DIFFERENTIAL RESPONSES OF EMBRYONIC AND POSTNATAL TENDON CELLS TO IL-1β TREATMENT: IMPLICATIONS FOR SCARLESS HEALING

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ABSTRACT

Adult and postnatal tendons heal poorly, forming scar tissue with aberrant properties. In contrast, embryonic tendons heal in a scarless manner, with restoration of native tissue properties. Investigation of scarless embryonic tendon healing may lead to strategies for improving adult tendon healing. We probed differences in embryonic and postnatal tendon cell responses to wound-related environmental factors, as well as potential mechanisms that may regulate their respective responses. Factors we tested included IL-1β treatment, substrate elastic modulus, and extracellular matrix composition. We found that postnatal tendon cells have higher expression of inflammatory mediators and matrix metalloproteinases than embryonic tendon cells, both at baseline and in response to IL-1β treatment, and that this disparity may be due to differences in IL-1 receptor profiles. The long-term goal of this project is to develop strategies to redirect adult scarred tendon healing outcomes toward more embryonic-like scarless tissue regeneration.

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DIFFERENTIAL RESPONSES OF EMBRYONIC AND POSTNATAL TENDON CELLS TO IL-1β TREATMENT: IMPLICATIONS FOR SCARLESS HEALING

CHAPTER 1: BACKGROUND

1.1 TENDON OVERVIEW

Tendon is an important musculoskeletal tissue whose major functions are to transmit forces from muscle to bone in order to allow for movement, as well as to stabilize joint structures. Tendons are primarily composed of collagen fiber bundles that are highly aligned along the major axis of the tendon. The collagen structure of tendon is hierarchically organized; triple-helical collagen molecules crosslink to form collagen fibrils, which aggregate to form collagen fibers, which in turn bundle to form fascicles, which then group to form tendon units (**Fig 1.1**). The collagen fibers that make up tendon exhibit a wavy crimp pattern (Diamant et al., 1972). The collagen bundles within tendons are surrounded by a connective tissue called endotenon, which contains the blood vessels and nerves, while the tendon units are surrounded by an outer layer called epitenon. While tendons are made up of mostly collagen type I (Col I), they also include other collagens, such as Col III, as well as additional extracellular matrix (ECM) molecules, such as proteoglycans and elastin (Kjaer, 2004). Also present in tendon are fibroblastic tendon cells, which are located within fascicles between collagen fibers and act to synthesize and maintain the ECM.

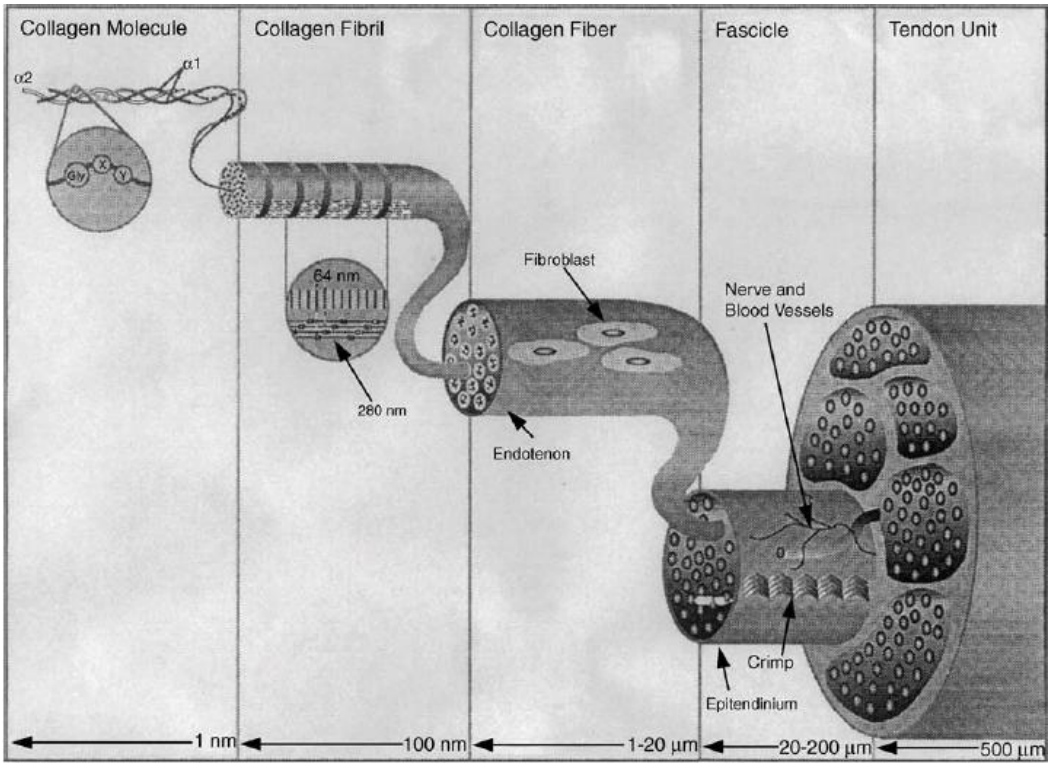

Figure 1.1: Hierarchical collagen structure of tendon. From Silver et al., 2003.

Because the main role of tendon is to transmit muscle-derived forces to bone, its mechanical properties are of utmost importance to its functionality. Tendon has an elastic modulus between that of muscle and bone, acting as a transition between the two tissue types, and its unique structure and composition lend it high tensile strength and viscoelastic properties. The stress-strain curve of tendon in tension is characterized by a toe region as the crimps in the collagen fibers straighten, a linear region as the fibers are pulled, and a plateau region at failure as the fibers begin to tear (**Fig 1.2**).

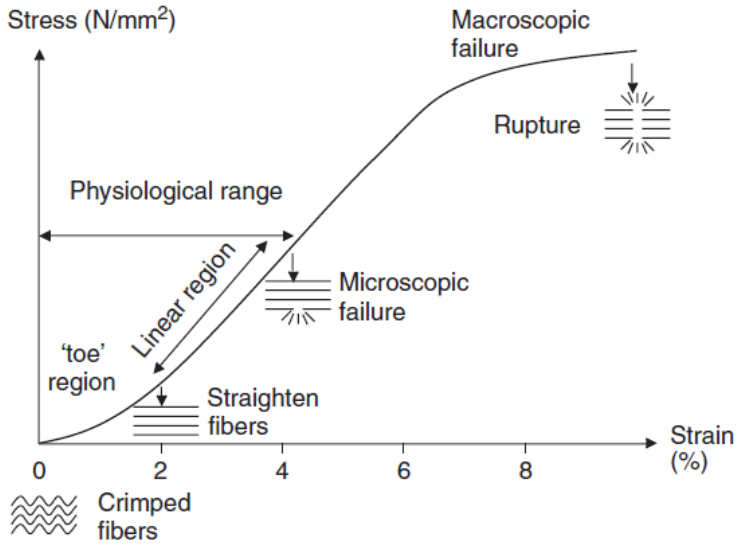

Figure 1.2: Stress-strain curve of tendon. From Wang, 2006.

1.2 TENDON INJURY

Tendon injuries are common and often debilitating; a survey of an orthopedic trauma unit in the United Kingdom found that the annual overall incidence of acute tendon/ligament injuries among their service population was 106.2/100,000 (Clayton and Court-Brown, 2008). Furthermore, a significant clinical problem is presented by the poor innate healing capability of tendons. Injured adult, including postnatal, tendons fail to heal or heal as scar tissue with aberrant mechanical and biochemical properties, leading to altered tendon structure and function. As a whole, healed tendons are weaker than normal tendons, limiting their functionality and making them prone to re-injury. Tendons can suffer from acute rupture injuries, which are the focus of this project, as well as long-term chronic pathologies.

1.2.1 Acute ruptures

When tendons are loaded very suddenly and/or with abnormally high strain, it can lead to a partial or full rupture of the tendon. Following such an injury, the tendon goes through several stages of healing that are very similar to that of skin (reviewed in Molloy et al., 2003; Schulze-Tanzil et al., 2011; and Voleti et al., 2012). The hemorrhagic healing phase, or hemostasis, occurs immediately after injury. When an adult tendon is wounded, the damage to the tissue causes blood vessels to rupture. This triggers the coagulation cascade, where a blood clot made up of aggregating platelets and fibrin begins to form at the site of injury. The platelets release a first wave of growth factors and cytokines, including PDGF, EGF, IGF-1, TGF-β1, TGF-β2, VEGF, bFGF, IL-1β, and TNFα. These released factors cause acute local inflammation and provide chemotactic signals for immune cells circulating in the blood supply to infiltrate the wound site, thereby initiating the inflammatory healing phase.

The inflammatory phase of healing begins within several hours after injury and lasts for approximately 2 to 3 days. Among the infiltrating immune cells, neutrophils are the "first responders," the most dominant immune cell type during the beginning of the inflammatory phase, after which the profile shifts toward monocytes and macrophages. These cells are monocytes when they in the blood stream, then they differentiate into macrophages once they arrive at the site of injury. Neutrophils and macrophages are both phagocytes that remove bacteria,

necrotic debris, and foreign particles present in the wounded area through phagocytosis. In addition, they, along with the resident tendon cells, release a second wave of growth factors and cytokines, including TGF-β1, FGF-2, EGF, IL-1β, TNFα, and IL-6.

The proliferative healing phase, or repair phase, begins several days after injury when the second wave of released factors causes recruitment of additional immune cells, neovascularization, proliferation of intrinsic tendon cells, and migration of extrinsic tendon cells to the wound site. The tendon cells deposit a temporary matrix that has a composition that differs from the normal matrix, with a higher proportion of Col III to Col I and more fibronectin (Juneja et al., 2013; Maffulli et al., 2000; Oshiro et al., 2003). By the end of the proliferative phase at approximately 4 weeks post-injury, a cell-rich granulation tissue has formed at the site of the tendon wound.

The final stage of healing is the remodeling phase, which begins about 4 weeks after injury and can persist for many months or even over a year. This phase involves the maturation of the newly formed tendon tissue at the wound site into healed scar tissue. The changes that occur during this transition include decreases in cellularity and vascularity, a reduction in overall matrix synthesis with a shift to an increased proportion of Col I to Col III production, and increases in the organization and alignment of the ECM. Unfortunately, the resulting repaired tendon tissue has aberrant properties and never completely regains its normal

tissue properties. Differences exhibited by scarred tendon compared to normal tendon include disorganized collagen structure with smaller fibril diameters, decreased mechanical properties, and an altered ECM composition with more Col III and proteoglycans (Alaseirlis et al., 2005; Ansorge et al., 2012; Birch et al., 1998; Bruns et al., 2000; Carpenter et al., 1998; Dyment et al., 2012; Ehrlich et al., 2005; Frank et al., 1997; Järvinen et al., 2004; Miyashita et al., 1997; Nagasawa et al., 2008).

1.2.2 Chronic tendinopathies

The term "tendinopathy" refers to chronic or long-term tendon issues that are often caused by overuse and are generally characterized by pain and decreased tendon function. The etiological factors involved in the development of tendinopathies are not well understood; there is not agreement on whether such conditions arise due to degeneration of the tendon, inflammation in the tendon, or a combination of both (Abate et al., 2009; Attia et al., 2013; de Mos et al., 2007; Jelinsky et al., 2011; Legerlotz et al., 2012; Millar et al., 2010; Millar et al., 2009; Pingel et al., 2013). Studies of the pathogenesis of tendinopathies are complicated by the fact that affected tendon samples are generally not obtained from patients until they undergo surgery at advanced stages of the disease. As such, animal models involving repetitive over-loading or collagenase injection of tendons are often used to study tendinopathy (Lake et al., 2008).

1.2.3 Current treatments for tendon injury

Because of their poor intrinsic healing capability, surgical intervention is usually required when a tendon is injured. Surgical tendon repair usually involves suturing together the ends of the ruptured tendon if it is still relatively intact, or replacing the damaged or missing tissue with autografts or allografts if it is not (Voleti et al., 2012; Yang et al., 2013). However, such treatment strategies have several shortcomings. Most importantly, none of them fully restore normal tendon tissue properties, so the repaired tendon has limited functionality and a high risk of re-injury. The grafts have their own additional issues, such as donor site morbidity and the potential for immunogenic responses. As such, there is a clear need for improved treatment strategies to repair tendons after acute injury.

1.3 EMBRYONIC SCARLESS HEALING

In contrast to adult wound healing, embryonic wounds heal without the formation of scar tissue. This embryonic scarless healing phenomenon was discovered in and has been mostly studied in skin, but has also been confirmed to exist in many other tissue types, including tendon.

1.3.1 Scarless healing in skin

Studies performed in skin have shown that scarless healing occurs during early and middle stages of embryonic/fetal development; at later developmental stages, healing begins to transition toward an adult-like scar-forming response. This

transition occurs at approximately the start of the third trimester for humans (Chen et al., 2007) and at approximately embryonic day (E) 18 for mice and rats (Colwell et al., 2006; Ihara et al., 1990). One of the most noticeable differences between embryonic and adult cutaneous wound healing is that there is minimal inflammation during embryonic healing, including less platelet aggregation, fewer infiltrating immune cells that are also not as activated and not present as long as in adult healing, and lower levels of released inflammatory mediators (Cowin et al., 1998; Liechty et al., 2000; Liechty et al., 1998; Olutoye et al., 1995; Olutoye et al., 1996; Wilgus et al., 2004).

1.3.2 Scarless healing in tendon

Like skin, embryonic tendons also heal in a scarless manner and regain normal tissue properties. Scarless embryonic tendon healing was first demonstrated by the Soslowsky group in fetal sheep (Beredjiklian et al., 2003). During healing from an acute injury, embryonic tendon has fewer inflammatory cells and lower levels of inflammatory mediators than injured adult tendon, similar to observations of embryonic vs. adult healing in skin (Beredjiklian et al., 2003; Favata et al., 2006). It was also found that, unlike adult tendon wounds, fetal tendon wounds regained their aligned collagen structure and normal mechanical properties after healing. However, there appears to be a limit to the size of wound that embryonic tendons can heal in a scarless manner (Herdrich et al., 2010).

1.3.3 Potential contributors to scarless healing

The ability of embryonic tissue to heal without scar formation was initially attributed to the sterile, amniotic fluid-filled intrauterine environment of developing embryos. However, it was found that such conditions are not necessary in order for embryonic tissue to heal scarlessly; a study conducted on opossum fetuses showed that, even though they develop in a pouch instead of in the womb, they still exhibited scarless healing of cutaneous wounds (Armstrong and Ferguson, 1995).

It was then thought that scarless vs. scarred healing outcomes were a function of immature embryonic vs. mature adult immune systems, based on observations of decreased inflammation and immune responses during embryonic wound healing compared to adult healing. However, transplant studies have demonstrated that a naïve immune system is not the sole contributor to scarless healing outcomes. Several studies have shown that fetal tissues transplanted into adult environments retained their scarless healing characteristics. For instance, when fetal and adult sheep tendon tissues were transplanted subcutaneously into adult severe combined immunodeficiency (SCID) mice, subsequently injured, and allowed to heal, the injured adult tendon grafts had significant granulation tissue, inflammatory infiltrate, disorganized collagen structure and poor mechanical properties, while fetal tendon grafts healed with no observable abnormalities (Favata et al., 2006). Similar results were found by a study performed in skin, where fetal human skin was transplanted subcutaneously onto adult athymic mice (Lorenz et al., 1992).

While SCID and athymic mice have greatly reduced numbers of lymphocytes, they can still mount a substantial inflammatory response to injury via macrophages and other immune cells, and yet the fetal tendon grafts still healed scarlessly. It has also been shown that adult sheep skin that had been transplanted onto fetal lambs, such that they experienced an embryonic environment, still healed wounds with scar formation (Longaker et al., 1994). These transplant studies suggest that scarless healing is an ability that is, at least in part, intrinsic to embryonic tissue itself. However, it is not known if scarless healing is due to the behavior or responses of the embryonic cells, the ECM of the embryonic tissue, or some combination of the properties of these tissue components.

Limited research has been conducted into how resident embryonic cells may be involved in giving rise to scarless healing outcomes. Various studies have been performed to investigate differences between normal (i.e. non-wounded, non-treated) embryonic and adult skin fibroblasts. These studies found several differences in cell behavior between embryonic and adult fibroblasts, including cell size, cell growth, cell adhesion, and collagen expression and deposition (Brink et al., 2009; Brink et al., 2005; Lorenz et al., 1995; Tang et al., 2014). There has also been a study comparing normal embryonic and adult ligament cells, which found that the embryonic ligament cells migrated faster and produced more Col I than the adult ligament cells (Stalling and Nicoll, 2008). However, such studies are lacking not only in tendon, but – more importantly – in investigating how the embryonic and adult cells differ in the context of healing by

actually performing treatment with inflammatory mediators and looking at wound-related cell responses.

1.4 TENDON INJURY ENVIRONMENTAL FACTORS

When an adult or postnatal tendon is injured, there are many different environmental factors affecting the healing tissue. These factors include inflammatory mediators and released soluble factors, as well as the biochemical and mechanical properties of the tissue itself.

1.4.1 Inflammatory mediators and soluble factors

Upon injury, resident tendon cells and infiltrating immune cells release numerous soluble inflammatory mediators, such as cytokines. Important cytokines released early in the inflammatory cascade are IL-1β and TNFα, both of which have been shown to be significantly upregulated in tendons and ligaments following injury (Berglund et al., 2007; Bigoni et al., 2013; Koshima et al., 2007; Manning et al., 2014; Millar et al., 2009). IL-1β is a particularly potent pro-inflammatory cytokine that is known to trigger further induction of inflammatory processes in a wide variety of tissues, including tendon. *In vitro* studies have found that adult tendon cells respond to treatment with pro-inflammatory cytokines such as IL-1β with increased expression and production of inflammatory mediators, including IL-6 and COX-2 (Archambault et al., 2002; Buhrmann et al., 2011; Busch et al., 2012; Corps et al., 2003; John et al., 2010; Thampatty et al., 2007; Tsuzaki et al.,

2003b; Yang et al., 2005). IL-6 is a cytokine that plays an important role in inflammation and wound healing and can act in both a pro-inflammatory and an anti-inflammatory manner (Scheller et al., 2011). COX-2 is an enzyme that catalyzes the conversion of arachidonic acid to prostaglandins such as PGE_2, which are lipid mediators of pain and acute inflammation. Both IL-6 and COX-2 are highly expressed and present at elevated levels *in vivo* following acute tendon and ligament injuries (Ackermann et al., 2013; Berglund et al., 2007; Bigoni et al., 2013; Higuchi et al., 2006; Koshima et al., 2007; Legerlotz et al., 2012; Manning et al., 2014; Millar et al., 2009).

In vitro adult tendon cell studies have found that IL-1β treatment also causes increased expression and production of matrix metalloproteinases (MMPs) (Archambault et al., 2002; Buhrmann et al., 2011; Busch et al., 2012; Corps et al., 2004; Hosaka et al., 2006; Thampatty et al., 2007; Tsuzaki et al., 2003b; Yang et al., 2005). MMPs are zinc-dependent catabolic enzymes that can cleave components of the ECM and play an important role in both physiological and pathological ECM remodeling. MMPs that are of particular interest to tendon and were found to be upregulated by tendon cells in response to IL-1β include MMP-3 and MMP-13. MMP-13, also known as collagenase-3, cleaves interstitial collagens including Col I and Col III (Burrage et al., 2006; Knäuper et al., 1996; Lemaître et al., 1997). MMP-3, also called stromelysin-1, is capable of cleaving Col III as well as non-collagen ECM molecules such as proteoglycans (Birkedal-Hansen et al., 1993; Burrage et al., 2006; Wu et al., 2010). MMPs, including

MMP-3 and MMP-13, have also been found to be more highly expressed and produced *in vivo* in injured and healing tendons and ligaments (Berglund et al., 2007; Chamberlain et al., 2011; Higuchi et al., 2006; Karousou et al., 2008; Loiselle et al., 2009; Manning et al., 2014; Oshiro et al., 2003).

Cellular responses to inflammatory cytokines released following injury, such as IL-1β, are initially regulated by mediators including receptors, co-receptors, and antagonists, which are all involved in binding and subsequent signal transduction into the cell. IL-1β has two receptors: IL-1R1 and IL-1R2. IL-1R1 is the functional receptor that transduces signaling into the cell (Sims et al., 1993). Increased IL-1R1 expression in mouse hepatocytes has been shown to lead to an increase in the level of IL-1β binding and subsequent signaling (Ito et al., 1999). IL-1R2 is a decoy receptor that binds IL-1β but does not activate intracellular signaling, thereby acting as a sink for IL-1β (Colotta et al., 1993). Overexpression of IL-1R2 in mouse epidermal keratinocytes has been shown to inhibit responses to IL-1α compared to normal keratinocytes (Rauschmayr et al., 1997). It has also been shown that inducing higher expression of IL-1R2 in mouse embryonic fibroblasts (MEF) causes a significant decrease in IL-1β induced signaling compared to normal MEF (Uchikawa et al., 2015). IL-1R2 also exists in a soluble form, which can prevent IL-1β signaling by binding free IL-1β, thereby preventing it from binding IL-1R1 and transducing a signal. Treatment with soluble IL-1R2 has been found to inhibit IL-1β induced TNFα production by mouse macrophage cells (Bessis et al., 2000). Another IL-1 mediator that is

necessary to induce IL-1β signaling is IL-1RAcP, which is a co-receptor that forms a complex with IL-1R1-bound IL-1β in order to recruit downstream signaling proteins(Sims and Smith, 2010). An additional mediator that can regulate IL-1β binding and activity is IL-1RA, which acts as an antagonist by competitively binding to IL-1R1 without causing signal transduction, thereby preventing IL-1β binding and signaling from occurring (Arend et al., 1990; Dripps et al., 1991). IL-1RA has been found to be upregulated during inflammation (Arend, 2002; Fischer et al., 1992; Galkowska et al., 2005). The balance between the various IL-1 mediators is important for the regulation of IL-1β activity, as are the interactions among the different mediators. It has been shown that increasing the cell surface IL-1R1 to IL-1R2 ratio of MEF by inducing shedding of IL-1R2 results in increased IL-1β induced signaling (Uchikawa et al., 2015). It has also been found that IL-1R2 binds IL-1β with much greater affinity than it binds IL-1RA, such that the IL-1β inhibitory effects of IL-1RA and IL-1R2 do not cancel each other out (Martin et al., 2013). Thus, it is important to examine the various IL-1 mediators when investigating IL-1β signaling.

The IL-1β signal transduction cascade (reviewed in Weber et al., 2010) begins when IL-1β binds to IL-1R1, which induces a conformational change in the receptor that allows recruitment of IL-1RAcP. This complex then activates intracellular signaling proteins including MyD88, IRAK4, IRAK1, and TRAF6, which leads to the activation of several intracellular signaling pathways. These pathways include the MAPK pathways (p38 MAPK, JNK, ERK) and the NF-κB

pathway. The MAPK and NF-κB pathways ultimately lead to the activation of transcription factors, which regulate gene expression of various molecules, such as inflammatory mediators and MMPs. There has been limited work done to investigate intracellular IL-1β signaling of tendon cells in particular. It has been shown that a p38 MAPK inhibitor could abrogate IL-1β induced upregulation of COX-2 and PGE_2 in adult human tendon cells (Thampatty et al., 2007), and that IL-1β both induced MMP expression and increased phosphorylation (activation) of p38 MAPK and JNK in adult human tendon cells (Corps et al., 2004). Responses of adult human tendon cells to IL-1β have also been found to be accompanied by phosphorylation of NF-κB (Buhrmann et al., 2011; Busch et al., 2012). IL-1β signaling has been more extensively studied in other adult cell types, including human chondrocytes (Ahmad et al., 2007; Liacini et al., 2002; Lim and Kim, 2011; Mengshol et al., 2000; Wang et al., 2011), human synoviocytes (Han et al., 1999; Han et al., 2001), human gingival fibroblasts (Kida et al., 2005), human smooth muscle cells (Laporte et al., 1999; Liang et al., 2007), rat hepatic stellate cells (Tang et al., 2013; Zhang et al., 2006), and human retinal cells (Liu et al., 2015). These studies, similar to those in tendon, have shown IL-1β signaling to be mediated by MAPK pathways (p38, JNK, ERK) and NF-κB, indicated by increased phosphorylation of these pathways in response to IL-1β treatment and attenuation of IL-1β induced responses when the pathways were chemically inhibited.

1.4.2 Biochemical properties of tissue

An important tendon tissue property that changes during wounding and throughout healing, and which may play an important role in regulating tendon cell responses and healing outcomes, is ECM composition. Col I is the most abundant ECM component in normal tendon tissue and is organized into highly-aligned fibers. However, after a tendon injury, the collagen fibers become disrupted at the site of the wound, and even after healing the collagen structure does not fully regain its alignment, remaining relatively disorganized (Bruns et al., 2000; Järvinen et al., 2004; Miyashita et al., 1997). At early stages of healing, before new ECM has had a chance to deposited, there is little Col I present in the provisional matrix of the blood clot and early granulation tissue. As healing progresses, there is an increasing amount of Col I in the new tissue as it forms and matures. Col III is another type of collagen that is present in significant amounts in normal tendon, although not nearly as abundant as Col I. During early healing stages, however, the proportion of Col III to Col I being produced and deposited at the wound site is higher than in normal tendon tissue (Juneja et al., 2013; Maffulli et al., 2000; Oshiro et al., 2003). The ratio between Col III and Col I shifts more toward a normal balance as healing progresses to later stages, but the healed tendon still ends up having more Col III than normal tendon (Birch et al., 1998). Fibronectin (Fn) is another ECM component whose prevalence changes throughout tendon healing; it is present is small amounts in normal tendon, but is abundant in the blood clot that forms at the beginning of wound healing (Lenselink, 2013). The amount of Fn in the wound then decreases as it is replaced

with collagen in the newly forming tissue. ECM composition in injured tendons can also be directly affected by inflammation during healing due to changes in the expression and production of various ECM molecules (Berglund et al., 2006; Boykiw et al., 1998; Karousou et al., 2008; Loiselle et al., 2009; Maffulli et al., 2000; Manning et al., 2014; Oshiro et al., 2003; Thomopoulos et al., 2002). ECM turnover events in healing tendon may additionally be influenced by MMPs, which are upregulated by inflammatory cytokines, through degradation of matrix components (Berglund et al., 2007; Chamberlain et al., 2011; Higuchi et al., 2006; Karousou et al., 2008; Loiselle et al., 2009; Manning et al., 2014; Oshiro et al., 2003). In addition to differences in ECM composition between wounded and normal tendon, there are also differences between embryonic and adult tendon ECM, which may play a role in determining scarless vs. scarred healing outcomes. For instance, embryonic tendons have relatively low collagen content (Marturano et al., 2013; McBride et al., 1988) and relatively high Fn staining intensity (Kuo et al., 2008).

1.4.3 Mechanical properties of tissue

In addition to the biochemical composition of the tissue ECM, tendon healing may also be influenced by changes in the mechanical properties of the tissue. For instance, after injury, wounded tendon tissue is much softer than normal tendon, with a significantly decreased elastic modulus (Ansorge et al., 2012; Carpenter et al., 1998; Dyment et al., 2012; Nagasawa et al., 2008). Even after the tendon is fully healed, the modulus does not completely return to normal values. In addition

to such differences in elastic modulus between injured and normal tendons, it has also been shown that embryonic tendons have relatively low elastic modulus values, while adult tendons have relatively high elastic modulus values, which may be somehow involved in scarless vs. scarred healing (Ansorge et al., 2011; Beredjiklian et al., 2003; McBride et al., 1988). While the effect of elastic modulus on healing or inflammatory responses has not been directly shown in tendon, it has been investigated for other tissues. One study showed that colonic fibroblasts grown on relatively stiff polyacrylamide (PA) gels, with a modulus corresponding to that of Crohn's disease-affected bowel strictures, have altered morphology and lower IL-1β, COX-2, MMP-1, and MMP-3 expression compared to fibroblasts grown on softer gels (Johnson et al., 2013). Another study found that when lung fibroblasts were grown on PA gels with modulus gradients that ranged from softer than normal lung to stiffer than fibrotic lung, they had increased cell spreading, proliferation, and expression and production of collagens, along with decreased MMP-1 and COX-2 expression and PGE_2 production, with increasing gel modulus (Liu et al., 2010). Substrate elastic modulus has also been shown to modulate the morphology, activation, adhesion, and migration of inflammatory cell types such as macrophages and neutrophils (Blakney et al., 2012; Hind et al., 2015; Irwin et al., 2008; Jannat et al., 2010; Oakes et al., 2009; Stroka and Aranda-Espinoza, 2009).

Wound healing is a complex process that involves many environmental factors and cues, as well as numerous intracellular pathways and mechanisms. As such,

an array of applied conditions and target molecules will need to be investigated in order to fully understand how scarless and scarred tendon healing outcomes arise.

CHAPTER 2: DIFFERENTIAL RESPONSES OF EMBRYONIC AND POSTNATAL TENDON CELLS TO IL-1β TREATMENT: IMPLICATIONS FOR SCARLESS HEALING

2.1 INTRODUCTION

2.1.1 Need

Tendon is an important musculoskeletal tissue whose major role is to transmit forces from muscle to bone in order to allow for movement. Tendon injuries are common and often debilitating; for instance, a survey of an orthopedic trauma unit in the United Kingdom found that the annual overall incidence of tendon/ligament injuries among their service population was 106.2/100,000 (Clayton and Court-Brown, 2008). The poor innate healing capability of tendons is a significant clinical problem, as injured adult (including postnatal) tendons heal with the formation of scar tissue that has aberrant mechanical and biochemical properties. As a result, healed tendons are weaker than normal tendons, limiting their functionality and making them prone to re-injury. The current standard of treatment for tendon injuries is surgical intervention, involving suturing to reconnect ruptured tendons or replacement with autografts or allografts in cases of more severe damage (Voleti et al., 2012; Yang et al., 2013). However, these methods do not fully restore normal tendon tissue properties, and they have associated risks such as donor site morbidity and potential

immunogenic responses. As such, there is a critical need for more effective strategies to treat acute tendon injuries.

2.1.2 Motivation for this study

Interestingly, while adult and postnatal tendons heal with scar formation, embryonic tendons heal in a regenerative, scarless manner with complete restoration of native tissue properties (Beredjiklian et al., 2003; Favata et al., 2006). This scarless embryonic healing phenomenon was first discovered in and has primarily been studied in skin. Skin studies found that the transition from scarless to scarred healing occurs during late embryonic development, at the beginning of the third trimester in humans and at embryonic day 18 (E18) in mice (Chen et al., 2007; Colwell et al., 2006). Investigations in both tendon and skin have consistently found that embryonic scarless healing is characterized by a generally decreased inflammatory response compared to scarred healing, including less inflammatory cell infiltration and lower levels of inflammatory mediators such as cytokines (Beredjiklian et al., 2003; Cowin et al., 1998; Favata et al., 2006; Liechty et al., 2000; Liechty et al., 1998). Based on such findings, scarless embryonic healing was initially attributed to a naïve immune system. However, various transplant studies performed for both skin and tendon suggest that the immune environment is not the sole determinant of scarless vs. scarred healing outcomes (Favata et al., 2006; Longaker et al., 1994; Lorenz et al., 1992). For instance, the Soslowsky group showed that when embryonic and adult sheep tendon tissues were subcutaneously transplanted into adult severe combined

immunodeficiency (SCID) mice, wounded, and allowed to heal, the injured adult tendon grafts healed with significant disruption in collagen fiber alignment, formation of granulation tissue, noticeable inflammatory infiltrate, and decreased mechanical properties (Favata et al., 2006). In contrast, the embryonic tendon grafts retained their scarless healing ability and regained normal tissue properties. These results suggest that the scarless healing is an ability that is intrinsic to the embryonic tissue itself. We propose that the tendon cells, in particular, are critical contributors to scarless vs. scarred tendon healing outcomes, perhaps due to differences between embryonic and adult (postnatal) tendon cells and their respective responses to various wound-related environmental factors. Such environmental factors include inflammatory mediators, biochemical matrix properties, and mechanical cues.

2.1.3 Molecular mediators involved in tendon wound healing

Following acute injury in an adult tendon, resident tendon cells and infiltrating immune cells release various inflammatory mediators. Key cytokines released early in this inflammatory cascade include interleukin (IL)-1β and tumor necrosis factor (TNF)α (Berglund et al., 2007; Manning et al., 2014), both of which play significant roles in inflammation and wound healing processes. IL-1β is a particularly potent pro-inflammatory cytokine that is known to trigger further induction of inflammatory mediators in a wide variety of cell types, including tendon cells. IL-1β activity is regulated by several IL-1 mediators (reviewed in Sims and Smith, 2010). IL-1β can bind to two receptors: IL-1 receptor (R)1,

which is the functional receptor that transduces signaling into the cell (Sims et al., 1993), and IL-1R2, which is a decoy receptor that functions as a sink by binding IL-1β without activating intracellular signaling pathways (Colotta et al., 1993). IL-1β signaling is also mediated by IL-1 receptor antagonist (RA), which is an antagonist that competitively binds IL-1R1 to prevent IL-1β signaling from occurring (Arend et al., 1990; Dripps et al., 1991).

Numerous *in vitro* studies have found that adult tendon cells increase their expression of several inflammatory mediators in response to IL-1β treatment, including IL-6 and cyclooxygenase (COX)-2 (Archambault et al., 2002; Thampatty et al., 2007; Tsuzaki et al., 2003b; Yang et al., 2005). IL-6 is a cytokine that is involved in inflammation and wound healing and can act in both a pro-inflammatory and an anti-inflammatory manner (Scheller et al., 2011). It is highly expressed *in vivo* following tendon injury (Legerlotz et al., 2012). COX-2 is an enzyme that catalyzes the formation of prostaglandins, which are lipid mediators of pain and inflammation, and it is also highly expressed *in vivo* after tendon injury (Berglund et al., 2007; Manning et al., 2014). *In vitro* tendon cell studies have found that IL-1β treatment also increases expression of matrix metalloproteinases (MMPs) (Archambault et al., 2002; Thampatty et al., 2007; Tsuzaki et al., 2003b; Yang et al., 2005), which are catabolic enzymes that can cleave components of the extracellular matrix (ECM). MMPs that are of particular interest in the context of tendon injury, and which were found to be upregulated by tendon cells in response to IL-1β treatment, include MMP-3 and MMP-13.

MMP-3 and -13 are capable of cleaving collagen molecules, including type I and III, as well as other ECM molecules such as proteoglycans (Birkedal-Hansen et al., 1993; Burrage et al., 2006; Knäuper et al., 1996; Lemaître et al., 1997; Wu et al., 2010).

2.1.4 Biochemical aspects of matrix environment of wounded tendon

In order to investigate potential contributors to scarless vs. scarred tendon healing responses, it is important to consider changes in the ECM of embryonic and postnatal tendons after injury because wounded and healing tendon tissues are characterized by altered matrix structure and composition. The most prevalent ECM component in normal tendon is collagen type I (Col I), which makes up a majority (~60%) of the tendon dry mass (Kjaer, 2004). Also present in the tendon ECM are other types of collagens, such as Col III, as well as various other molecules including proteoglycans and elastin. When a tendon is injured, the ECM composition of the tissue changes significantly from that of normal tendon tissue. For instance, immediately after injury, a blood clot containing significant amounts of fibronectin (Fn) forms at the tendon wound site (Lenselink, 2013). Inflammation during healing can also impact ECM composition and turnover events in injured tendons by directly altering expression and production of ECM molecules as well as MMPs (Berglund et al., 2006; Boykiw et al., 1998; Karousou et al., 2008; Loiselle et al., 2009; Manning et al., 2014; Oshiro et al., 2003; Thomopoulos et al., 2002).

2.1.5 Mechanical aspects of matrix environment of wounded tendon

In addition to ECM composition of the tissue, changes in mechanical cues such as substrate elastic modulus may affect resident tendon cell behavior at an injury site. Wounded and healing tendons have significantly lower elastic modulus values than normal tendon, and even after healing is complete, the elastic modulus of injured tendons never completely returns to that of normal tissue (Ansorge et al., 2012; Carpenter et al., 1998; Dyment et al., 2012; Nagasawa et al., 2008). The effect of tissue elastic modulus on inflammatory responses of tendon cells has not been studied. However, substrate elastic modulus has been shown to affect the expression of inflammation and healing-related genes, including IL-1β, COX-2, MMP-1, MMP-3, Col I, and Col III, in both human lung fibroblasts and human colonic fibroblasts (Johnson et al., 2013; Liu et al., 2010). Substrate elastic modulus has also been shown to modulate the behavior of inflammatory cell types such as macrophages and neutrophils (Blakney et al., 2012; Hind et al., 2015; Irwin et al., 2008; Jannat et al., 2010; Oakes et al., 2009; Stroka and Aranda-Espinoza, 2009).

2.1.6 Study objectives and general approach

We propose that resident tendon cells are significant contributors to scarless vs. scarred tendon healing, with such differential healing outcomes arising at least in part due to intrinsic differences between embryonic and adult (postnatal) tendon cells and how they respond to wound-related environmental factors. Therefore, our focus for this study was to investigate the responses of embryonic and

postnatal tendon cells to pro-inflammatory cytokine (IL-1β) treatment along with changes in ECM composition (Col vs. Fn) and substrate elastic modulus, as well as to begin to probe mechanisms that may cause differing responses between embryonic and postnatal tendon cells. We hypothesized that postnatal tendon cells respond to IL-1β with higher expression of inflammatory mediators and MMPs than embryonic tendon cells, and that these respective behaviors are maintained on various substrates. We also hypothesized that such embryonic vs. postnatal differences in tendon cell responses are due to differences in expression of IL-1 mediators. This study represents the first step toward our long-term goal of developing cell-targeted strategies to redirect adult scarred tendon healing toward a more embryonic-like scarless healing outcome.

2.2 MATERIALS AND METHODS

2.2.1 Tendon cell harvest and culture

Tendon cells were isolated as previously described (Brown et al., 2014). Briefly, postnatal day (P) 7 and pregnant Scx-GFP mice were sacrificed according to IACUC guidelines. Embryonic day (E) 16 embryos were harvested from the pregnant mice and staged (Theiler, 1989). Limbs were isolated, minced, and digested in 1% type II collagenase in PBS at 37°C for 45 minutes under agitation at 200 rpm. The collagenase was neutralized with growth medium (Dulbecco's Modified Eagle Medium, DMEM; 10% fetal bovine serum, FBS; 1% penicillin/streptomycin, P/S), and the suspensions were strained through 40 μm cell strainers (BD Biosciences). The cells were pelleted, washed with Ca/Mg-free PBS, resuspended in growth medium, plated, and grown to 80% confluency. Cells were then trypsinized and sorted by GFP signal using a MoFlo Legacy cell sorter (Beckman Coulter) to isolate tendon cells. Three independent limb cell pools were harvested. Tendon cells were expanded to passage 3-5 in growth medium at 37°C and 5% CO_2 for experiments.

2.2.2 Polyacrylamide (PA) gels

PA gels were fabricated and simultaneously functionalized with Col I or Fn according to a method similar to that previously described by (Leach et al., 2007). Briefly, 22 mm square glass coverslips were activated by sequentially soaking them in 0.5% 3-aminopropyltriethoxysilane and 0.5% glutaraldehyde. Pre-

polymerized acrylamide gel solutions were made by mixing acrylamide, bis-acrylamide, PBS, distilled water, Col I or Fn, TEMED, hydrochloric acid, acrylic acid N-hydroxysuccinimide ester, and ammonium persulfate. 60 μL of the resulting solution was then immediately pipetted onto the center of each activated coverslip and covered with an 18mm circular glass coverslip to spread the solution evenly. The gels were allowed to polymerize for 30 min at room temperature before the circular top coverslips were removed. The gels were transferred to 6-well tissue culture plates and soaked overnight in PBS at 4°C to rinse off any un-polymerized PA solution before cell seeding. The PA gels used in experiments had compositions of 4% acrylamide and 0.1% bis-acrylamide (elastic modulus = 19 kPa via force-volume atomic force microscopy (FV-AFM) (Marturano et al., 2013)) or 15% acrylamide and 0.5% bis-acrylamide (elastic modulus = 120 kPa via FV-AFM).

2.2.3 IL-1β treatment

Tendon cells were seeded at 30,000 cells/cm^2 in growth medium on tissue culture plastic (TCP) or PA gels and allowed 24 h to attach and grow before IL-1β treatment. At 0 h (after 24 h attachment), cells were washed with PBS and fed with reduced-serum (1% FBS) growth medium with or without 100 pM recombinant mouse IL-1β (R&D Systems). Cells were harvested and medium collected after 24 h of IL-1β treatment.

2.2.4 Proliferation assay

Cell number was determined using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Cells were washed with PBS and 0.05% Triton X-100 was added before freezing and storing at -20°C until use. After thawing, the samples were sonicated for 6 s using a Q125 sonicator (Qsonica) and combined with PicoGreen solution in the wells of a black 96-well plate. The plate was read on a SpectraMax M2 microplate reader (Molecular Devices) at 480 nm excitation and 520 nm emission. To calculate DNA concentrations, a calibration curve was generated using the lambda dsDNA standard provided in the kit. Results were divided by the approximate amount of DNA in a murine diploid cell (5.6 pg/cell (Morton, 1991; Serth et al., 2000; Waterston et al., 2002)) and by the culture surface area to obtain final cell density values.

2.2.5 Metabolic assay

A resazurin solution, prepared using resazurin sodium salt (Sigma-Aldrich) in growth medium, was used to evaluate cell metabolic activity. Cells were incubated at 37°C in a 0.01 mg/mL resazurin solution, and sample solution aliquots were pipetted into a black 96-well plate. References were taken from unseeded TCP wells or wells with unseeded PA gels incubated with the resazurin solution. The plate was read on a SpectraMax M2 microplate reader (Molecular Devices) at 530 nm excitation and 590 nm emission. Results were normalized to respective cell counts (measured using the PicoGreen assay) to obtain per-cell relative metabolic activity values.

2.2.6 qPCR

Cells were homogenized in TRIzol reagent (Life Technologies), and total RNA was isolated and reverse-transcribed using the SuperScript III First-Strand Synthesis System (Life Technologies). QPCR was performed with Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies) on a Stratagene Mx3000P qPCR system (Agilent Technologies). Mouse-specific primers for 18s, IL-6, TNFα, COX-2, MMP-3, MMP-13, Col I, Col III, IL-1R1 and IL-1RA were used. Fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to 18s housekeeping gene.

2.2.7 ELISA

Sample culture medium was collected and centrifuged, and the supernatant was aliquotted, snap frozen on dry ice, and stored at -80°C until use. IL-6, MMP-3, and IL-1RA protein concentrations in the supernatant samples were determined using mouse-specific Quantikine ELISA kits (R&D Systems). Results were normalized to respective cell counts (measured using the PicoGreen assay).

2.2.8 Immunostaining

Tendon cells were seeded on glass coverslips and treated with IL-1β, as described in previous section. Cells were fixed in 4% paraformaldehyde and washed three times with PBS. Cells were permeabilized with PBST (PBS with 0.1% Triton X-100), blocked with 10% goat serum in PBS, then incubated in 5% bovine serum

albumin (BSA) in PBS with primary antibodies against IL-1R1 or IL-1R2 (1:100; Abcam). Following three washes with PBS, cells were incubated in 5% BSA in PBS with the appropriate secondary Alexa Fluor 488 antibody (1:1000; Life Technologies) and co-stained with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Following three washes with PBS, coverslips were mounted with ProLong Gold mounting medium (Life Technologies) and imaged on a confocal microscope (Leica Microsystems). Negative controls without primary antibodies (not shown) exhibited minimal background fluorescence.

2.2.9 Statistics

Statistical analyses were performed using GraphPad Prism software. Significance ($p < 0.05$) was determined by *t*-test or 1-way ANOVA with Tukey's post-hoc test.

2.3 RESULTS

To examine how embryonic and adult (postnatal) tendon cells compare in their responses to IL-1β, we treated E16 and P7 tendon cells with IL-1β and conducted various assays to probe how the cells differed both at baseline and in response to the treatment. Cell responses were tested on various substrates, including TCP and PA gels with varying elastic moduli (19 and 120 kPa) and functionalized ECM proteins (Col I and Fn).

2.3.1 Proliferation and metabolic activity

We first investigated if cell proliferation or metabolic activity differed between embryonic and postnatal tendon cells or were affected by IL-1β treatment or substrate properties.

2.3.1.1 Proliferation

Cell counts were normalized to culture surface area in order to obtain cell density values (cells/cm^2) for each experimental condition.

2.3.1.1.1Baseline proliferation

Because the tendon cells were seeded for experiments 24 h before beginning the IL-1β treatment at the 0 h time point, we examined the proliferation of the embryonic and postnatal tendon cells at 0 h to determine baseline cell density values at the start of treatment.

Effect of substrate material: In order to determine whether the substrate material affected baseline (0 h) cell density values for the E16 tendon cells or for the P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 cell density values in order to test whether the TCP values were significantly different compared to any of the PA gel values. There were no significant differences in baseline cell density values between TCP and any of the tested PA gels for E16 cells or P7 cells (**Fig 2.1**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected baseline cell density values for the E16 tendon cells or for the P7 tendon cells on either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa cell density values on either Col I or Fn PA gels for each cell stage. There were no significant differences in baseline cell density values between 19 and 120 kPa PA gels for Col I or Fn gels in E16 cells or P7 cells (**Fig 2.1**).

Effect of substrate ECM: In order to determine whether the ECM composition affected baseline cell density values for the E16 tendon cells or for the P7 tendon cells on either the 19 kPa or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn cell density values on either 19 or 120 kPa PA gels for each cell stage. There were no significant differences in baseline cell density values between Col I and Fn PA gels for 19 or 120 kPa gels in E16 cells or P7 cells (**Fig 2.1**).

E16 vs, P7: In order to determine whether E16 and P7 tendon cells had significantly different baseline cell density values, we performed t-tests to

compare E16 vs. P7 cell density values on each substrate. There were no significant differences in baseline cell density values between E16 and P7 cells on any of the tested substrates (**Fig 2.1**).

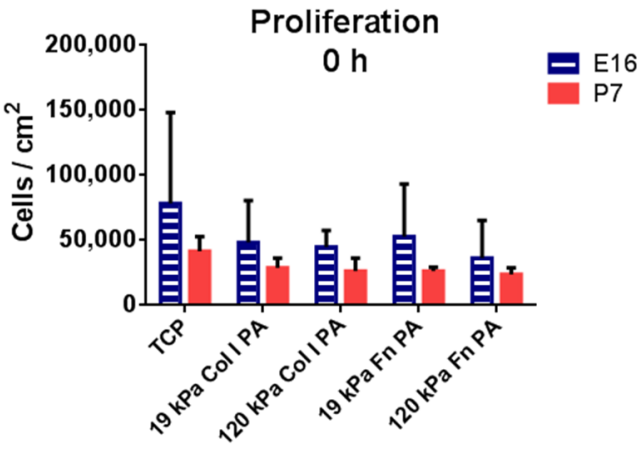

Figure 2.1: Proliferation of E16 and P7 tendon cells at 0 h baseline (without IL-1β treatment, 24 h after cell seeding) on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Cell counts were measured using PicoGreen assay and normalized to culture surface area to yield cell density values. Cells were seeded 24 h before treatment at 30,000 cells/cm^2. (No statistically significant differences between conditions for N=3)

2.3.1.1.2 Change in proliferation over time

In order to determine whether E16 or P7 cell density values changed over time, we performed t-tests to compare cell density values at 0 h vs. 24 h (without IL-1β treatment) for each cell stage on each substrate. There were no significant changes in cell density values over time for E16 or P7 cells on any of the tested substrates (**Fig 2.2**).

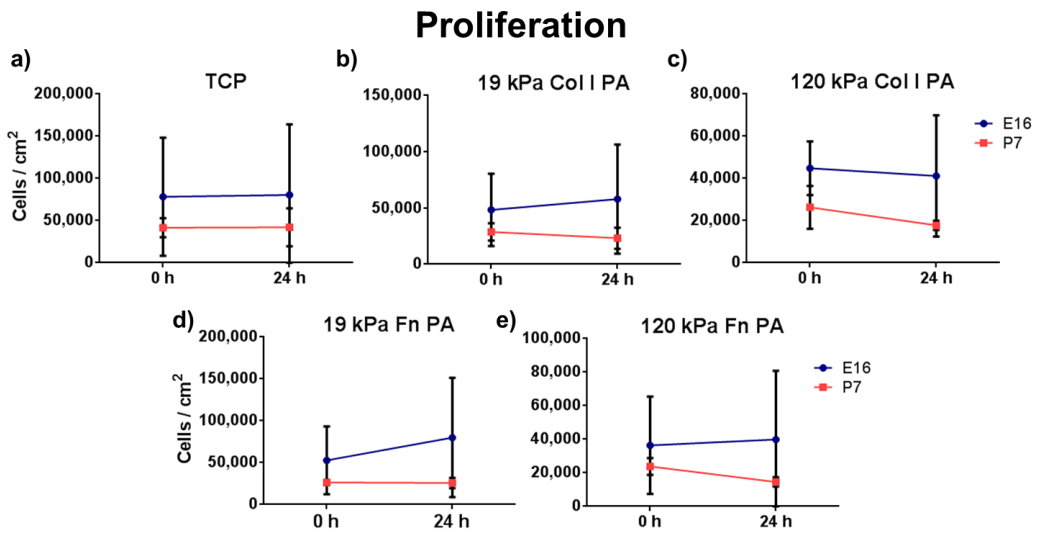

Figure 2.2: Proliferation of E16 and P7 tendon cells from 0 to 24 h, without IL-1β treatment, on TCP (**a**), 19 kPa Col I-functionalized PA gels (**b**), 120 kPa Col I-functionalized PA gels (**c**), 19 kPa Fn-functionalized PA gels (**d**), and 120 kPa Fn-functionalized PA gels (**e**). Cell counts were measured using PicoGreen assay and normalized to culture surface area to yield cell density values. Cells were seeded 24 h before treatment at 30,000 cells/cm^2. (No statistically significant differences between conditions for N=3)

2.3.1.1.3 Proliferation in response to IL-1β treatment

Effect of IL-1β treatment on proliferation: In order to determine whether IL-1β treatment affected cell density values for the E16 tendon cells or for the P7 tendon cells, we performed t-tests to compare IL-1β treated vs. non-treated cell density values for each cell stage on each substrate. There were no significant changes in cell density values in response to IL-1β treatment for E16 or P7 cells on any of the tested substrates (**Fig 2.3**).

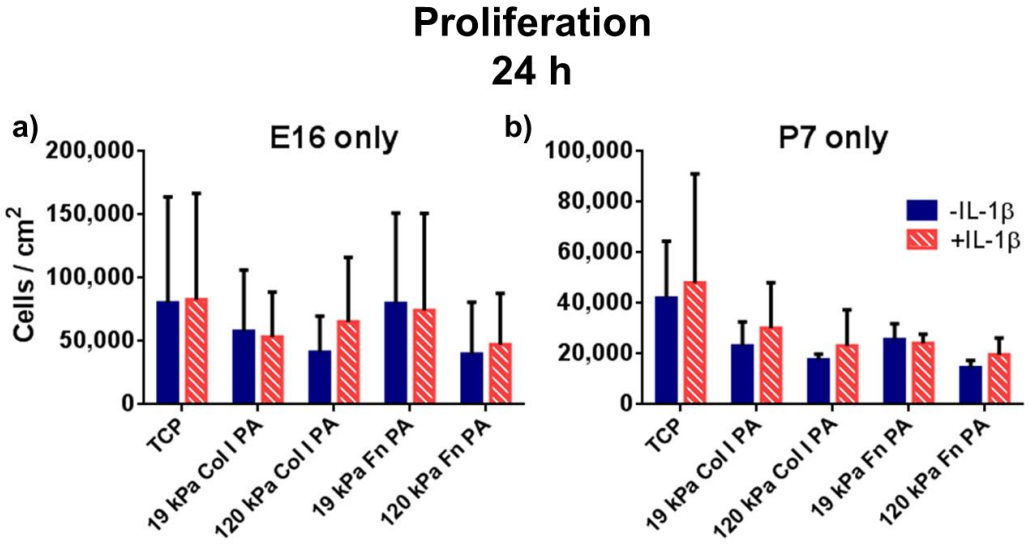

Figure 2.3: Proliferation of E16 (**a**) and P7 (**b**) tendon cells at 24 h, with and without IL-1β treatment, on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Cell counts were measured using PicoGreen assay and normalized to culture surface area to yield cell density values. Cells were seeded 24 h before treatment at 30,000 cells/cm^2. (No statistically significant differences between conditions for N=3)

Effect of substrate material: In order to determine whether the substrate material affected cell density values for IL-1β treated E16 or P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 cell density values in order to test whether the TCP values were significantly different compared to any of the PA gel values. There were no significant differences in cell density values between TCP and any of the tested PA gels for IL-1β treated E16 or P7 cells (**Fig 2.4**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected cell density values for IL-1β treated E16 or P7 tendon cells on

either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa cell density values on either Col I or Fn PA gels for each cell stage. There were no significant differences in cell density values between 19 and 120 kPa PA gels for Col I or Fn gels in IL-1β treated E16 cells or P7 cells (**Fig 2.4**).

Effect of substrate ECM: In order to determine whether the ECM composition affected cell density values for IL-1β treated E16 or P7 tendon cells on either the 19 kPa or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn cell density values on either 19 or 120 kPa PA gels for each cell stage. There were no significant differences in cell density values between Col I and Fn PA gels for 19 or 120 kPa gels in IL-1β treated E16 cells or P7 cells (**Fig 2.4**).

E16 vs. P7: In order to determine whether IL-1β treated E16 and P7 tendon cells had significantly different cell density values, we performed t-tests to compare E16 vs. P7 cell density values on each substrate. There were no significant differences in cell density values between IL-1β treated E16 and P7 cells on any of the tested substrates (**Fig 2.4**).

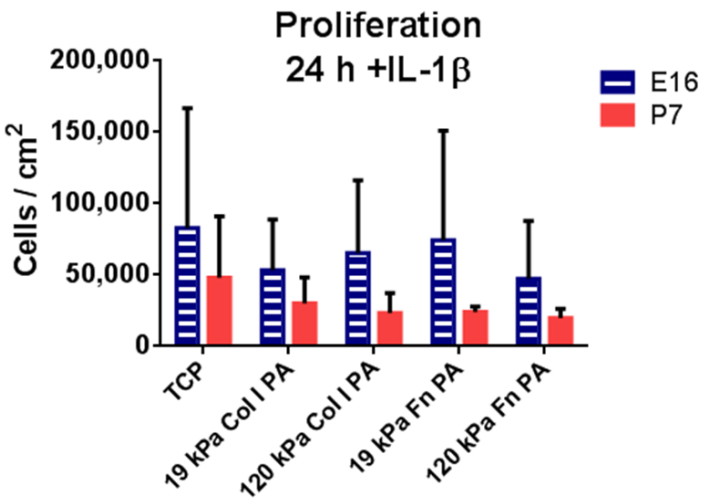

Figure 2.4: Proliferation of E16 and P7 tendon cells at 24 h with IL-1β treatment on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Cell counts were measured using PicoGreen assay and normalized to culture surface area to yield cell density values. Cells were seeded 24 h before treatment at 30,000 cells/cm^2. (No statistically significant differences between conditions for N=3)

Overall, our data indicate that proliferation is not different between E16 and P7 tendon cells and is not affected by the IL-1β treatment or the different tested substrates.

2.3.1.2 Metabolic activity

The relative fluorescent readings were normalized to cell count for each condition in order to obtain per-cell metabolic activity values.

2.3.1.2.1 Baseline metabolic activity

Because the tendon cells were seeded for experiments 24 h before beginning the IL-1β treatment at the 0 h time point, we examined the metabolic activity of the embryonic and postnatal tendon cells at 0 h to determine baseline metabolic activity at the start of treatment.

Effect of substrate material: In order to determine whether the substrate material affected baseline (0 h) metabolic activity for the E16 tendon cells or for the P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 per-cell metabolic activity in order to test whether the TCP values were significantly different compared to any of the PA gel values. There were no significant differences in baseline metabolic activity between TCP and any of the tested PA gels for E16 cells or P7 cells (**Fig 2.5**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected baseline metabolic activity for the E16 tendon cells or for the P7 tendon cells on either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa per-cell metabolic activity on either Col I or Fn PA gels for each cell stage. There were no significant differences in baseline metabolic activity between 19 and 120 kPa PA gels for Col I or Fn gels in E16 cells or P7 cells (**Fig 2.5**).

Effect of substrate ECM: In order to determine whether the ECM composition affected baseline metabolic activity for the E16 tendon cells or for the P7 tendon cells on either the 19 kPa or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn per-cell metabolic activity on either 19 or 120 kPa PA gels for each

cell stage. There were no significant differences in baseline metabolic activity between Col I and Fn PA gels for 19 or 120 kPa gels in E16 cells or P7 cells (**Fig 2.5**).

E16 vs. P7: In order to determine whether E16 and P7 tendon cells had significantly different baseline metabolic activity, we performed t-tests to compare E16 vs. P7 per-cell metabolic activity on each substrate. The only significant E16 vs. P7 difference observed for baseline metabolic activity was that E16 cells had lower metabolic activity than P7 cells on the 19 kPa Fn PA gel (**Fig 2.5**).

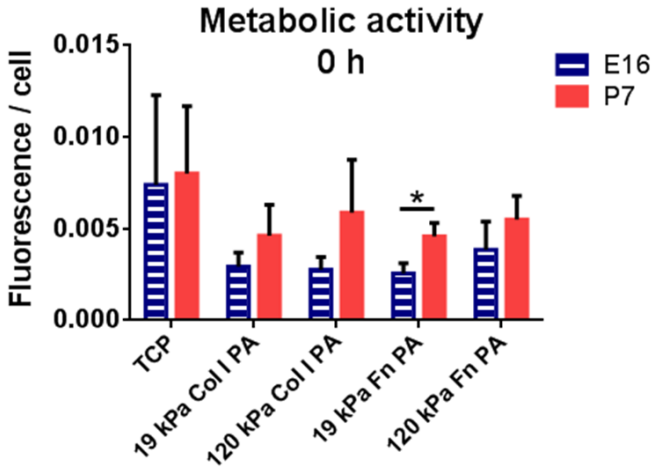

Figure 2.5: Metabolic activity of E16 and P7 tendon cells at 0 h baseline (without IL-1β treatment, 24 h after cell seeding) on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Relative fluorescence was measured using resazurin assay and normalized to cell count to yield the relative fluorescence per cell. (* = $p < 0.05$ for E16 vs. P7; N=3)

2.3.1.2.2 Change in metabolic activity over time

In order to determine whether E16 or P7 metabolic activity changed over time, we performed t-tests to compare 0 h vs. 24 h (without IL-1β treatment) per-cell metabolic activity for each cell stage on each substrate. There were significant increases in metabolic activity over time for E16 cells on the 19 kPa and 120 kPa Col I PA gels (**Fig 2.6b,c**).

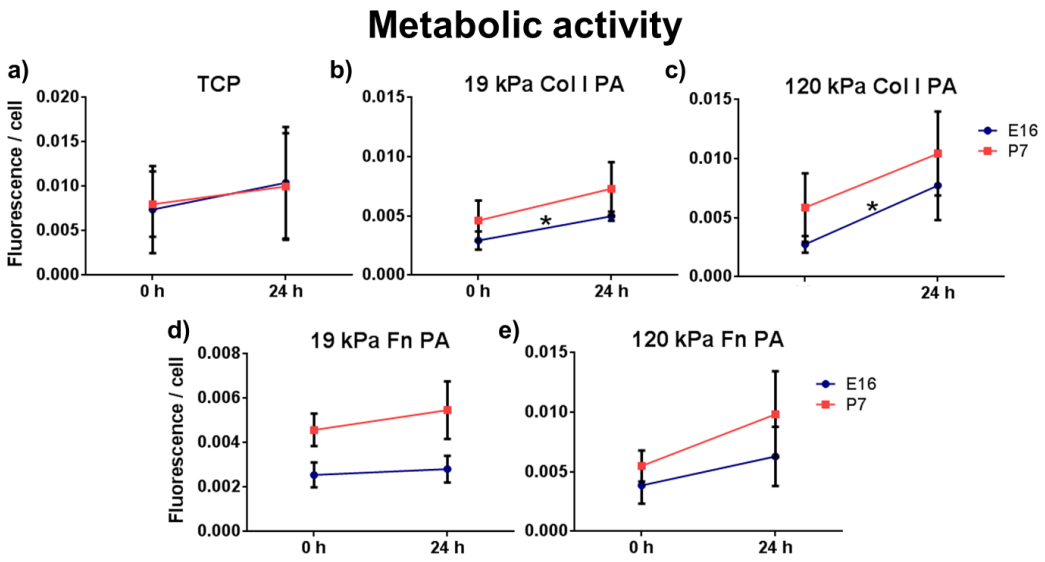

Figure 2.6: Metabolic activity of E16 and P7 tendon cells at 0 and 24 h, without IL-1β treatment, on TCP (**a**), 19 kPa Col I-functionalized PA gels (**b**), 120 kPa Col I-functionalized PA gels (**c**), 19 kPa Fn-functionalized PA gels (**d**), and 120 kPa Fn-functionalized PA gels (**e**). Relative fluorescence was measured using resazurin assay and normalized to cell count to yield the relative fluorescence per cell. (* = $p < 0.05$ between 0 and 24 h for E16; N=3)

2.3.1.2.3 Metabolic activity in response to IL-1β treatment

Effect of IL-1β treatment on metabolic activity: In order to determine whether IL-1β treatment affected metabolic activity for the E16 tendon cells or for the P7 tendon cells, we performed t-tests to compare IL-1β treated vs. non-treated per-cell metabolic activity for each cell stage on each substrate. There were no significant changes in metabolic activity in response to IL-1β treatment for E16 or P7 cells on any of the tested substrates (**Fig 2.7**).

Effect of substrate material: In order to determine whether the substrate material affected metabolic activity for IL-1β treated E16 or P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 per-cell metabolic activity in order to test whether the TCP values were significantly different compared to any of the PA gel values. There were no significant differences in metabolic activity between TCP and any of the tested PA gels for IL-1β treated E16 or P7 cells (**Fig 2.8**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected metabolic activity for IL-1β treated E16 or P7 tendon cells on either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa per-cell metabolic activity on either Col I or Fn PA gels for each cell stage. There were no significant differences in metabolic activity between 19 and 120 kPa PA gels for Col I or Fn gels in IL-1β treated E16 cells or P7 cells (**Fig 2.8**).

Effect of substrate ECM: In order to determine whether the ECM composition affected metabolic activity for IL-1β treated E16 or P7 tendon cells on either the 19 kPa or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn per-cell

metabolic activity on either 19 or 120 kPa PA gels for each cell stage. There were no significant differences in metabolic activity between Col I and Fn PA gels for 19 or 120 kPa gels in IL-1β treated E16 cells or P7 cells (**Fig 2.8**).

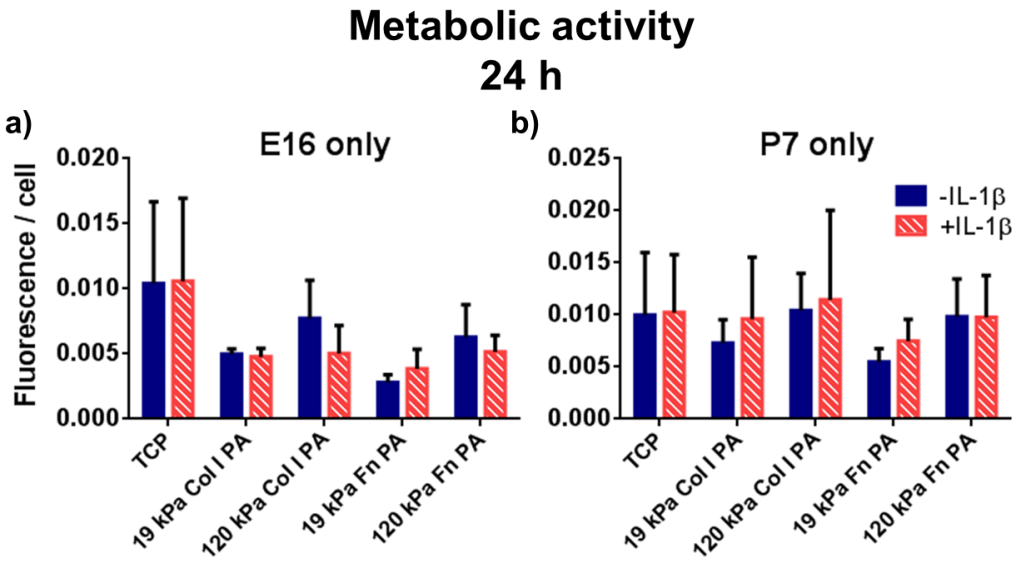

Figure 2.7: Metabolic activity of E16 (**a**) and P7 (**b**) tendon cells at 24 h, with and without IL-1β treatment, on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Relative fluorescence was measured using resazurin assay and normalized to cell count to yield the relative fluorescence per cell. (No statistically significant differences between treated and non-treated for N=3)

E16 vs. P7: In order to determine whether IL-1β treated E16 and P7 tendon cells had significantly different metabolic activity, we performed t-tests to compare E16 vs. P7 per-cell metabolic activity on each substrate. There were no significant differences in metabolic activity between IL-1β treated E16 and P7 cells on any of the tested substrates (**Fig 2.8**).

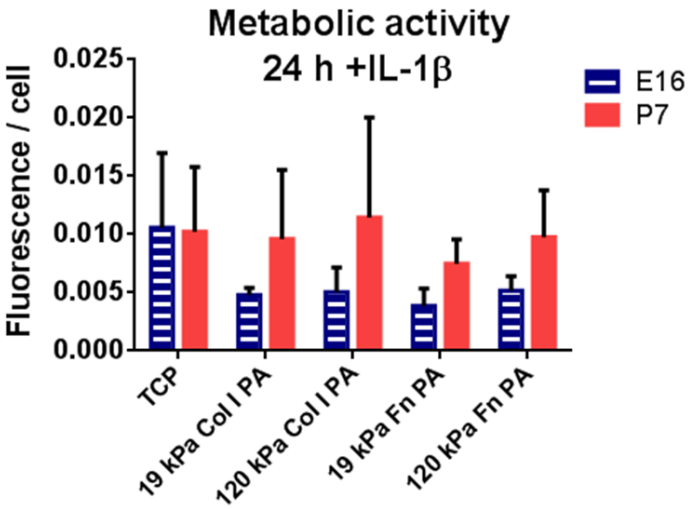

Figure 2.8: Metabolic activity of E16 and P7 tendon cells at 24 h with IL-1β treatment on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Relative fluorescence was measured using resazurin assay and normalized to cell count to yield the relative fluorescence per cell. (No statistically significant differences between conditions for N=3)

Overall, our data indicate that metabolic activity is generally not different between E16 and P7 tendon cells and is not affected by the IL-1β treatment or the different tested substrates.

2.3.2 Inflammatory mediators and MMPs

We investigated E16 and P7 tendon cell gene and protein expression of inflammatory mediators and MMPs, due to their involvement in regulating inflammation and healing-related processes.

2.3.2.1 Inflammatory mediator and MMP gene expression

We measure E16 and P7 tendon cell inflammatory mediator and MMP gene expression at baseline and in response to IL-1β treatment on the various substrates.

2.3.2.1.1 Baseline inflammatory mediator and MMP gene expression

To examine the baseline (0 h) gene expression of inflammatory mediators and MMPs by embryonic and postnatal tendon cells, we normalized 0 h expression data to the 18s housekeeping gene.

Effect of substrate material: In order to determine whether the substrate material affected baseline (0 h) inflammatory mediator or MMP gene expression for the E16 tendon cells or for the P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 gene expression values in order to test whether the TCP values were significantly different compared to any of the PA gel values. The only significant effect of substrate material on baseline gene expression was for COX-2 expression in P7 cells, which was lower on TCP compared to all of the tested PA gels (**Fig 2.9c**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected baseline inflammatory mediator or MMP gene expression for the E16 tendon cells or for the P7 tendon cells on either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa gene expression values on either Col I or Fn PA gels for each cell stage. There were no significant

differences in baseline gene expression between 19 and 120 kPa PA gels for Col I or Fn gels in E16 cells or P7 cells (**Fig 2.9**).

Effect of substrate ECM: In order to determine whether the ECM composition affected baseline inflammatory mediator or MMP gene expression for the E16 tendon cells or for the P7 tendon cells on either the 19 kPa or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn gene expression values on either 19 or 120 kPa PA gels for each cell stage. There were no significant differences in baseline gene expression between Col I and Fn PA gels for 19 or 120 kPa gels in E16 cells or P7 cells (**Fig 2.9**).

E16 vs. P7: In order to determine whether E16 and P7 tendon cells had significantly different baseline inflammatory mediator or MMP gene expression, we performed t-tests to compare E16 vs. P7 gene expression values on each substrate. At baseline, P7 cells generally had greater gene expression of inflammatory mediators and MMPs than E16 cells on all substrates. While these trends did not reach significance for IL-6 (**Fig 2.9a**), they were significant for TNFα on both Col I PA gels (**Fig 2.9b**), for COX-2 on all tested PA gels (**Fig 2.9c**), for MMP-3 on all tested PA gels (**Fig 2.9d**), and for MMP-13 on all tested substrates (**Fig 2.9e**). Collagen gene expression levels were also significantly higher in P7 cells than E16 cells; this was true for Col I on both Col I PA gels and on the 19 kPa Fn PA gel (**Fig 2.9f**), and for Col III on both Col I PA gels (**Fig 2.9g**).

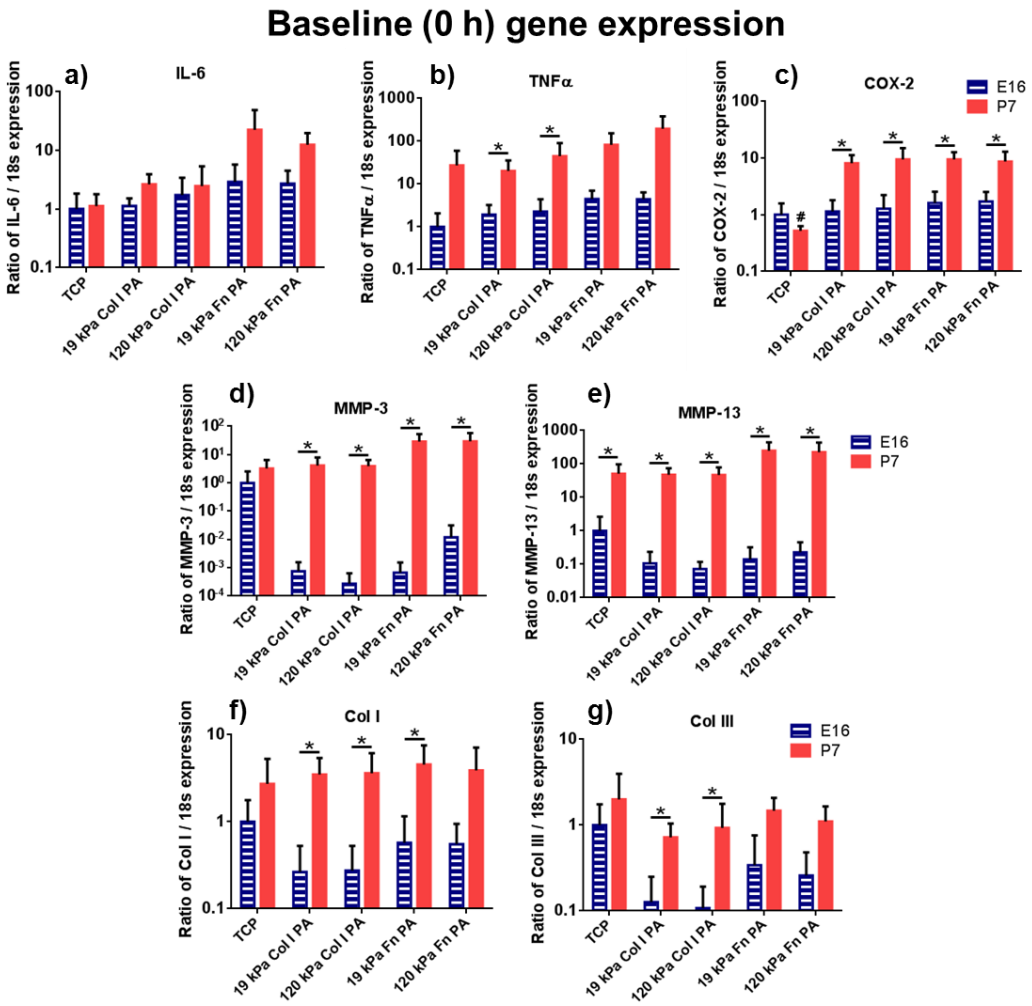

Figure 2.9: Inflammatory mediator, MMP, and collagen gene expression of E16 and P7 tendon cells at 0 h (baseline, without IL-1β treatment), on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Results analyzed as fold-change over 18s and then normalized to E16 TCP condition for each gene. (* = $p < 0.05$ for E16 vs. P7; # = $p < 0.05$ compared to all P7 PA gels; N=3)

2.3.2.1.2 Inflammatory mediator and MMP gene expression in response to IL-1β treatment

To assess how embryonic and postnatal tendon cells regulate their gene expression in response to IL-1β treatment, we normalized 24 h expression data of IL-1β treated E16 or P7 tendon cells to the corresponding control (non-treated) condition.

Effect of substrate material: In order to determine whether the substrate material affected inflammatory mediator or MMP gene expression responses to IL-1β treatment for the E16 tendon cells or for the P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 gene expression values in order to test whether the TCP values were significantly different compared to any of the PA gel values. There were no significant differences in gene expression responses to IL-1β between TCP and any of the tested PA gels for E16 cells or P7 cells (**Fig 2.10**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected inflammatory mediator or MMP gene expression responses to IL-1β treatment for the E16 tendon cells or for the P7 tendon cells on either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa gene expression values on either Col I or Fn PA gels for each cell stage. There were no significant differences in gene expression responses to IL-1β between 19 and 120 kPa PA gels for Col I or Fn gels in E16 cells or P7 cells (**Fig 2.10**).

Effect of substrate ECM: In order to determine whether the ECM composition affected inflammatory mediator or MMP gene expression responses to IL-1β treatment for the E16 tendon cells or for the P7 tendon cells on either the 19 kPa

or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn gene expression values on either 19 or 120 kPa PA gels for each cell stage. There were no significant differences in gene expression responses to IL-1β between Col I and Fn PA gels for 19 or 120 kPa gels in E16 cells or P7 cells (**Fig 2.10**).

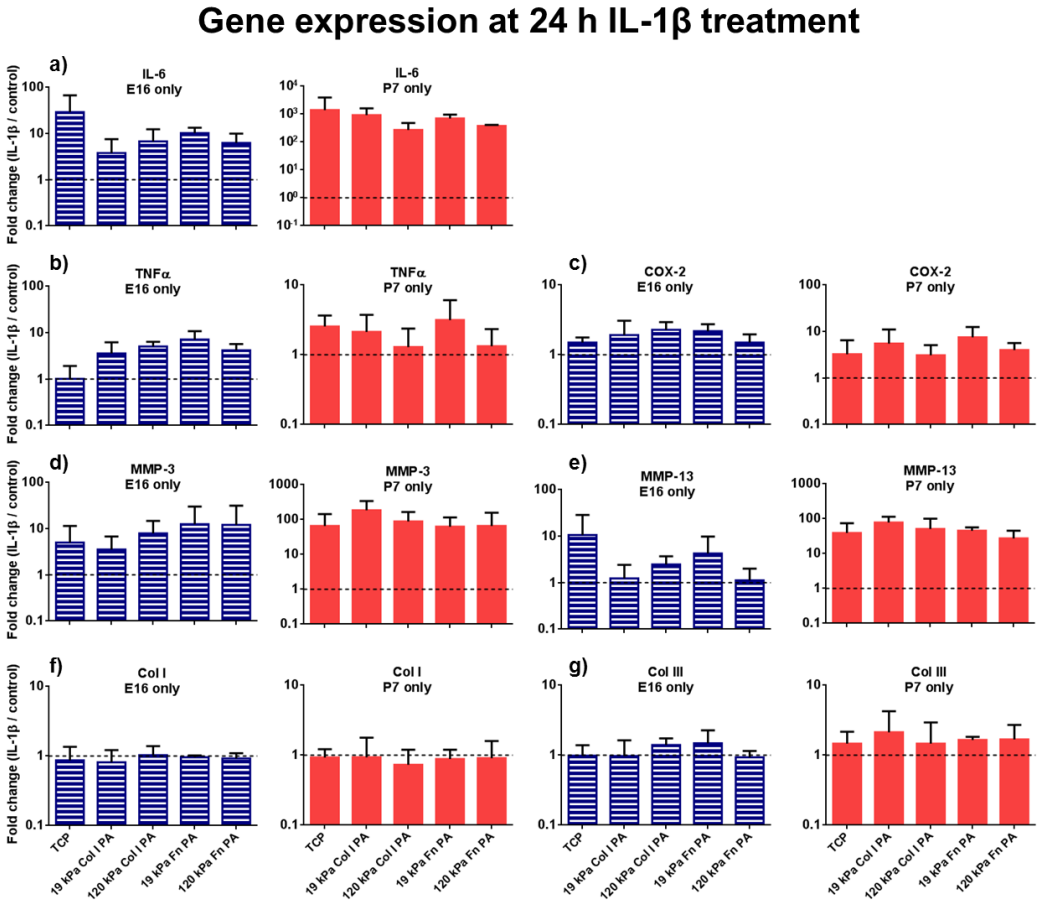

Figure 2.10: Inflammatory mediator, MMP, and collagen gene expression of IL-1β treated E16 and P7 tendon cells at 24 h on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Results normalized to respective control (non-treated) condition, indicated by dashed line at 1. (No statistically significant differences between substrates for N=3)

Effect of IL-1β treatment on cells cultured on TCP: Because embryonic and postnatal tendon cell gene expression responses to IL-1β treatment were independent of substrate (TCP vs. PA gels), we focused on experiments performed on TCP only to further examine differences in E16 vs. P7 tendon cell gene expression responses to IL-1β treatment. To determine whether E16 or P7 cell gene expression responses to IL-1β were significant, we performed t-tests to compare IL-1β treated vs. non-treated gene expression values for E16 cells or P7 cells. We found that P7 cells responded to IL-1β with upregulation of several inflammatory mediators and MMPs, while E16 cells generally did not. At 24 h on TCP, IL-1β treated P7 cells had significantly higher expression compared to control for IL-6, TNFα, MMP-3, and MMP-13 (**Fig 2.11b**), while treated E16 cells only had significantly higher expression of COX-2 compared to control (**Fig 2.11a**). Furthermore, the magnitudes of the P7 gene upregulation trends were higher than that of the E16 COX-2 upregulation, especially for IL-6, MMP-3, and MMP-13. IL-6 was upregulated 1,400-fold, TNFα was upreguated 2.5-fold, MMP-3 was upregulated 65-fold, and MMP-13 was upregulated 40-fold by P7 cells in response to IL-1β treatment (**Fig 2.11b**), while E16 cells upregulated COX-2 only 1.5-fold in response to IL-1β (**Fig 2.11a**). Expression of Col I and Col III was not significantly affected by IL-1β treatment at 24 h on TCP in the E16 cells (**Fig 2.11a**) or in the P7 cells (**Fig 2.11b**).

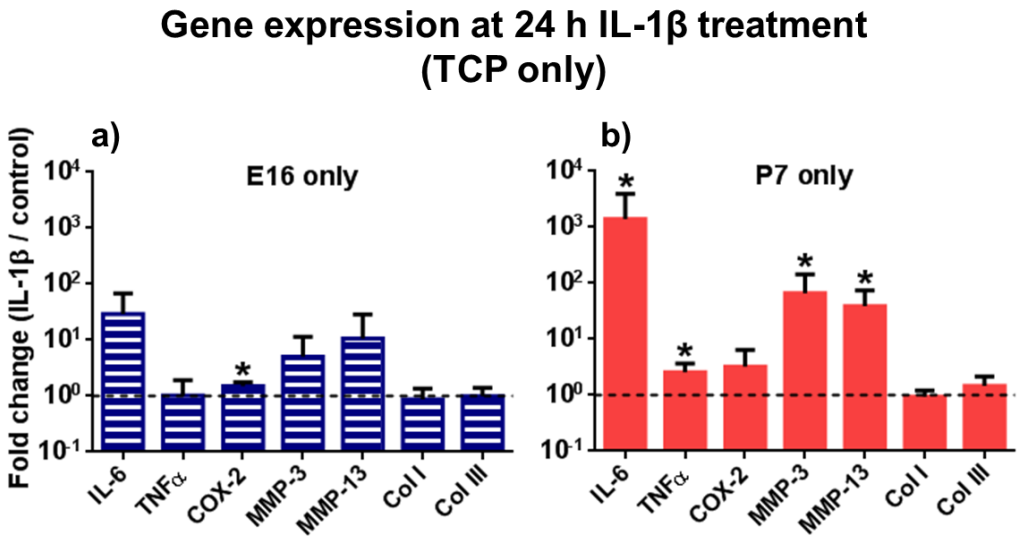

Figure 2.11: Inflammatory mediator, MMP, and collagen gene expression of IL-1β treated E16 (**a**) and P7 (**b**) tendon cells at 24 h on TCP. Results normalized to respective control (non-treated) condition, indicated by dashed line at 1. (* = $p < 0.05$ compared to control; N=3)

2.3.2.2 Inflammatory mediator and MMP protein expression

Because proliferation, metabolic activity, and gene expression were generally independent of substrate conditions (i.e. TCP vs. PA gels,substrate elastic modulus, ECM proteins), we chose to use TCP as the cell substrate for subsequent experiments. To investigate whether the gene expression trends we observed for E16 and P7 tendon cells were maintained at the protein level, we performed ELISAs on cell culture supernatant for IL-6 and MMP-3, both of which were expressed at significantly higher levels in postnatal compared to embryonic tendon cells. The measured amount of IL-6 or MMP-3 in the supernatant was normalized to cell count for each condition.

2.3.2.2.1 Baseline inflammatory mediator and MMP protein expression

E16 vs. P7: In order to determine whether E16 and P7 tendon cells had significantly different baseline IL-6 or MMP-3 protein expression, we performed t-tests to compare E16 vs. P7 protein expression values at 0 h. Similar to gene expression results, IL-6 and MMP-3 protein expression was higher in P7 cells than E16 cells at baseline, although the trend was significant only for MMP-3 (**Fig 2.12**).

2.3.2.2.2 Inflammatory mediator and MMP protein expression in response to IL-1β treatment

E16 vs. P7: In order to determine whether IL-1β treated E16 and P7 tendon cells had significantly different IL-6 or MMP-3 protein levels, we performed t-tests to compare E16 vs. P7 protein levels at 24 h with treatment. Again, similar to gene expression results, IL-6 and MMP-3 protein levels were higher in IL-1β treated P7 cells than E16 cells, with both trends consistent but not significant (**Fig 2.12**).

Effect of IL-1β treatment: In order to determine whether IL-1β treatment affected IL-6 or MMP-3 protein levels for the E16 tendon cells or for the P7 tendon cells, we performed t-tests to compare IL-1β treated vs. non-treated protein levels for each cell stage. Once again similar to gene expression results, 24 h of IL-1β treatment caused a greater upregulation of IL-6 and MMP-3 protein levels compared to control (non-treated) in P7 cells than in E16 cells. The IL-6 protein level of 24 h IL-1β treated E16 cells was 50-fold greater than the 24 h E16

control, while the IL-6 protein level of 24 h IL-1β treated P7 cells was 30,000-fold greater than the 24 h P7 control (**Fig 2.12a**). The MMP-3 protein level of 24 h IL-1β treated E16 cells was 10-fold greater than the 24 h E16 control, while the MMP-3 protein level of 24 h IL-1β treated P7 cells was 30-fold greater than the 24 h P7 control (**Fig 2.12b**).

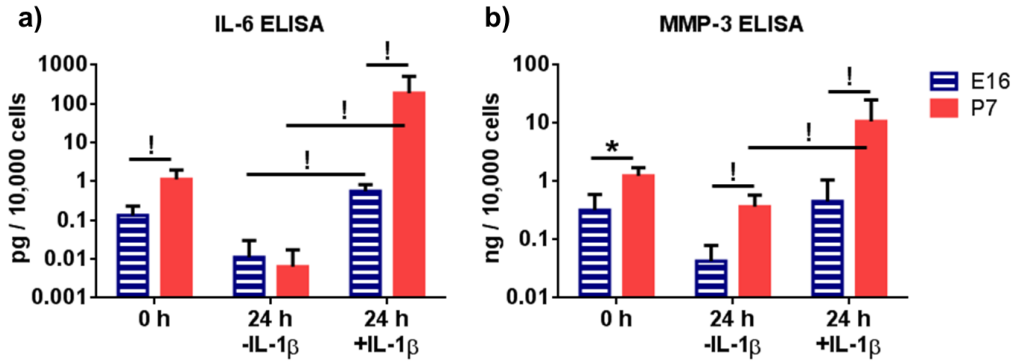

Figure 2.12: IL-6 (**a**) and MMP-3 (**b**) protein expression of E16 and P7 tendon cells at 0 and 24 h, with and without IL-1β treatment, on TCP. Results measured using ELISA, normalized to cell count, and expressed as pg/10,000 cells or ng/10,000 cells. (* = $p < 0.05$; ! = consistent for N=3)

Overall, we found that the protein expression data for both IL-6 and MMP-3 corresponded with the gene expression data, although most of the trends, while consistent among N=3, did not reach statistical significance.

2.3.3 IL-1 mediators

Since we found that embryonic and postnatal tendon cells had different gene and protein expression responses to IL-1β treatment, we investigated how E16 and P7

tendon cells might be mediating IL-1 signaling through IL-1 mediators by examining expression of IL-1R1, IL-1R2, and IL-1RA. In addition to gene and protein expression for IL-1R1 and IL-1RA, we also performed immunofluorescent staining for IL-1R1 and IL-1R2.

2.3.3.1 IL-1 receptor IF staining

2.3.3.1.1 Baseline IL-1 receptor IF staining

E16 vs. P7: At baseline (0 h), P7 cells stained more intensely for IL-1R1, while E16 cells stained more intensely for IL-1R2 (**Fig 2.13a**).

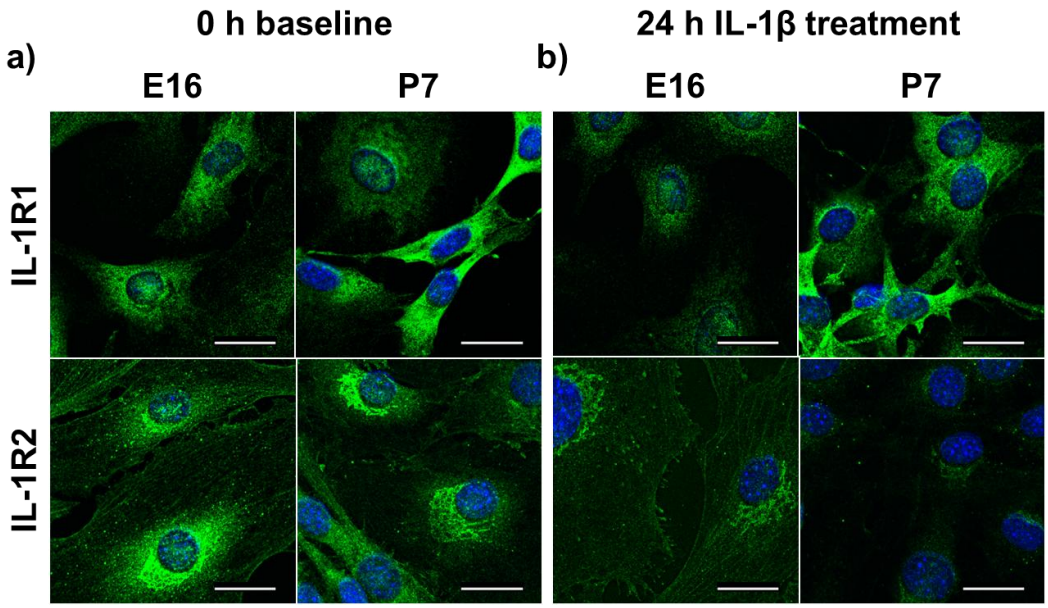

Figure 2.13: Representative images of IL-1R1 and IL-1R2 immunofluorescence staining for E16 and P7 tendon cells at 0 h baseline (**a**) and with 24 h IL-1β treatment (**b**) on glass. Scale bar = 25 μm.

2.3.3.1.2 IL-1 receptor IF staining with IL-1β treatment

E16 vs. P7: After 24 h of IL-1β treatment, P7 cells stained more intensely for IL-1R1, while E16 cells stained more intensely for IL-1R2 (**Fig 2.13b**).

2.3.3.2 IL-1 mediator gene expression

2.3.3.2.1 Baseline IL-1 mediator gene expression

To examine the baseline (0 h) gene expression of IL-1 mediators by embryonic and postnatal tendon cells, we normalized 0 h expression data to the 18s housekeeping gene.

Effect of substrate material: In order to determine whether the substrate material affected baseline (0 h) IL-1 mediator gene expression for the E16 tendon cells or for the P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 gene expression values in order to test whether the TCP values were significantly different compared to any of the PA gel values. There were no significant differences in baseline IL-1R1 or IL-1RA gene expression between TCP and any of the tested PA gels for E16 cells or P7 cells (**Fig 2.14**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected baseline IL-1 mediator gene expression for the E16 tendon cells or for the P7 tendon cells on either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa gene expression values on either Col I or Fn PA gels for each cell stage. There were no significant differences in baseline IL-1R1

or IL-1RA gene expression between 19 and 120 kPa PA gels for Col I or Fn gels in E16 cells or P7 cells (**Fig 2.14**).

Effect of substrate ECM: In order to determine whether the ECM composition affected baseline IL-1 mediator gene expression for the E16 tendon cells or for the P7 tendon cells on either the 19 kPa or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn gene expression values on either 19 or 120 kPa PA gels for each cell stage. There were no significant differences in baseline IL-1R1 or IL-1RA gene expression between Col I and Fn PA gels for 19 or 120 kPa gels in E16 cells or P7 cells (**Fig 2.14**).

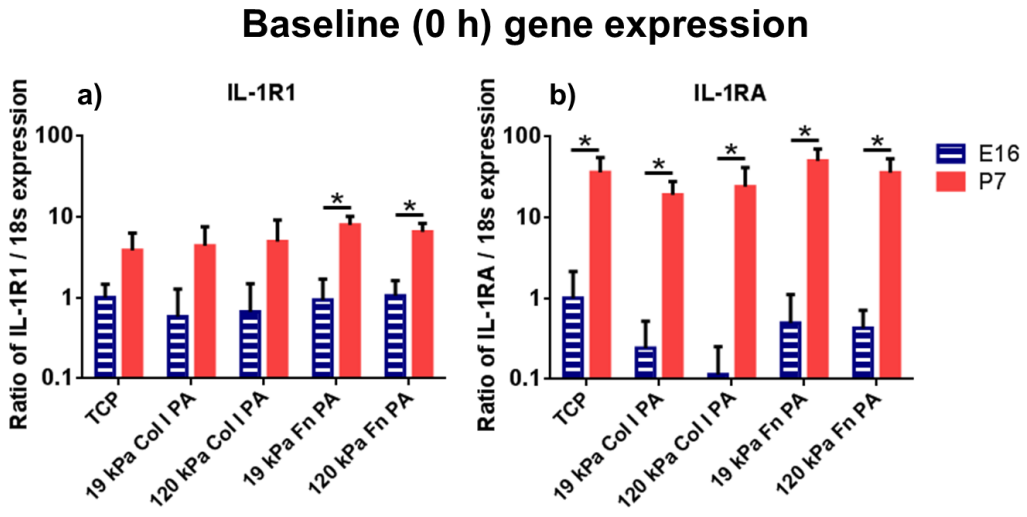

Figure 2.14: IL-1 mediator gene expression of E16 and P7 tendon cells at 0 h (baseline, without IL-1β treatment), on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Results analyzed as fold-change over 18s and then normalized to E16 TCP condition for each gene. (* = $p < 0.05$ for E16 vs. P7; N=3)

E16 vs. P7: In order to determine whether E16 and P7 tendon cells had significantly different baseline IL-1 mediator gene expression, we performed t-tests to compare E16 vs. P7 gene expression values on each substrate. At baseline, gene expression levels were significantly higher in P7 cells than in E16 cells for IL-1R1 on both Fn PA gels (**Fig 2.14a**) and for IL-1RA on all tested substrates (**Fig 2.14b**).

2.3.3.2.2 IL-1 mediator gene expression in response to IL-1β treatment

To assess how embryonic and postnatal tendon cells regulate their IL-1 mediator gene expression in response to IL-1β treatment, we normalized 24 h expression data of IL-1β treated E16 or P7 tendon cells to the corresponding control (non-treated) condition.

Effect of substrate material: In order to determine whether the substrate material affected IL-1 mediator gene expression responses to IL-1β treatment for the E16 tendon cells or for the P7 tendon cells, we performed 1-way ANOVAs for E16 or P7 gene expression values in order to test whether the TCP values were significantly different compared to any of the PA gel values. There were no significant differences in IL-1R1 or IL-1RA gene expression responses to IL-1β between TCP and any of the tested PA gels for E16 cells or P7 cells (**Fig 2.15**).

Effect of substrate stiffness: In order to determine whether the substrate stiffness affected IL-1 mediator gene expression responses to IL-1β treatment for the E16 tendon cells or for the P7 tendon cells on either the Col I or Fn PA gels, we performed t-tests to compare 19 kPa vs. 120 kPa gene expression values on

either Col I or Fn PA gels for each cell stage. There were no significant differences in IL-1R1 or IL-1RA gene expression responses to IL-1β between 19 and 120 kPa PA gels for Col I or Fn gels in E16 cells or P7 cells (**Fig 2.15**).

Effect of substrate ECM: In order to determine whether the ECM composition affected IL-1 mediator gene expression responses to IL-1β treatment for the E16 tendon cells or for the P7 tendon cells on either the 19 kPa or 120 kPa PA gels, we performed t-tests to compare Col I vs. Fn gene expression values on either 19 or 120 kPa PA gels for each cell stage.The only effect of ECM protein on IL-1 mediator gene expression response to IL-1β was for IL-1RA in E16 cells on 120 kPa PA gels, for which expression was higher on the Col I than the Fn gel (**Fig 2.15b**).

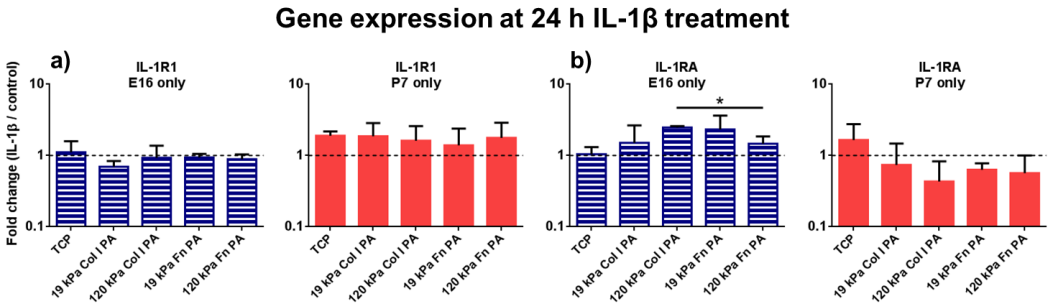

Figure 2.15: IL-1 mediator gene expression of IL-1β treated E16 and P7 tendon cells at 24 h on TCP and on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Results normalized to respective control (non-treated) condition, indicated by dashed line at 1. (No statistically significant differences between substrates for N=3)

Effect of IL-1β treatment on cells cultured on TCP: Because embryonic and postnatal tendon cell IL-1 mediator gene expression responses to IL-1β treatment were independent of substrate (TCP vs. PA gels), we focused on experiments performed on TCP only to further examine differences in E16 vs. P7 tendon cell IL-1R1 and IL-1RA gene expression responses to IL-1β treatment. To determine whether E16 or P7 cell gene expression responses to IL-1β were significant, we performed t-tests to compare IL-1β treated vs. non-treated gene expression values for E16 cells or P7 cells. At 24 h on TCP, P7 cells significantly upregulated IL-1R1 in response to IL-1β treatment (**Fig 2.16b**), while E16 cells did not (**Fig 2.16a**). Expression of IL-1RA was not significantly affected by IL-1β in either cell stage (**Fig 2.16**).

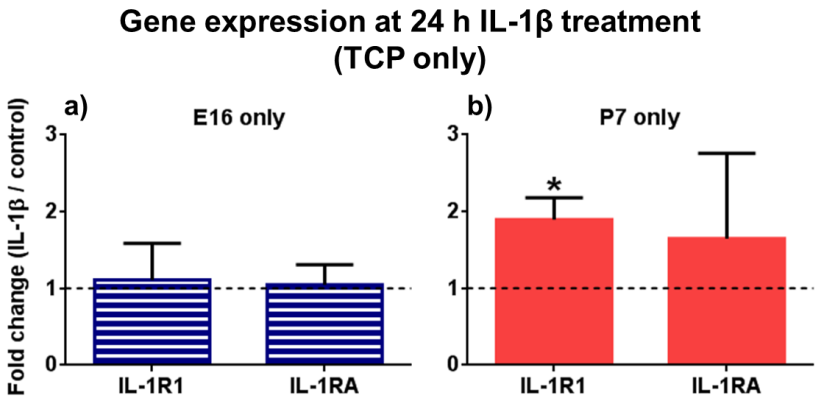

Figure 2.16: IL-1 mediator gene expression of IL-1β treated E16 (**a**) and P7 (**b**) tendon cells at 24 h on TCP. Results normalized to respective control (non-treated) condition, indicated by dashed line at 1. (* = $p < 0.05$ compared to control; N=3)

2.3.3.3 IL-1 mediator protein expression

To investigate whether the IL-1RA gene expression trends we saw for E16 and P7 tendon cells were maintained at the protein level, we performed ELISAs on cell culture supernatant. The measured amount of IL-1RA in the supernatant was normalized to cell count for each condition.

2.3.3.3.1 Baseline IL-1 mediator protein expression

E16 vs. P7: In order to determine whether E16 and P7 tendon cells had significantly different baseline IL-1RA protein levels, we performed a t-test to compare E16 vs. P7 protein levels at 0 h. Similar to gene expression results, IL-1RA protein levels were higher in P7 cells than E16 cells at baseline, although the trend did not reach significance (**Fig 2.17**).

2.3.3.3.2 IL-1 mediator protein expression in response to IL-1β treatment

E16 vs. P7: In order to determine whether IL-1β treated E16 and P7 tendon cells had significantly different IL-1RA protein levels, we performed a t-test to compare E16 vs. P7 protein levels at 24 h with treatment. IL-1RA protein levels were significantly higher in IL-1β treated P7 cells than E16 cells (**Fig 2.17**).

Effect of IL-1β treatment: In order to determine whether IL-1β treatment affected IL-1RA protein levels for the E16 tendon cells or for the P7 tendon cells, we performed t-tests to compare IL-1β treated vs. non-treated protein levels for each cell stage. We found that E16 cells slightly increased IL-1RA protein levels

in response to IL-1β treatment, while P7 cells decreased IL-1RA protein levels in response to IL-1β (**Fig 2.17**). Both trends were consistent but not significant.

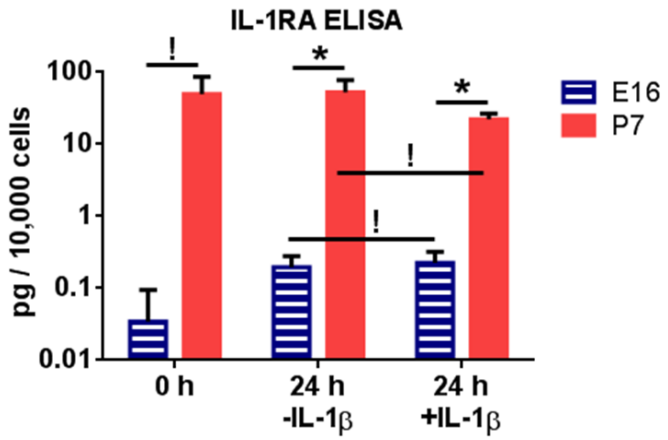

Figure 2.17: IL-1RA protein expression of E16 and P7 tendon cells at 0 and 24 h, with and without IL-1β treatment, on TCP. Results measured using ELISA, normalized to cell count, and expressed as pg/10,000 cells. (* = $p < 0.05$; ! = consistent for N=3)

2.4 DISCUSSION

2.4.1 Findings

This study investigated the responses of embryonic and postnatal tendon cells to IL-1β treatment in order to shed light on potential differences that may contribute in some way to the scarless vs. scarred healing responses observed in embryonic and adult (including postnatal) tendon, respectively. We found that embryonic and postnatal tendon cells have distinctly different baseline gene and protein expression profiles of inflammatory mediators, MMPs, and collagens, with postnatal cells generally having higher baseline expression of these targets than embryonic cells on the various tested substrates (differing materials, elastic moduli, and functionalized ECM proteins). Among the inflammatory mediators, we found that TNFα expression was significantly higher in postnatal than embryonic tendon cells on both moduli of Col I-functionalized PA gels, and COX-2 expression was significantly higher in postnatal than embryonic on all tested PA gels. These results for baseline COX-2 expression are in keeping with findings in skin, where it was observed that mouse skin fibroblasts from a scarred healing stage had higher baseline COX-2 expression compared to fibroblasts from a scarless healing stage (Wilgus et al., 2004); similar results were found for COX-1 in the same cell type and stages (Wulff et al., 2013). While the trends we observed for higher baseline IL-6 expression in postnatal compared to embryonic tendon cells did not reach statistical significance, they were consistent with literature in skin, which found that adult human skin fibroblasts had higher

baseline gene and protein expression of IL-6 than fetal fibroblasts (Liechty et al., 2000). We found that postnatal tendon cell expression levels of MMP-3, MMP-13, Col I and Col III were also consistently higher than embryonic, especially on PA gels. Similarly, in literature it has been found that baseline expression levels of MMPs, including MMP-3, are higher in human and rat skin and dermal fibroblasts from scarred healing stages than from scarless healing stages (Chen et al., 2007; Gosiewska et al., 2001; Peled et al., 2002). Our baseline expression results may indicate that, in a normal, non-inflammatory environment, embryonic and postnatal tendon cells have different "set point" levels of inflammatory mediators and MMPs, which might be why they respond to inflammation differently and, in turn, somehow contribute to the scarless vs. scarred healing outcomes in their respective tissues.

In addition to baseline differences, we also found that embryonic and postnatal tendon cells respond differently to IL-1β treatment, with changes in the substrate properties having no effect on the responses for either cell stage. More specifically, postnatal tendon cells responded to 24 h of IL-1β treatment with upregulation of IL-6, TNFα, MMP-3, and MMP-13, while embryonic tendon cells only responded with a small upregulation of COX-2. It should be noted that this embryonic tendon cell upregulation trend did not alter the COX-2 expression enough from baseline levels to actually make it significantly higher than that of the postnatal tendon cells. Results from various studies on adult tendon cells corroborate our finding that IL-1β treatment causes upregulation of inflammatory

mediator and MMP gene expression in postnatal cells. Such studies have found that adult human and rabbit tendon cells respond to IL-1β with increased expression of inflammatory mediators and MMPs, including IL-6, MMP-3 and MMP-13 (Archambault et al., 2002; Buhrmann et al., 2011; Corps et al., 2004; Thampatty et al., 2007; Tsuzaki et al., 2003b). While there have not been any studies done in tendon comparing inflammatory mediator responses during scarless vs. scarred healing, such studies have been performed in skin. For instance, it was found that adult human skin wounds more persistently produced IL-6 compared to fetal wounds and that administration of exogenous IL-6 to fetal wounds induced scar formation, indicating that overexpression of IL-6 may contribute to scarred healing outcomes (Liechty et al., 2000). Another group showed that mouse skin wounds from a scarred healing stage have greater COX-2 gene and protein expression levels than mouse skin wounds from a scarless healing stage (Wilgus et al., 2004). It was also found that MMP-3 gene expression was higher in healing wild-type mouse skin wounds, which heal with scar, than in healing nude mouse skin wounds, which heal scarlessly (Gawronska-Kozak, 2011). All of these findings in skin were in keeping with our results for tendon cells. The upregulation of inflammatory mediators and MMPs in response to IL-1β treatment that we observed in postnatal tendon cells but not embryonic cells may be indicative of greater inflammatory and catabolic responses of scarred healing tendons to inflammation following injury compared to scarlessly healing tendons. Such differing responses may contribute in some way to these respective

wound healing outcomes, perhaps with increased inflammation and catabolic activity leading to scar formation in postnatal and adult tendons.

Of particular interest among the target molecules we investigated are MMP-3 and MMP-13, expression levels of which were consistently much higher in postnatal tendon cells than embryonic cells both at baseline and in response to IL-1β. As mentioned in the previous paragraph, the MMP upregulation in response to IL-1β that we observed for the postnatal tendon cells is in keeping with findings in literature for adult tendon cells. This IL-1β induced upregulation of MMPs is well-documented in many other adult cell types as well, including chondrocytes and synoviocytes (Ahmad et al., 2007; Fan et al., 2006; Gosset et al., 2010; Han et al., 1999; Han et al., 2001; Liacini et al., 2002; Lim and Kim, 2011; Mengshol et al., 2000; Wang et al., 2011). Gene and protein expression levels of MMPs, including MMP-3 and MMP-13, have also been found to be elevated in injured adult tendons and ligaments (Berglund et al., 2007; Chamberlain et al., 2011; Higuchi et al., 2006; Manning et al., 2014; Oshiro et al., 2003). This has also been observed for adult skin wounds (Madlener et al., 1998; Soo et al., 2000; Wysocki et al., 1993). Interestingly, it has been found that MMP-3 can upregulate the expression of certain MMPs, as well as trigger release of various pro-inflammatory mediators (Steenport et al., 2009). As such, expression and production of MMP-3 during injury may contribute to further inflammatory responses through such positive feedback mechanisms. Overall, the strong embryonic vs. postnatal MMP expression trends that we saw for the tendon cells

at baseline and in response to IL-1β may indicate that MMPs in particular play an important role in determining scarless vs. scarred tendon healing outcomes.

Considering the very different gene and protein expression profiles of embryonic and postnatal tendon cells at baseline and in response to IL-1β, we decided to examine how embryonic and postnatal tendon cells might be mediating IL-1 signaling differently by investigating their expression of IL-1 mediators, including IL-1R1, IL-1R2, and IL-1RA. We found that embryonic and postnatal tendon cells have differing expression of IL-1 mediators, both at baseline and in response to IL-1β treatment, with postnatal cells expressing higher levels of IL-1R1 and IL-1RA and embryonic cells expressing higher levels of IL-1R2. The higher postnatal expression of IL-1RA that we observed is interesting, since IL-1RA is an antagonist that can counteract IL-1β signaling. However, an increase in IL-1RA is a natural response to inflammation (Arend, 2002; Fischer et al., 1992; Galkowska et al., 2005), so the higher expression levels in postnatal tendon cells may simply be a response caused by the greater inflammatory and catabolic responses to IL-1β that we also observed in the postnatal cells. Little work has been performed to investigate how IL-1 mediator expression changes in response to inflammation in tendon or in scarless healing. One paper showed that treatment of adult human tendon cells with IL-1β did not affect IL-1R1 gene expression (Tsuzaki et al., 2003b). However, it has been shown in certain adult cell types, including mouse T cells (Chung et al., 2009), mouse and rat hepatocytes (Ito et al., 1999; Teshima et al., 2004) and rat Schwann cells (Skundric et al., 1997), that

treatment with inflammatory molecules such as IL-1β and IL-6 can cause an increase in IL-1R1 gene expression, which was in keeping with our results for IL-1β treated P7 cells on TCP. The embryonic vs. postnatal IL-1 mediator expression differences we observed may be partially responsible for causing the differences in inflammatory mediator and MMP expression in response to IL-1β that we saw between the two cell stages. More specifically, our results may indicate that a higher amount of IL-1β signaling occurs in postnatal tendon cells due to increased number of IL-1R1 for IL-1β to bind, while IL-1β signaling is reduced in embryonic tendon cells due to IL-1β being bound to IL-1R2 without transducing intracellular signaling. Thus, more IL-1β signal transduction in postnatal cells than embryonic cells may have led to the greater pro-inflammatory and catabolic responses we observed. We plan to test this further by manipulating expression of IL-1R1 and IL-1R2 (by performing knockouts, knockdowns, overexpression, etc.) in each cell type and examining how their responses to IL-1β are affected.

2.4.2 Experimental parameters

For this study, we conducted experiments on tendon cells from embryonic day 16 (E16) and postnatal day 7 (P7) mice. These developmental stages were chosen because E16 and P7 are stages at which scarless and scarred healing occur, respectively, in mice (Colwell et al., 2006). It will also be important in future studies to examine tendons from adult stages of development as well (see Future Directions 3.4).

Since the purpose of this study was to investigate how embryonic and postnatal tendon cells respond to inflammation, we treated tendon cells *in vitro* with a representative pro-inflammatory cytokine. IL-1β was chosen because it is a key cytokine released early in the inflammatory cascade following injury (Mills and Dunne, 2009; Weber et al., 2010), and because it has been found to be particularly highly upregulated (4000-fold) after tendon injury (Manning et al., 2014). Furthermore, numerous studies have shown IL-1β to be a reliable and potent inducer of inflammatory responses in tendon cells *in vitro* (Archambault et al., 2002; Buhrmann et al., 2011; Corps et al., 2003; Thampatty et al., 2007; Tsuzaki et al., 2003b; Yang et al., 2005). In the future, it will also be important to investigate the effects of other soluble factors involved in inflammation and healing (see Future Directions 3.2).

For these experiments, we used a 100 pM dose for the IL-1β treatment. Human serum levels of IL-1β in cases of injury and pathology have been found to range from approximately 0.01 to 10 pM (Antonelli et al., 2009; Catterall et al., 2010; Di Iorio et al., 2003; Hasdai et al., 1996; Jablonska et al., 1997). In addition, IL-1β has been shown to be produced by torn rabbit rotator cuff tendon cultures at 1 day after injury at a concentration of approximately 10 pM (Koshima et al., 2007). However, some studies have found that IL-1β doses in this physiological range are not sufficient to induce significant inflammatory responses from tendon cells *in vitro* (Archambault et al., 2002; Tsuzaki et al., 2003b), so we chose a dose that was one order of magnitude higher to use for our experiments. The 100 pM dose

we used was also similar to what has been used in *in vitro* tendon cell experiment literature (Archambault et al., 2002; Corps et al., 2003; Thampatty et al., 2007; Tsuzaki et al., 2003b).

Since we were interested in acute inflammatory responses, which occur within hours after injury and persist for about 2 to 3 days (Molloy et al., 2003; Schulze-Tanzil et al., 2011; Voleti et al., 2012), we chose a 24 h time point for our experiments. We also checked tendon cell responses after only 1 h of IL-1β treatment and found that cells responded significantly even at 1 h (**Fig 3.1**). It will be important in future studies to investigate later time points in order to determine how embryonic and postnatal tendon cell responses differ during later phases of healing when activity shifts from acute inflammation to active tissue repair/regeneration (see Future Directions 3.4).

2.4.3 Conclusions and future work

This study represents the first step toward investigating intrinsic differences in embryonic and adult tendon cell that may contribute to scarless vs. scarred differential tendon healing by 1) specifically focusing on the responses of the tendon cells to pro-inflammatory cytokine treatment, and 2) beginning to probe mechanisms causing such differential responses by examining cytokine receptor profiles. For this study, we performed *in vitro* experiments on tendon cells because our goal here was to investigate embryonic and postnatal tendon cell responses to specific controlled variables (cytokine treatment, substrate elastic

modulus, ECM composition). We focused on acute inflammatory responses in particular, using applied IL-1β treatment, because it has been observed in both tendon and skin literature that scarless healing and scarred healing are characterized by noticeable differences in inflammatory activity (Beredjiklian et al., 2003; Cowin et al., 1998; Favata et al., 2006; Liechty et al., 2000; Liechty et al., 1998; Wilgus et al., 2004). We demonstrated that embryonic and postnatal tendon cells have distinctly different expression profiles of inflammatory mediators and MMPs at baseline and in response to cytokine treatment, which may contribute to or be a factor involved in the differential scarless vs. scarred tendon healing outcomes observed at these two developmental stages. We also showed that these disparate embryonic vs. postnatal responses are accompanied by differences in cytokine receptor profiles, which is a potential mechanism that may give rise to the differential responses between the two cell stages.

Moving forward, we plan to conduct further investigation into potential causes of the differential embryonic vs. postnatal tendon cell responses by manipulating IL-1 mediator expression to determine if the responses of one cell type can be redirected to become more like the other. We also plan to investigate how embryonic and postnatal tendon cells activate different downstream intracellular signaling pathways in response to IL-1β treatment. In addition, we plan to investigate how other factors and cues are involved in differential tendon healing, both in isolation and in combination. Such cues include different soluble factors, such as other inflammatory molecules and growth factors, as well as additional

mechanical cues, such as static and dynamic loading. We also plan to expand testing to include additional developmental stages by using cells from adult and elderly animals, as well as to include longer-term time points and additional target molecules.

This study represents the first step in a larger project to learn about embryonic scarless tendon healing in order to improve adult scarred tendon healing outcomes. Our results here will help inform future studies through the identification of potential targets for further investigation. In turn, our work here and in future studies to identify differences between embryonic and postnatal tendon cells and the potential causes of these differences may provide insight that will bring us closer to our eventual goal of developing treatment strategies to redirect scarred healing of adult tendon toward regenerative, scarless healing outcomes.

CHAPTER 3: FUTURE DIRECTIONS

3.1 FURTHER INVESTIGATION OF CURRENTLY IDENTIFIED FACTORS

An important next step for this project will be to further investigate the potential mechanism of embryonic vs. postnatal tendon cell differences that we identified in this study, namely the differential expression of IL-1 receptors and IL-1RA by embryonic and postnatal tendon cells. By doing so, we can confirm and more fully understand the involvement of these IL-1 mediators in determining scarless vs. scarred healing outcomes. This can be done by manipulating the expression of the IL-1 mediators and examining how the responses of the embryonic and postnatal tendon cells are affected. Such manipulations could include selectively inhibiting or overexpressing IL-1R1 or IL-1R2 using antibodies, siRNA, vectors, or knockout mice. In this way, we could examine whether such manipulations can make the postnatal tendon cells respond to IL-1β more like the embryonic tendon cells, or vice versa.

Similarly, it may also be beneficial to further examine the particular molecular mediators we have identified thus far as targets of interest in differential embryonic vs. postnatal tendon cell responses, such as the MMPs. For instance, MMPs are secreted as proenzymes, and in order for them to actually degrade ECM molecules, they must first be activated through removal of their pro-peptide

domain. As such, it may be useful to conduct MMP activity assays in order to investigate whether MMP action truly differs between embryonic and postnatal tendon cells. We could also investigate additional target molecules related to our current targets. *In vivo*, the activity of MMPs is counteracted by tissue inhibitors of metalloproteinases (TIMPs), so the physiological balance between MMPs and TIMPs is important in determining ECM turnover. As such, it could be useful to look at TIMP expression and production in the embryonic and postnatal tendon cells and determine how it compares to MMP expression, production, and activity. Furthermore, we could also further explore the role of targets such as MMPs in affecting embryonic vs. postnatal tendon cell responses by conducting inhibition or addition studies to examine how the cells respond. This could be accomplished for MMPs in particular by adding MMP inhibitors for loss-of-function or treating with activated MMPs for gain-of-function.

These studies would represent first steps toward actually redirecting adult tendon healing outcomes toward a more embryonic-like, scarless phenotype by providing a concrete idea of what type of adjustments to cell activity/responses can effectively alter inflammatory and other healing-related responses. Such insight would help us move toward our goal of developing treatment strategies or techniques to improve adult tendon healing.

3.2 INVESTIGATION OF ADDITIONAL WOUND-RELATED FACTORS

In moving forward with this project, we also plan to investigate how additional factors and cues are involved in or may contribute to scarless vs. scarred tendon wound healing. So far, we have looked at IL-1β treatment, substrate elastic modulus, and ECM protein composition, so there may be other important cues that we are missing. For instance, there are many other soluble factors present during inflammation and healing besides IL-1β that may play important roles in scarless vs. scarred healing. These include various additional cytokines, as well as numerous growth factors. In this study, we chose to treat tendon cells with IL-1β due to its potency and its importance early in the inflammatory cascade (Mills and Dunne, 2009; Weber et al., 2010), as well as its presence during tendon injury and healing (Berglund et al., 2007; Bigoni et al., 2013; Koshima et al., 2007; Manning et al., 2014). However, there are also other cytokines that are involved in tendon inflammation and healing, such as TNFα and IL-6; as such, it will be important to look at these additional cytokines as well in future work. As for growth factors, the isoforms of transforming growth factor β (TGF-β) are of particular interest to scarless vs. scarred healing due to the specific differential embryonic vs. adult patterns of each that have been observed during cutaneous wound healing; TGF-β1 and TGF-β2 have been found to be present at higher levels in adult skin healing, while TGF-β3 is present at a higher level in embryonic skin healing (Chen et al., 2005; Hsu et al., 2001; Shah et al., 1995; Sullivan et al., 1995). Perhaps embryonic and adult tendon cells react differently to several soluble

factors, such that it is the particular profile of multiple soluble mediators present in the environment that leads to scarless vs. scarred healing outcome.

There are also additional mechanical cues besides elastic modulus that may play a significant role in scarless vs. scarred tendon healing. Tendons are subjected to frequent loading, including during healing and physical therapy after injury, so it will be critical to understand how mechanical loading influences embryonic and postnatal tendon cell responses to pro-inflammatory cytokines. Previous studies have shown that cyclic tensile loading can modulate the effects of pro-inflammatory cytokines on adult tendon cells. One study showed that loading of rabbit tendon cells enhanced IL-1β induced upregulation of MMP-1 and MMP-3 (Archambault et al., 2002). Another study found that high magnitude loading of human tendon cells enhanced IL-1β induced upregulation of MMP-1, COX-2, and PGE_2, while low magnitude loading abrogated these IL-1β induced responses (Yang et al., 2005). Loading alone has also been shown to be capable of altering adult tendon cell expression of inflammatory mediators and MMPs (Asundi and Rempel, 2008; Legerlotz et al., 2013; Qi et al., 2011; Skutek et al., 2001; Sun et al., 2008; Tsuzaki et al., 2003a; Wang et al., 2003). However, the effect of loading on embryonic scarless healing has not been yet been studied in tendon or in other tissues. It will therefore be informative to investigate how mechanical loading is involved in scarless vs. scarred tendon healing, as well as how it modulates tendon healing outcomes, by subjecting embryonic and postnatal tendon cells to cyclic tensile loading using a mechanical loading system.

It will also be important to investigate how embryonic and postnatal tendon cells respond to various applied factors and cues in combination, rather than to only one cue at a time in isolation, since there are likely multiple factors working together *in vivo* to give rise to scarless or scarred tendon healing outcomes. Overall, investigation of additional wound-related environmental factors, whether alone or in combination, will give us a more complete picture of the various influences that govern scarless vs. scarred tendon healing and how they do so, as well as potentially allow us to identify useful targets for manipulation in order to improve adult tendon healing outcomes.

3.3 INVESTIGATION OF ADDITIONAL MECHANISMS AND PATHWAYS

In addition to investigating additional wound-related factors, we also plan to further probe other potential causes of the differential embryonic vs. postnatal tendon cell responses to IL-1β treatment that we observed, besides differences in expression of IL-1 mediators. For instance, we could investigate the role of IL-1β signaling in regulating differential tendon wound healing outcomes by examining embryonic and postnatal tendon cell activation of downstream intracellular signaling pathways that are involved in transduction of IL-1β signals. While there has been limited work done to investigate downstream intracellular signaling of tendon cells in response to IL-1β, such studies have found that responses of adult tendon cells to IL-1β seem to involve activation of the MAPK (p38 MAPK, JNK)

and NF-κB pathways. The Wang group showed that a p38 MAPK inhibitor could abrogate IL-1β induced upregulation of COX-2 and PGE_2 in adult human tendon cells (Thampatty et al., 2007), and the Riley group showed that IL-1β both induced MMP expression and increased phosphorylation of p38 MAPK and JNK in adult human tendon cells (Corps et al., 2004). In addition, adult human tendon cells have been found to respond to IL-1β treatment with both upregulation of COX-2 and MMPs and an increase in NF-κB phosphorylation (Buhrmann et al., 2011; Busch et al., 2012). There have also been few studies looking at MAPK/NF-κB signaling in scarless healing. One paper found that wounded rat skin from a scarred healing stage had greater c-fos expression than wounded skin from a scarless healing stage (Gangnuss et al., 2004). c-fos is a downstream regulator of the ERK pathway. The wounded rat skin from the scarred healing stage also required more ERK inhibitor than the wounded skin from the scarless healing stage in order to abrogate wounding-induced ERK phosphorylation. Both of these findings indicate stronger and more persistent ERK signaling in scar-forming wounds than in scarlessly healing wounds.

We could investigate IL-1β signaling by performing western blots to examine the protein expression and phosphorylation of p38 MAPK, JNK, ERK, and NF-κB in IL-1β treated embryonic and postnatal tendon cells. Since phosphorylation of signaling proteins represents activation of the corresponding signaling pathway, the resulting data would indicate to what extent embryonic and postnatal tendon cells activate each IL-1β signaling pathway. Perhaps differences in IL-1β

signaling pathway activation contributed to the greater expression of inflammatory mediators and MMPs in response to IL-1β that we observed for postnatal compared to embryonic tendon cells, which may, in turn, contribute in some way to scarless vs. scarred tendon healing outcomes. We could further investigate the involvement of downstream IL-1β signaling pathways in different embryonic vs. postnatal tendon cell responses by inhibiting the pathways using chemical inhibitors or siRNA knockdown to examine how the responses of each cell type to IL-1β are affected, as well as to test if we can make the postnatal tendon cells respond to IL-1β more like the embryonic cells, or vice versa. Additional knowledge about potential mechanisms that may contribute to scarless vs. scarred tendon healing outcomes may provide strategies for manipulating adult tendon cells in order to redirect healing toward a regenerative, scarless phenotype.

3.4 EXPANSION OF TESTING TO INCLUDE ADDITIONAL DEVELOPMENTAL STAGES, TIME POINTS, AND TARGET MOLECULES

Another next step that will be important for this project is to expand testing to include additional cell stages, such as adult and aging/elderly. Using an adult stage will be important due to its direct relevance to ages at which acute tendon injuries are likely to be seen in human patients (Thomopoulos et al., 2015). Also, an adult, rather than postnatal, stage may yield more insight into factors involved

in scarless vs. scarred wound healing, perhaps due to more definite or drastic differences compared to embryonic stages. Furthermore, an older, elderly developmental stage may be interesting in terms of probing how healing occurs in aging tendons and in investigating chronic tendinopathies. Findings from studies involving these older developmental stages may provide more insight into how tendon healing processes change into adulthood and old age, potentially leading to the development of strategies to improve tendon healing outcomes and to treat tendinopathy.

It will also be informative to expand experimental time points in future studies. For this first study, we chose a relatively short time point of 24 h because we were investigating acute inflammatory responses, which occur on this time scale (Molloy et al., 2003; Schulze-Tanzil et al., 2011; Voleti et al., 2012). It will also be important to include longer time points in order to examine how embryonic and postnatal tendon cells differ in their responses and activities, such as ECM production, during later stages of healing, including the repair and remodeling phases. Such investigation would provide more information about how scarless and scarred tendon healing outcomes arise by revealing more about how regenerated tissue or scar tissue, respectively, forms and matures throughout healing. In addition, based on data we collected, it may be informative to probe shorter time points as well. At 1 h on TCP, we found that only E16 tendon cells responded significantly to IL-1β treatment, while P7 tendon cells did not (**Fig 3.1**); the IL-1β treated E16 tendon cells significantly upregulated IL-6 and TNFα

compared to control at 1 h (**Fig 3.1a**). This was in contrast to results at 24 h, when the P7 tendon cells were more responsive to treatment than the E16 cells (**Fig 2.11**). Expression of IL-1 mediators, however, was not significantly affected by IL-1β for either cell stage at 1 h (**Fig 3.2**). These data suggest that embryonic tendon cells may respond to cytokines on a shorter time scale than postnatal tendon cells, perhaps indicating that their inflammatory responses occur more rapidly and are over sooner compared to postnatal. Future experiments including time points of less than 24 h will allow us to probe into this further.

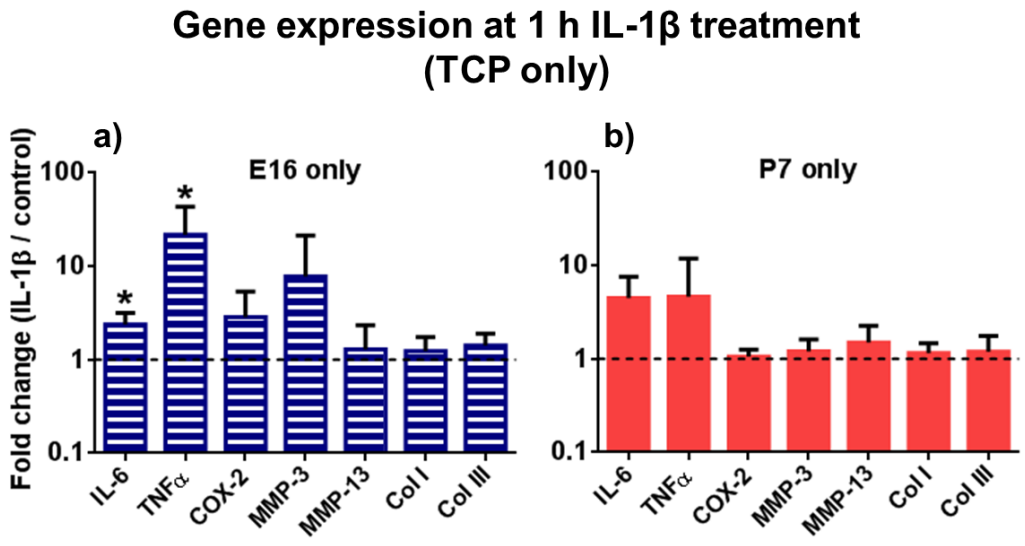

Figure 3.1: Inflammatory mediator, MMP, and collagen gene expression of IL-1β treated E16 (**a**) and P7 (**b**) tendon cells at 1 h on TCP. Results normalized to respective control (non-treated) condition, indicated by dashed line at 1. (* = $p < 0.05$ compared to control; N=3)

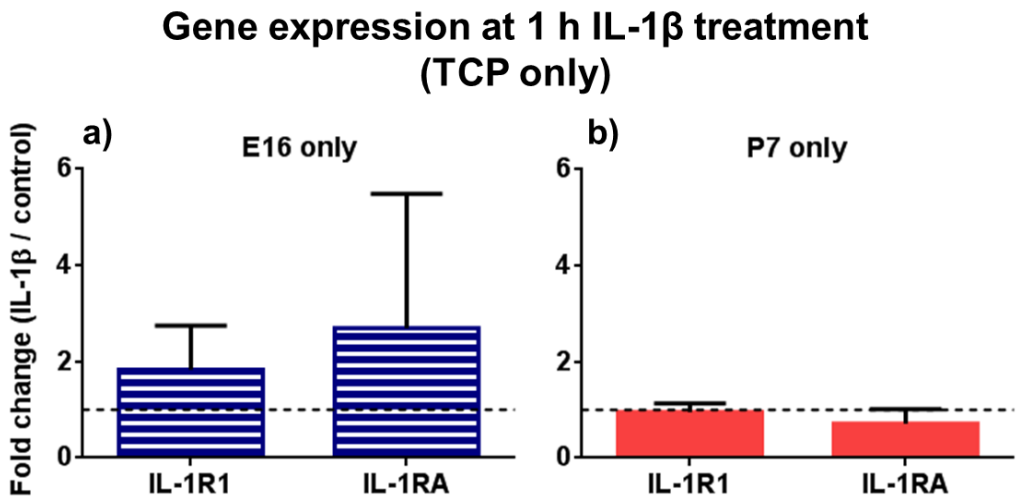

Figure 3.2: IL-1 mediator gene expression of IL-1β treated E16 (**a**) and P7 (**b**) tendon cells at 1 h on TCP. Results normalized to respective control (non-treated) condition, indicated by dashed line at 1. (No statistically significant differences compared to control for N=3)

In addition, it may be useful to examine expression of additional target molecules besides inflammatory mediators, MMPs, and IL-1 mediators, especially in investigating later stages of tendon healing. For example, we could examine expression of tendon markers, as the differentiation state of tendon cells may play a role in whether the tissue regenerates without scar or repairs with scar formation during healing. To begin to probe this, we examined embryonic and postnatal tendon cell expression of scleraxis (Scx) at baseline and in response to 24 h of IL-1β treatment on PA gels of varying elastic modulus and ECM composition. Scleraxis is a transcription factor that is the most widely-used marker for tendon cells (Schweitzer et al., 2001). We found that baseline (0 h) expression of Scx was not significantly different between E16 and P7 tendon cells, nor was it

significantly affected by substrate elastic modulus or ECM composition for either cell stage (**Fig 3.3**). After 24 h of IL-1β treatment, Scx expression was significantly affected by treatment only in the P7 tendon cells, with significant downregulation on the 120 kPa Col I PA gel and significant upregulation on the 19 kPa Fn PA gel (**Fig 3.4b**). E16 tendon cell Scx expression was not significantly affected by IL-1β treatment (**Fig 3.4a**). In addition, Scx expression by E16 tendon cells was not significantly affected by substrate properties (**Fig 3.4a**), while P7 tendon cell Scx expression was significantly higher on 19 kPa compared to 120 kPa Fn PA gels (**Fig 3.4b**). It is noteworthy that Scx expression is affected by IL-1β treatment in postnatal tendon cells but not embryonic tendon cells; perhaps changes in the differentiation state of postnatal tendon cells during healing somehow contributes to scarred healing outcomes. Further investigation of embryonic and postnatal tendon cell expression of Scx, as well as other tendon markers, may yield valuable insights into differences that contribute to scarless vs. scarred tendon healing outcomes.

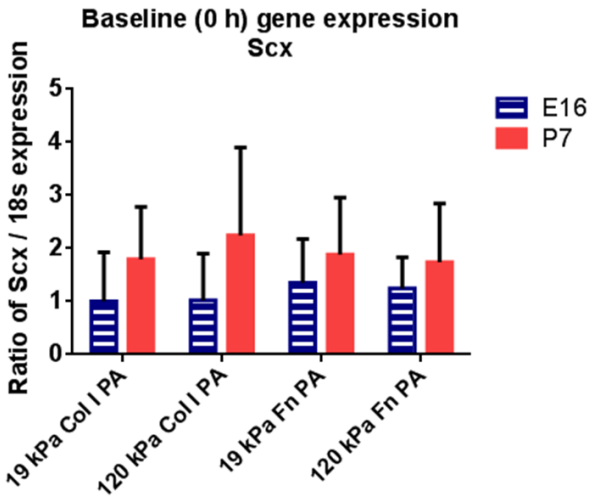

Figure 3.3: Scleraxis gene expression of E16 and P7 tendon cells at 0 h (baseline, without IL-1β treatment), on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Results analyzed as fold-change over 18s and then normalized to E16 19 kPa Col I PA gel condition. (No statistically significant differences between conditions for N=3)

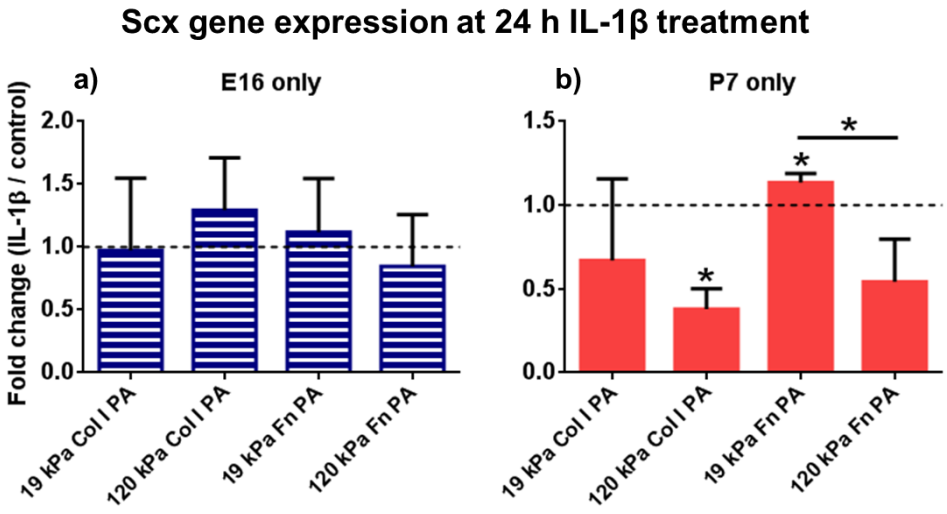

Figure 3.4: Scleraxis gene expression of IL-1β treated E16 (**a**) and P7 (**b**) tendon cells at 24 h on 19 kPa and 120 kPa Col I- and Fn-functionalized PA gels. Results

normalized to respective control (non-treated) condition, indicated by dashed line at 1. (* = $p < 0.05$; N=3)

3.5 *IN VIVO* INVESTIGATIONS

In addition to the future *in vitro* studies outlined above, it will be crucial to perform *in vivo* experiments in order to determine whether the information we gather and findings we make *in vitro* can be applied to actual tendons to successfully redirect healing outcomes, as per our long-term goal. We could accomplish this by manipulating tendon cell activity or behavior *in vivo*, according to our findings from *in vitro* cell studies, and examine whether tendon healing outcomes are altered and in what way. For example, if our future *in vitro* studies further support our conclusion that increased MMP activity in postnatal tendon cells may contribute to scarred tendon healing, we could perform an *in vivo* study to investigate the effect on applied/injected MMP inhibitors on adult/postnatal tendon healing and determine if such an intervention has the ability to improve healing outcomes. Such studies would represent the first direct testing of strategies to functionally improve scarred tendon healing outcomes, bringing the project closer to the ultimate goal of developing effective treatments to improve clinical outcomes for acute adult tendon injuries.

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