### Appendix A: Supplementary Table for Chapter 2

#### Supplementary Table 2.1: Regional Comparison of Growth Plate Thickness and Tensile Strength of Immature (6-9 months) Bovine VB-OAF-VB Samples.

Differences for growth plate thickness \( (p = 0.95) \) and strength \( (p = 0.18) \) did not reach statistical significance.

<table>
<thead>
<tr>
<th>Region</th>
<th>Growth Plate Thickness (mm)</th>
<th>Tensile Strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior ( (n=10) )</td>
<td>1.30 ± 0.22</td>
<td>2.07 ± 0.61</td>
</tr>
<tr>
<td>Posterior ( (n=10) )</td>
<td>1.08 ± 0.14</td>
<td>1.32 ± 0.36</td>
</tr>
<tr>
<td>Average</td>
<td>1.17 ± 0.18</td>
<td>1.69 ± 0.49</td>
</tr>
</tbody>
</table>
Supplementary Figure 2.1: Collagen Type I and Type II Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope. (B,C,D) Co-staining of collagen type I and collagen type II or (E,F,G) collagen type I alone in the (B,E) outer annulus fibrosus, (C,F) cartilage endplate and (D,G) calcifying region immediately adjacent to the vertebral body. (H) Negative control (40x magnification). Section is 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. These are representative images from one experiment done in triplicate and repeated three times.
Supplementary Figure 2.2: Collagen Type III Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope. (B,C,D) Co-staining of collagen type III and collagen type II or (E,F,G) collagen type III alone in the (B,E) outer annulus fibrosus, (C,F) cartilage endplate and (D,G) calcifying region immediately adjacent to the vertebral body. (H) Negative control (40x magnification). Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type II (Col II); Collagen Type III (Col III); Outer Annulus Fibrosus (OAF); Vertebral Body (VB). Scale bars represent 100 µm. These are representative images from one experiment done in triplicate and repeated three times.
Supplementary Figure 2.3: Collagen Type X Distribution in the Native Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope. (B,C,D) Co-staining of collagen type X and collagen type II or (E,F,G) collagen type X alone in the (B,E) outer annulus fibrosus, (C,F) cartilage endplate and (D,G) calcifying region immediately adjacent to the vertebral body. (H) Negative control (40x magnification). Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type II (Col II); Collagen Type X (Col X); Outer Annulus Fibrosus (OAF); Vertebral Body (VB). Scale bars represent 100 µm. These are representative images from one experiment done in triplicate and repeated three times.
Supplementary Figure 2.4: Versican Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate.  (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope.  (B,C,D) Co-staining of collagen type II and versican or (E,F,G) versican alone in the (B,E) outer annulus fibrosus, (C,F) cartilage endplate and (D,G) calcifying region adjacent to the vertebral body.  (H) Negative control (40x magnification).  Interlamellar space (arrow).  Section is counterstained with DAPI (blue) to visualize nuclei.  Cartilage Endplate (CEP); Collagen Type II (Col II); Outer Annulus Fibrosus (OAF); Vertebral Body (VB).  Scale bars represent 100 µm.  These are representative images from one experiment done in triplicate and repeated three times.
Supplementary Figure 2.5: Biglycan Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope. (B,C,D) Co-staining of collagen type II and biglycan or (E,F,G) biglycan alone in the (B,E) outer annulus fibrosus, (C,F) cartilage endplate and (D,G) calcifying region adjacent to the vertebral body. (H) Negative control (40x magnification). Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type II (Col II); Outer Annulus Fibrosus (OAF); Vertebral Body (VB). Scale bars represent 100 µm. These are representative images from one experiment done in triplicate and repeated three times.
Supplementary Figure 2.6: Decorin Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope. (B,C,D) Co-staining of collagen type II and decorin or (E,F,G) decorin alone in the (B,E) outer annulus fibrosus, (C,F) cartilage endplate and (D,G) calcifying region adjacent to the vertebral body. (H) Negative control (40x magnification). Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type II (Col II); Outer Annulus Fibrosus (OAF); Vertebral Body (VB). Scale bars represent 100 µm. These are representative images from one experiment done in triplicate and repeated three times.
Supplementary Figure 2.7: Aggrecan Distribution in the Bovine Outer Annulus Fibrosus Interface with the Cartilage Endplate. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*) as seen by light microscope. (B,C,D) Co-staining of collagen type I (red) with aggrecan or (E,F,G) aggrecan alone in the (B,E) outer annulus fibrosus, (C,F) cartilage endplate and (D,G) calcifying region adjacent to the vertebral body. (H) Negative control (40x magnification). Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type I (Col I); Outer Annulus Fibrosus (OAF); Vertebral Body (VB). Scale bars represent 100 µm. These are representative images from one experiment done in triplicate and repeated three times.
Appendix C: Supplementary Figures for Chapter 3
Supplementary Figure 3.1: Optimization of 3-Dimensional Co-Culture Conditions. (A-C) Haematoxylin and eosin staining of interface model formed by placing 2 week old 3-layer annulus fibrosus (AF) tissue on top of (B) 3 day or (C) 2 week old calcifying cartilage. (D-F) Influence of nutrient availability on AF and cartilage tissue integration during 2 week static co-culture. (F) Increased media mixing compared to (E) constructs cultured inside the manufacturer membrane holder. Scale bars represent 100μm.
Supplementary Figure 3.2: Effect of Mechanical Stimulation on the Attachment of In Vitro-Formed Multilayer AF Tissue and Cartilage after 2 Week of 3D Co-Culture. In vitro-formed AF-cartilage interface model mechanically stimulated at various frequencies (0.1, 0.5, 1.0 Hz) and forces (1 KPa, 2KPa) for 1800 cycles 1 week after co-culture and harvested 1 week post-stimulation. (A-H) Constructs stimulated with 2 kPa of force at (A-C) a frequency of 0.1Hz, (D-F) 0.5 Hz or (G-H) 1 Hz had a low rate of attachment upon harvest. (I-Q) Constructs stimulated at 1 kPa had a higher rate of attachment at (I-K) 0.1 Hz, (L-N) 0.5 Hz and (O-Q) 1 Hz. (L-N) The most reliable integration was achieved at 1 kPa and 0.5 Hz and this condition was used for subsequent experiments. Scale bars represent 100μm. (n=2)
Supplementary Figure 3.3: Uniform Circumferential Expansion of an Agarose Disc. (A) Experimental schematic. (B) Elastic moduli of a 2% and 3% agarose disc determined by uniaxial, unconfined compression (1 kPa) at 10% strain. Experimental values were used to estimate radial deformation of the agarose disc under 1 kPa of uniaxial compression. (C) Confocal imaging used to visualize diameter changes in the lower 30% of the agarose disc when compressed on a glass slide or calcifying cartilage.
Supplementary Figure 3.4: Absence of Mineral and Alkaline Phosphatase Activity in the Human Fetal Outer Annulus Fibrosus Interface with the Cartilage Endplate. Outer annulus fibrous interface (*) with the cartilage endplate as seen by light microscopy. (A-B) Toluidine blue (blue) and von Kossa (black) staining shows proteoglycans but no mineral at the interface. (C-D) No alkaline phosphatase activity visualized by azo dye stain (blue) and (eosin counter stain). (E) In the positive control alkaline phosphatase activity is seen in the primary ossification center of the fetal disc. (F) Calcium deposition seen in the positive control of bovine adolescent interface. Scale bars represent 100 μm. Cartilage Endplate (CEP); Outer Annulus Fibrosus (OAF); Vertebral Body (VB). Images are representative of one experiment done in triplicate and repeated three times.
Supplementary Figure 3.5: Collagen Type I and Type II Distribution in the Human Outer Annulus Fibrosus Interface and Cartilaginous Vertebral Body.  (A) Haematoxylin and eosin stained annulus fibrosus-cartilage endplate interface (*).  (B,C) Co-staining of collagen type II (red) and collagen type I (green) or (D,E) Col I alone in the (B,D) in the outer annulus fibrosus and (C,E) interface.  (F) Collagen type I and collagen type II negative control (40x magnification) in the human fetal interface. Section is 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 from one experiment done in triplicate and repeated three times.
Supplementary Figure 3.6: Collagen Type III Distribution in the Human Outer Annulus Fibrosus Interface with the Cartilaginous Vertebral Body. (A) Haematoxylin and eosin stained of annulus fibrosus-cartilage endplate interface (*). (B,C) Co-staining of collagen type II (red) and collagen type III (green) or (D,E) Col III alone in the (B,D) in the outer annulus fibrosus and (C,E) interface. (F) Collagen type II and III negative control (40x magnification) in human fetal interface. Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type II (Col II); Collagen Type III (Col III); Outer Annulus Fibrosus (OAF); Vertebral body (VB). Scale bars represent 100 µm. Images are representative from one experiment done in triplicate and repeated three times.
Supplementary Figure 3.7: Versican Distribution in the Human Outer Annulus Fibrosus Interface with the Cartilaginous Vertebral Body. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*). (B, C) Co-staining of collagen type II (red) and versican (green) or (D, E) versican alone (green) in the (B, D) outer annulus fibrosus and (C, E) interface. (F) Versican and collagen type II negative control (40x magnification) in human fetal interface. Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type II (Col II); Outer Annulus Fibrosus (OAF); Vertebral body (VB). Scale bars represent 100 µm. Images are representative from one experiment done in triplicate and repeated three times.
Supplementary Figure 3.8: Biglycan Distribution in the Human Outer Annulus Fibrosus Interface with the Cartilaginous Vertebral Body. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*). Co-staining of (B,C) collagen type II (red) and biglycan (green) or (D,E) biglycan alone (green) in the (B,D) outer annulus fibrosus and (C,E) interface. (F) Biglycan and Col II negative control (40x magnification) in human fetal interface. Cartilage Endplate (CEP); Collagen Type II (Col II); Outer Annulus Fibrosus (OAF); Vertebral body (VB). Scale bars represent 100 µm. Images are representative from one experiment done in triplicate and repeated three times.
Supplementary Figure 3.9: Decorin Distribution in the Human Outer Annulus Fibrosus interface with the Cartilaginous Vertebral Body. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate (OAF-CEP) interface (*). (B,C) Co-staining of collagen type II (Col II; red) and decorin (green) or (D,E) decorin alone (green) in the (B,D) OAF and (C,E) CEP. (F) Decorin and collagen type II negative control (40x magnification) in human fetal interface. Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type II (Col II); Outer Annulus Fibrosus (OAF); Vertebral body (VB). Scale bars represent 100 µm. Images are representative from one experiment done in triplicate and repeated three times.
Supplementary Figure 3.10: Aggrecan Distribution in the Human Outer Annulus Fibrosus Interface with the Cartilaginous Vertebral Body. (A) Haematoxylin and eosin stained outer annulus fibrosus-cartilage endplate interface (*). (B, C) Co-staining of collagen type I (green) and aggrecan (red) or (D, E) aggrecan alone (red) in the (B, D) outer annulus fibrosus and (C, E) interface. (F) Aggrecan and collagen type I negative control (40x magnification) in human fetal interface. Section is counterstained with DAPI (blue) to visualize nuclei. Cartilage Endplate (CEP); Collagen Type I (Col I); Outer Annulus Fibrosus (OAF); Vertebral body (VB). Scale bars represent 100 µm. Images are representative from one experiment done in triplicate and repeated three times.
Appendix D: Mechanical Properties of 3% Agarose Disc

A.1 Methods

The radial expansion of a 3% agarose disc under compression (1 kPa and 2 kPa) was calculated from experimentally derived elastic moduli and then verified by confocal microscopy.

COMPRESSIVE MODULUS: To determine the elastic modulus, an agarose disc was submersed in culture media (DMEM) at 37°C and subject to uniaxial, unconfined compression (1 kPa and 2 kPa). Discs were preloaded (10mN) and then subjected 10 cycles of stepwise compression of 10% strain until equilibrium was reached (less than 2mN/min change in force). The equilibrium stress for each sample was plotted as a function of the applied strain.

CONFOCAL MICROSCOPY: Confocal microscopy was used to quantify the actual diameter change of the compressed agarose disc. To enable visualization, Hoescht dye (1.0 μM) was added to molten agarose, cooled and placed on top of a glass slide (n=10) or 7 day old in vitro-formed cartilage (n=10) in a humidified chamber. Sequential images of the loaded and unloaded disc (1 kPa and 2 kPa) were taken at four levels in the lower 1/3 of the 3% agarose disc: 0 μm, 300 μm, 600 μm, 900 μm from the bottom. The diameter was measured using Adobe Photoshop CS5 (Adobe Systems Incorporated). The radial expansion of the agarose disc was defined as the diameter of the loaded disc minus the diameter of the unloaded disc.
A.2 **Results: Expansion of Agarose Disc Under Compression**

**ESTIMATION OF AGAROSE DISC EXPANSION:** The compressive properties of 2% and 3% agarose disc were determined to permit estimation of the radial expansion of the agarose disc in the 3D co-culture system. The tensile moduli significantly increased as the agarose concentration increased, 65 + 3.8 MPa to 167 + 4.9 MPa (Appendix C; Supplementary Figure 3.3, n=3). To calculate the radial expansion of a 3% agarose discs (4mm height x 6mm diameter) under 1 kPa of static compression, the strain of the agarose disc ($\varepsilon_A$) was set to equal to the applied stress ($\sigma$) divided its elastic modulus (E).

$$\varepsilon_A = \frac{\sigma}{E} = \frac{1 \text{ kPa}}{167 \text{ kPa}} = 0.006$$

The applied strain of the disc was then used to calculate the change disc height ($\Delta H$) where $\varepsilon_A$ is the strain of the agarose and $H_i$ is the height of the unloaded agarose disc.

$$\Delta \text{Height} = \varepsilon_A \times H_i = 0.006 \times 4 \text{ mm} = 0.024 \text{ mm}$$

Thus, a 3% agarose disc is expected to have a final height of 3.98 mm under 1 kPa of compression. Assuming agarose is incompressible, the radius of the compressed agarose disc is given by equating the discs initial and final volumes where $V$ is the volume, $r$ the radius, $H$ the height and $\pi$ the diameter of the disc.

$$\text{Volume}_{\text{Initial}} = r_i^2 \times \pi \times H_i$$
$$\text{Volume}_{\text{Final}} = r_F^2 \times \pi \times (H_i - \Delta H)$$

Appendix D-2
Appendix D-3

\[ \text{Volume}_i = \text{Volume}_F \]

\[ r_i^2 \times \pi \times H_i = r_F^2 \times \pi \times (H_F - \Delta H) \]

\[ r_F = \frac{r_i^2 \times \pi \times H_i}{\pi \times (H_i - \Delta H)} \]

\[ r_F = (3\text{mm})^2 \times 3.14 \times 4\text{mm} \]

\[ r_F = 3.14 \times 3.98 \text{mm} \]

\[ r_F = \sqrt{9.05} \text{mm} \]

\[ r_F = 3.01 \text{mm} \]

Thus, the final diameter of a compressed disc is estimated to be 6.02 mm.

RADIAL EXPANSION OF AGAROSE DISC VERIFIED BY CONFOCAL MICROSCOPY:

Confocal microscopy verified the agarose disc expanded to a maximum of 6.08 mm which is within 1% of calculated values. When compressed (1 kPa) on glass, the agarose disc radially expanded by an average of 0.054 mm to a final diameter of 6.05. Radial expansion at the base of the agarose disc contacting the glass slide was significantly lower that the radial expansion measured at the 900 µm levels (lower: 0.034 ± 0.01 mm vs upper: 0.071 ± 0.008 mm; Appendix C Supplementary Figure 3.3). This discrepancy was reduced when 3% agarose discs were compressed on 7 day old \textit{in-vitro} formed cartilage (0.081 ± 0.007 mm). Agarose discs subject to 2 kPa compression repeatedly failed during compressive loading and were not used in experiments.

\textbf{A.3 Discussion}

These studies provide insight into how a 3% agarose disc may radially expand when compressed on in vitro-formed cartilage, such as would occur when placed at the center of the OAF-CEP interface model. While the cartilage appeared to uniformly expand when compressed on in vitro-formed cartilage, it is unknown how the circumferential constraint of the multilayer AF tissue
will alter the expansion of the disc. Furthermore, while 1 kPa is applied to the agarose disc, this system is unable to quantify the magnitude and types of forces experienced in each individual component tissue of the model OAF-CEP interface.
Supplementary Figure 4.1: Optimization of Seeding and Culturing Methods to form a 3-Layer Annulus Fibrosus Tissue on Polyurethane Scaffolds. (A-C) The effect of static or dynamic seeding and culture on tissue formation. Haematoxylin and eosin staining of 2 week old tissue formed by (B) static and (C) dynamic seeding and culture of single passaged outer annulus fibrosus (OAF) cells. (D-G) The effect of speed on tissue formation. Single passaged OAF cells seeded and cultured on multilayer PU-ADO constructs at (E) 15 rpm, (F) 35 rpm and (G) 65 rpm. Scale bars represent 100μm.