following a single dose of cyclic compression at 1 kPa and 0.5 Hz for 1800 cycles. While the mechanically stimulated interface contained Col X, it is unknown if mineral formation was influenced as mineral content in the tissue was not quantified. It remains unclear from these findings if the ECM changes observed following mechanical stimulation will improve the mechanical stability of the immature in vitro-formed interface. A key future experiment is to quantify the tensile strength of stimulated and unstimulated bioengineered tissues. Furthermore, it is unknown if the current level of tissue formation is sufficient for the interface to remain stable upon implantation. To begin establishing minimal thresholds, future studies should couple the mechanical properties of the in vitro-formed interface with quantification of proteoglycan and collagen accumulation. Future studies should also investigate the effect of frequency and duration of mechanical stimulation on ECM gene expression, DNA content and the synthesis and retention of proteoglycans and collagens. The interface could also be characterized by Western blot in addition to immunostaining.

While preliminary results suggest that cyclic compression may stimulate tissue, separation of the AF and cartilage tissues was common after mechanical stimulation (Appendix C; Supplementary Figure 3.2). At the tendon-to-bone interface, changes in substrate stiffness and ECM help maintain cell phenotypes and regulate tissue composition and mechanical properties (Benjamin et al., 1998; Docheva et al., 2005; Kuo et al., 2008; Attia et al., 2011; Smith et al., 2012). Similarly, it may be
helpful to alter the scaffold to improve mechanical stability of immature in vitro tissue. Studies have demonstrated increased tensile properties of bioengineered ligament-bone interfaces by introducing a mineral and protein gradient on the scaffold material (Li et al., 2009; Shi et al., 2010; He et al., 2012; Kolluru et al., 2013). Similarly, a graded ECM could be incorporated into the design of a PU-ADO scaffold to increase the stability of the immature AF-cartilage interface during mechanical stimulation.

4.4 Conclusion

Tissue engineering an IVD construct may be a suitable method to overcoming the shortcomings associated with current treatment options for LBP. The successful development of a composite IVD tissue will require the functional integration of disc tissues: the NP, IAF, OAF and CEP. The 3D AF-cartilage co-culture system presented here provides evidence that integration can occur. It also suggests that compressive forces can affect this process; an observation that warrants more thorough investigation.
4.5 References


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