INVESTIGATION OF ANTERIOR CRUCIATE LIGAMENT FIBROBLAST BIOMARKERS FOR CELL CHARACTERIZATION BY IMMUNOHISTOCHEMISTRY

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CHAPTER 1

INTRODUCTION TO THE STUDY

Background

The anterior cruciate ligament (ACL) in humans is one of the primary ligaments of the knee joint, and is biomechanically responsible for stability and preventing the translocation of the femur anteriorly. It is commonly ruptured or injured during physical activity, and is also the most commonly injured ligament in the knee joint (Cheng M T 2009). There is an estimated 200,000 ACL injuries in the United States per year with treatment costs ranging from $17,000 to $25,000 per patient (Pennisi E 2002; Loës M 2008). Although this injury is common in athletes, at least 70% of ACL injuries are non-contact, which is described as making a sudden stop, twisting, or jumping (Boden B P 2000). Once partially or completely ruptured, its ability to heal is low due to its poor vascularization (Kleiner J B 1995; Zigang G 2005). Surgical reconstruction of the ACL by arthroscopy has been the preferred method of repair by orthopaedic surgeons using hamstring tendon autograft (53.1%) and patellar tendon autograft (22.8%) to replace the ligament (Middleton K K 2013). These autografts are taken from the patient. Another common replacement method is utilizing an allograft from a cadaver. Although arthroscopy is referred to as a minimally invasive procedure, it involves multiple incisions around the knee and drilling of the femoral bone at the distal end for graft positioning and attachment. The recovery period may vary, but typically takes 6 months or more for full recovery. When the ligament is torn, 55-65% of the time the meniscus is also torn due to the attachment site as shown in figure 1 (Wyatt R W B 2014). Ligament injury has been shown to cause up to a 13% prevalence of knee osteoarthritis

(OA), and up to 48% when both the meniscus and ACL are injured (Øiestad B E 2010). Additionally, research has shown that the rupture of the ACL will put a patient at risk for cartilage degradation and OA development (Roos H 1995). Potentially, a less invasive and more promising method of reconstruction is through ligament engineering using gene therapy, stem cells, and a growth matrix (Petrigliano F A 2006; Laurencin C T 2005).

Anatomy of the Anterior Cruciate Ligament

The ligament is located in a synovial joint, the knee joint, encapsulated by a synovial tissue (Zhou et. al 2005). There is debate on whether the ligament consists of two bundles, the anteromedial and posterolateral bands, or having an additional intermediate bundle (Girgis et. al 1974; Otsubo et. al 2012). The attachment site from posterior of the lateral femoral condyle to the tibial tubercle, aids its function in preventing the translocation of the tibia past the femur (Figure 1).

Methods of Construct Engineering

To date, the endeavor to create an engineered construct that can replace an allograft or autograft ACL has not been achieved. Mainly the coarse of study has focused on investigating the types of cells that can be used for the construct, the matrix or scaffold that cells can be seeded into, and the use of polymers for ligament replacement.

Materials for constructs have ranged from a simple scaffolding system made of native extracellular matrix components, to synthetic three-dimensional constructs with emphasis on organization and cellular content similar to that of the human ACL. Laurencin et. al are currently working on a patent for a polymeric fiber-based braided scaffold as a material for ligament and

tendon repair. The advantages claimed are that it is three-dimensional, biodegradable, and allows for organization (Laurencin et. al, 2015). The 3D aspect of this construct allows for cells to be seeded within the material and encourage similar organizational patterns like that of a ligament (Laurencin et. al, 2015). Silkworm fibers have also been used as a source for matrix development (Altman et. al 2002). Using silkworm fibers, Altman et. al found that the mechanical properties and stem cells combination could be used for ligament engineering (Altman et. al 2002).

Hydrogels have also been popular among engineered constructs. Particularly because of the 3D versus 2D aspect of growing cells on a flat surface for ligament growth. This allows for cells seeded into the hydrogel to form an environment more similar to the native environment (Ruedinger et. al 2014). Zschenker et. al showed that a microarray analysis of a human lung tumor cell lines experienced upregulation of gene expression in ECM proteins such as fibronectin and collagen when using a hydrogel (Zschenker et. al 2012). Hydrogels have been used for engineering purposes for periodontal ligaments as well (Saminathan et. al 2013). Hydrogels containing ECM components such as collagen I seeded with mesenchymal stem cells have also been used for construct engineering (Noth et. al 2005; Haddad-Weber et. al 2014). Although promising, as Noth et. al and many others have concluded, the constructs are still too fragile to withstand the stress and strain ACLs experience.

Scaffolds comprised of ECM, collagen, collagen and silk, and collagen and glycosaminoglycans have also been used for cell seeding (Dunn et. al 1995; Bellincampi et. al 1997; Murray et. al 2001; Panas-Perez et. al 2013; Mizutani et. al 2013). Synthetic scaffolds made of biodegradable materials such as poly-L-lactic acid (PLLA) and poly-lactic and glycolic acid have also been tested (Ge et. al 2005). However, the mechanical properties and integrity of

the construct remain a challenge. The difficulty appears to lie in making the characteristics and integrity of the construct similar to or better than the ACL ligament.

Several different types of cells have been used for engineering purposes and have included mesenchymal stem cells, skin fibroblasts, periodontal ligament fibroblasts, and ACL cells. Mesenchymal stem cells and ACL cells have both shown morphological changes after passaging (Ge et. al 2005).

Tissue Composition

The ACL tissue is composed of collagens, elastin, proteoglycans, water, and fibroblasts (Hadjicostas P 2008; Laurencin C T 2005). It consists of fibroblast-like cells, which are known to be present in elastic tissue (Murray M M 2000). The fibroblasts are responsible for the production of the extracellular matrix, which is important for the integrity of the ligament. The ECM is comprised mainly of collagens I, II, III, V, elastin, and proteoglycans among other components (Laurencin C T 2005; Petersen W 1999). Collagen makes up the majority of the dry weight of the normal ligament (80%), and this tissue is mainly composed of collagens type I and III (Haut R C 1993; McDougall J J 1999). Specifically, the ACL is composed of collagen I type bundles and type III collagen fibrils, which separate the bundles (Petersen W 1999). These collagens not only play a role in the structure of the ECM and integrity of the tissue, but also are necessary for the stretching of the ligament during locomotion (Noth et. al 2005).

The ligament consists of a top layer of tissue often referred to as the, epiligament (Amiel et. al 1990; Frank et. al 1988). It contains vessels and loose connective tissue, different from the dense connective tissue of the ligament. This epiligamentous layer is thought to possibly support the viability of the tissue, however this is still up for debate. The vascularization of the

epiligament arises from mainly the middle geniculate artery, which is a branch of the popliteal artery (Tillmann et. al 1999). The ligament itself is not vascularized and is therefore incapable of repairing or healing (Duthon et. al 2006).

This tissue is very complex in its mechanical and morphological properties. The characteristic of crimp within the tissue has been suggested to help with the mechanical properties of the ACL (Rigby et. al 1959; Viidik 1968). This crimp phenotype is found throughout the ACL ligament and tendons as well. Although this tissue has a predominant population of phenotypically elongated fibroblasts, several other shapes have also been found (Murray et. al 1999; Tillman et. al 1999). These include: ovoid, fusiform, and spheroid (Murray et. al 1999; Tillmann et. al 1999). Some ligament cells have even portrayed chondrocyte-like phenotypes (Tillmann et. al 1999). However, the chondrocyte appearance may be linked to aging which causes morphological changes in the ACL (Hasegawa et. al 2012 and 2013). Therefore, various phenotypes of fibroblasts may be found within the tissue. To our knowledge, no engineered construct created using ACL cells, has taken into consideration the differences that may exist between these fibroblast isoforms.

Biomarkers of Interest

An important step in using ACL fibroblasts for construct engineering, is to establish biomarkers that are reliable in the characterization of these specific cells. No single biomarker has been consistently used as a means of characterizing ACL fibroblasts. Commonly found in the literature, is the use of biomarkers chosen at the discretion of the researchers and more often than not, no specific reasoning is expressed behind the chosen biomarker(s). Biomarkers are especially important for fibroblasts because they can experience differentiation under

environmental changes during tissue repair, stress, and fibrosis (Darby I 2007). Additionally, if ACL cells are isolated from human ACL, depending on the area they are harvested from, they may show chondrocyte characteristics due to changes in tissue that may resemble fibrocartilage (Petersen W 1999). For construct engineering purposes, it is essential to characterize fibroblasts native to the ACL tissue. This would allow for a streamlined process for the characterization of ACL fibroblasts in construct formation whether primary ACL or cell-line fibroblasts are used.

For example, alpha-smooth muscle actin (α-SMA) has been used as an ACL fibroblast biomarker in studies investigating cellular and ECM changes in the ACL, as well as for engineering purposes (Hasegawa A 2013; Brune T 2007; Zigang G 2005). Previous studies have hypothesized that fibroblast-like cells, express this particular biomarker and these cells may be found within the ACL (Grinelll F 1994; Weiler A 2002). Alpha-smooth muscle actin is designated as a myofibroblast biomarker primarily; however, studies suggest that the α-SMA may be a characteristic of fibroblasts in connective tissue as well (Murray M M 1999). This protein is best known for its role in the contractile apparatus, responsible for contraction in smooth muscle tissue. It has also been claimed to be present in myofibroblasts and may play a role in wound healing (Darby I 1990). Additionally, this biomarker is also used to characterize smooth muscle cells such as those that are found in vessels and some organs (Gabbiani et al. 1981).

Fibroblasts are derived from the mesenchyme of embryos, and are therefore a type of mesenchymal stromal cell. The most common biomarker used to characterize mesenchyme-derived cells is vimentin (Lee C H 2010). Mesenchyme-derived cells, also called Mesenchymal Stem Cells (MSCs) include: chondrocytes, fibroblasts, myocytes, osteoclasts, and adipocytes. Vimentin is a type III, Intermediate Filament (IF) protein responsible for cell integrity, and is

therefore significantly present in these types of cells (Steinert et. al 1981). Vimentin has been commonly used as a biomarker for skin fibroblasts (Dooley et. al 2003). It has also been shown to be the predominant intermediate filament present in vascular smooth muscle cells as well (Gabbiani et. al 1981). Therefore, it is not exclusive to ACL tissue, but has been used to characterize other mesenchyme-derived cells.

Fibronectin is a glycoprotein in the extracellular matrix (ECM) and binds to collagen, fibrin, and heparin. Due to fibronectin's abundance in the ECM, it has often been used for fibroblast characterization (O-Brian M 1997; Haddad-Webber M 2010; Brune T 2007). Fibronectin is abundantly expressed in a variety of tissues, and is therefore a popular biomarker.

Additionally, Collagen type I and III are commonly used together as cell markers for ACL tissue due to their abundant expression (Hadjicostas PT 2008; Zigang G 2005; Mizutani N 2013). The biomarker used in this study is collagen type III. Collagen type III separates the bundles of collagen type 1 (Tillmann et. al 1999). Collagen III is of particular interest to us due to its role in the mechanics of the ligament as well as healing. During cruciate ligament induced stretching, the mRNA levels of collagen III were observed to increase significantly compared to collagen I indicating the necessity for this ECM component for normal function and for ligament stress (Kim et. al 2002).

In addition to these markers, an investigation on the reliability of the less commonly used biomarker, Fibroblast Growth-Factor Receptor 1 (FGFR1), will also be conducted. To our knowledge, no previous study has used this biomarker for ACL fibroblast characterization. This protein is part of a family of four highly conserved transmembrane tyrosine kinase receptors (Grose et. al, 2010). Fibroblast Growth Factor Receptors play a major role in development. Specifically, FGFR1 has been established to play a role in mesoderm patterning and formation in

vertebrates (Yamaguchi et. al 1991). These receptors are involved in would healing, development, angiogenesis, proliferation, and many more processes. Of particular interest to this study is Fibroblast growth-factor receptor 1 (FGFR1). This signaling molecule is composed of an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic domain (Jaye M 1992). This lead us to deduce that due to the formation of mesenchyme by mesoderm during development, the expression of highly conserved proteins, such as FGFR1, will exist in mesenchymally derived tissue. Due to this receptors' highly conserved nature across a number of species, this study aims to investigate the reliability of this protein as a potential and novel biomarker for characterizing ACL fibroblasts.

Hypothesis

There is no combination or single biomarker designated for the characterization of anterior cruciate ligament fibroblasts. By investigating the expression of a specific set of biomarkers, we aim to establish a reliable combination to characterize anterior cruciate ligament fibroblasts. The central hypothesis of this study is that the biomarkers of interest, vimentin, fibronectin, alpha-smooth muscle actin(α-SMA), Fibroblast Growth Factor Receptor 1 (FGFR1), and collagen III, will be a suitable and reliable combination to characterize anterior cruciate ligament fibroblasts because 100% of the cells are expected to stain positive for these biomarkers by the method of immunohistochemistry.

Significance

There is a need to further refine characterization of ACL fibroblasts for their use in ligament engineering. This is particularly important because there is no single biomarker that has been consistently used as a means of characterizing ACL fibroblasts. Biomarkers currently used may lead to the selection of fibroblast-like cells that may not specifically be ACL fibroblasts, which are the cells responsible for giving the tissue its mechanical properties and integrity. This study will test a combination of reliable biomarkers that can be used to specifically characterize human ACL fibroblasts for their use in the creation of an engineered ligament construct using the method of immunohistochemistry. In addition, this study will introduce FGFR1as a potential ACL fibroblast biomarker that has not been previously used for characterization purposes. The findings of this study could be used to characterize fibroblasts isolated from the ACL for the purposes of engineering an ACL construct.

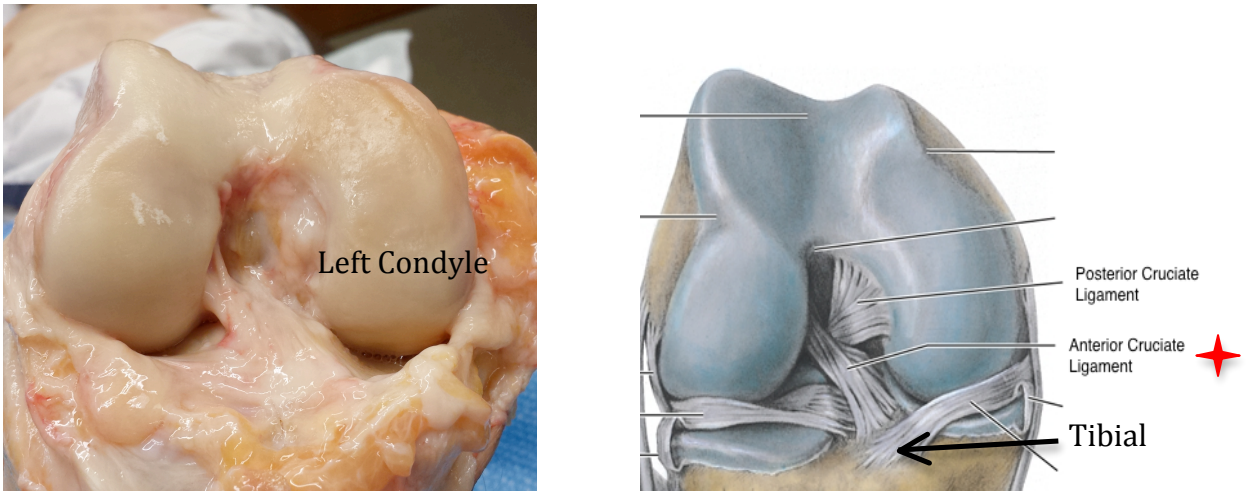

Figure 1. Anatomy of the anterior cruciate ligament. A) Distal end of right femur from 48 year old male with condyles shown anteriorly. Anterior cruciate ligament can be seen from inner right condyle to attachment site on tibial tubercle. B) Adapted anatomical drawings of the human knee joint from Wolters Kluwer Health, 2010. The ligament can be seen stretching from the posterior region of the lateral (right) condyle to the tibial tubercle.

CHAPTER 2

RESEARCH DESIGN AND METHODS

Tissue Acquisition and Processing

Anterior cruciate ligaments were harvested and processed within 2 hours of cadaver arrival. This allowed an appropriate window for this tissue to be used without the risk of tissue degeneration to occur. Cadavers were kept in a cadaver cooler (37 degrees F) until the dissection procedure. ACL ligaments were collected from male and female cadavers that were approved for research use. For this study, a total of six ligaments from four donors were examined.

In 2013, the Willed Body Program at UNTHSC received 127 cadavers, 82 of which information on age was acquired (Table 1). The majority of donors were over the age of 60. Since this was not an age-related study, no age group was excluded. Upon beginning the dissection, donors were inspected for evident knee trauma, osteoarthritis, or for visible signs of previous surgery. If any of the above criteria pertained to the donor, the ligament(s) were excluded from this study for the possibility of having caused changes in the tissue.

The ligament was dissected from each cadaver and processed immediately for fixation. ACLs were carefully cut from the distal and proximal ends connected to the tibia and femur, respectively (Figure 1). This procedure was carried out by a vertical midline skin incision over the patella about 4 to 6 inches long, followed by a lateral parapatellar arthrotomy. This allowed for the patella to be dislocated medially with the knee extended fully. The knee was then hyper-flexed and the ligament was cut at the insertion points with a sterile scalpel. The ligament was then immediately submerged in betadine for two minutes. Once removed from the betadine, the

ligament was then cut into 3 equal parts, and fixed in 10% neutral buffered formalin for 24 hours. Once fixed, the tissue was submerged in 1X PBS (Sigma) for histological processing.

Immunohistochemistry

Paraffin embedded tissue was cut to 5 μm sections. The microscopic evaluation of the tissue included hematoxylin and eosin staining to confirm the typical phenotype of the tissue. To begin the process of evaluating the fibroblast biomarkers of interest, paraffin embedded ACL slides were baked. Slides designated for detection of FGFR1 were baked for 14 hours overnight at 60 degrees Celsius. This allowed the sectioned tissue to adhere well to the slide and prevent the loss of tissue during antigen retrieval. This was found to be the best method due to the high pH of the buffer used for the retrieval of this particular antigen. All other slides were baked for 1 hour at 60 degrees Celsius. Following baking, the sections were deparaffinized in xylene and ethanol (100%, 95%, 50%) for 3 minutes each before rehydration in water. Sections were rinsed in deionized water, and then subjected to heat-mediated antigen retrieval process using appropriate buffers for each primary antibody (Table 2). Heat-mediated antigen retrieval was carried out in the 2100 Retriever (Electron Microscopy Sciences) for approximately 12 minutes. To block non-specific staining, Superblock Blocking Buffer-Blotting in PBS (Thermo Scientific) or goat serum will be applied for 1-2 hours at room temperature. Once blocking solution was removed after three PBS washes, sections were incubated overnight with primary antibodies in humid chamber at 4 degrees Celsius.

The following primary antibodies were used: Rabbit anti-Vimentin (D21H3, Rabbit, Cell Signaling Technology, Danvers, MA, USA; 1:100 dilution), rabbit anti-α-smooth muscle actin (ab5694, Abcam, Cambridge, MA, USA; 1:200), mouse anti-fibronectin (ab2413, Abcam,

Cambridge, MA, USA; 1:100), goat anti-collagen III (133001, AbD Serotec, Raleigh, North Carolina, USA; 1:50), and rabbit anti-fibroblast-growth factor receptor 1 (D8E4, Cell Signaling Technology, Danvers, MA, USA; 1:100 dilution). Dilutions were determined by empirical experimentation.

The following secondary antibodies were used: Donkey anti-rabbit IgG, Alexa Fluor ® conjugate 488 (A21207, Life Technologies, Carlsbad, California, USA; 1:200), donkey anti-goat IgG, Alexa Fluor ® conjugate 488 (A11029, Life Technologies, Carlsbad, California, USA; 1:200). Secondary antibodies were applied for 2 hours at room temperature in humid dark chamber. Table 2 displays the dilutions, blocking solution, and buffer for antigen retrieval.

Following three PBS washes, slides were counterstained with Prolong Gold Antifade with DAPI (Life Technologies) for nuclear counterstain. Slides designated for FGFR1 detection were washed once with PBS due to their vulnerability to detachment of the tissue. Slides were then mounted, and allowed to dry in 4 degrees celsius, overnight. Slides were then observed by fluorescence microscopy using a Nikon eclipse Ti microscope and images were acquired by the Nuance Imaging System 3.0.2 (Perkin Elmer). Furthermore, the images acquired were superimposed to show both DAPI and biomarker staining using Adobe Creative Cloud Photoshop CC. This procedure was performed to show the localization and distribution of the components of interest in the tissue. This protocol was applied to all ACL samples acquired.

Regions of Interest (ROI) from tissue tested for each biomarker by immunohistochemistry, were captured under a magnification of 400X. Each ROI was analyzed by counting the total number of cells visibly stained by DAPI, and the total number of positive-stained cells, identified by the secondary antibody with the fluorescence conjugate. Although we adhered to choosing three ROIs at random for all ligaments, some portions of the tissue were

heavily damaged during the antigen retrieval process or were folded. Particularly for those samples tested for FGFR1 due to the buffer, and for samples that were more prone to folding when sectioned.

Detection of vimentin, FGFR1, and apha-SMA positive cells was determined by counting for positive-stained cells and observing for their localization such as in the cytoplasm and/or in the plasma membrane. To quantify the percentage of positively cells for each biomarker, the number of total positively stained cells was divided by the total number of cells within the examined histological area (ROI). The measurements for a given ROI were 353.3 μm X 263.96 μm. This proportion was then converted to a percentage. The value for total number of cells was determined by the visible nuclei stained by DAPI in blue fluorescence. Cells were determined to be positive for the biomarker of interest by the presence of green fluorescence staining (Alexafluor 488). An observation for typical morphology, organization, and localization of the biomarker was also performed. For example, when observing the biomarker vimentin, we looked for its presence within the cell in congruence with its properties as a cytoskeletal intermediate filament (Brulet et. al 1980; Mendez et. al 2010).

The percent of total positive-stained cells/total number of cells within the examined area was calculated for each ROI. A total weighted average of the three ROIs was then quantified. This corrected for an imbalance in the distribution of cells in ROIs caused by areas more dense or populated with cells than other less dense areas. This weighted average was converted to a percentage, and graphed for each ACL that was examined. The method behind this protocol was adapted from Murray et al. (Murray M M 1999).

Additionally collagen III and fibronectin were analyzed by using ImageJ software (NIH.GOV) to quantify the fluorescence intensity. These two biomarkers were analyzed

differently because they are part of the extracellular matrix, and are widely expressed throughout the tissue. This makes it difficult to determine how much of the component was produced by a specific cell. Using ImageJ to quantify the fluorescence intensity allowed us to gather data on the average distribution of the biomarker within a given ROI. To analyze this data, the total fluorescence intensity in the histological region was divided by the total number of cells in a ROI. A total weighted average of the ROIs were then quantified. This weighted average was represented as a percent, and graphed for each ACL that was examined.

Data Representation

Data were represented by box and whisker plot (box-plot) to show the range of the percentage of positive stained cells when the results of all ligaments were combined for each individual biomarker: alpha-SMA, vimentin, and FGFR1. Box-plot was also used to represent data from fibronectin and collagen III. Additionally, graphs showing results of the percentages of positive stained cells for each of the corresponding biomarkers per individual ligament was presented by histogram.

Donor information	Females	Males
Cadavers Acquired in 2013	40	43
Age range (Low-High)	47 - 105	29 – 99

Table 1. 2013 Body donor count for females and males and age range.

Primary Antibody	Dilution	Antigen Retrieval Buffer	Blocking Buffer
Rb anti Alpha-SMA	1:200	Tris and EDTA, pH 9	Super Block Blocking Buffer
Gt anti Collagen -III	1:50	Tris and EDTA, pH 9	Super Block Blocking Buffer
Rb anti Fibronectin	1:100	Citrate, pH 6	Serum
Rb anti FGFR1	1:100	1 M Tris, pH 10	Serum
Rb anti Vimentin	1:100	Tris and EDTA, pH 9	Super Block Blocking Buffer

Table 2. Summary of immunohistochemistry materials. This table shows the information for each of the primary antibodies used in this study. The designated buffers, blocking buffers, and dilutions were determined by empirical testing.

RESULTS

Histological Evaluation of ACL Tissue

Microscopic evaluation of ACL Tissue was achieved by H&E staining. Figure 2 shows typical characteristics of the ACL. To characterize the tissue, the samples were examined for crimping, a wavy texture, characteristically found in ACL tissue. In addition, elongated nuclei were also observed within the tissue, and due to their morphological state, these cells were most likely fibroblasts. However, given that this staining does not identify specific cells, it is difficult to make conclusions based soley observations. Other shapes of cells were also observed such as ovoid, and triangular.

Also observed in a few of the ACL tissues were the presence of vessels. The ACL is known to be an avascular tissue. However, in the samples acquired, the occasional vessel was present within the tissue (Figure 2), especially as we got closer to the epiligamentous layer. The epiligament is shown in figure 3. In figure 3, the transition from the dense connective tissue from the ACL tissue to the loose connective tissue of the epiligament layer is observed. In this layer, we observed the abundance of vessels and occasional adipocytes. Observing these changes and characteristics allowed us to choose ROIs within the ACL sections that were consistent in morphology to the anterior cruciate ligament as well as excluding areas that were not consistent with the typical phenotype of the tissue. The epiligament was not included in this study.

Skin samples were used as a positive control for the biomarkers of interest. H&E staining and microscopic observations of this tissue were also conducted (Figure 4). Skin harbors fibroblasts in the dermal layer among the loose connective tissue. Although these fibroblasts are present in a different tissue (skin), it allowed for an appropriate positive control for the detection

of positive staining of the biomarkers of interest. For example in Figure 5, the IHC showed fluorescence of vimentin biomarker along the border of the epidermis and dermal layer, and more heavily in the dermal layer itself. All other biomarkers were also confirmed by the use of skin as a positive control.

Immunohistochemistry of ACL

The biomarkers of interest: vimentin, alpha-SMA, FGFR1, fibronectin, and collagen III were investigated in six total ACL samples. The samples were acquired from a 48 year old male, a 67 year old male, 64 year old female, and a 71 year old male. Both the 48 and 71 year old males had both right and left ligaments included in this study. For other experimental purposes outside of our study, the left ACL was not dissected from the remaining donors.

The expression of α-SMA protein was inconsistently expressed among all ligaments. Two out of the six ligaments showed no indication of expression by fibroblasts. Although no fluorescence was detected, we are confident that this was not attributed to a problem with the antibodies used due to nearby staining of α-SMA in the neighboring epiligamentous tissue for both ligaments three and four (Figure 6). To check for a possible error in staining or antibody malfunctions, additional experiments were carried out following the same protocol. However, the same results were obtained. Among the other ligaments (1, 2, 5, and 6) the percentage of positive stained cells for α-SMA ranged from 45.8% to 86% (Figure 7). This component was observed to express in the cytoplasm.

FGFR1 expression was found to be localized on both the plasma membrane as well as in the cytoplasm among samples. The percentage of positively stained cells ranged among the six ligaments (Figure 8). Ligaments three and four, showed the highest percentage of positively

stained cells, and all other ligaments had a higher than 60% positive staining for FGFR1 (Figure 8). Vimentin was localized intracellular across all samples due to it being an intermediate filament. All samples had percentages higher than 50% for vimentin positive cells. (Figure 9).

Figure 10 shows results for both collagen III and fibronectin measured by fluorescence intensity. It must be noted that we are not comparing antibodies, therefore no comparison was made between these biomarkers. Being that collagen III is one of the predominant components of the ECM, the fluorescence intensity was higher than 500000 across all samples. Sample three showed the highest amount of fluorescence intensity, while ACL 6 had the lowest. The fluorescence intensity across all ligaments detected for fibronectin was higher than 200000.

Cumulative data was represented by box-plots in figure 11. The fluorescence intensity of fibronectin ranged between 300000 and 600000 (min and max). The range of fluorescence intensity for collagen III was 50000000 and 170000. Collagen III was overall widely expressed in the ROIs, therefore explaining the high fluorescence intensity.

When looking at the images taken of the ROIs for the biomarkers (Figures 12-17), we were able to look at the localization, morphology, and overall characteristics of the ligaments that were examined. We especially saw how the composition of the ACL changes between not only samples, but also regions of the ligament. Ligament 1 exhibited dense, yet non-homogenous staining of collagen III. Tight and loosely coiled collagen fibrils can be seen stained throughout the ligaments (figure 12 and 13). Nuclei can be found interspersed throughout the regions. It is difficult to declare with confidence whether these nuclei are those of fibroblasts found within the ECM in the images for collagen III and fibronectin. Fibronectin staining, although faint in some regions and in some ligaments more than others, is more vibrant on the marginal portion of the

collagen fibers it is bound to in all ROIs. It was also observed that not all nuclei were surrounded by both collagen III staining and fibronectin. Overall some ligaments had areas where no staining, faint, and dense staining occurred.

Ligament three had very abundant collagen III staining throughout the tissue and more homogenous than other ligaments. This ligament was that of a 48 year old male. This same cadaver had non-homogenous staining of fibronectin as is shown in figure 14.

ACL five and six, which came from the same donor showed similar collagen III staining in that the texture of the tissue appeared similar, as well as the shapes and formation of the collagen fibers. Fibronectin expression was non-homogenous in these samples.

Among all ligaments, nuclei can be seen partially covered, "powdered", adjacent to areas of fluorescent staining alpha-SMA, FGFR1, and vimentin. Cells were counted as testing positive for the expression of the biomarker of interest by complying with the following criteria: the green fluorescence partially covered, completely covered, or "powdered" (meaning-a dusting of fluorescence). Staining that was faint or had clear localization was not considered as positive staining.

Our results indicate that none of the biomarkers were detected in cells at a rate of 100%. Fibronectin and collagen III abundance in the ECM made it difficult to determine specific production by an isolated fibroblast. However, both biomarkers were detected in abundance by fluorescence intensity. FGFR1 and vimentin stained positive in over 50% of cells, but less than 95%. Alpha-SMA was more variable due to two of the ligaments having 0% of cells stain positive for this biomarker. However, samples that did show expression of alpha-SMA stained over 46%.

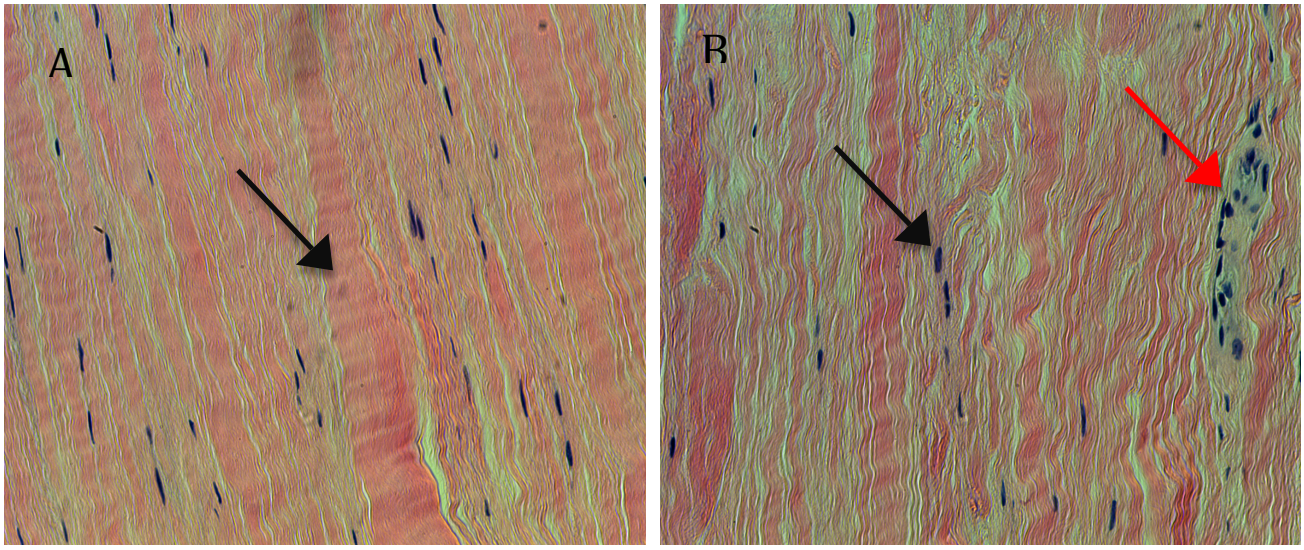

Figure 2. H&E of ACL tissue showing phenotype of ACL tissue. A) Crimping is shown throughout tissue. B) Black arrow showing elongated nuclei within crimped ACL tissue. This is characteristic of fibroblast cells. Red arrow is showing vessels found in ACL tissue. Image was acquired at a magnification of 400x.

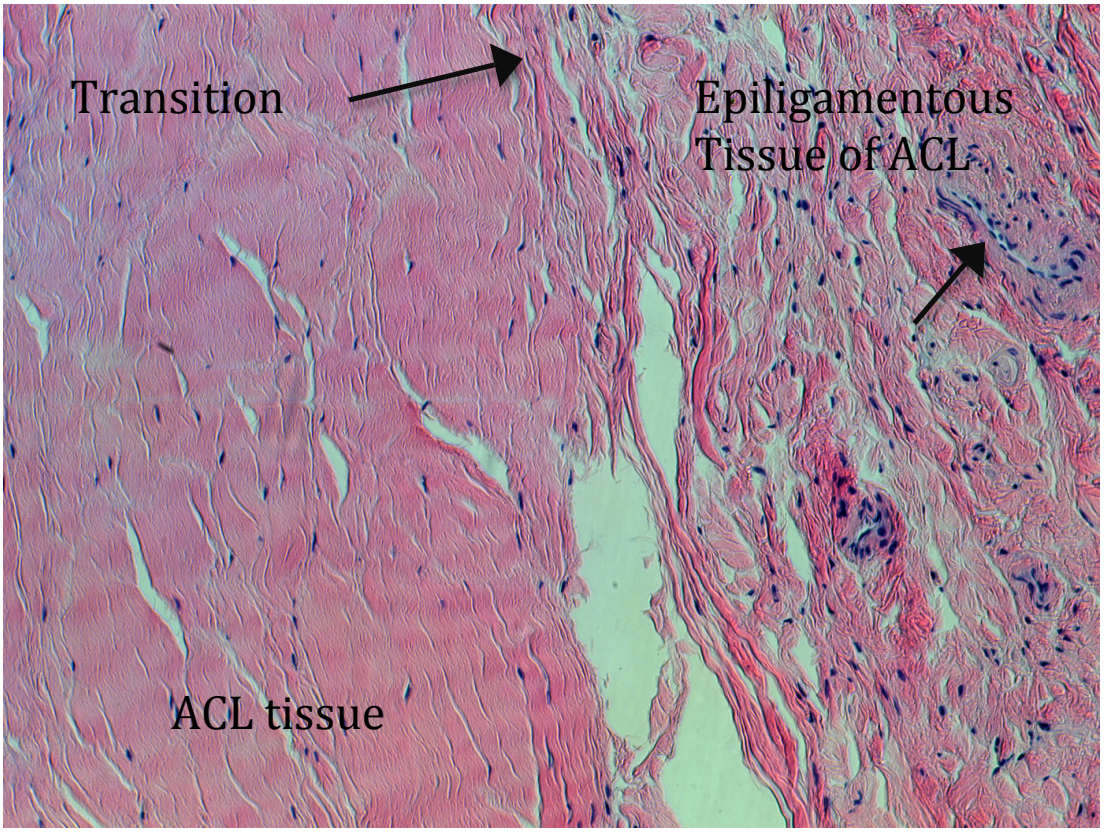

Figure 3. H&E staining of the ACL with visible epiligament layer. Black arrow on right side indicating vessels present in layer. Image was acquired at a magnification of 400x.

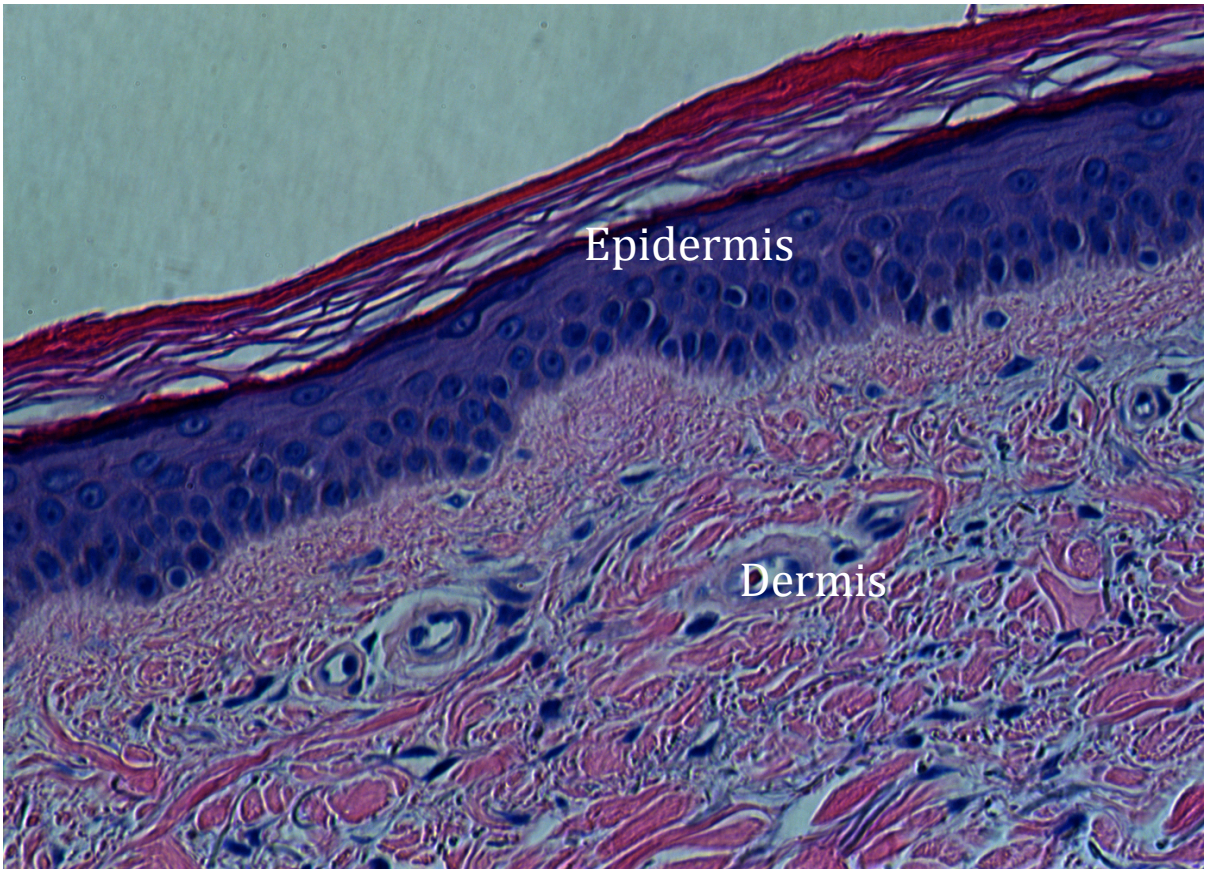

Figure 4. H&E of skin showing epidermis and dermis. Image was acquired at a magnification of 400x.

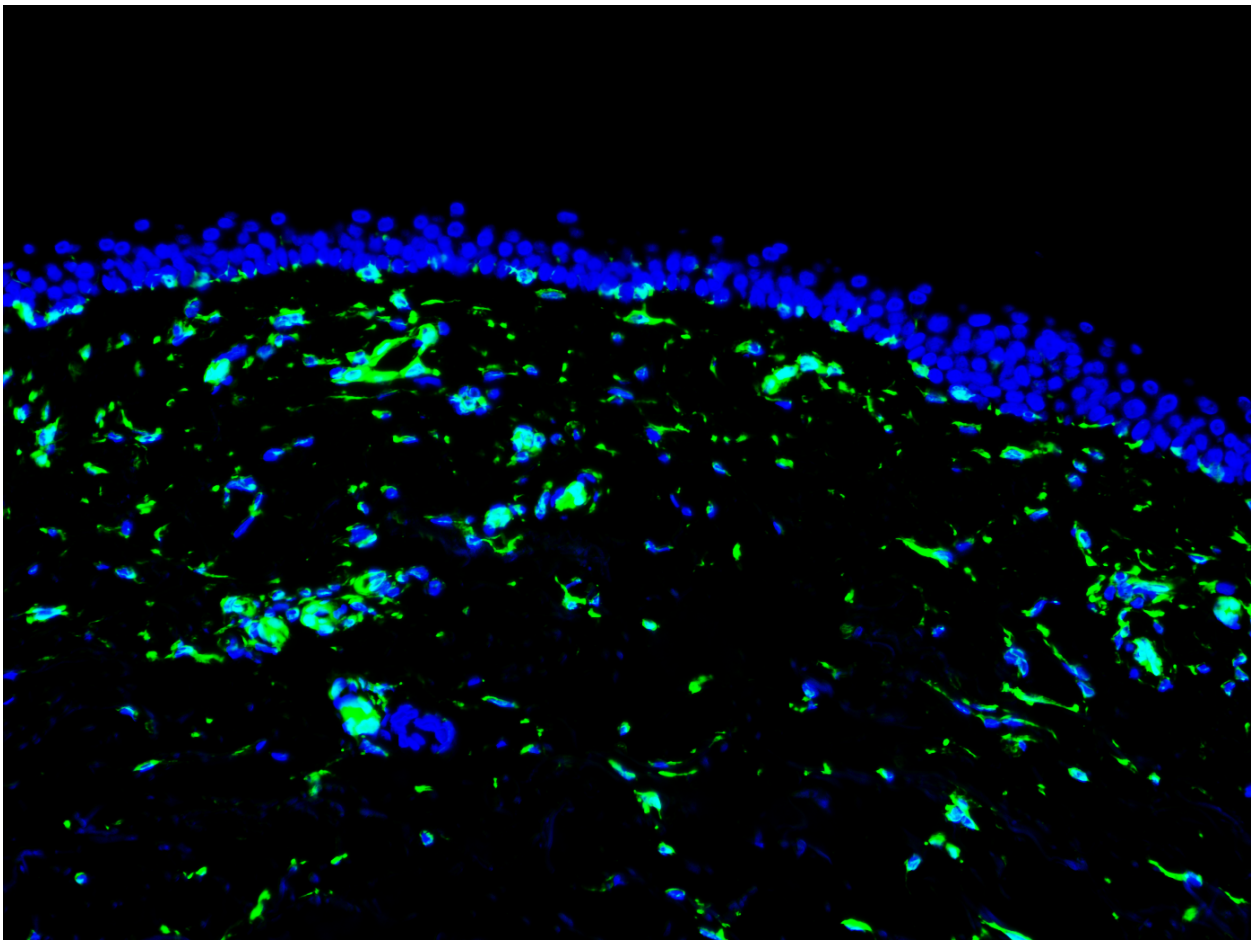

Figure 5. Skin (positive control): Vimentin shown in green fluorescence and nuclei are seen blue staining. Vimentin can be seen where blood vessels and fibroblasts exist. Image was acquired at a magnification of 400x.

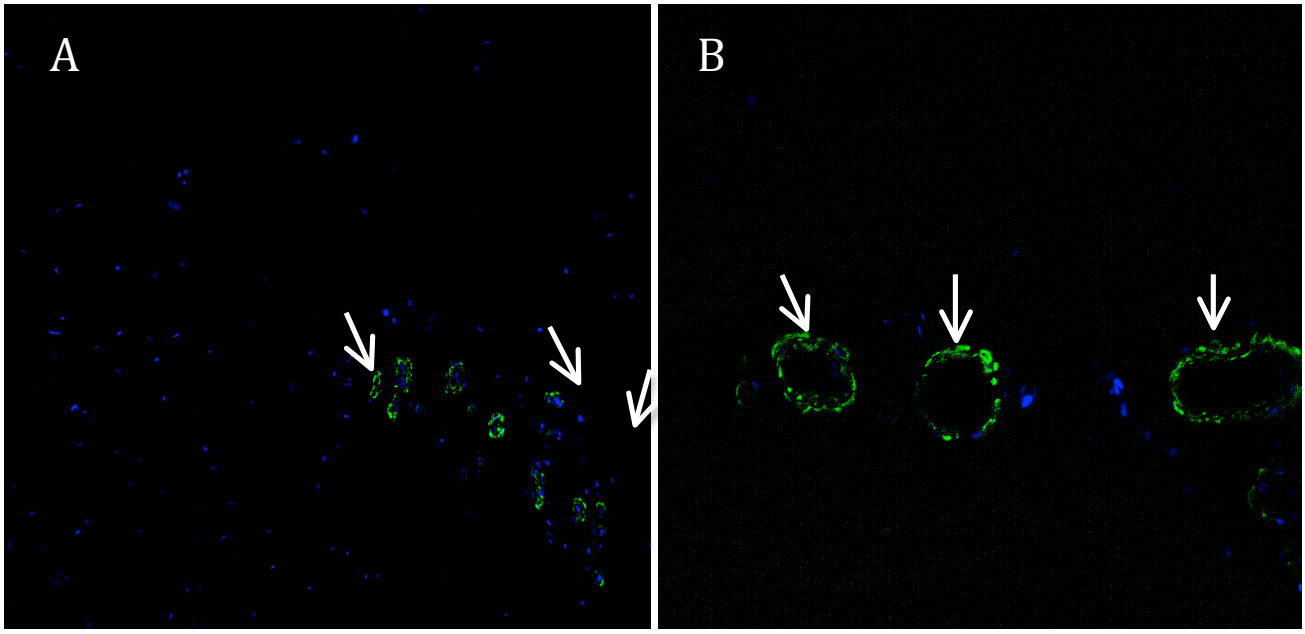

Figure 6. Presence of alpha-smooth muscle actin staining on vessels. **A)** Ligament 3 showing no staining of alpha-smooth muscle actin in fibroblasts, but positive staining is found around blood vessels. **B)** Blood vessels positive for alpha-smooth muscle actin in ligament 4.

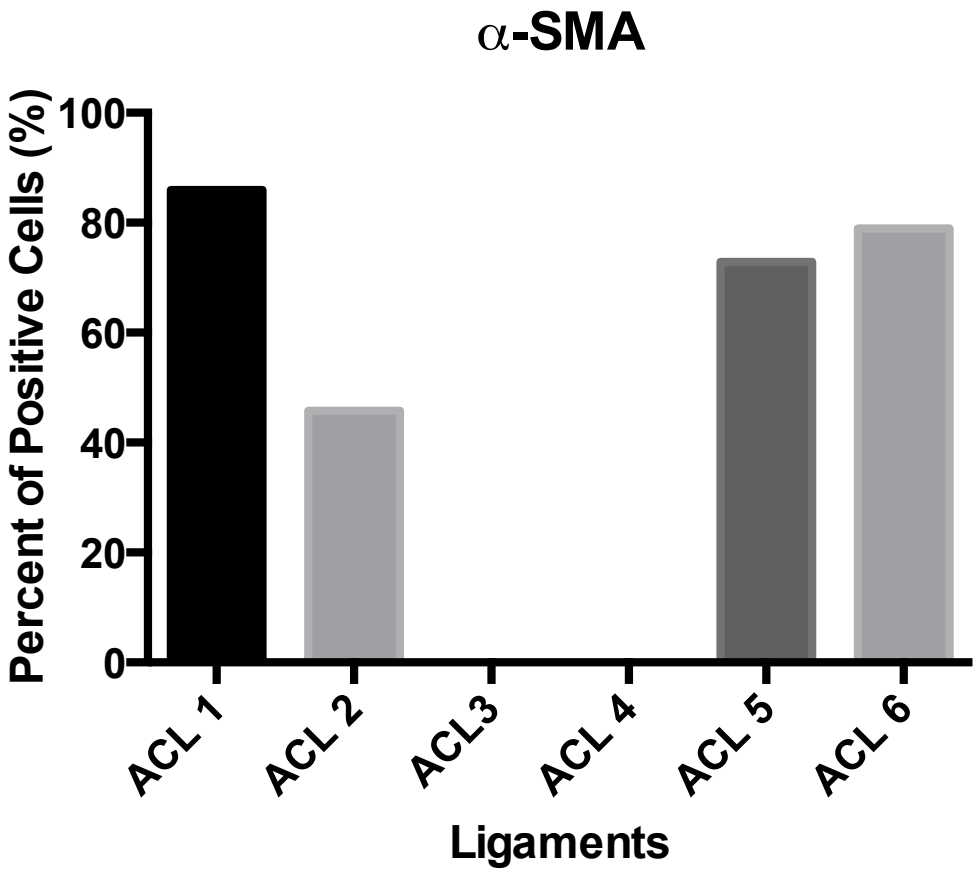

Figure 7. Percentage of cells positive for alpha-smooth muscle actin across all samples. Two of the ligaments did not have positive staining for this component (ACL 3 and 4).

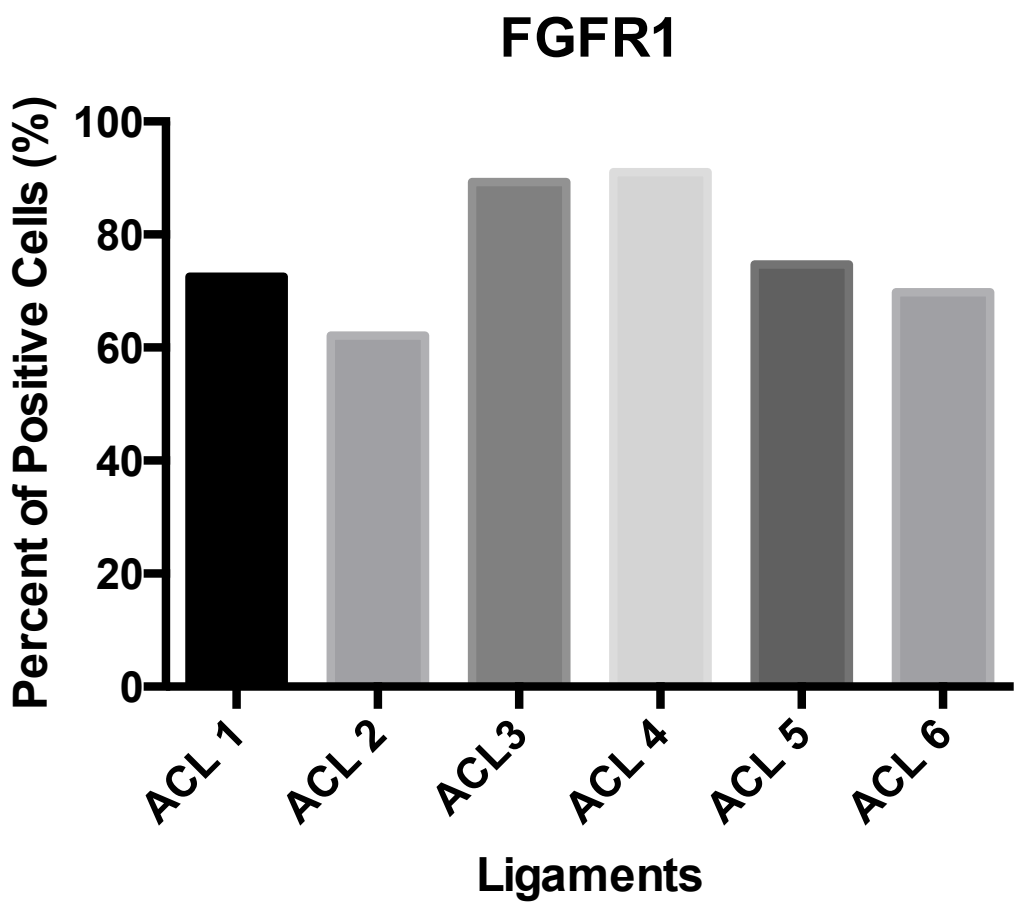

Figure 8. Percentage of cells positive for FGFR1 across all ligament samples.

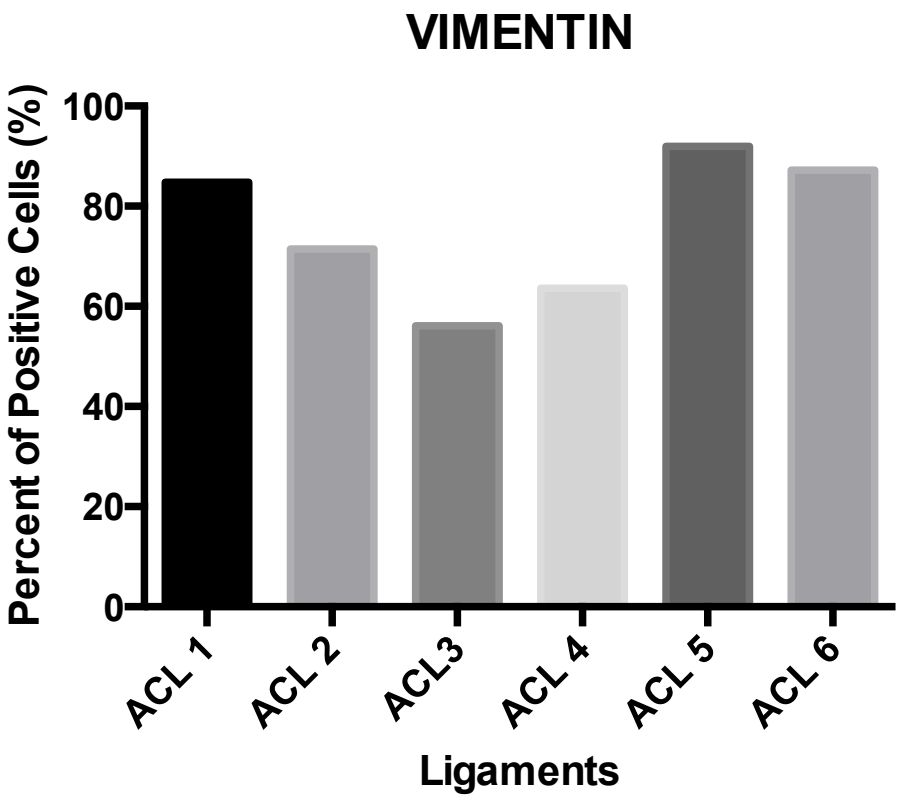

Figure 9. Percentage of cells positive for vimentin across all ligament samples.

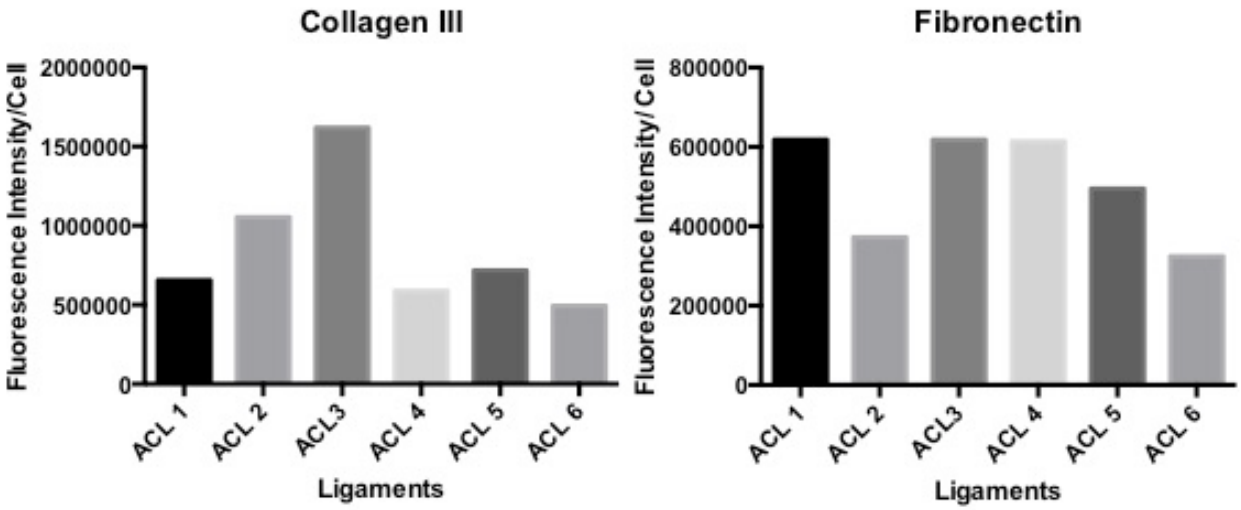

Figure 10. A.) Fibronectin was quantified using fluorescence intensity across all ligaments. B.) Collagen III was quantified using fluorescence intensity across all ligaments.

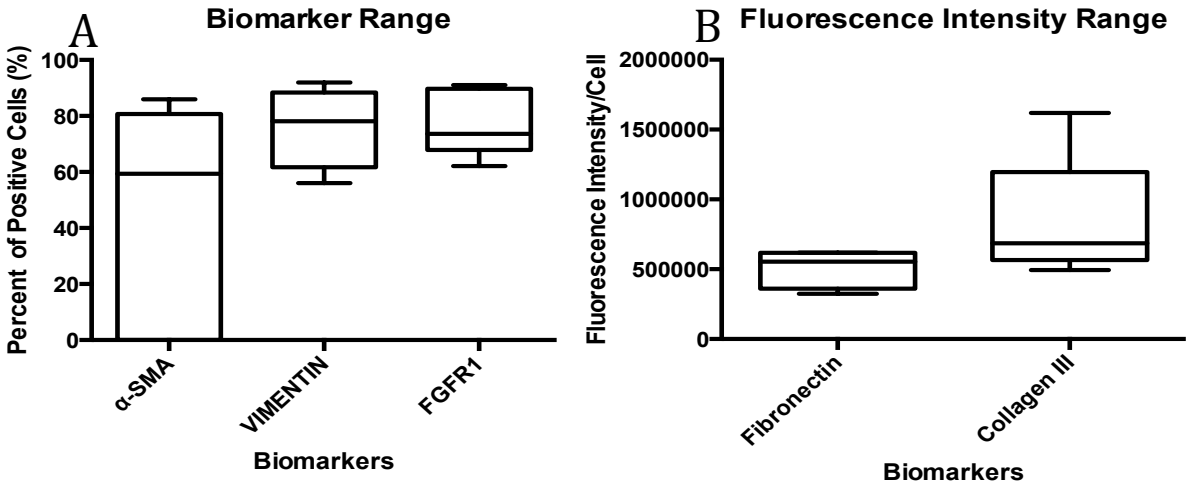

Figure 11. A.) Box-plot representation of fibronectin and collagen III showing the range of fluorescence intensity within combined ACL samples. B.) Box-plot representation of alpha-SMA, vimentin, FGFR1 showing range of percent of positive cells obtained within combined ACL samples.

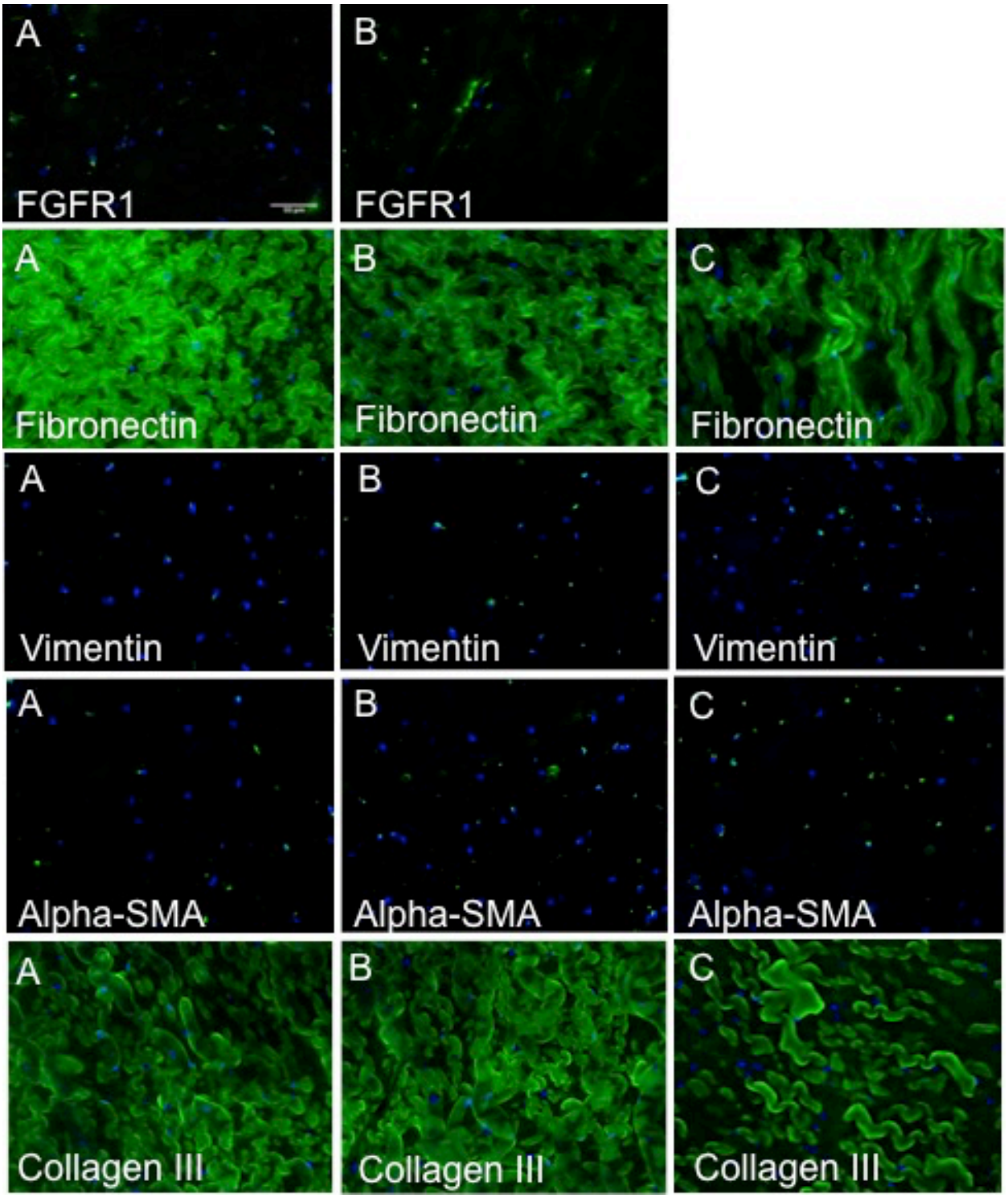

Figure 12. Regions of Interest (ROI) for biomarkers of interest detected in anterior cruciate ligament one. Blue staining is indicative of nuclei found in ligament tissue. Green fluorescence staining indicates component expression and presence in ROI. All images were acquired at a magnification of 400x.

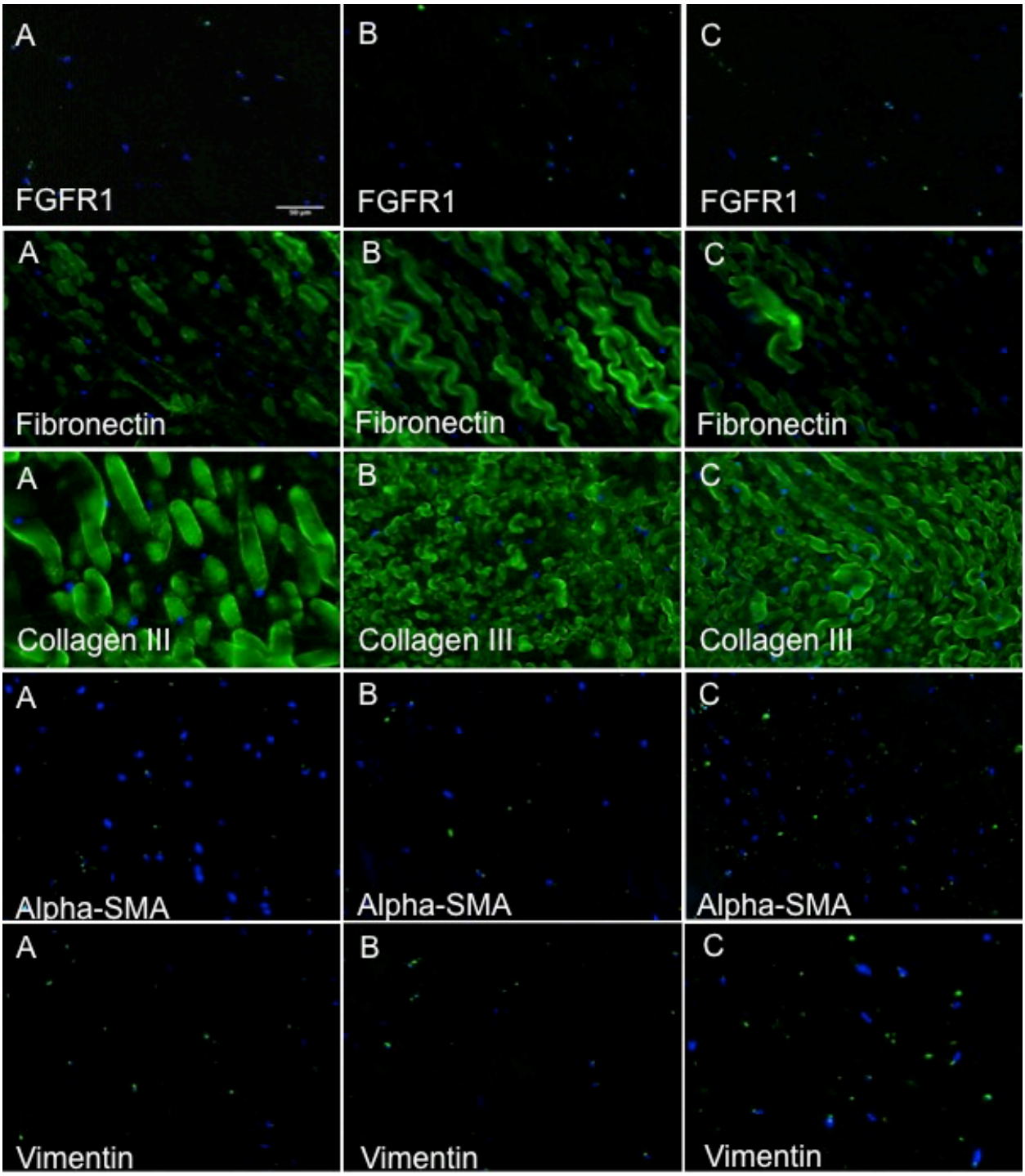

Figure 13. Regions of Interest (ROI) for biomarkers of interest detected in anterior cruciate ligament two. Blue staining is indicative of nuclei found in ligament tissue. Green fluorescence staining indicates component expression and presence in ROI. All images were acquired at a magnification of 400x.

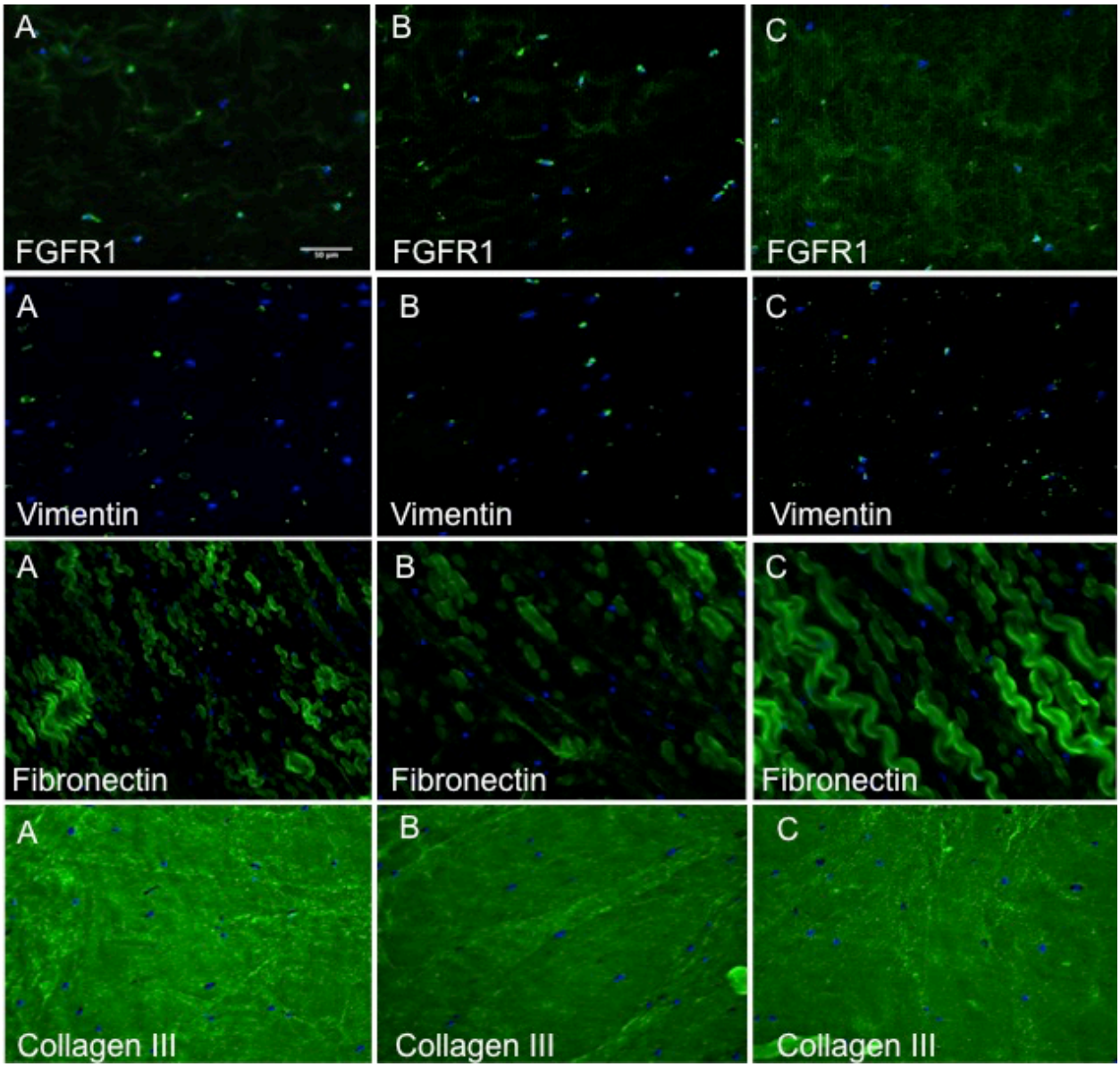

Figure 14. Regions of Interest (ROI) for biomarkers of interest detected in anterior cruciate ligament three. Blue staining is indicative of nuclei found in ligament tissue. Green fluorescence staining indicates component expression and presence in ROI. There was no alpha-smooth muscle actin staining in this ligament. All images were acquired at a magnification of 400x.

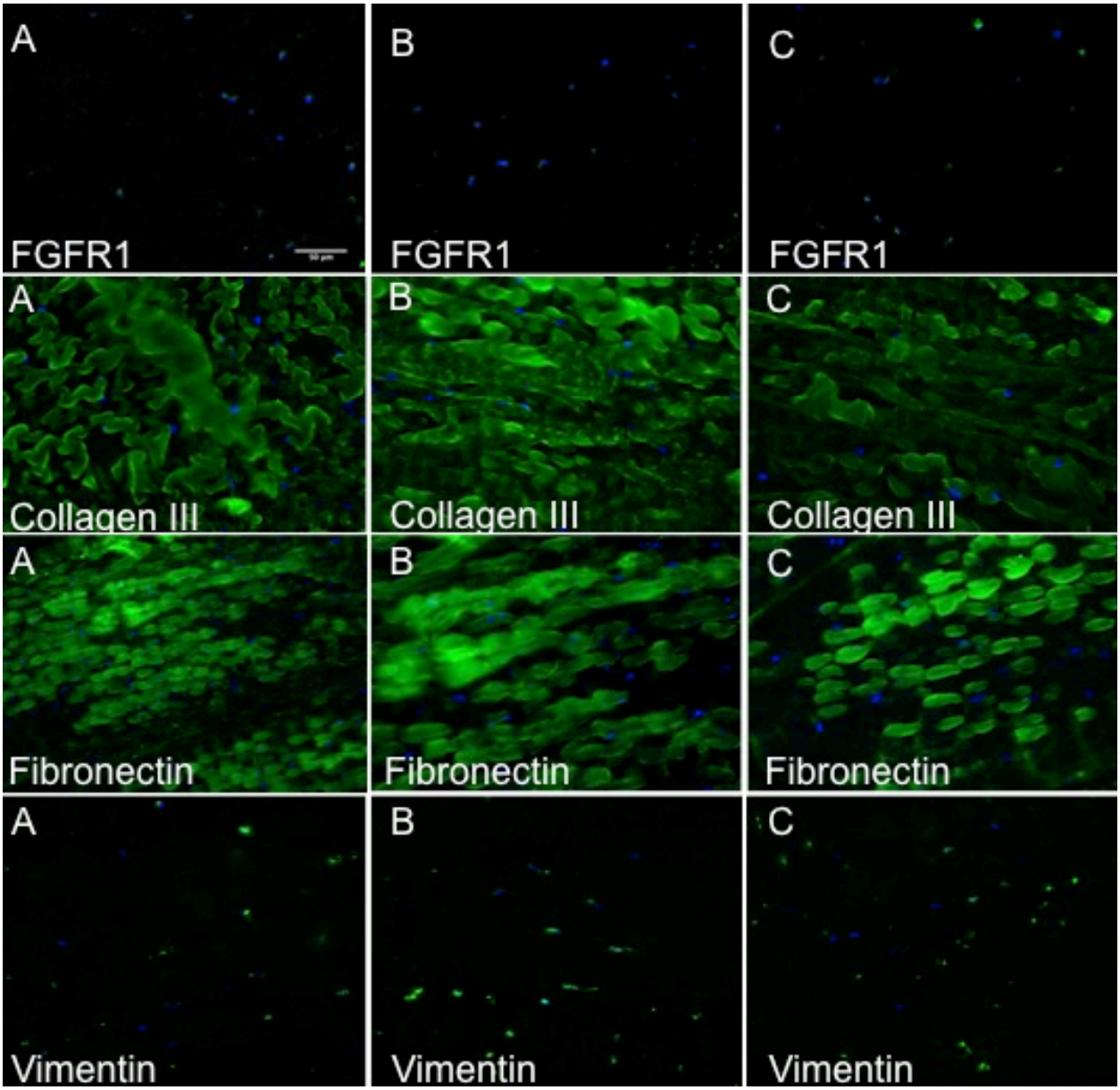

Figure 15. Regions of Interest (ROI) for biomarkers of interest detected in anterior cruciate ligament four. Blue staining is indicative of nuclei found in ligament tissue. Green fluorescence staining indicates component expression and presence in ROI. There was no alpha-smooth muscle actin staining in this ligament. All images were acquired at a magnification of 400x.

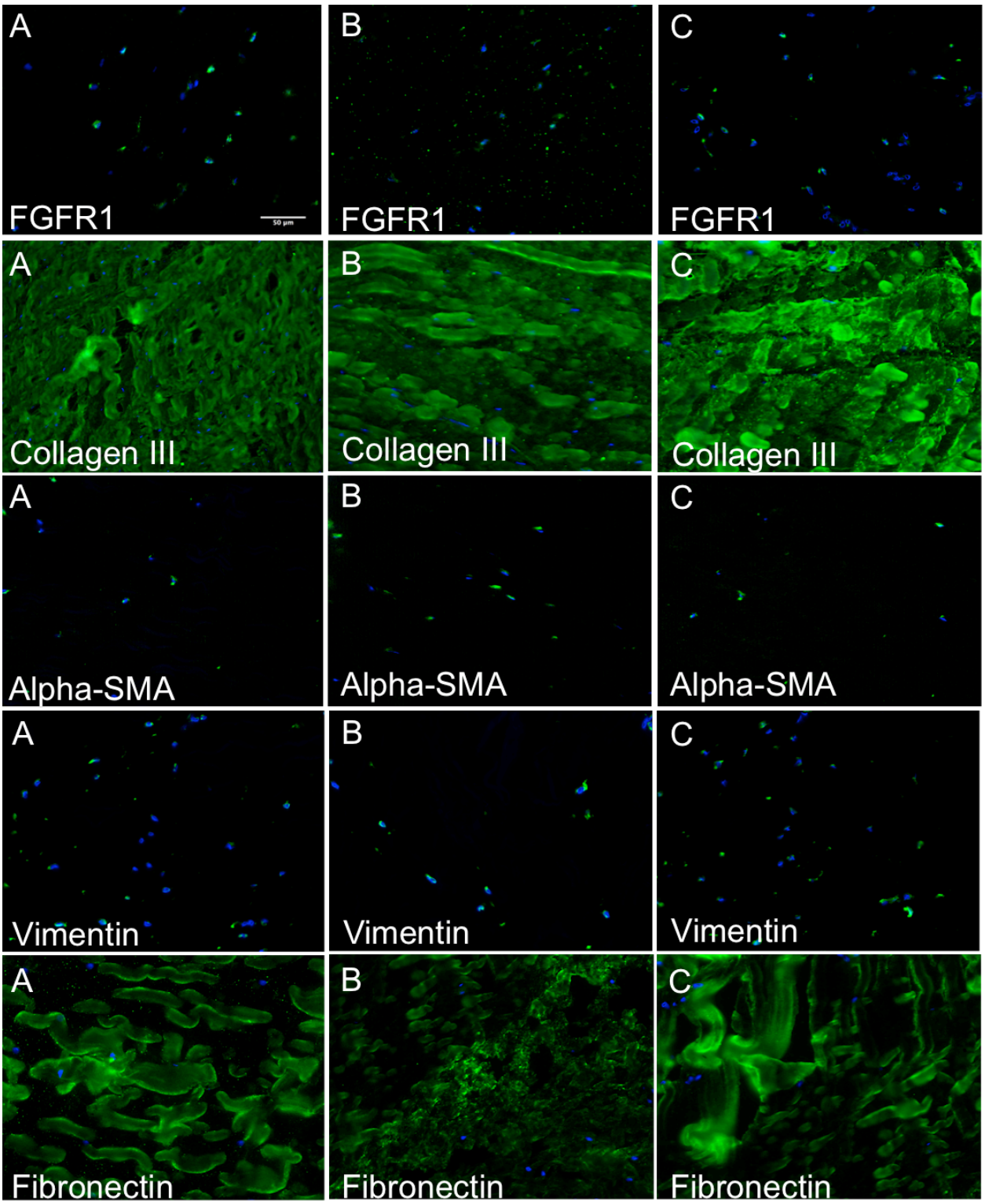

Figure 16. Regions of Interest (ROI) for biomarkers of interest detected in anterior cruciate ligament five. Blue staining is indicative of nuclei found in ligament tissue. Green fluorescence staining indicates component expression and presence in ROI. All images were acquired at a magnification of 400x

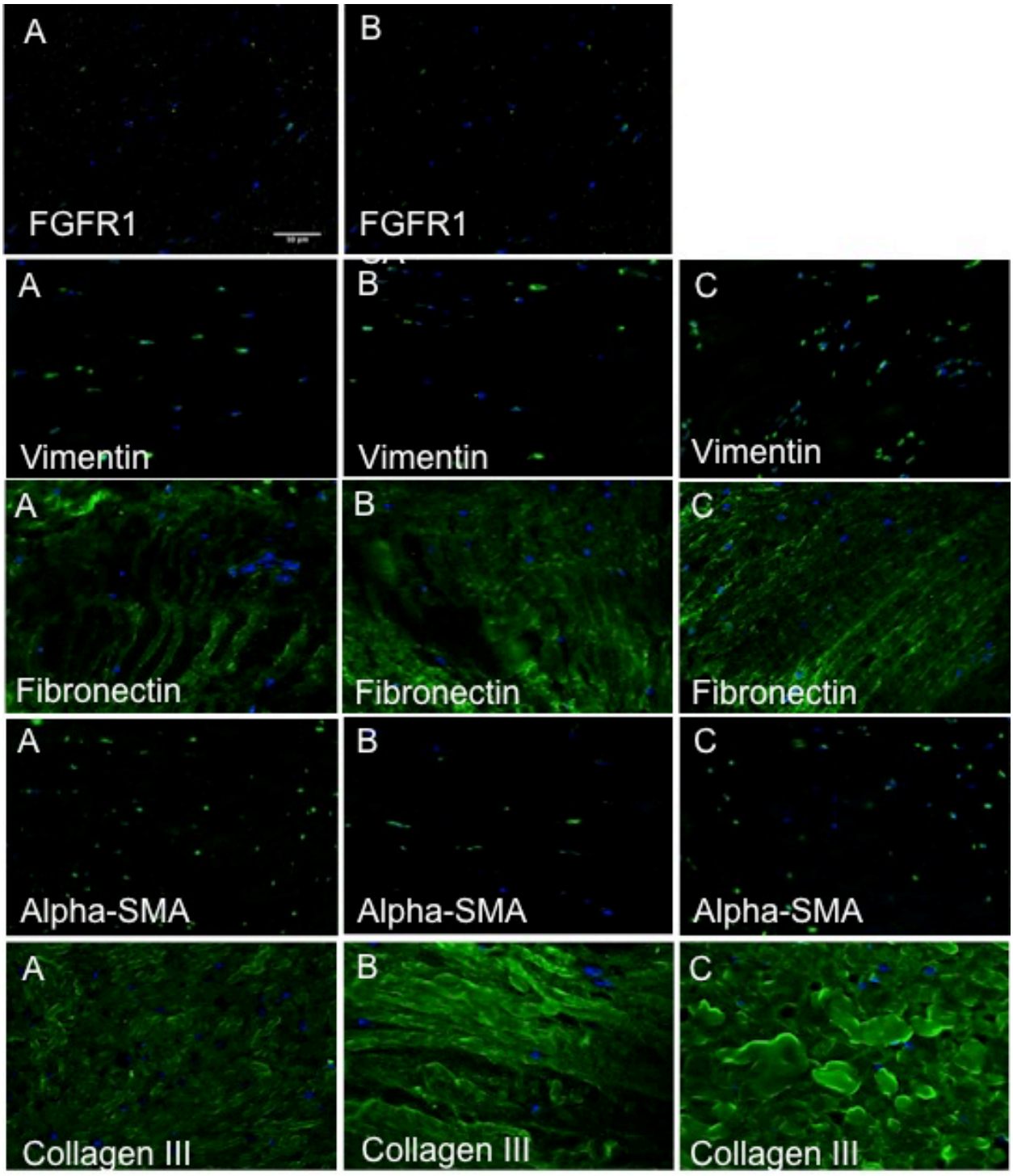

Figure 17. Regions of Interest (ROI) for biomarkers of interest detected in anterior cruciate ligament six. Blue staining is indicative of nuclei found in ligament tissue. Green fluorescence staining indicates component expression and presence in ROI. All images were acquired at a magnification of 400x.

CHAPTER 3

DISCUSSION

Many things must be factored and considered in the production of engineered ligaments. Selection of appropriate materials for the construct to withstand its role in the knee joint is extremely important. For example, West et. al tested engineered ligaments for the response of collagen content and tensile strength by exposing the construct to serum obtained after exercise. These investigations led this group to conclude that the serum may be increasing collagen expression and improving overall tensile strength, which may be due to Growth Hormone or Insulin-Like Growth Factor-1 present in serum after exercise (West et. al, 2015). Lee et. al studied the role of estrogen in engineered ligaments to determine if a rise in the hormone decreases the mechanical properties of an engineered ligament. They found that the rise in estrogen in serum during menstruation, may lead to decreased integrity of the ligament (Lee et. al, 2015).

Research studies like those previously described bring into perspective the multitude of environmental conditions the construct will have to adapt to. Our study puts into perspective the importance of cell selection and the testing that must take place to utilize cells that will be used for engineering purposes.

An advantage of this study is the characterization of fibroblasts using fresh anterior cruciate ligament tissue from humans. This allowed us to probe for the biomarkers of interest, and to observe how they may be naturally expressed within this tissue. These results may be different from characterizing a cell line or primary fibroblasts from the ACL using mRNA or protein expression of these components because the tissue has not been altered. When the tissue is homogenized to isolate cells, or when these cells are cultured, there is a chance of obtaining

false positive results such as in the case of alpha-smooth muscle actin, which may be absent in the ligament tissue, but present in vessels found in the epiligament. To our knowledge, this has not been previously reported. Based on our findings, we recommend investigators using this particular biomarker, to consider using additional biomarkers for characterization purposes to avoid false positive results. Although four out of six of the ligaments showed alpha-smooth muscle actin positive staining within fibroblasts, there may be an underlying mechanism, possible differentiation, or phenotype change that could be associated with the expression or non-expression of this type of actin.

As has been previously investigated, the expression of alpha-smooth muscle actin is common to myofibroblasts, which are predominant in vessel (Gabbiani et. al 1981; Goodpaster et. al 2008). Whether differentiation or phenotype changes occurred in the two ligaments that had no staining of alpha-SMA, can not be determined by our results. However, because engineering a suitable construct requires taking into consideration any and all variables and factors, perhaps this biomarker may not be well suited to be used as the only biomarker for characterization.

The use of FGFR1 has not been previously reported to be used as a biomarker for characterization of ACL fibroblasts. Based on our findings FGFR1 did not show as much variability in expression as alpha-SMA. Although it did not test positive in all ACL fibroblasts 100% of the time as was hypothesized, the range at which it did test positive was as comparable as vimentin; between a range of 62.1% to 91.1% of cells positive for FGFR1 compared to vimentin detection at 56% to 92%. Although the sample size is a limitation in our study, with further acquisition of samples, we hypothesize that FGFR1 could potentially be used as a biomarker for ACL fibroblast characterization.

Additionally, the use of fibronectin and collagen III are abundantly expressed and are therefore an attractive biomarker for characterization. However, as previously mentioned, it is difficult to know which fibroblast is responsible for producing either biomarker due to the morphology of the tissue and the overall distribution of the components.

Taking consideration of the variation that may exist in ACL tissue is extremely important for construct engineering applications. As was shown in our study, the characterization process must be well thought out to isolate cells that will respond well and similar to native cells in the ACL. Additionally, we propose that the use of this combination of biomarkers is suitable for characterization purposes with careful precaution for alpha-smooth muscle actin false positives.

Future Directions

These preliminary findings have provided the foundation for further investigations pertaining to construct engineering. We would like to investigate the expression of these biomarkers using an ex-plant method to culture ACL fibroblasts. Using this method, we aim to use primary ACL fibroblasts for immunohistochemistry staining of this combination of biomarkers to study their effectiveness for characterization purposes.

Although not a focus in this study, aging is a component we would like to add to this project. To do so we would like to further collect ligaments and incorporate age groups as an additional variable. Specifically, we are looking to investigate if age is playing a role in the changes of the components we focused on. ACL investigations have focused more on the younger, more physically active population, so it would be beneficial to provide insight on the cellular changes that occur in these components in those above the age of 60.

Yet another layer of complexity we would like to add to this project is that of the affects of osteoarthritis on the cruciate ligaments of the knee. In this study we excluded donors who showed signs of osteoarthritis such as articular cartilage degradation and eburnation. However, we would like to incorporate this variable into our future studies with particular interest on the influence of the different degrees of osteoarthritis.

Pertaining to our interest in construct engineering, we would like to look further into the different phenotypes of fibroblasts in the ACL and methods of isolation. Specifically, using flow cytometry, we would like to investigate if using this combination of biomarkers, we are able to select for the different isoforms of fibroblasts in the ACL. If this is achieved, it would be interesting to investigate if there are differences in the production of these biomarkers, other ECM components, and growth factors.

BIBLIOGRAPHY

Amiel D, Billings E, Jr., Harwood FL. Collagenase activity in anterior cruciate ligament: Protective role of the synovial sheath. J Appl Physiol 1990; 69(3): 902-906.

Bellincampi LD, Closkey RF, Prasad R, Zawadsky JP, Dunn MG. J Orthop Res. 1998 Jul; 16(4):414-20.

Boden BP, Dean GS, Feagin JA Jr, Garrett WE Jr. Mechanisms of anterior cruciate ligament injury. Orthopedics. 2000;23(6):573–578.

Brulet, Philippe et al.. "Monoclonal Antibodies Against Trophectoderm-specific Markers During Mouse Blastocyst Formation". *Proceedings of the National Academy of Sciences of the United States of America* 77.7 (1980): 4113–4117.

Brune, T., et al. "In vitro comparison of human fibroblasts from intact and ruptured ACL for use in tissue engineering." *Eur Cell Mater* 14 (2007): 78-90.

Cheng, M-T., et al. "Isolation and characterization of multipotent stem cells from human cruciate ligaments." Cell proliferation 42.4 (2009): 448-460.

Darby, I., O. Skalli, and G. Gabbiani. "a-Smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing." *Lab Invest* 63.1 (1990): 21-29. Grinnell F. Fibroblasts, myofibroblasts and wound contraction. *J Cell Biol* 1994;124:401 4.

Darby I A, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol. 2007;257:143–179.

Dooley, Thomas, Ernest Curto, and Richard Davis. "Skin cell biomarkers and methods for identifying biomarkers using nucleic acid microarrays." U.S. Patent Application 10/361,006.

Dunn MG, Liesch JB, Tiku ML, Zawadsky J. J Biomed Mater Res. 1995 Nov; 29(11):1363-71.

Duthon et. al: Anatomy of the anterior cruciate ligament. Knee Surg Sports Traumatol Arthrosc. 2006; 14: 204-213.

Goodpaster, Tracy, et al. "An immunohistochemical method for identifying fibroblasts in formalin-fixed, paraffin-embedded tissue." *Journal of Histochemistry & Cytochemistry* 56.4 (2008): 347-358.

Hadjicostas PT, Soucacos PN, Koleganova N, Krohmer G, Berger I (2008) Comparative and morphological analysis of commonly used autografts for anterior cruciate ligament reconstruction with the native ACL: an electron, microscopic and morphologic study. Knee Surg Sports Traumatol Arthrosc 16:1099–1107.

Hasegawa et al.: Anterior cruciate ligament changes in the human knee joint in aging and osteoarthritis. Arthritis Rheum 2012, 64: 696-704.

Hasegawa, Akihiko, et al. "Cellular and extracellular matrix changes in anterior cruciate ligaments during human knee aging and osteoarthritis." Arthritis Res Ther 15.1 (2013): R29.

Jaye, M., Schlessinger, J., Dionne, C.A. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. Biochim Biophys Acta, 1135 (1992), pp. 185–199.

Kim, Sung-Gon, et al. "Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells." *Cell structure and function* 27.3 (2002): 139-144.

Kleiner, Jeffrey B., et al. "Origin of replacement cells for the anterior cruciate ligament autograft." Journal of orthopaedic research 4.4 (1986): 466-474.

Laurencin CT, Freeman JW: Ligament tissue engineering: an evolutionary materials science approach. *Biomaterials* 2005, 26: 7530-7536.

Laurencin, Cato T., et al. "Ligament and tendon replacement constructs and methods for production and use thereof." U.S. Patent No. 8,945,218. 3 Feb. 2015.

Lee, C. H., Shah, B., Moioli, E. K. & Mao, J. J. CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model. *J. Clin. Invest.* **120**, 3340–3349 (2010).

Loës, M., Dahlstedt, L. J. and Thomée, R. (2000), A 7-year study on risks and costs of knee injuries in male and female youth participants in 12 sports. Scandinavian Journal of Medicine & Science in Sports, 10: 90–97.

Mendez, Melissa G., Shin-Ichiro Kojima, and Robert D. Goldman. "Vimentin Induces Changes in Cell Shape, Motility, and Adhesion during the Epithelial to Mesenchymal Transition." *The FASEB Journal* 24.6 (2010): 1838–1851.

Middleton, K. K., et al. "Anatomic anterior cruciate ligament (ACL) reconstruction: a global perspective. Part 1." Knee Surgery, Sports Traumatology, Arthroscopy (2014): 1-16.

Mizutani, Naoki, et al. "The behavior of ligament cells cultured on elastin and collagen scaffolds." Journal of Artificial Organs 17.1 (2014): 50-59.

Mohammadi, M et al. "Identification of Six Novel Autophosphorylation Sites on Fibroblast Growth Factor Receptor 1 and Elucidation of Their Importance in Receptor Activation and Signal Transduction." *Molecular and Cellular Biology* 16.3 (1996): 977–989. Print.

Murray, M. M., and M. Spector. "Fibroblast distribution in the anteromedial bundle of the human anterior cruciate ligament: The presence of (alpha-smooth) muscle actin-positive cells." *Journal of orthopaedic research* 17.1 (1999): 18.

Murray MM, Martin SD, Martin TL, Spector M: Histological changes in the human anterior cruciate ligament after rupture. J Bone Joint Surg Am 2000, 82A: 1387-1397.

Nau, Thomas, and Andreas Teuschl. “Regeneration of the Anterior Cruciate Ligament: Current Strategies in Tissue Engineering.” *World Journal of Orthopedics* 6.1 (2015): 127–136. *PMC*. Web. 31 Oct. 2015.

Nöth, U., et al. "Anterior cruciate ligament constructs fabricated from human mesenchymal stem cells in a collagen type I hydrogel." Cytotherapy 7.5 (2005): 447-455.

Otsubo, Hidenori, et al. "The arrangement and the attachment areas of three ACL bundles." *Knee Surgery, Sports Traumatology, Arthroscopy* 20.1 (2012): 127-134.

Panas-Perez E, Gatt CJ, Dunn MG. J Mater Sci Mater Med. 2013 Jan; 24(1):257-65.

Pennisi, Elizabeth. "Tending tender tendons." Science 295.5557 (2002): 1011-1011.

Petersen W, Tillmann B: Structure and vascularization of the cruciate ligaments of the human knee joint. *Anat Embryol (Berl)* 1999, 200: 325-334.

Petrigliano, Frank A., David R. McAllister, and Benjamin M. Wu. "Tissue engineering for anterior cruciate ligament reconstruction: a review of current strategies." Arthroscopy: The Journal of Arthroscopic & Related Surgery 22.4 (2006): 441-451.

Roos H, Adalberth T, Dahlberg L, Lohmander LS: Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. Osteoarthritis Cartilage 1995, 3:261-267.

A. Saminathan, K. J. Vinoth, H. H. Low, T. Cao, and M. C. Meikle, “Engineering three-dimensional constructs of the periodontal ligament in hyaluronan-gelatin hydrogel films and a

mechanically active environment," Journal of Periodontal Research, vol. 48, no. 6, pp. 790–801, 2013.

Skalli, Omar, et al. "A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation." *The Journal of Cell Biology* 103.6 (1986): 2787-2796.

Steinert P M, Idler W W, Cabral F, Gottesman M M, Goldman R D. In vitro assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblastic cells. Proc Natl Acad Sci U S A. 1981;78:3692–3696.

Weiler, A., Unterhauser, F. N., Bail, H.-J., Hüning, M. and Haas, N. P. (2002), α-Smooth muscle actin is expressed by fibroblastic cells of the ovine anterior cruciate ligament and its free tendon graft during remodeling. J. Orthop. Res., 20: 310–317.

West, Daniel WD, et al. "The exercise‐induced biochemical milieu enhances collagen content and tensile strength of engineered ligaments." *The Journal of physiology* 593.20 (2015): 4665-4675.

Wyatt, Ronald WB, et al. "Prevalence and Incidence of Cartilage Injuries and Meniscus Tears in Patients Who Underwent Both Primary and Revision Anterior Cruciate Ligament Reconstructions." The American Journal of Sports Medicine (2014): 0363546514536020.

Yamaguchi, Terry P., et al. "fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation." *Genes & development* 8.24 (1994): 3032-3044.

Zigang, G., Cho Hong Goh, J., Hin Lee, E. "Selection of cell source for ligament tissue engineering." *Cell transplantation* 14.8 (2005): 573-583.

Zschenker O, Streichert T, Hehlgans S, Cordes N (2012) Genome-wide gene expression analysis in cancer cells reveals 3D growth to affect ECM and processes associated with cell adhesion but not DNA repair. PLoS One 7(4):e34279.