Exploring Mechanical and Biochemical Regulators of the Collagen Crosslinking Enzyme, Lysyl Oxidase, in Tenogenically Differentiating Stem Cells

A Thesis
Presented in Partial Fulfillment of the Requirements for the
Degree of Master of Science
with a
Major in Biological Engineering
in the
College of Graduate Studies
University of Idaho
by

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August 2021

Authorization to Submit Thesis

This thesis of Allison J. Ellingson, submitted for the degree of Master of Science with a Major in Biological Engineering and titled "Exploring Mechanical and Biochemical Regulators of the Collagen Crosslinking Enzyme, Lysyl Oxidase, in Tenogenically Differentiating Stem Cells," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Collagen crosslinking impacts normal tendon mechanics. This thesis first reviews the major types of collagen crosslinks found in tendon (enzymatic crosslinking and non-enzymatic), and their contributions to tendon mechanical properties, as well as their mechanisms of formation. Enzymatic crosslinking that occurs during normal development is mediated by the enzyme lysyl oxidase (LOX), which plays a role in collagen matrix stiffening and strain attenuation in tendon mechanical loading. However, the cellular mechanisms regulating LOX production remain largely unknown. Several possible regulators of LOX have been identified, and experiments were conducted to, explore transforming growth factor (TGF)β2, hypoxia-inducible factor (HIF)-1α, and mechanical stimuli through applied shear stress as potential LOX regulators in mesenchymal stem cells (MSCs). TGFβ2 significantly upregulated both LOX and HIF-1α in MSCs, suggesting that, in addition to inducing tenogenesis, TGFβ2 may play a role in regulation of enzymatic crosslinking. Although hypoxic environments have been shown to upregulate LOX, HIF-1α, a transcription factor that occurs in hypoxia, does not have any significant effects on LOX, although it is upregulated in early TGFβ2-induced tenogenesis. Shear stress applied at 25 mPa did not impact LOX production, although it may be explained by magnitude-dependent regulation. A different level of mechanical stimuli, perhaps one that imitates loading experienced physiologically, could induce LOX, as seen in previous studies using different levels of stress. Other types of collagen crosslinking that occur include nonenzymatic crosslinking, or advanced glycation endproducts (AGEs) and might also be worth exploring in future studies. Due to the slow turnover of collagen, free glucose reacts to form collagen crosslinks between amino acids in the collagen fibrils. This occurs over time during the aging process, but much more quickly in hyperglycemic environments like those seen in diabetes. AGEs typically contribute to tissue stiffening and can be detrimental to tissue healing. Overall, collagen crosslinking is critical for tendon formation and homeostasis. Our results suggest that TGFβ2 may play a role in regulating enzymatic crosslinking by MSCs, and these findings could be used in future studies to improve tendon mechanical properties and advance regenerative medicine.

Acknowledgements

This work was made possible by the NASA EPSCoR Research Initiation Grant, which funded my tuition and stipend here at the University of Idaho. I would also like to acknowledge the Department of Chemical and Biological Engineering for providing me with the opportunities to better myself as a scientist and open doors in my career. Specifically, Judy Vandergrift who helped me make sure everything was put together correctly, and for being a good friend.

A special thank you to my committee members, Dr. Nathan Schiele, Dr. Deborah Stenkamp, and Dr. Matthew Bernards for their flexibility with scheduling, and all their help in coordinating my defense and improving my manuscript.

I would also like to recognize all the help and support I got from Dr. Nathan Schiele, both with questions in lab and editing my writing, but also in my graduate school applications, GRFP application, and overall helping me become a better scientist. I would also like to think you for your trust that I would be able to complete this degree, despite the time constraints. It would not have been possible without you.

I am also grateful for all the members of Dr. Schiele's lab, especially Nick Pancheri for coauthoring both papers that will be produced from this thesis. I would also like to thank Jett Murray, Alex Cunningham, Annie Carper, Kaitlyn Harvey, and all the new members that we have added this year. Without you, the lab would not have been the fun and welcoming place it is. You will all go on to do great things. A special thanks to Nico Yensen, who quickly became very good at Western blots and was always down to run a million of them at a time. Without your help, there's no doubt I would have had to defend much later.

I am especially grateful to Sophia Theodossiou, who answered every phone call and every question, even though she had zero obligation to. You are a great scientist and an even better mentor, and I am so grateful to have had the pleasure of sharing an office with you, even if it was for a short time. Every piece of advice you have given me has been extremely helpful, and I am lucky to know you as both a person and a scientist.

Dedication

I would not have made it through these tumultuous years without the help and support of my friends and family. I would like to dedicate this to the following people. To Quinn, Genna, Johnny, Taylor, Cameron, and Haley, my COVID pod in Moscow, for keeping me sane, even if it was at the cost of some productivity at times. To my twin sister, Holly, who always makes sure I have my head screwed on straight, even in the most difficult of situations. To my partner, Corey Knapp, who inspired me to take this path in life, who supported me unconditionally, and struggled with me from 350 miles away. And to my parents who have believed and supported me since the first day of my life, even when I didn't believe in myself. I am eternally grateful for my parents who are so amazing.

I could not have done this without any of these people.

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Statement of Contribution

Chapter 2 was co-authored by Nicholas Pancheri, an undergraduate researcher in Dr. Nathan Schiele's lab, and his contributions consisted of the sections covering tendon fibrillogenesis, lysyl oxidase like proteins (LOXL) and their contribution to collagen crosslinking, mechanisms of lysyl oxidase crosslinking formation, and editing. He also participated in some data collection for mechanical loading experiments from chapter 3 and contributed to editing that chapter as well. He is listed as a co-author on these chapters and will be a co-author on the submitted manuscripts resulting from this work. I had the primary responsibility of all data collection, analysis, and interpretation, as well as manuscript drafting and revision, so I am listed as the primary author for every chapter of this dissertation, and I am responsible for all errors.

Chapter 1: Introduction

Tendons are important contributors to normal physiological movement and are responsible for the transfer of force from muscle to bone. However, tendons experience high levels of injury, and most clinical treatment options either do not restore full function of tendon or they result in a high chance of reinjury[1]. Furthering our understanding of the mechanisms regulating tendon formation and tenogenesis[2], the process of stem cell differentiation towards tendon lineage, could contribute to knowledge of how tendons develop and withstand mechanical force. Information on how tendon forms and tenogenesis is regulated could be used to advance regenerative therapies and improve current clinical treatment options.

Tendon is composed mainly of collagen. Crosslinking of the collagen molecules may play an important role in tendon formation and contribute to the functional mechanical properties of tendon[3,4]. When mature tendon is loaded in tension, the collagen fibrils and fibers in tendon are also loaded, but experience relatively less tensile strain, compared to the whole tendon[3]. The sliding that occurs between collagen fibrils and fibers is a large contributor to this strain attenuation and may allow tendons to withstand a lifetime of physiological forces[5,6]. Sliding between collagen fibers is a major contributor to this strain attenuation in tendons[3,7], and the shear stresses experienced by resident tendon cells and crosslinking may play a key role in this[8–10]. A key regulator of collagen crosslinking is lysyl oxidase (LOX). LOX is an enzyme produced by the cells that has been found to impact embryonic tendon development and is typically used as a marker of crosslinking levels[11,12]. Because of the importance of LOX in tendon development and proper mechanical function, understanding and identifying regulators of LOX is imperative to furthering understanding of how tendons form. Two important potential regulators of LOX that have been identified are mechanical stimuli[13,14] and hypoxia[15]. Mechanical loading during development from embryonic movement is specifically known to influence the mechanical properties of tendon[16]. Hypoxia, or a low oxygen environment, may affect LOX production by cells[15], but the exact cellular mechanisms through which it does this are unknown. One possible mechanism is through hypoxia-inducible factor (HIF)-1α, which has been found to be activated by both hypoxic conditions and mechanical loading[15,17].

Tenogenesis through treatment of stem cells with transforming growth factor (TGF)β2, a tenogenesis-inducing agent[2], upregulated tendon marker proteins, such as scleraxis and tenomodulin. TGFβ2 treatment was found to upregulate LOX and LOX-like proteins in non-tendon cells (i.e., a trabecular meshwork cell)[18,19]. Exploring the effect of TGFβ2-induced tenogenesis on tendon crosslinking through LOX will contribute to understanding of mechanisms of tenogenesis.

To address these gaps in knowledge on the regulators of LOX production, *the overall goal of this thesis is to explore how LOX production by mesenchymal stem cells (MSCs) is impacted by tenogenic induction, mechanical stimuli, and HIF-1α (**Fig. 1.1**).*

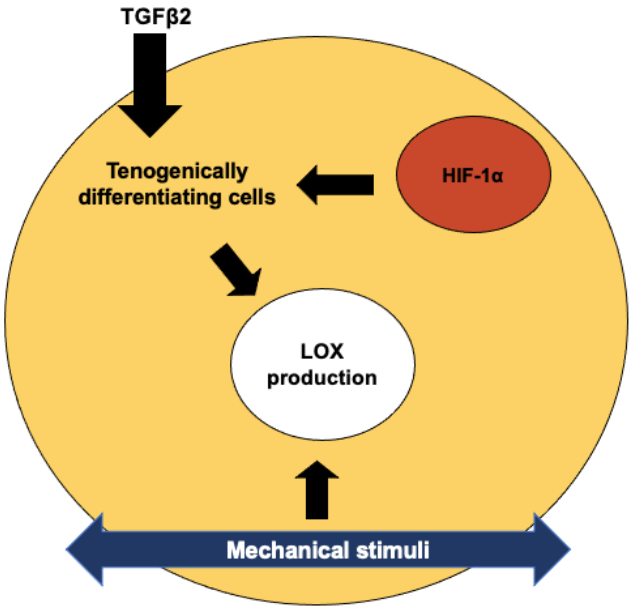

Figure 1. Hypothesis overview

The **central hypothesis** is that LOX production is induced by tenogenically differentiating MSCs and is regulated by mechanical stimuli and HIF-1α activation.

The research aims of this thesis are summarized below:

Aim 1: Test how TGFβ2-induced tenogenesis of MSCs affects LOX production.

Hypothesis: Stem cells undergoing tenogenesis will have increased LOX production.

Since TGFβ2 increases LOX in other cell types[18,19], we proposed that MSCs treated with TGFβ2 to induce tenogenesis would also see an upregulation of LOX levels. To test this, MSCs were treated with TGFβ2 for up to 14 days to induce tenogenesis, and protein levels were measured to determine the impacts on LOX production. *Results will determine if TGFβ2-induced tenogenesis contributes to the production of cellular mediators of collagen crosslinking.*

Aim 2: Determine how HIF-1α impacts LOX production in tenogenically differentiating MSCs.

Hypothesis: HIF-1α activation upregulates LOX production in tenogenically differentiating MSCs.

The cell signaling pathways that are involved in LOX-mediated collagen crosslinking are unknown in tendon cells. Considering hypoxia has been shown to contribute to tissue stiffening through regulation of LOX, HIF-1α is a potential cell signaling candidate[15]. Through loss-of-function studies, HIF-1α was tested as a possible contributor to LOX production. *Results will determine the role HIF-1α plays in the regulation of LOX, an important cellular mediator of collagen crosslinking, as well as in tenogenesis.*

Aim 3: Test how mechanical loading impacts LOX and HIF-1α production in tenogenically differentiating MSCs.

Hypothesis **Test how mechanical loading impacts LOX and HIF-1α production in tenogenically differentiating MSCs.**

Mechanical stimuli contributes to embryonic tendon development[16], and in other cell types has been found to increase levels of both LOX and HIF-1α[13,14,17]. Recent studies have suggested that tendon cells are exposed to shear stress. Therefore, we expect that tenogenically differentiating MSCs exposed to shear stress mechanical stimuli will increase levels of LOX and HIF-1α. Tenogenically differentiating MSCs were exposed to shear stress for up to 14 days, and LOX and HIF-1α levels were compared to static controls. *Results will show how collagen crosslinking mediators are regulated by mechanical stimuli.*

Results of these studies will determine the cellular mechanisms that regulate collagen crosslinking through LOX production. With the completion of these aims, *we enhanced the understanding of how mechanical stimuli, tenogenesis, and cellular factors affect the regulation of LOX.* Ultimately, a better understanding of factors that regulate LOX can contribute to a better understanding of the development of tendon mechanical properties.

Chapter 2: Collagen crosslinking in developing and adult tendons

Abstract

Crosslinking of collagen contributes to the mechanical function of tendon. Enzymatic and non-enzymatic crosslinks can be affected by a number of factors and play a role in the formation of tendon as well as pathology. Lysyl oxidase (LOX), which regulates the formation of enzymatic crosslinks, and advanced glycation endproducts (AGEs), which regulate the formation of non-enzymatic crosslinks, appear at different developmental stages and their accumulation significantly impacts the collagen network and has profound functional implications. Despite the importance of crosslinking, there is limited information regarding how crosslinks are regulated during tendon development. Here, we review recent studies on the role of enzymatic crosslinking in development, potential regulators of LOX and how those play a role in tendon development, and possible mechanisms and impacts of AGE accumulation.

Introduction

Tendon is predominantly composed of collagen, which is hierarchically arranged in fibrils, fibers, and fascicles[20]. The stretching of the collagen fibrils and fibers of tendon, and sliding that occurs between them and the fascicles plays a role in the ability of tendon to transfer forces between muscles and bones, and ultimately contributes to the strength and deformation mechanisms of tendon[3,4,7,21,22]. Collagen crosslinking may be a critical regulator of tendon mechanics and is essential for tendons to withstand physiologic forces over an entire lifetime[5,6]. However, the mechanisms that regulate the formation of collagen crosslinking, as well as the types and extent of crosslinking, are not well understood. As collagen crosslinking impacts tendon development, injury, and aging, an improved understanding of the regulators of crosslinking has the potential to significantly impact strategies focused on tendon tissue engineering, homeostasis, and healing.

The interfibrillar and intrafibrillar crosslinks between collagen molecules are formed by both enzymatic and non-enzymatic processes, which have unique contributions to tendon function. Collagen crosslinking that occurs during embryonic tendon development appears to be mainly regulated by enzymatic processes, such as through lysyl oxidase (LOX)[23,24]. LOX enzymatically catalyzes the reaction of collagen hydroxylysine and lysine residues into reactive aldehyde species which subsequently spontaneously ‘link’ to aldehyde species on adjacent fibers[25,26]. These LOX-mediated crosslinks lead to the formation of trivalent and divalent links between collagen fibers and the formation of a mechanically competent tendon[11,27,28]. Global LOX knockout mice are perinatal lethal due to compromised cardiovascular networks and also exhibit abnormal fiber morphology and organization within the dermis[29–31]. No tendon-specific LOX knockouts have been conducted to our

knowledge. Inhibiting LOX via ligands such as β-aminopropionitrile (BAPN) leads to mechanically inferior tendons. Non-enzymatic crosslinks, such as advanced glycation endproducts (AGEs) and glucosepane, are spontaneously formed through glycation processes and yield divalent crosslinks between neighboring fiber residues[32]. AGE accumulation is associated with aging and diseases, such as diabetes, and may contribute to increased brittleness and limited remodeling ability. Together, enzymatic and non-enzymatic crosslinks play an important role in elaboration of tendon mechanical properties by reinforcing the collagen matrix and possibly facilitating the transfer of mechanical forces throughout the tissue[8].

Impacts of enzymatic and non-enzymatic collagen crosslinks on tendon mechanical properties have been explored in a number of recent reviews[33–36]. Despite the important and complex role crosslinking plays in tendon formation and pathology, it is poorly understood how these crosslinks are regulated. Therefore, this review aims to highlight recent findings on the potential regulators of enzymatic and non-enzymatic collagen crosslinks in tendon as well as discuss the limitations of our knowledge of collagen crosslinking in development and disease of tendon tissue. To further develop novel effective treatments for damaged tendons, an improved understanding of collagen crosslinking, and the mechanisms governing their formation are needed.

Collagen Fibrillogenesis and Role of Enzymatic Crosslinking

Collagen fibrillogenesis

Embryonic tendon is characterized by a transition from a dense cellular network with relatively minimal collagen content to a structure defined by an organized collagen matrix[37–40]. This process of collagen fibrillogenesis has been previously described[41–45] but will be briefly reviewed along with the formation of the two primary forms of enzymatic crosslinks, hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP).

Collagen fibrillogenesis begins with the translation of collagen prepro-α-polypeptide chains, which subsequently undergo hydroxylation of proline and lysine residues. Lysine hydroxylation is mediated by the enzyme lysyl hydroxylase (LH). Consisting of three distinct isoforms, LH1 primarily hydroxylates lysine along the helical domain of the α-chains to form hydroxylysine (Hyl)[33]. Together, glycosylation of Hyl residues, hydroxylation of proline via the complex P4HA/protein disulfide isomerase (PDI) and Prolyl-3-hydroxylase (P3H) isoforms, and formation of disulfide bonds initiates aggregation of α-chains into a triple helix to form a procollagen molecule that is secreted by a collagen producing cell into the extracellular matrix (ECM) via a vacuole. This secreted molecule is

cleaved at the N-terminal and C-terminals by procollagen peptidases to produce mature tropocollagen[33](Figure 2). Remaining telopeptide lysine residues can be hydroxylated through LH isoforms, possibly LH2[33,34]. Notably, LH1 and LH2 (consisting of spliced isoform LH2a and LH2b) are primarily associated with the major form of collagen (types I, II, and III), whereas LH3 is primarily associated with the minor collagen forms (e.g., types IV and V), and therefore may be less relevant to tendon[33,34,46]. LH1 primarily hydroxylates lysines in the helical domain of procollagen, whereas LH2 functions within the telopeptidyl domain[34,47,48]. In a mouse model of development, LH isoforms were found expressed at various levels during embryogenesis and in a tissue dependent manner, expressed more in the heart and muscle tissues and less in other tissues. In mice organogenesis, all LH isoforms were expressed in the mesoderm at E7.5 and had continued expression in later mesoderm-derived tissues. LH1 is highly expressed through embryonic development of mice within collagen type I, II, and III. LH2a is expressed in whole mouse embryos until E11.5, and then localizes to the brain, kidney, and testis[49,50]. LH2b (long splice form) expression begins at E11.5 and is the primary form in mechanically relevant tissues, such as muscle, lung, and connective tissue through adulthood. LH3 appears constitutively expressed, suggesting a housekeeping function.[51]

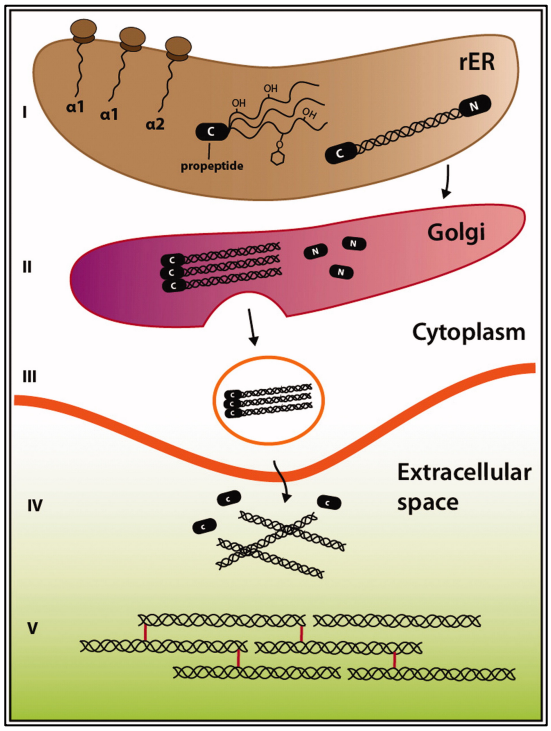

Figure 2. LOX precursors.

Fibrillogenesis begins with production and processing of procollagen-α-chains in the Rough Endoplasmic Reticulum and Golgi apparatus. These molecules are then secreted into the extracellular space for maturation and eventual crosslinking. *Reprinted from Gjatema and Bank 2016.*

Following secretion and hydroxylation of the triple-helical procollagen, LOX catalyzes the formation of hydroxylysine and lysine residues into reactive aldehyde species. In turn, these species spontaneously 'link' to aldehydes on adjacent collagen fibers with covalent bonds to form immature divalent crosslinks[25,26,52]. Immature divalent crosslinks may subsequently link with further residues on another telopeptidyl residue to create a single mature trivalent crosslink[11,24]. The most abundant type of enzyme-mediated crosslinks within adult tendon are trivalent hydroxylysyl pyridinoline (HP or HylPyr or pyridinoline), composed of three hydroxylysines, and to a lesser extent, trivalent lysyl pyridinoline (LP or LysPyr or deoxypyridinoline) crosslinks, composed of two hydroxylysines and one lysine (Figure 3)[53,54].

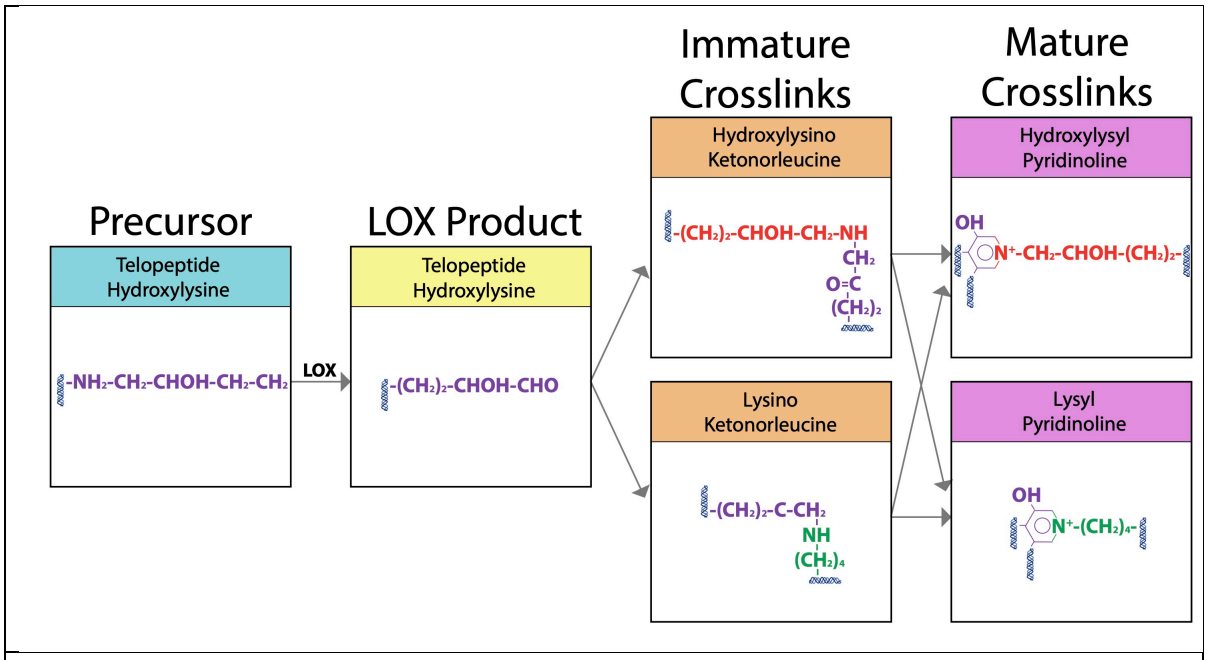

Figure 3. LOX mediated crosslinking

Lysyl oxidase (LOX) mediated formation of immature divalent crosslinks and mature trivalent crosslinks in collagen fibrils in tendons. *Modified from Makris+ 2013.*

A recent study suggested that these LOX-mediated crosslinks are actually too large to diffuse within assembled collagen fibrils, suggesting that these crosslinks are formed at the fibril surface during the collagen fibril development[55]. Differences in HP and LP forms (e.g., hydroxylysine vs lysine) are driven by LH1 and LH2 during fibrillogenesis[56,57]. However, the specific mechanisms governing LH mediated processes are not well understood in tendon or ligament. Other types of enzymatic crosslinking are more or less prevalent among tendons of different function, such as hydroxylysine pyrrole, lysyl pyridinoline, and lysyl pyrrole, but these were recently discussed in detail [36,15,33].

Crosslinks identified in mature tendons

Functionally distinct tendons contain different amounts of mature trivalent and immature divalent crosslinks. Rat Achilles tendons and tail tendons have different quantities of immature and mature crosslinks[4,58]. A possible explanation for these disparate quantities in crosslinking is the functional differences between these tendon types. For example, positional tendons, such as rat tail tendons, are predominantly composed of immature divalent quantities instead of mature trivalent crosslinks[4,58]. Crosslinking and its specific contributions to mechanical properties varies between

tendon type. Flexor tendons and extensor tendons have been shown to have different crosslink types and crosslink densities, with flexor tendons containing more thermally stable crosslinks (i.e., more mature crosslinks) and a greater crosslink density. Despite this, extensor tendons are significantly stronger and tougher than flexor tendons[59]. The role of crosslinking and its contribution to these mechanical properties requires further investigation, but it is proposed that there is a trade-off between strength and fatigue resistance, and crosslinking may play a role in it.

Crosslinking may also be unique between tendons and ligaments. Rabbit anterior cruciate ligament (ACL) has been shown to have a higher total number of enzymatic crosslinks than the medial collateral ligament (MCL) and patellar tendon (PT), as well as increased LOX and LH gene expression, suggesting functionally distinct ligaments and tendons have unique crosslink maturation processes[60,61]. Mechanisms governing these maturation processes are not well characterized.

Equine superficial digital flexor tendons (SDFT)s are commonly used as a functional equivalent to human Achilles tendon[62]. It is also the most commonly injured tendon in horses[63]. Using HPLC, a significant positive correlation between pyrrole crosslinking densities and mechanical properties, such as elastic modulus, ultimate stress, and yield stress has been found in SDFTs[63]. However, the predominant crosslink, HP, does not correlate with any change in mechanical properties. Considering the contribution of advanced glycation endproducts (AGE)s that accumulate over time, collecting SDFTs from horses of an age range of (11.6 ± 7.2) years ignores the difference in LOX-mediated crosslinking and AGEs.

Crosslinks at the tendon-bone interface

Tendon properties and characterization change throughout the substance of the tissue. The tendon-bone interface (enthesis) is particularly pertinent because tendon evulsion from this interface is a primary cause of rotator cuff injury. The enthesis demonstrates a lower modulus than other regions of the tendon, possibly leading to increased deformation and energy absorption prior to failure, creating an overall tougher tissue[64]. Mineral content, collagen orientation, and protein gradients all vary throughout the enthesis[65,66]. Specifically, collagen fibers appear more oriented on the tendon side of the gradient, along with localization of decorin and biglycan. As the tendon becomes ossified (transitions to bone), collagen becomes less oriented and localized aggrecan (a proteoglycan localized in other regions of the tendon that experience high compressive forces) content becomes prominent[66,67]. Several studies have identified an increase of glycosaminoglycans (GAGs) within the enthesis[68,69]. Adult mouse Achilles tendons treated with chondroitinase-ABC, to deplete GAG content, showed a softening in the tendon-bone insertion area, but no change in the

central and proximal thirds of the tendon constructs, emphasizing the varied proteoglycan content and function within tendon.[51] A significant knowledge gap is the heterogeneity of collagen crosslinking and associated mechanisms (including types of crosslinks, LOX, lysyl hydroxylase, etc.), particularly within critical regions like the enthesis. A better understanding of these phenomena is necessary for the development of tissue engineered constructs and regenerative therapies targeted at treating evulsion injuries.

LOX and crosslinks in tendon development

The copper-dependent amine oxidase LOX, coded by the *LOX* gene, is first transcribed as a pre-proprotein. Pre-proLOX immediately undergoes post-translational modifications within the ER and then subsequently experiences N-glycosylation of the N-terminal propeptide chain (147 aa residues) and folding of the C-terminal containing the mature protein (249 aa residues) to form 3 or more disulfide bonds[70,71]. ProLOX is then secreted into the ECM where the glycosylated N-terminal is proteolytically cleaved by the metalloproteinase (MMP) bone morphogenetic protein-1 (BMP-1), also known as procollagen-C-proteinase (PCP), to release the active mature LOX protein and generate a LOX-propeptide (LOX-PP)[72,73]. The mechanisms regulating BMP-1 are not currently known, but considering the role they play in LOX generation, they would be worth investigation. LOX-PP may help govern LOX activity by mediating secretion of proLOX into the ECM, and glycosylation of LOX-PP aids in efficient protein folding[11]. The specific role of LOX-PP in tendon has not been characterized and investigation could enhance understanding of LOX regulation. Notably, LOX incorporates a copper cofactor and organic peptidyl cofactor (lysyltyrosine quinone or LTQ) for proper function[74,75]. As a copper-dependent enzyme, the addition of copper sulfate to cell culture has been shown to increase the total HP crosslinks and tensile properties (which can be further enhanced with additional treatment of exogenous hydroxylysine) in engineered neocartilage constructs through enhanced LOX activity[76].

The LOX superfamily also consists of four additional isoforms, LOXL1-4. A copper-binding domain, LTQ domain, and cytokine receptor-like (CRL) domain on the C-terminal are conserved through all five paralogues[77]. LOXL1, while the most morphologically like LOX, appears to primarily target elastin and tissues undergoing elastogenic events, making it less relevant to tendon collagen crosslinking[78,79]. Whereas LOX and LOXL1 both require proteolytically cleavage for activation, LOXL2-4 are active in both processed and non-processed forms[70,80]. LOXL3 is sensitive to BAPN treatment while LOXL2 is not, however, both isoforms exhibit amine oxidase activity towards collagen and elastin[77,81]. Knockdown of LOXL2 in chondrogenic cell lines blocks further chondrogenesis through increases in SNAIL, a transcriptional repressor, and decreases in SOX9, a

transcription factor and key regulator of chondrogenesis[82]. Application of exogenous LOXL2 to chondrogenic constructs and *in vivo* mouse models promoted tissue maturation and enhanced tensile mechanical properties, however specific mechanisms were not explored[15]. LOXL3 appears to have a critical role in facilitating development of the myotendinous junction and proper formation of the fibronectin matrix within mouse embryos[83]. Increases in LOXL2-4 have been found to correlate with fibrotic conditions in skin and lung, often identified by abnormal material stiffness via excessive ECM and collagen deposition and crosslinking, but need further investigation within tendon[84–86]. Together, the impact of LOX-Like isoforms on tissue development and physiology justifies additional investigation into these topics within tendon tissue engineering, however, subsequent review of enzymatic crosslinkers will primarily attend to the most prevalent isoform, LOX.

Complete suppression of the *LOX* gene results in perinatal murine death[29–31]. To date, selective inactivation of *LOX* gene during adulthood has not been assessed and would be a beneficial model to study wound healing. To our knowledge, no known animal models have been developed that allow for selective activation of the *LOX* gene. As such, current techniques rely on the use of various ligands to inhibit LOX activity. These inhibitors act through two mechanisms: 1. Interaction with the copper-dependent cofactor (e.g., thiram, disulfiram, 2-mercaptopyridine-N-oxide (MCP)) or 2. Interaction with the LTQ prosthetic group at the LOX active site (e.g., BAPN, trans-2phenylcylopropylamine (TCP))[87]. BAPN is the most widespread irreversible inhibitor of LOX in tendon, and while a few recent studies suggest conflicting efficacy of the compound, will be the inhibitor primarily focused upon in this review[87]. Although BAPN is broadly recognized as the field standard, some conflicting evidence may suggest the treatment is not effective against LOXL2[81]. Others point to BAPN as an inhibitor of the entire LOX superfamily due to its affinity for amine oxidases[88,89]. Few studies have focused upon elucidating the specific effect of BAPN on individual LOX isoforms, and more work should be conducted on how these molecules specifically interact within tendon tissue. As such, interpretations of current studies may be limited by a lack of understanding surrounding the specificity of BAPN inhabitation. Conclusions regarding the effects of LOX that have been surmised via experimentation with BAPN do not directly consider the potential activity of LOXL2-4 within tendon.

A number of studies using BAPN in a chick model have shown that LOX is essential during maturation for tendons to develop their characteristic mechanical properties. Embryonic chick has been used as a model to study LOX and BAPN. Previous work has found embryonic chick treated with varying concentrations of BAPN (5 mg/g, 15 mg/g) resulted in a decreased nanoscale elastic modulus from Hamburger-Hamilton (HH) 28 through HH 43 without identified changes in GAG/dry

mass, HP/dry mass, cell viability, or proliferation[23]. A higher concentration of BAPN (15 mg/g) resulted in a greater decrease of elastic modulus than a lower concentration (5 mg/g). BAPN has also been found to decrease the ratio of HP/collagen and LP/collagen compared to controls but increased the ratio of HP/dry mass[24]. The HP/LP ratio remained constant, suggesting BAPN acts without substrate preference towards HP or LP crosslinks[24]. Using chick embryo calcaneal tendon as a model, LOX gene expression changes between HH38-45, with peak expression at HH42. LOX activity steadily increased. Interestingly, proLOX activity peaked at HH43, which is when chick embryos exhibit peak motility. Also, each LOX isoform exhibited a distinct gene expression profile. LOXL2 and LOXL4 decreased relative to HH38, and LOXL1 and LOXL3 remained relatively constant, emphasizing each isoform has a distinct role in tendon development[12].

Less work on LOX has utilized human or mammalian tendon models. A 2015 study employed a unique tendon-construct model that incorporated tendon derived fibroblasts in a silk suture and fibrinogen construct. Constructs immediately treated with BAPN (day 0) resulted in a ruptured construct and were untestable. Constructs first cultured for 14d and then treated with BAPN, showed increased collagen type I monomers and dimers (i.e., less crosslinked) compared to controls at 21 days of culture. BAPN treatment resulted in a weaker construct (decreased failure stress, failure strain, and tensile modulus) and abnormal collagen fibrils that resembled Ehlers-Danos collagen phenotypes[12]. Treatment did not appear to affect COL1A1 gene expression, collagen type V, decorin, fibromodulin, or tenascin-X proteins.

Based on these studies, LOX appears to be an important regulator in the formation of tendon mechanical properties. However, more work is needed in mammalian systems to determine specifically how LOX is regulated.

Potential Regulators of Enzymatic Crosslinking

LOX and LH enzymes are necessary for the development of tendon mechanical properties. Therefore, understanding the mechanisms that regulate LOX and LH production and activity are needed to understand how tendon forms. While there is limited information on specific signaling pathways or external factors that contribute to LOX and LH regulation, possibilities include mechanical loading, hormones, hypoxia, and glycoproteins (Table 1).

Mechanical Stimuli:

A study utilizing embryonic chicks explored LOX gene expression in relation to tendon development through a variety of loading and unloading techniques. Chick embryos exhibiting a

hypermotile (i.e., excessive mechanical loading) phenotype (via 4-aminopyridine) had an increased calcaneal tendon elastic modulus relative to controls without changes in the LOX gene expression profile. Embryos treated with rigid and flaccid paralysis (unloading) phenotypes (via decamethonium bromide, DMB, and/or pancuronium, PB) had a decreased calcaneal tendon elastic modulus that corresponded to decreases in LOX activity. Interestingly, a hypermotility plus paralysis phenotype did not significantly alter the elastic modulus[16]. Further research into frequency and type of movement and mechanical loading is necessary to better understand the role of motility in LOX regulation.

Human periodontal ligament (hPDL) cells embedded within a collagen gel have been shown to respond uniquely to different levels of mechanical loading. Low tensile strain magnitudes (3%) upregulated the gene expression of collagen type I (COL I), collagen type III (COLIII), LOX, LOX activity, and the production of secreted collagen. In contrast, high tensile strain magnitudes (10%) did not affect LOX, but downregulated COL1, upregulated COL3 expression and matrix metalloproteinases (MMP)2 and tissue inhibitors of MMPs (TIMP)2 expression. One potential explanation of this phenomena is that low level mechanical stimulation facilitated extracellular matrix (ECM) deposition and stabilization via LOX-mediated crosslinking, whereas high-level stimulation may favor ECM degradation and tissue remodeling[13]. A similar study on hPDL cells applying varying magnitudes of compressive mechanical loading, found LOX and LH2 expression increased at lower loading magnitudes but decreased at high levels. This same study explored *in vivo* effects of loading on rats using an excessive occlusal loading model (i.e., steel wire was added to the left first molar to induce excessive contact between teeth). Excessive loading increased collagen maturation and LOX and LH2 gene expression and excessive loading plus BAPN treatment suppressed the relative increases of LOX and LH2 expression and collagen maturation[14]. This suggests hPDL collagen maturation in an excessive loading model is at least partially driven by mechanosensitive enzymatic crosslinker post-translational activity. Together, these studies on the mechanosensitive behavior of hPDL tissue and cells justify further investigation in tendon tissue, specifically on the impacts of varying level of mechanical load and the correlation between ECM remodeling proteins, like matrix metalloproteinases (MMPs) and LOX.

Further evidence for a mechanosensitive crosslinking driven mechanism comes from the different types of crosslinks identified in positional versus energy-storing tendons. Rat Achilles tendons (an energy-storing tendon) and tail tendons (a positional tendon) have different quantities of immature and mature crosslinks[4,58]. A possible explanation for these disparate quantities in crosslinking is the functional differences between these tendon types and the different loading

magnitudes. Achilles tendons are repeatedly exposed to a greater mechanical load than tail tendons, suggesting LOX and crosslinks are mechanically regulated. Recent studies have begun investigating the role of LOX and its inhibition in mechanical properties and its effect on crosslinking density. However, it is possible that differences observed in tendon types are not related to mechanical loading, but underlying differences in formation due to unknown growth factors, signaling, genetic variation, etc. This should be investigated in future studies.

Hypoxia:

The exact oxygen tension of tendon *in vivo* is not well documented, but the minimal vasculature of tendon suggests it is a relatively hypoxic tissue with O_2 concentrations under 7% (the estimated pO2 of bone marrow)[90]. A hypoxic environment has significantly increased LOX gene expression, Young's modulus, and the concentration of HP crosslinks in bovine articular cartilage explants after culturing for 4 weeks[15](Figure 4). Interestingly, a hypoxic environment combined with BAPN treatment did not affect Young's modulus or HP crosslinks but did increase LOX gene expression (at a level lower than just the hypoxia treatment) relative to controls. This study later applied these treatments to bovine ACL, PCL, patellar tendon, and knee meniscus explants, and the same trends continued, although LOX expression was not assessed[15]. Hypoxic environments have also been shown to increase the self-renewal capacity and tenogenic capability of human-derived supraspinatus tendon cells[91].

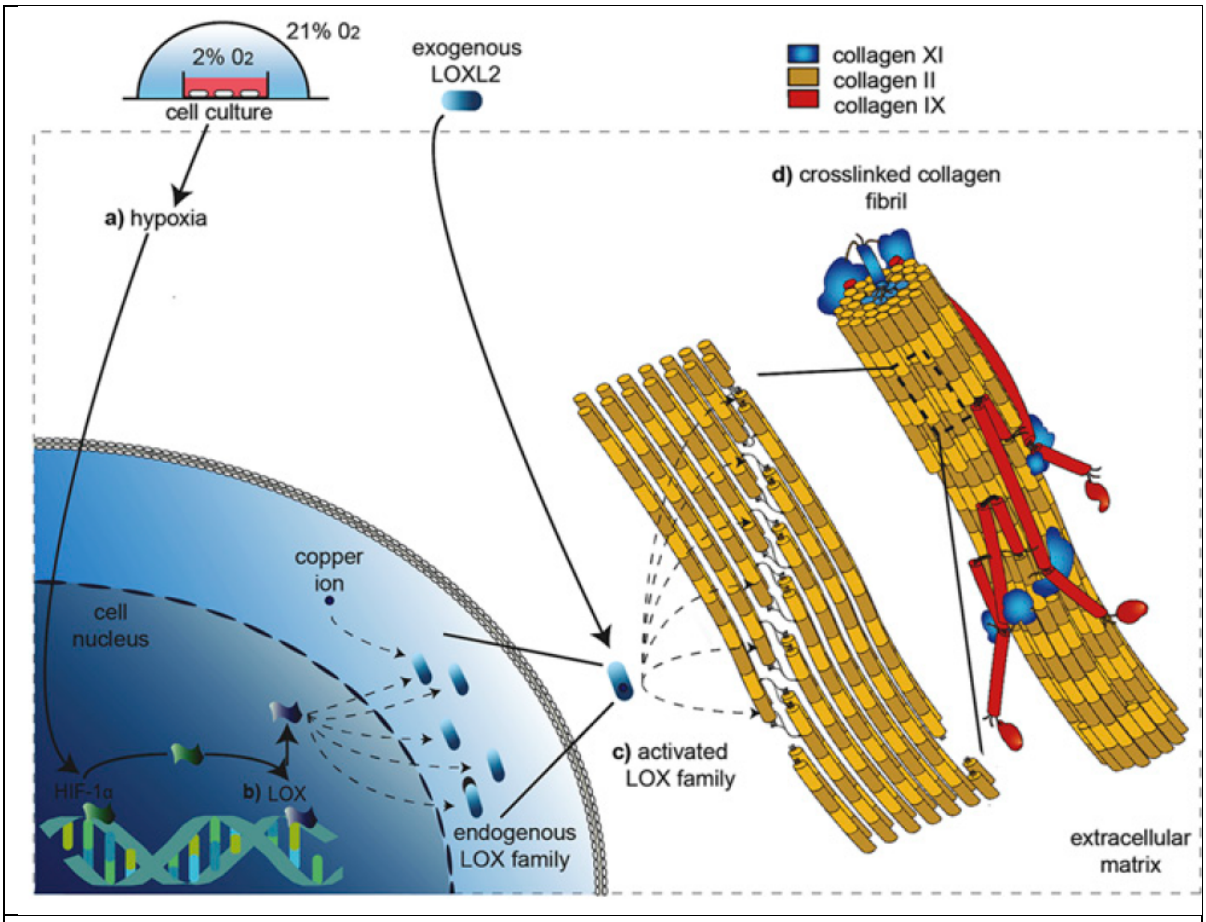

Figure 4.Effect of hypoxia on crosslinking

A potential mechanism through which hypoxic environments may be driving LOX mediated collagen crosslinking. (A) In vitro cell cultures exposed to hypoxia may upregulate LOX production via the HIF-1 pathway. (B) Promotion of LOX and LOXL genes increases activity of respective isoforms (LOX, LOXL1-4). (C) LOX and LOXL isoforms drive collagen maturation by catalyzing the formation of intermolecular bonds within and between collagen fibers to form HP and LP crosslinks. (D) Fibrillar collagen crosslinks catalyzed by LOX enhance the mechanical competency of the tissue. *Figure reprinted from Makris et. al. 2014.*

Treatment of rat lung fibroblasts with Cobalt chloride (a hypoxia mimic) induced activation of the LOX gene through the hypoxia inducible factor (HIF)-1 pathway, specifically, via the hypoxia-response element located in the promoter region of the LOX gene[92]. Treatments combining Cobalt and Cadmium chloride (an inducer of reactive oxygen species) inhibited HIF-1 expression and binding with the LOX gene[92]. A study in dermal fibroblasts found that hypoxia promoted collagen

deposition via HIF-1α and TGF-β/Smad signaling, and a HIF-1α knockdown inhibited TGF-β/Smad signaling[93]. Further work found that silencing Smad4 decreased HIF-1α significantly when combined with hypoxia[93]. Furthermore, multiple studies have identified LH2 expression and activity to increase under hypoxic conditions[94–97]. Mouse embryonic fibroblasts cultured in a hypoxic environment resulted in a time-dependent five- to 12-fold up-regulation of transcription of the PLOD1 and PLOD2 genes that code for LHs, but embryonic fibroblasts lacking the HIF-1α subunit were not affected[97].

While LOX appears to be impacted by hypoxia, the specific cellular pathways are poorly understood, particularly in tendon. Further investigation into this topic has potential to significantly impact our understanding of the mechanisms governing tendon crosslinking and has merit in the field of tissue engineering.

Glycoprotein Interactions:

The KGHR Sequence is a highly conserved amino acid binding sequence unique to fibrillar collagen and is involved in collagen crosslinking[98,99]. Currently, Thrombospondin-1 (TSP-1) and Fibromodulin are the best understood ECM proteins that specifically interact with the KGHR binding motif in collagen. Thbs1 (the gene that codes for TSP-1)-null mice, have been shown to experience altered crosslinking in the dermis, less abundant levels of proLOX and mature LOX, and an increased ratio of mature LOX to proLOX. Interestingly, this same study found TSP-1 inhibits maturation of proLOX via interactions with BMP-1 in *in vitro* COL I-III, and that TSP-1 binds to both intracellular and extracellular collagen through the KGHR binding motif[100,101]. TSP-1 is a particularly intriguing indirect regulator of enzymatic crosslinking because it regulates fibrillogenesis at the intracellular procollagen level, extracellular fibrillar collagen level, and through regulation of LOX maturation.

Fibromodulin deficient mice (Fmod-null) have altered tendons (mechanically weaker and morphologically altered) that exhibit increased LOX induced mature crosslinks within COL I C-telopeptide domains, but LOX itself did not appear to have been affected through post-translational modifications[102]. Fibromodulin, a small leucine-rich proteoglycan (SLRP), appears to alter crosslinking behavior through interactions with LOX at the surface molecules of growing fibrils that do not directly affect LOX quantity or processing, emphasizing the importance of not only stability and quantity of crosslinks, but also the location on the collagen fibril[101,102].

Together, these studies on TSP-1 and Fibromodulin highlight the importance of further investigation into the interactions between glycoproteins that bind to the surface of collagen fibrils (e.g., the KGHR motif) and collagen crosslinking. The dual binding of these exemplars to fibrillar

surface domains demonstrates that LOX mediated crosslinking is modulated through a variety of interactions that are not well understood.

One study on Cyclophilin-B (CypB), an ER-resident chaperone, null mice, used as a model to study recessive osteogenesis imperfecta, showed tail tendons exhibited poorly organized and abnormal morphology. CyB contributes, but does not appear to be essential to the P3H complex that regulates lysyl hydroxylase isoforms and precollagen crosslinking[103]. Inhibiting CyB in tendon, appears to impair fibrillogenesis and collagen morphology by altering Lys hydroxylation of collagen molecules, potentially through modulation of LH1-3 selectivity and activity, alteration of the P3H complex, or modification of collagen-SLRP interactions[102,104]. The role of chaperone proteins on regulation of enzymatic crosslinking needs to be further explored in tendon tissue.

Estrogen:

Women may be at a reduced risk of Achilles tendon rupture compared to men, suggesting an unknown protective mechanism in female tendons[105]. A previous study identified female rat Achilles tendons had increased material properties (linear and dynamic moduli) and decreased viscoelastic properties (hysteresis, percent relaxation) and failure load, suggesting female tendons may have greater resistance to deformation under load and more efficient energy transfer. Comparing the male and female tendons, there appeared no differences in tendon organization, cell shape, cellularity, proteoglycan content, or muscle fiber type, indicating an unknown mechanism driving these disparities[106]. Ligament models using fibroblasts derived from human ACLs and treated with peak physiologically relevant estrogen levels (i.e., 3-4 days before female ovulation) showed reduced mechanical properties (ultimate tensile stress, modulus) and LOX activity, but no changes in collagen content. Interestingly, LOX activity was more severely inhibited with estrogen treatment than LOX mRNA, suggesting estrogen may affect LOX primarily post-translationally[107]. When comparing the results of these studies, it is important to note that while female Achilles tendons appear more protected from injury than males, female ACLs are at an increased risk of rupture, especially during menstruation[105,108]. Unique sex-related differences between ligaments (e.g., ACL) and tendons (e.g., Achilles) may be attributed to the different roles of the tissues (ACLs primarily experience shear and compression forces, whereas Achilles tendons experience higher levels of tensile stress). Together, these results suggest LOX may be partially controlled post-translationally by estrogen, and it justifies further investigation into the effects of estrogen on collagen crosslinking (e.g., LOX, lysyl hydroxylase, BMP-1, etc.) within tendon tissue.

Tissue Engineering Applications of LOX:

Manipulation of LOX and associated proteins for tendon tissue engineering applications shows promise and recent interest. A 2020 patent was filed titled, “Enhancing Tissue Mechanical Properties”. This patent proposed to enhance tissue mechanical properties by increasing LOX or LOXL(1-4) activity using techniques including mechanical stimulation to treat injuries and related musculoskeletal conditions[109].

Though currently less investigated in tendon tissue engineering applications, scaffold-free approaches that explore enhancing collagen crosslinking for skin and cartilage are potential strategies for improved tissue formation. A previously established method studied in human-like skin models showed inhibition of LOX and LOXL4 produced antifibrogenic phenotypes in TGF-β1-induced fibrotic constructs[84]. Accordingly, inhibition of LOX and/or associated proteins within tissues that experience post-injury fibrosis (e.g., an excessive amount of collagen often leading to scar formation), like tendon, has the potential to facilitate restoration of pre-injury mechanical function. Similarly, injection of BAPN combined with control exercise has been used to improve fiber alignment and reduce the risk of reinjury of adult horse tendons, potentially through a similar mechanism relating LOX/LOXL4 and antifibrogenic phenotypes[110]. For cartilage application, LOXL2 has been used in combination with TGF-β1 and chondroitinase-ABC to form 3D neocartilage from expanded articular chondrocytes (Figure 5). This novel strategy combining manipulation of enzymatic crosslinkers, glycoproteins, and growth factors appears a promising technique for engineering more physiologically relevant cartilage cellular constructs[111]. This technique has not been applied to tendon tissue.

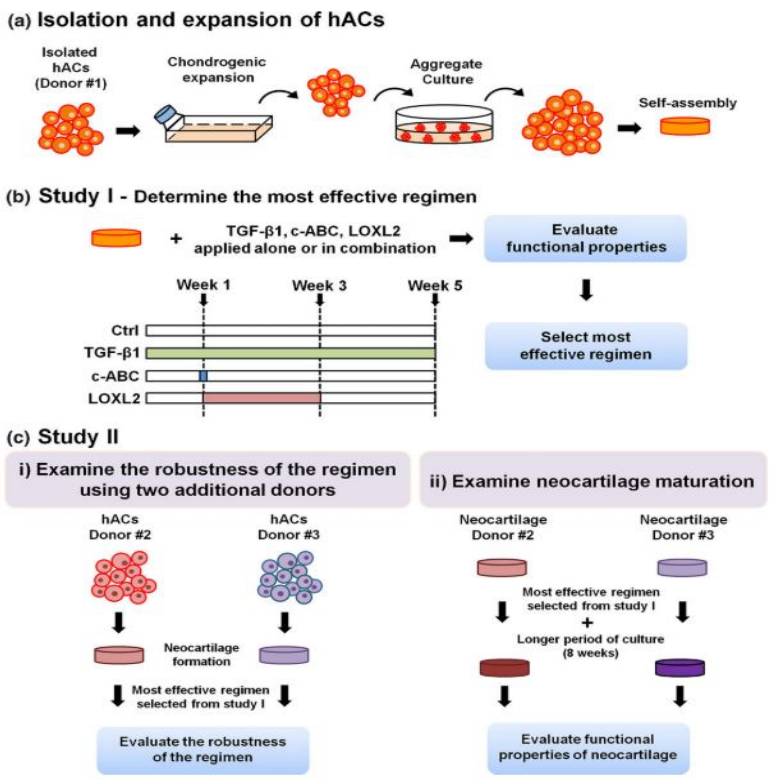

Figure 5. Self-assembly of neocartilage.

Graphical illustration of experiment (a) Human articular chondrocytes (hACs) were isolated and experienced expansion and aggregation prior to self-assembly and formation of neocartilage. (b) Neocartilage treated with either transforming growth factor-β1 (TGF-β1), chondroitinase-ABC (c-ABC), lysyl oxidase-like 2 (LOXL2), or a combination of the three. Samples were then evaluated to assess functional properties. (c) The TGF-β1 + c-ABC + LOXL2 treatment regimen was later applied to additional donors samples for further evaluation. *Reprinted from Kwon et. al. 2019*

LOX and associated collagen crosslinks have been used in tissue engineering applications but have yet to be fully explored for tendon applications. Further understanding of the effect of LOX on mechanical properties and LOX regulation will prove beneficial for developing novel tendon tissue engineering techniques.

Table 1. Enzymatic crosslinking in recent studies

Age	Species/Tendon	LOX modification	Evaluation	Outcome	Source
14-21 day incubation	Human fibroblast tendon constructs	BAPN mediated inhibition	Tensile mechanical testing	BAPN had significantly lower failure stress and elastic modulus than controls, as well as a higher collagen solubility due to fewer crosslinks	Herchenahan+ 2015
5-18 days pregestational	Embryonic chick tendon	BAPN mediated inhibition	HPLC, mass spectrometry to determine collagen crosslinking content	Changes in enzymatic crosslink density during embryonic chick tendon development served as a marker for mechanical properties	Marturano+ 2014
4-8 week tissue from calves cultured for 4 weeks	Bovine cartilage explants	Hypoxia-induced LOX and exogenous LOXL4 treatment	Tensile mechanical testing and quantitative biochemistry	Increased tendon crosslinking and improved mechanical properties	Makris+ 2014
20-24 year old ligament samples from young adults	Human ligament constructs	Estrogen, BAPN	Mechanical testing,	Estrogen decreases enzymatic collagen crosslinking and reduces tensile mechanical properties	Lee+ 2015
HH28-HH45 embryonic tendon explants	Chick embryo	Paralysis, hypermotility, BAPN	Mechanical testing, protein assays, photon imaging	Hypermotility increases LOX production and elastic modulus. Paralysis does the opposite.	Pan+ 2018

Non-enzymatic Crosslinking

Unlike enzymatic crosslinking, non-enzymatic crosslinks typically form after adolescence and occur throughout adulthood[112,113]. It is proposed that slow turnover of collagen in tendon allows for non-enzymatic crosslinking to accumulate[9,114] with the most prevalent type of non-enzymatic crosslinking being AGEs (Table 2). AGEs form through Maillard reactions when reducing sugars, such as glucose, react with proteins.[115] The Maillard reaction is the same reaction that causes bread and meat to brown under high heat. Interestingly, tendons that have accumulated a high number of AGEs appear yellow due to this reaction (Figure 6). Glucosepane is an AGE derived from D-glucose that covalently links the amino acids lysine and arginine. Glucosepane is the most commonly found AGE in tendons, occurring 1000 times more frequently than other AGEs.[116] However, pentosidine is a similar AGE and its presence can be used as a quantitative biomarker for AGEs in studies. It is formed from ribose and occurs between lysine and arginine.

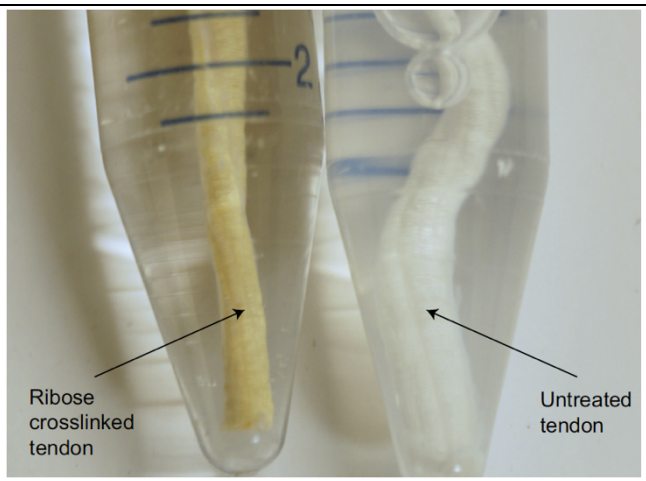

Figure 6. Yellowing tendons from AGEs.

Representative image of a ribose-treated bovine tail tendon compared to an untreated control after 28 days of incubation. The Maillard reaction that occurs between amino acids and reducing sugars is responsible for the yellow color. *Reprinted from Lee et. al. 2018.*

Originally, AGEs were thought to result in spontaneous and non-specific crosslinking between helical regions of the collagen molecules[117] (Figure 7). This differs from enzymatic crosslinks, which form at the telopeptides of collagen molecules[118]. However, a recent study has shown that AGE-mediated crosslinks occur on the same helical domain lysine sites as enzymatic crosslinking[119], but remain partially formed and unstable in the presence of free glucose[119,114] (Figure

8). While this study found evidence that the majority of AGE crosslinking occurs on the same active sites that LOX-mediated crosslinking occurs, it does not completely rule out nonspecific AGE crosslinking that have been the recent precedent. The focus only on glycation of Hyl is a major limitation. Understanding the location of glycation of both Hyl and Pyr crosslinks needs continued investigation. Collagen crosslinking content observed from diabetic C57BL/6 mice as they aged over 4 weeks showed that the total level of collagen crosslinking, both AGE and enzymatic, decreased over time[120]. These findings contrast with the predominant hypothesis that accumulation of AGE crosslinks contributes to increased stiffness found in aged and diabetic tendons and ligaments. However, this same study demonstrated lysine glycation to potentially contribute to tendon stiffening as well. Further studies and revised techniques to better determine levels of crosslinking are necessary to elucidate the role of lysine glycation and accumulation of AGEs in aged and diabetic tendons.

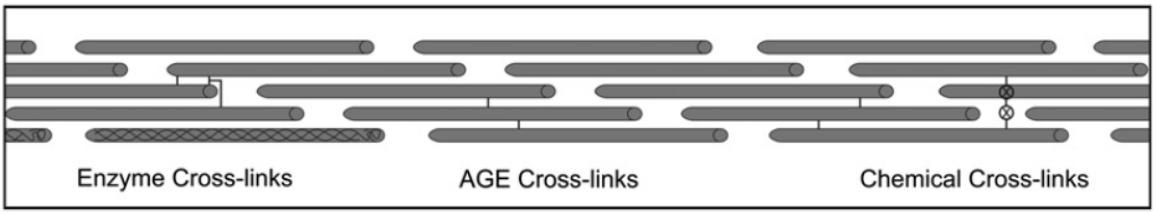

Figure 7. Crosslinking between helixes in AGEs.

Lysyl oxidase (LOX) mediated crosslinks connect the telopeptidyl domain of a collagen molecule to the helical region of neighboring molecules. Glycation end product (AGEs) crosslinks occur between the helical regions of adjacent collagen molecules. on the other hand, form between the helical regions of the collagen molecules. Collagen molecules may also have other chemical crosslinks within and around them that may form an array of polymers (denoted by an "x"). *Reprinted from Fessel et. al. 2012.*

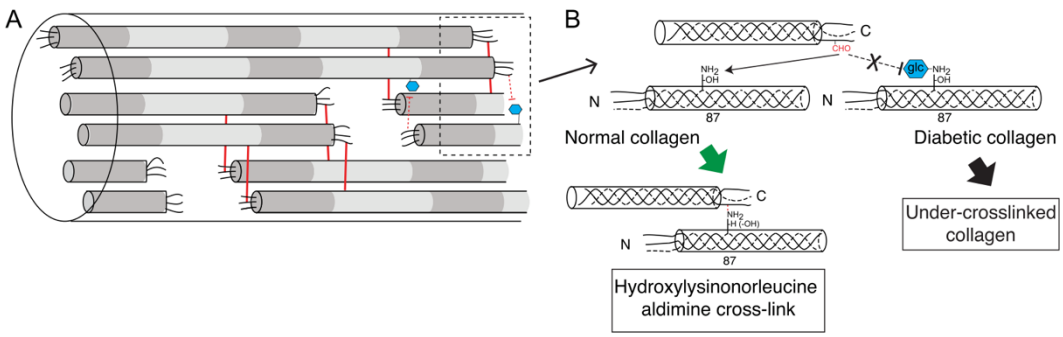

Figure 8. Under-crosslinked collagen in AGEs.

Graphical illustration of the collagen crosslinking profile of a glycated tendon. A) Collagen fibrils are spatially organized to favor intermolecular crosslinking. B) In healthy murine tendon, aldimine crosslinks are formed between the helical domain hydroxylysine and telopeptidyl lysine aldehydes within type I collagen. However, diabetic collagen is partially glycated on the helical domain hydroxylysine, hindering its involvement in normal collagen crosslinking. This may potentially result in inferior material properties relative to healthy tissue. *Reprinted from Hudson et. al. 2018.*

AGE accumulation due to aging

AGEs increase as a function of age and may play a role in the changes of tendon mechanical properties that occur with aging. One study explored old (ages 67 ± 3 years) and young (ages 27 ± 2 years) human patellar tendons mechanical properties[61]. Biochemical analysis identified aged tendons had less total collagen but had higher levels of HP and LP enzymatic crosslinks and pentosidine (as a representative AGE), compared to young tendons (Figure 9). The increased crosslinking seen in the older tendons corresponded to a mechanically weaker tendon, specifically a decreased maximum force response (5161 ± 737 N (old) versus 7415 ± 2184 N (young)). Strain, cross sectional area, and common force responses (i.e. 50% of max. force) were constant between the two groups[121]. The increased crosslinking of the aged tendon can likely be explained by the low turnover rate, allowing both enzymatic and non-enzymatic crosslinks to accumulate. This suggests that differences in mechanical properties of tendons seen over aging may be partially explained by the accumulation of AGEs, but collagen content indicates that it is not the only factor in these changes. This study was the first to compare AGE content to human patellar tendon mechanical properties.

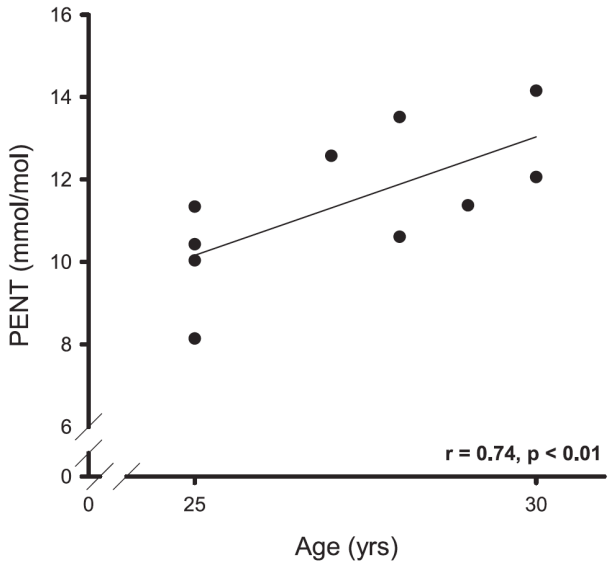

Figure 9. Pentosidine positively correlated with age in maturing men.

Reprinted from Couppe et. al. 2009.

Another study used equine superficial digital flexor tendons (SDFTs) that were creep tested to failure as well as fatigue loaded. Aged (17-20 years) SDFTs demonstrate reduced ability to withstand cyclic loading in fatigue testing (4062 ± 927 (old) versus 16825 ± 6104 cycles (young)), without differences in average fascicle diameter or stress compared to young (3-6 years) SDFTs[112]. Interestingly, at strains above 2%, fatigue loading resulted in a significant decrease in helix-related rotation for tendons from younger horses, but not increased fiber sliding. In tendons from older horses, fiber sliding was increased at strains above 2% in fatigue-loaded tendons, but no change in helix-related rotation, suggesting older tendons are less capable of helix-related rotation (Figure 10)[112]. The fiber sliding behavior observed in old tendons before fatigue loading is similar to the behavior observed in young tendons after fatigue loading. This suggests the helical twist in the tendon collagen structure is compromised in older tendons and is unable to rotate to account for the fatigue loading. Although the exact role of AGEs is not investigated in this paper, AGEs accumulate in older tissues,[121] and are therefore a possible contributor to the change in mechanical properties of older tendons observed here.

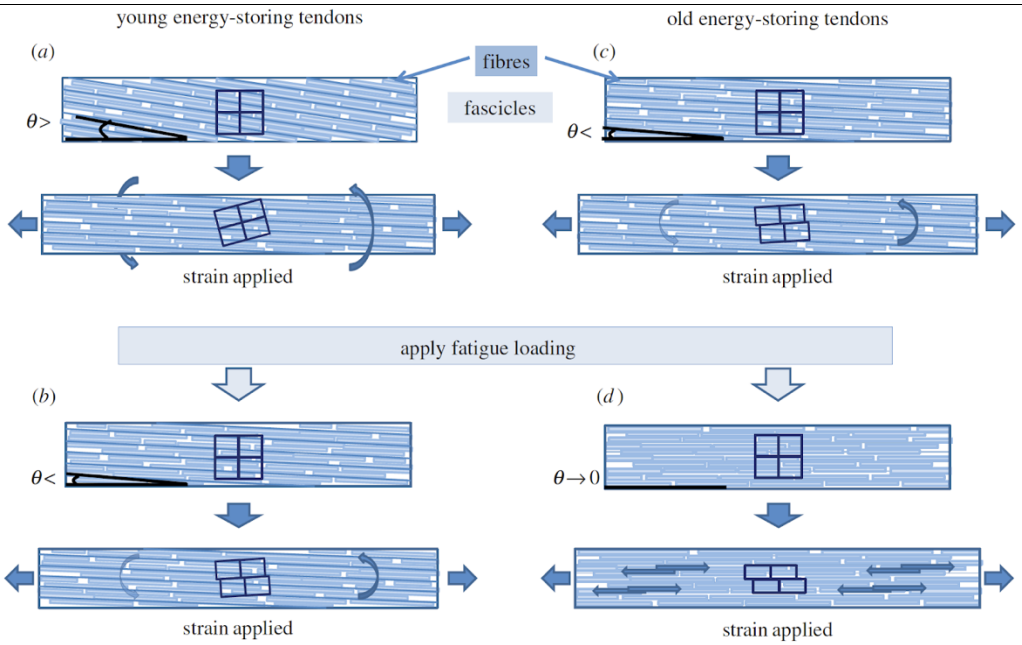

Figure 10. Helix-related rotation.

Graphical illustration demonstrating the effects of fatigue loading combined with aging on the helical twist of collagen. A) Extension of superficial digital flexor tendon (SDFT) fascicles in young horses occurs as a result of helical unwinding instead of simple rotation. B) Fatigue loading of SDFTs leads to reduced capacity for helical rotation. (C) Aged SDFT fascicles demonstrate a reduced ability to respond to loading through a compromised ability to helically unwind (a response similar to young SDFTS after FL). (D) Accordingly, aged tendon fascicles poorly respond to mechanical loads and fatigue loading leads to increased fiber sliding. *Reprinted from Thorpe et. al. 2014.*

AGE accumulation due to diet and diabetes

Due to the slow turnover rate of collagen molecules, hyperglycemia in diabetic patients results in excess free glucose and may induce AGE accumulation at significantly higher rates than non-diabetic counterparts[113,122]. Achilles tendons of diabetic patients showed signs of advanced aging, such as increased stiffness when compared to their healthy equivalent-age counterparts[121]. In a study on diabetic-induced mice, collagen solubility decreased relative to non-diabetic mice, suggesting that AGE accumulation may limit collagen remodeling and leave the tendon more susceptible to microdamage accumulation, although this was partially mitigated by a green tea treatment[123]. Specifically, a green tea extract decreased Ehlrlich-positive material, a marker of pyrrolic

crosslinking, in diabetic mice, suggesting mitigation of damage induced by hyperglycemic exposure is possible.

Diabetes-related AGE accumulation also corresponds to alteration in the intermolecular spacing of collagen fibrils in 11-day-glycated rat tail tissues, showing an increase of 0.05 nm and a decrease in D-period length of 0.04 nm[124]. Changes to the molecular structure of collagen fibrils may contribute to the increased stiffness associated with diabetic tendons.

Diet is another factor that may also affect AGE accumulation. When mice were fed either a high-fat low-AGE diet (HFD) or a normal-diet with high AGE content (ND) throughout their lifespan, those fed with the ND showed significantly higher levels of the AGEs carbosymethllysine (CML), methylglyoxal-derived hydroimidazolone and pentosidine in both Achilles and tail tendons[125]. This suggests that diet could be another factor in the rate of AGE accumulation. Further investigating how diet changes, such as switching from a high-AGE to a low AGE-diet, could alter the accumulation of AGEs, and if this change could protect tendons from further accumulation or even reverse the process.

In vitro evaluation of AGE accumulation on tendon mechanics

A common technique to study the impacts of non-enzymatic crosslinkers on tendon mechanics is to induce AGEs through either ribose or metabolite methylglyoxal (MGO) treatments *in vitro*. Natural AGE accumulation from Glucose is a slow process, thus alternative treatments are used to speed up the process, like Ribose or methylglyoxal (MGO) treatment, to create a high-AGE model quickly.

Ribose-glycated rabbit Achilles tendons show increased mechanical properties, including maximum stress, load, strain, as well as Young's modulus, stiffness, and energy absorption[126]. Biochemical analysis demonstrates a decrease in total collagen and an increase in pentosidine (168%) (as a representative AGE). However, study limitations included a small sample size and excessive ribose-induces crosslinks, making direct comparison to naturally occurring AGEs from hyperglycemia or aging difficult. A different application of ribose treatments was used to explore the effect of AGEs on collagen hydration and its role in AGE associated mechanical changes. Ribose-treated fibrils had increased hydration levels and decreased transverse stiffness in two-month old mouse tail tendon[127]. Because tissue hydration can affect its mechanical properties[128], any effects AGE accumulation may have on collagen hydration may also be contributing to the mechanical effects resulting from AGE accumulation. Although this did not significantly affect any common measurements of mechanical properties, it significantly reduced the extensibility of the tendons after

the yield point[122]. Further research into the effect of AGE-induced hydration on collagen fibrils will highlight its possible contribution to the mechanical effects seen from AGE accumulation.

Other methods to induce AGE crosslinking include an *in vitro* methylglyoxal (MGO) treatement[126]. MGO is a dicarbonyl derived as a metabolic by-product of glycolysis, and unlike ribose, does not induce excessive crosslinking. MGO interacts with ECM proteins to rapidly form AGEs, at a quicker rate than physiological glucose reactions. MGO-derived AGEs cause diminished fiber sliding, but not fiber-specific "stiffening" in rat tail tendons, compared to untreated tendons[129].

These findings corroborated a later study also using MGO-derived AGEs in rat tail tendons which investigated the effect of this reduced sliding on collagen fibril damage mechanisms[129]. The mechanical testing run on these MGO-treated rat tail tendons supported the claim that tissue stiffening observed in AGE-heavy tissues must occur in the tendon at a higher architecture than individual fibrils. Small-angle X-ray scattering (SAXS) analysis following mechanical loading also revealed molecular deformation in MGO treated fibrils[129]. Specifically, higher levels of strain reduced collagen fibril capacity for stress relaxation. This explanation for reduced stress relaxation could also be applied to age-related differences in stress relaxation through helix-related rotation found in old tendons (Figure 7). However, these findings contradict a similar study on fiber sliding in tendon using diabetic-induced AGEs in the murine Achilles tendon[123]. More research into the comparability of diabetic- and age-induced AGEs and MGO-induced crosslinking is needed to determine if this is an appropriate way to model physiologically relevant AGEs, specifically looking at mechanically loaded tendons like the Achilles, in addition to the tail tendons discussed in these MGO-studies discussed.

Glutaraldehyde is the most clinically relevant AGE crosslink in collagen. Using glutaraldehyde as a model for high levels of AGEs, a comparison with the native mechanical properties of tendon showed that glutaraldehyde crosslinked tendon fibrils do not show stiffening at low strains (0-15%), and strain softening at high strains (15-25%) of untreated tendon fibrils. Instead, the tissue is significantly stiffer and does not exhibit strain stiffening, regardless of strain[130]. This stiffening of individual collagen fibers after being treated with an AGE-crosslinker is in disagreement with earlier studies using MGO-derived AGE crosslinking[129,129], which showed that stiffening occurs at a higher level in the tissue. This could be due to a difference in crosslinking through MGO versus Glutaraldehyde.

A finite element analysis (FEA) computer model was used to investigate the mechanical effects of glucosepane, the most abundant lysine-arginine derived crosslinking AGE[131]. In the model, glucosepane accumulation results in significant changes to mechanical properties in the low-strain

range (0-15%). The tensile modulus was increased between 2.9 % and 60.3 % in this low-strain range. This increase in modulus may contribute to tendon stiffening that occurs with age when AGEs accumulate[132]. Glucosepane is not the only intra-collagen crosslink that accumulates with age. However, a study using a similar FEA model focusing on arginine-lysine methylglyoxal-, glyoxal-, and 3-deoxyglucosone-derived imidazolium crosslinks (MODIC, GODIC, and DOGDIC) failed to show the same mechanical effects as glucosepane[133]. Understanding exactly where these different AGE crosslinks bind on collagen fibrils would greatly increase the accuracy of these computer models. These results suggest that various non-enzymatic crosslinks can contribute to the effect of AGEs on mechanical properties in dissimilar ways, which is worth investigating in the future[133].

Regulation of AGEs by mechanical loading

AGE accumulation has been reported to increase tendon susceptibility to enzyme degredation[134]. Rat tail tendons treated with ribose for 3 to 7 days to induce AGE crosslinking resisted degradation longer than their untreated counterparts while unloaded, but when mechanically deformed from an applied tensile force (1.3% to 3.4% equilibrium strain), became highly susceptible to enzymatic degredation[134]. This is surprising because mechanical loading[135,136] and AGE crosslinks[137] are both known to have protective effects against enzymatic degradation. It is proposed that a mechanically-induced micro-unfolding is responsible for this susceptibility to enzyme cleavage[134]. Tail tendons harvested from mature mice and fixed with sodium borohydride to induce reversible bonds showed altered crosslinking after exposure to mechanical stretch[138]. Specifically, at 4% strain, there was a noted decrease in glycated lysine residues in collage, indicating that some of these AGEs are stress liabile[138]. As such, mechanical stimulation may affect glycation levels and, therefore AGE crosslinking, and serves as a potential method to modulate AGE accumulation. Further investigation into the mechanisms through which mechanical stretching may mitigate AGEs is necessary.

Overall, non-enzymatic crosslinks, specifically AGEs, are physiologically relevant in the process of aging, diabetes, and other diseases. AGEs form through the relatively slow process of glycation that, when coupled with the slow turnover rate of collagen in tendon, leads to an accumulation of crosslinks during aging that can affect tendon mechanical properties. AGE accumulation appears to reduce fiber-fiber and fibril-fibril sliding, and AGE-linked conditions, like diabetes and aging, can negatively affect fatigue loading, healing capacity, and elastic modulus. Further investigation is needed to determine the specific mechanical effects of AGEs and identify mechanisms that regulate AGE accumulation and mitigation and will benefit the field of tendon tissue engineering

Conclusion

Collagen crosslinking is a major contributor to the mechanical properties of tendon. However, the function and regulation of crosslinking changes as a function of time, injury, and disease. Beginning in the embryo, LOX mediates crosslinking during fibrillogenesis, allowing maturation of mechanically contributing trivalent crosslinks. These crosslinks are essential to the unique mechanical properties of tendon tissue and are imperative to proper function. The mechanisms governing enzymatic crosslinking are diverse and complex and may include hypoxia, estrogen fluctuation, mechanical stimulation, and a number of factors that remain undiscovered.

Crosslinks derived from non-enzymatic interactions resulting from free glucose, known as AGEs, are considered to have an overall negative effect on tendon mechanical properties. AGEs also accumulate during the normal process of aging, but can be accelerated in diabetic conditions, and may also be affected by diet. The specific impact of AGE accumulation is not entirely understood, but it has been shown to alter fibril sliding and fiber sliding. The exact location and process of formation is unclear, but further investigation of AGEs will identify how these crosslinks attach and how they affect fibril and fiber sliding. Evidence suggests that AGEs can be mitigated with certain techniques like green tea or low-AGE diets, but no specific or proven treatments have been approved for clinical use. Furthering our understanding of enzymatic and non-enzymatic crosslinking and associated regulatory mechanisms can contribute to clinical treatments for disease, injury, and age-related tendon tissue changes.

Table 2. Advanced glycation endproducts in recent studies

Age	Species/Tendon	Source of AGEs	Evaluation	Outcome	Source
17-24 weeks	Rat-tail tendon	MGO	Mechanical testing	Altered sliding caused reduced inherent collagen fibril viscoelasticity	Fessel+ 2014
12-16 weeks	Rabbit Achilles	Ribose	Mechanical testing	Increased matrix stiffness, Young's modulus, etc.	Reddy+ 2004
>17 weeks	Rat-tail tendon	MGO	Mechanical testing, collagen associated fluorescence	Decreased fibular sliding, increased stiffness, compensation by collagen fiber stretching	Li+ 2013
3, 12, and 22 months	Rat-tail tendon	MGO	TTBT, enzymatic analysis	Novel MGO-derived amide AGE crosslink identified; age increased AGEs linearly	Jost+ 2018
3-6 years and 17-20 years	Horse SDFT	N/A	Mechanical testing,	Older tissue suffered more matrix damage and increased fiber sliding	Thorpe+ 2014
9-10 weeks	Rat-tail tendon	Diabetes	Collagen solubility and Elrich positive material	A green tea treatment reduced the amount of collagen (AGEs) in diabetic mice, but made no difference in the control group	Babu + 2007
40 weeks	Rat-tail and Achilles tendon	High AGE diet	UPLC/MS-MS, HPLC	Diets high in AGEs formed more AGEs in both tendons than a normal diet	Skovgaard+ 2015

67 ± 3 years 27±2 years	Human patellar tendon	N/A	Mechanical testing, collagen crosslink densities	Old men had 7 times as much pentosidine, and 34% less overall collagen, mechanical properties were not significantly different between young and old men	Couppe+ 2009
58-60 years	Human Achilles Tendon	Diabetes	Mechanical testing, collagen composition	Patients with diabetes had increased Achilles stiffness, but no significant increase in collagen composition	Couppe+ 2016
Adult	Bovine tail tendons	Ribose	Mechanical testing	AGEs formed from ribose had a negative effect on discrete plasticity of collagen fibrils	Lee+ 2019

Chapter 3: Potential regulators of lysyl oxidase in tenogenically differentiating MSCs

Abstract

Lysyl oxidase (LOX) is an enzyme that plays a key role in enzymatic crosslinking between collagen fibrils in tendon. However, how LOX production is regulated by differentiating cells in tendon during formation is largely unknown. This study investigates transforming growth factor (TGF)β2, hypoxia-inducible factor (HIF)-1α, and mechanical stimuli as potential regulators of LOX in mesenchymal stem cells (MSCs). At earlier timepoints (1 day), TGFβ2 treatment not only increased LOX, but also increased HIF-1α. Further timepoints had some significant increases in LOX production, and some trending in both HIF-1α and LOX production. Mechanical stimuli in the form of shear stress was applied via a shaker plate running at 1 Hz. Mechanical stimuli did not appear to impact either LOX or HIF-1α. An siRNA knockout of HIF-1α showed that it had little to no effect on LOX production, even though hypoxia has been shown to upregulate LOX in other tissues. These results show that TGFβ2 significantly upregulated LOX levels in MSCs, while 1 Hz of shear stress mechanical loading and HIF-1α had no effect on LOX production. These findings suggest that TGFβ2 may play a role in matrix stiffening during tenogenesis, that hypoxia may regulate LOX production independently of HIF-1α, and mechanical stimuli may have limited impact on regulating LOX in early tenogenesis.

Introduction

Tendons are responsible for transferring force from muscle to bone. They play an imperative role in normal physiological movement and have unique mechanical properties. They are comprised of a hierarchical structure of collagen fibrils[20], which may slide against each other when the tissue is mechanically loaded[3]. This sliding attenuates the strain throughout the entire tissue by distributing it among individual fibril bundles (fibers). Collagen crosslinking has been found to influence this sliding and may be a potential regulator of tendon mechanical properties[3,7]. The enzyme lysyl oxidase (LOX) is the primary mediator of collagen crosslinking and is often used to assess enzymatic crosslinking levels[24,36,139]. However, the specific mechanisms governing LOX by tendon cells are poorly understood. Investigating and identifying regulators of LOX will further our understanding of tendon formation and provide insight on the development of tendon mechanical properties in tendon.

A full genetic knockout of LOX is lethal in mice[30]. Therefore, β-aminopropionitrile (BAPN), a LOX activity inhibitor, is commonly used to study the role of LOX in various tissues[12,16,24,107].

BAPN-mediated LOX inhibition causes a significant decrease in tendon mechanical properties during embryonic development, suggesting that LOX-mediated crosslinking is imperative for normal mechanical properties of tendon[12,63]. Thus, a thorough understanding of the specific cellular mechanisms that regulate LOX production in tendon development is necessary for tendon tissue engineering applications.

Recent studies have investigated several potential regulators of LOX, including mechanical loading[13,14] and hypoxia[15]. Hypoxia, or low oxygen conditions, can affect LOX production. In a study using bovine cartilage explants, a continuously applied hypoxic environment contributed to a 40-fold increase in LOX gene expression[15]. However, specific cellular mechanisms that occur during hypoxia are not well characterized. A potential transcription factor involved in this phenomenon is hypoxia-inducible factor (HIF)-1α, which is upregulated by hypoxic conditions, and increases under mechanical stimuli in tendon fibroblasts[17]. Mechanical unloading has been shown to affect LOX levels. Specifically, rigid and flaccid paralysis during embryonic chick development has been shown to reduce LOX production in the calcaneal tendon, while excessive loading (hypermotility) corresponded to an increase in LOX production[16].

Tenogenesis, or the differentiation of stem cells toward the tendon lineage, also may be involved in enzymatic crosslinking through regulating LOX production. Transforming growth factor(TGF)β2 is an inducer of tenogenesis[2] and necessary for tendon formation[140,141]. It has been shown to upregulate LOX levels in non-tendon cells, such as trabecular meshwork cells[18,19], but has not yet been explored in tendon or stem cells. Thus, TGFβ2 is a potential regulator of LOX, and therefore a contributor to collagen crosslinking.

The objective of this current study was to investigate the role of mechanical stimuli and HIF-1α on LOX regulation in tenogenically differentiating mesenchymal stem cells (MSCs), as well as the role of TGFβ2, which induces tenogenesis. We hypothesized that tenogenically differentiating MSCs will show increased LOX production, which is regulated by both mechanical stimuli and HIF-1α activation. We demonstrated that tenogenesis increases both LOX and HIF-1α production, that an applied shear stress of 25 mPA as mechanical stimuli on tenogenically differentiating MSCs affects neither LOX nor HIF-1α production, and a knockout of HIF-1α does not affect LOX production in tenogenically differentiating MSCs.

Methods and Materials

Cell Culture and Supplementation

Murine mesenchymal stem cells (MSCs), specifically C3H10T1/2, were cultured in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% Penicillin/Streptomycin). Cells were passaged at 80% confluence and used between the 3rd

and 13th passage. The MSCs were trypsinized, and cells were seeded into each well of a 6 well plate at a density of 5000 cells/cm^2. MSCs were then incubated for 24 h to allow for initial cell attachment. Cells were washed with warmed phosphate buffered saline (PBS) (Gibco, Grand Island, NY), and switched to cell-starving medium (DMEM, 1% FBS, 1% Penicillin/Streptomycin), 24 h or equilibration time was allowed before treatment. After another rinse with warmed PBS, MSCs were treated with 50 ng/mL of recombinant human TGFβ2 (PeproTech, Rocky Hill, NJ) to induce tenogenesis[2], or an equivalent amount of sterile water for the controls, in cell-starving media). Cells were cultured for 1, 3, 7, and 14 days (d) and the media was changed every third day. Experiments were repeated a minimum of 3 times.

Mechanical Loading

To induce shear stress, cells were mechanically loaded using an orbital shaker plate running at 1 Hz (Lab-Line Instruments Inc., Melrose Park, IL). Liquid shear stress was calculated using the following equations:

$$V_s = 2\pi \cdot f \cdot r$$

$$\tau = \frac{\mu 2\pi \cdot f \cdot r}{h_{BL}} = \frac{\mu \cdot V_s}{h_{BL}}$$

Terminal velocity (*Vs*) was calculated assuming dynamic viscosity (μ) of the media is pure water, using the provided radius (*r*) of an individual well in a 6 well culture plate and the frequency of the orbital shaker plate, (*f*). The calculated fluid shear stress (τ) exerted on the bottom of the well plate (assuming no-slip boundary condition) was calculated in Pa using V_s from equation (1), as well as frequency (*f*), radius (*r*), and the experimentally derived maximum height of the fluid boundary layer (h_{BL}). Fluid shear stress (τ) was found to be 0.024 Pa, which was rounded to 25 mPa.

RNAi transfection

HIF-1α was selectively silenced using IDT Technologies TriFECTa RNAi (IDT Technologies, Coralville, IA). From the three different siRNA constructs provided, each was tested at 3 different concentrations of 0.1 nM, 1 nM, and 10 nM. The most effective siRNA construct at knocking down HIF-1α was selected for the siRNA/loading experiment combinations. Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) was used to transfect MSCs plated in 6-well plates at a density of 5000 cells/cm^2. A universal scrambled construct (NC) was used as a control. HIF-1α knockdown was compared to controls treated with cobalt(II) chloride (Sigma-Aldrich, St. Louis, MO), with both the negative control (NC), and HIF-1α knockdown (HIF-1α siRNA).

Western Blot Analysis

Cells were collected using RIPA cell lysis buffer and HALT protease inhibitor (Invitrogen, Carlsbad, CA) for western blot (WB) analysis. A 1:1 ratio of Sodium dodecyl sulfate (SDS) was

added to the sample, the samples were then sonicated three times for 15 seconds each and heated to 95° C for 5 minutes. Samples were loaded into Novex Wedgewell 4-20% Tris Glycine Mini Gels (Invitrogen). Total protein content was normalized due to increased cell proliferation from TGFβ2. 20 μL of control was loaded in each lane, but 5 μL per timepoint was decreased for each TGFβ2 treated sample. For example, 1d was loaded with 20 μL for each treatment, 3 d was loaded with 15 μL per lane, and so on. Each treatment group had two technical replicates, which were run in duplicate and are included in the densitometry data. Gels were transferred to nitrocellulose membranes (Invitrogen) following electrophoresis. They were then blocked in 5% milk in tris buffered saline (Boston Bioproducts, Ashland, MA) with 0.1% Tween20 (TBST) (Acros Organics, Morris Plains, NJ), and incubated overnight on an orbital shaker at 4° C with appropriate primary antibodies in 5% bovine serum albumin (BSA) in TBST. Primary antibodies were purchased for Lysyl Oxidase (LOX), hypoxia-inducible factor (HIF)-1α (Cell Signaling Technologies, Danvers, MA), and β-actin (Abcam, Cambridge MA) for normalization. All antibodies were raised in rabbit. Concentrations were used between 1:1,000 and 1:10,000. Using TBST, blots were washed 3x for 5min and incubated for 1 hour with goat anti-rabbit HRP-linked secondary antibody (Invitrogen) at room temperature. Blots were again washed with TBST and developed using ECL chemiluminescence reagents (Invitrogen), imaged (Syngene, Frederick, MD), and densitometry was performed in ImageJ (NIH, Bethesda, MD), with all intensities normalized to β-actin bands.

Statistical Analysis

After proteins were normalized to their respective β-actin bands, they were all normalized to their respective controls. Technical replicates were averaged for each biological replicate. A one-way ANOVA test was conducted followed by a Tukey's multiple-comparison test (Prism 9, GraphPad, LaJolla, CA). Significance was considered for $p<0.05$ and results are reported as mean ± standard deviation.

Results

TGFβ2-induced tenogenesis increases LOX and HIF-1α production

Morphology changes typically associated with TGFβ2 treatment, such as increased proliferation and elongation, were observed in all timepoints (Fig. 1). TGFβ2 treatment significantly increased levels of LOX and HIF-1α compared to untreated controls. Specifically, one day after treatment, HIF-1α levels showed a 3-fold increase ($p<0.01$), and LOX levels showed an 8-fold increase ($p<0.01$) (Fig. 2A). Levels of HIF-1α and LOX were trending higher ($p<0.2$) in all other time points (Fig.2B-D), and LOX levels were increased over 6-fold in the 7 d treated group ($p<0.05$)(Fig. 2C).

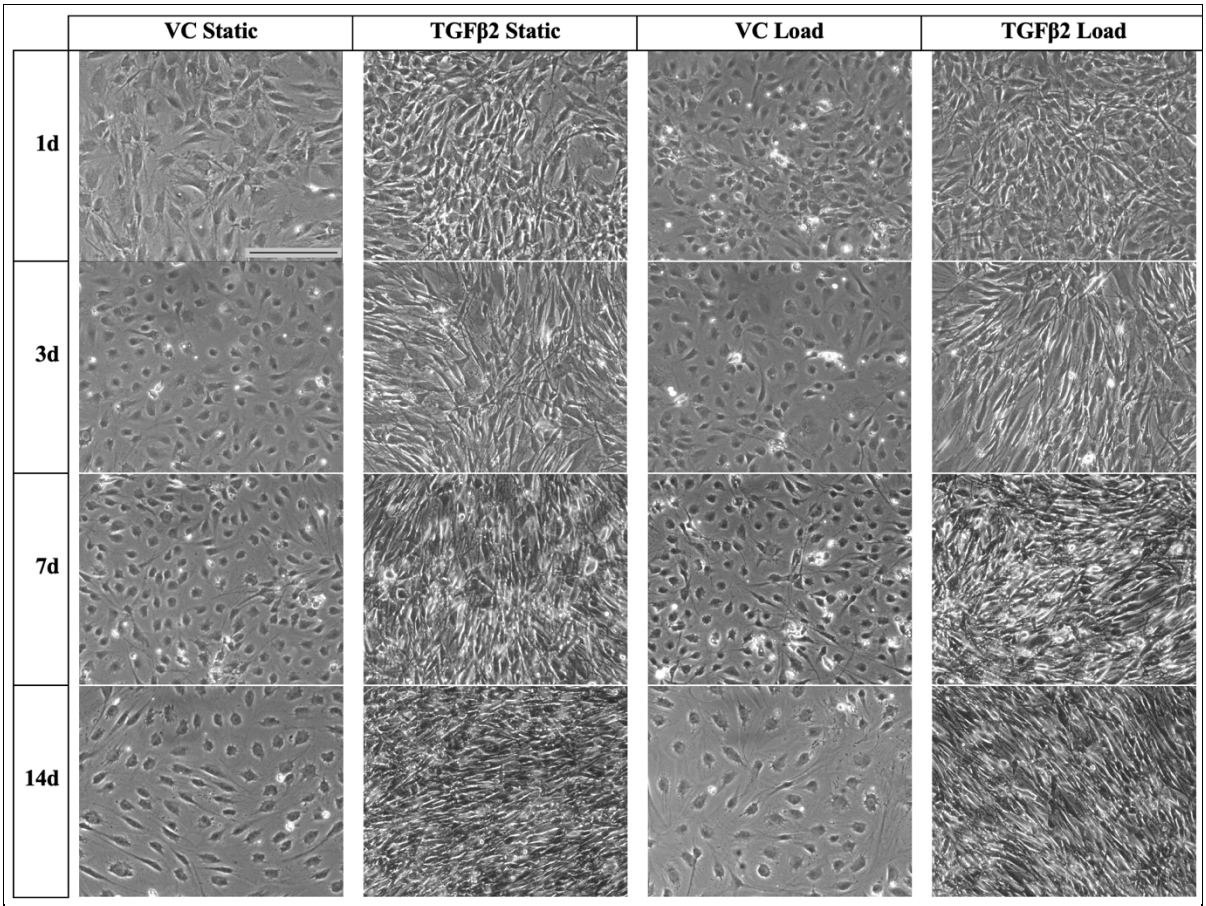

Figure 11. Cell images for loading

Representative cell images (10x) showing morphology for a vehicle control and TGFβ2-treated cells that were either static or loaded over 1, 3, 7, and 14 day timepoints. There are TGFβ2-associated morphology changes, such as increased proliferation and elongation, but no obvious morphology changes from loading. Scale bar = 400 μm

Mechanical stimuli at 25 mPa has no significant effect on LOX or HIF-1α production

There are no noticeable differences in cell morphology when comparing mechanically loaded cells and their static controls (Fig. 1). When comparing the levels of LOX and HIF-1α, there are no significant differences between mechanical stimuli and static controls (Fig. 2).

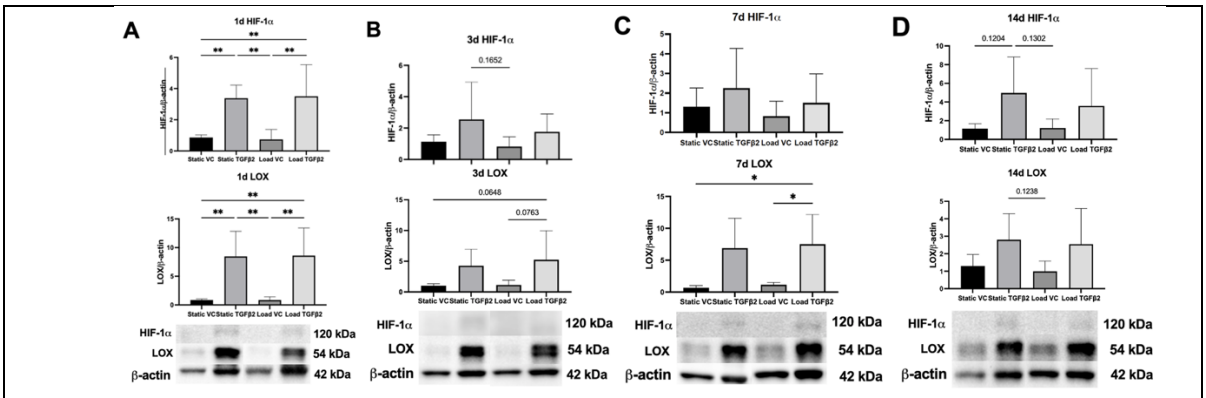

Figure 12. Western Blot data for loading

Quantified band densitometry is shown above representative Western Blot images. Protein names and weights can be found to the left and to the right of their bands, respectively. There were significant ($p<0.01$) increases in HIF-1α and LOX in TGFβ2-treated cells in 1 day timepoints (A), and a significant ($p<0.05$) increase in LOX in TGFβ2-treated cells on day 7 (C). Most other TGFβ2-treated cells were trending ($p<0.2$) upwards for HIF-1α and LOX (B-D), but there were no differences between loading and static treatments.

Loss-of-function experiments reveal that HIF-1α has no significant impact on LOX production

As a proof of concept for the RNAi transfection, MSCs were transfected with a negative control (NC) scrambled peptide, as well as a HIF-1α knockout. Cells were later treated with Cobalt Chloride ($CoCl_2$) II to chemically induce HIF-1α and analyzed to determine HIF-1α content (to validate knockout) (Fig. 3A,C). $CoCl_2$ was not used to induce HIF-1α as a gain-of-function experiment due to cell death resulting from long term exposure (>3 days) (Fig.3B). Cells that were treated with an RNAi transfection to block HIF-1α production, and a negative control, were treated for 3 d with TGFβ2 or water as a control. To ensure the transfection was successful, the same experiment included two technical replicates treated with $CoCl_2$. The effect of $CoCl_2$-induced HIF-1α on LOX production was insignificant, and the siRNA HIF-1α knockout also did not produce any effects (Fig 4).

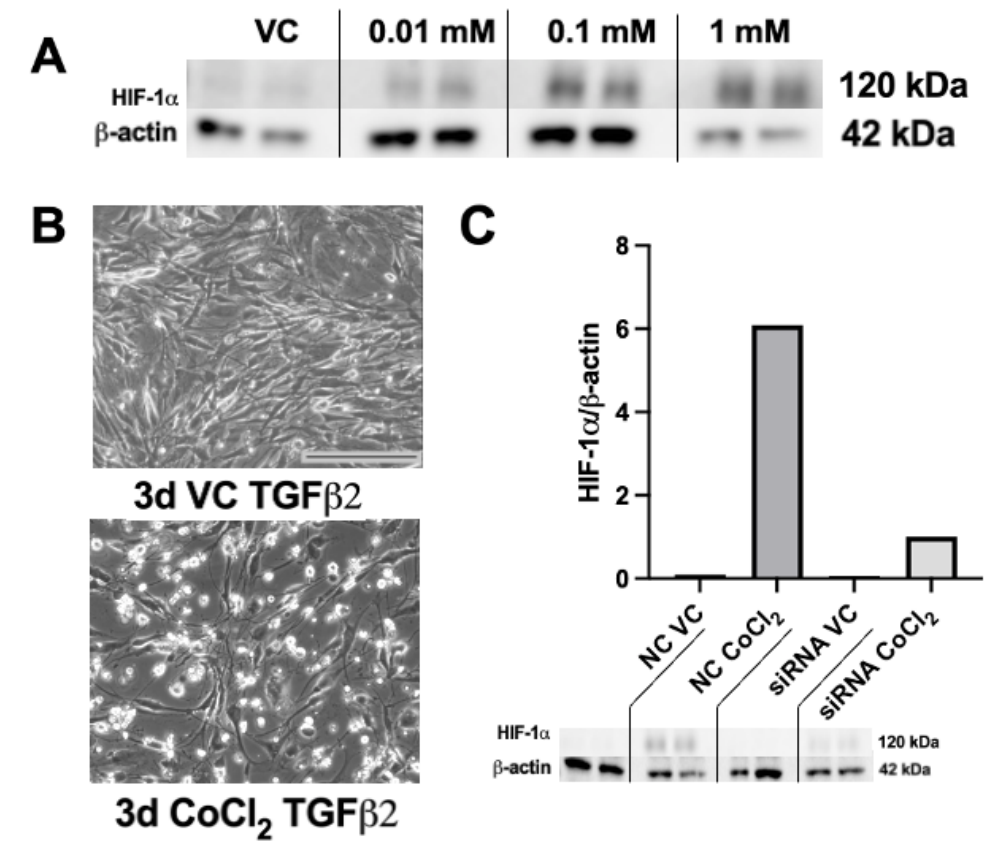

Figure 13. Activation and knockout of HIF-1α.

Different concentrations of Cobalt Chloride ($CoCl_2$) were investigated for HIF-1α activation. A 0.1 mM treatment was proven to be the most effective without causing excessive cell death (A). However, prolonged $CoCl_2$ treatment causes cell death, and shown here are representative images of the resulting decline in cell health. (B). Treatment longer than 3 days caused total cell death. To evaluate HIF-1α knockout, cells were treated with $CoCl_2$ or a control, and transfected with either a scrambled construct (NC), or a HIF-1α knockdown (siRNA). Quantitative band densitometry is shown above its respective representative western blot image to evaluate knockdown of HIF-1α even in the presence of a chemical inducer (C). Scale bar = 400 μm.

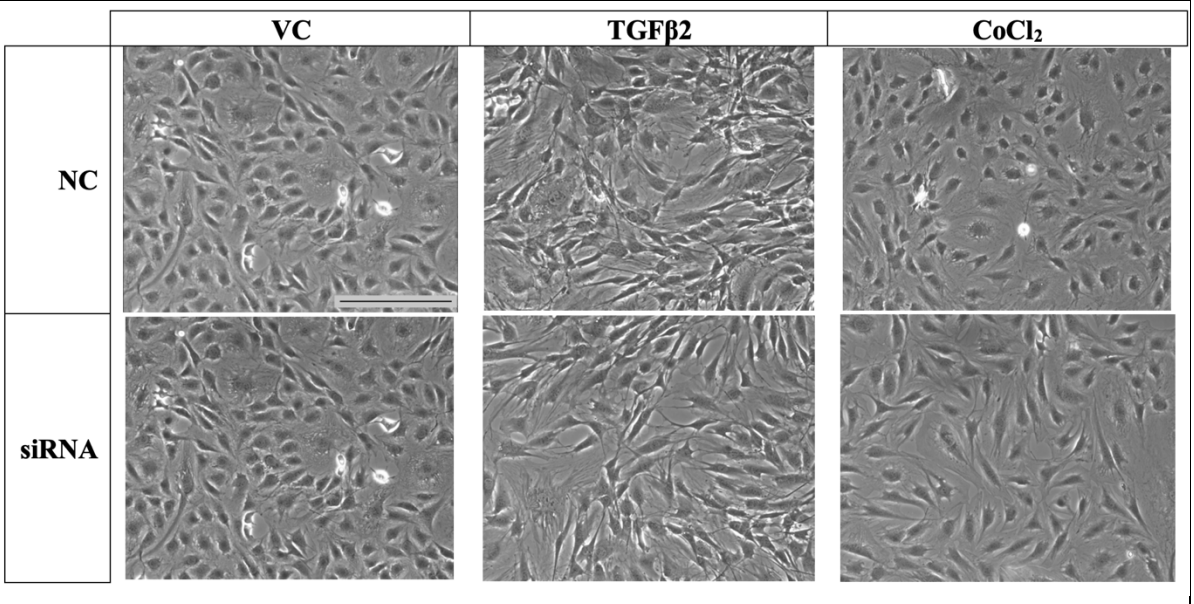

Figure 14. Images of siRNA

Representative images (10x) of 1 d siRNA experiments. The top row was treated with the negative control scrambled peptide (NC), while the bottom row was treated with the HIF-1α siRNA knockout. The first column was treated with sterile water as a control (VC), the second row was treated with TGFβ2 to induce tenogenesis, and the last column was treated with $CoCl_2$ to induce HIF-1α production. There are some morphology changes from early TGFβ2-induced tenogenesis. Scale bar = 400 μm.

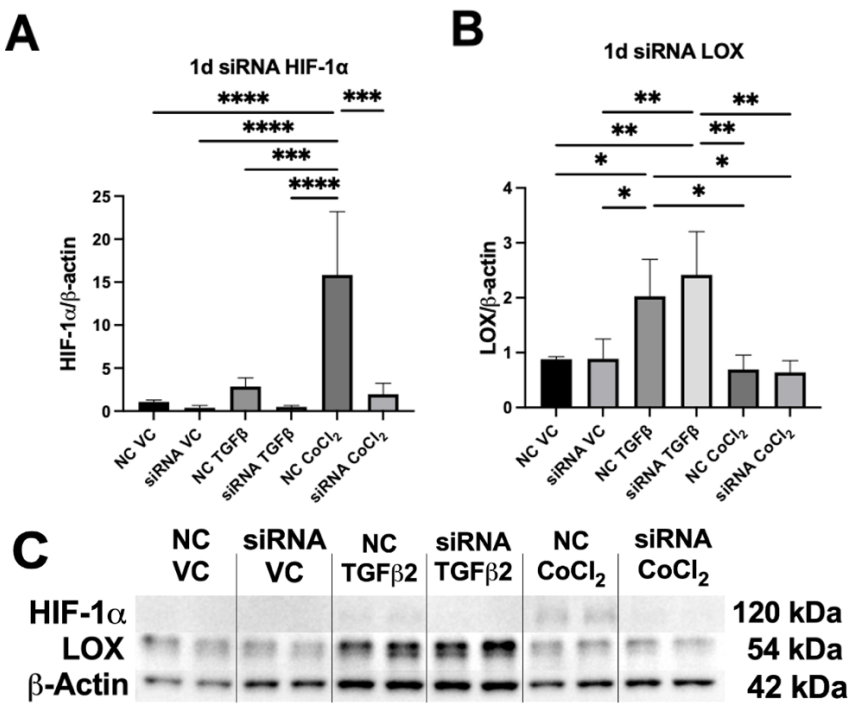

Figure 15. Western Blot data of siRNA

Quantitative densitometry from the 1d siRNA experiments shows that HIF-1α was both properly induced by the chemical inducer, $CoCl_2$, and knocked out in the siRNA treatments. The negative control treated $CoCl_2$ cells had significantly higher ($p<0.001$) HIF-1α than any other treatment, while there was no difference between the siRNA cells treated with $CoCl_2$ (A). There are no significant effects of either HIF-1α induction or HIF-1α loss-of-function on LOX production. However, TGFβ2 still significantly increases LOX production (B). Representative images from the western blots (C).

Discussion

Collagen crosslinking is important for normal tendon mechanical properties, but regulators of the crosslinking enzyme, LOX, are unknown. In this study, we showed that TGFβ2-induced tenogenesis in MSCs significantly upregulates LOX production, as well as HIF-1α production at early time points, and continues to trend upwards in later timepoints. Our results demonstrate that mechanical stimulus (shear stress at 25 mPa) has no detectable impact on LOX activity in MSCs. In loss-of-function experiments, HIF-1α knockdown had no effect on LOX production, but TGFβ2 still significantly increased LOX production.

TGFβ2 and its relationship with LOX production has been studied in other cell types and environments, but not in tendon cells or tenogenically differentiating MSCs. TGFβ1 is a well-known

inducer of LOX and found in scar[142], but all three TGFβ isoforms have been found to induce LOX and all 4 LOX-like proteins (LOXL1-4)[18] in trabecular meshwork (TM) cells, which are cells found in the sponge-like connective tissue found near the front of the eye. However, gremlin, a BMP antagonist that induces trabecular meshwork (TM) matrix proteins, was found to induce LOX and LOXL1-4 specifically through the TGFβ2/SMAD pathway[143], illustrating the importance of the pathway activated by TGFβ2 in LOX regulation. In the development of a chick embryo, the loss of TGFβ2 and TGFβ3 in mouse embryos resulted in the loss of all tendons and ligaments[140]. TGFβ2 has also been found to play an important role in chick embryo tendon development[144], and perhaps the role TGFβ2 plays in LOX production is a contributor to matrix stiffening during tenogenesis. This finding has the potential to contribute to the mechanical properties of engineered tissues, potentially by increasing stiffness in a seeded scaffold by inducing crosslinking through LOX production.

While tendons are exposed to tensile loads, the tendon cells may be exposed to high levels of shear stress due to sliding between collagen fibrils[3,10]. Therefore, in this study, mechanical stimuli was applied *in vitro* via fluid shear stress. In contrast to our hypothesis, mechanical loading through shear stress at a frequency of 1 Hz, which results in an estimated shear stress of 25 mPa, did not significantly impact LOX or HIF-1α levels in MSCs. There are several possible explanations for the lack of changes observed during mechanical loading. Primarily, the shear stress magnitude (25 mPa) may not be appropriate for inducing changes on LOX and HIF-1α. Previous studies exploring the impact of loading on collagen crosslinking have identified magnitude-depending relationships. Specifically, human periodontal ligament (hPDL) cells embedded in a collagen gel showed an upregulation of LOX at lower, more physiologically relevant mechanical stretching (3%), and no effect on LOX during higher mechanical stretching (10%)[145]. Additionally, 3% stretch upregulated collagen type 1 gene expression (COL1A1), collagen type 3 gene expression (COL3A1), and LOX gene expression (*LOX*), whereas higher stretch downregulated COL1A1, but upregulated COL3A1 and MMP2 (an ECM degradation protein)[145]. Similarly, embryonic chicks with induced hypermotility (e.g., excessive loading), do not correspond to increased LOX activity levels[16]. However, paralysis did lead to reduced LOX activity levels. It is also possible that TGFβ2 present in the embryonic tendon could override any loading impacts[144,146], but hypermotility did increase tendon elastic modulus even after LOX inhibition with BAPN. This suggests that mechanical stimuli has the potential to impact functional tendon properties, possibly through an unknown mechanism. Together, these previous studies suggest that higher level (excessive) mechanical loading may promote ECM degradation and remodeling and that low level loading may stimulate ECM production and stabilization, potentially via enzymatic crosslinking mediated by LOX. Since shear stress experienced

by tendon is attenuated to a much smaller stress in individual tendon fibrils[145], perhaps experiments in tenogenically differentiated MSCs should explore lower shear stresses.

A recent study also identified that calcium signaling through the ion channel PIEZO1 in tendon cells may be mechanosensitive and upregulate collagen crosslinking in response to shear stress[10]. Although LOX was not investigated in that study, it would be worthwhile to investigate in the future. It is unknown if tenogenically differentiating MSCs have PIZEO1, but future experiments can show if MSCs are a good model to investigate PIZEO1's relationship with LOX. Further investigation of the role of mechanical loading can contribute to understanding of potential effects of antigravity on tendon development.

Although hypoxia induces higher levels of LOX[15], our results suggest that HIF-1α may not directly influence LOX activity in MSCs. While HIF-1α is a transcription factor activated by hypoxia, other channels affected by hypoxia are likely involved in the signaling cascade. Additional investigations into the impacts of LOX could help identify alternative roles of HIF-1α during tenogenesis. Perhaps a study focusing on the longer-term role of HIF-1α or hypoxia on tenogenically differentiating MSCs could provide further insight.

Limitations of this study include induction and progression of tenogenesis within MSCs. Specifically, the results found in this study could vary in MSCs further on in the tenogenic process (21+ d), fully differentiated tendon cells or tenocytes. As a future direction, using BAPN and other ways to inhibit LOX without impacting cell or organism health could prove beneficial to help understand the role TGFβ2-induced LOX production and its effects on cell morphology.

Also, the mechanical loading experienced by these cells was predominantly fluid shear stress. While tendon cells likely experience some level of fluid shear stress *in* vivo, this loading model may not effectively mimic physiological movement-induced loading (i.e., walking, running, or jumping) that may result in tensile loading of tendon and stretching of tendon cells. This study also only uses a 2D model to study mechanical forces, rather than 3D models, such as a collagen sponge. Investigating mechanical loading within a 3D environment may produce different results.

A final limitation included undesirable cell death following long-term culture with Cobalt II Chloride ($CoCl_2$), making long-term HIF-1α gain-of-function studies inconclusive. Future studies using a hypoxia chamber to create an artificial hypoxic environment would allow longer term studies without sacrificing cell viability. While HIF-1α does not appear to be directly regulating LOX production in this study, hypoxia may still contribute to tenogenesis, and future studies should explore the effect hypoxia has on tenogenic markers, such as scleraxis and tenomodulin, as well as its effect on lysyl oxidase-like isoforms.

Chapter 4: Conclusion and future directions

Whether an average person walking to work, a professional athlete playing a sport, or an astronaut in zero gravity completing a task, tendons are imperative for normal movement. Unfortunately, they are also frequently injured and slow to heal, with high rates of reinjury[1]. Collagen crosslinking has been implicated as a critical regulator of tendon mechanical properties[5,6] and furthering our understanding of crosslinking during development and disease can contribute to ways to improve current clinical treatments and further tissue engineering and regenerative medicine approaches.

Interfibrillar sliding that occurs in tendons during mechanical loading[3] of the tissues is responsible for attenuating strain experienced by the tissue through distributing it among individual fibril bundles (fibers)[3,7]. Collagen crosslinking has been shown to influence this sliding, and therefore is an important contributor to mechanical properties in tendon tissue and may be essential for tendons to withstand physiological forces experienced over an entire lifetime[5,6]. The enzyme lysyl oxidase (LOX) is primarily responsible for this enzymatic crosslinking, and can be used as a marker to determine the level of crosslinking present in the tissue[24,36,139]. However, the specific cellular mechanisms that regulate LOX are poorly understood. In order to understand the complete role crosslinking plays in tendon mechanical properties, the exact formation of enzymatic crosslinking and regulation of LOX must be studied further.

LOX-mediated crosslinking has proven to be a relatively complicated process to study, partly due to the inability to globally knockout the LOX gene without organism death[30]. However, the use of β-aminopropionitrile (BAPN), a LOX activity inhibitor, has allowed investigation into the role of LOX without damaging organism health. The use of BAPN has led to the discovery that LOX is essential for tendons to develop their normal mechanical properties, leading to a decreased elastic modulus[26,114]. Regulation of LOX has been also been difficult to study, and potential regulators that have been studied include mechanical stimuli[117,119,120,147], hypoxia[15,148], estrogen[107], and in this thesis, transforming growth factor (TGF)β2, hypoxia-inducible factor (HIF)-1α, and mechanical stimuli in the form of liquid shear stress. However, other than those studied in this thesis, these regulators have only been explored in non-tendon tissues, such as cartilage, or ligament. This thesis used these findings to investigate potential LOX regulators in tendon using tenogenically differentiating MSCs as a model.

In our study, we found that TGFβ2 significantly upregulated LOX levels in early tenogenic timepoints (1 d) in MSCs and trended upwards in later time points. TGFβ2 also significantly upregulated HIF-1α in early time points (1 d). In other tissue types, like trabecular meshwork (TM) cells, TGFβ isoforms have been shown to induce LOX and LOX-like proteins (LOXL1-4)[57]. Specifically, TGFβ2 has been linked to an upregulation of LOX through a study focusing on the BMP antagonist protein gremlin, which induces TM matrix proteins. This protein has been found to use the TGFβ2/SMAD pathways specifically to induced LOX as well as LOXL1-4[67]. TGFβ2 has also been shown to be detrimental to proper tendon development in chick embryo[144], and in a mouse embryo study, when it was knocked out with TGFβ3[140], it resulted in the loss of most tendons and ligaments. Our findings that LOX levels are directly increased following TGFβ2 treatments could impact the tissue engineering world by providing a way to modulate stiffness in a seeded scaffold, especially for tendon engineering. In the future, studies on how BAPN-mediated LOX inhibition during treatment with TGFβ2 could affect tenogenesis by either visible changes in cell morphology or a change in expression of tenogenic markers, such as tenomodulin or scleraxis.

Although our study found that TGFβ2-induced tenogenesis also increases HIF-1α at early timepoints, we also found that neither HIF-1α loss-of-function nor chemical induction impacted cell morphology or LOX levels. This is surprising since HIF-1α is an transcription factor activated by hypoxia and hypoxic environments have been shown to increase LOX levels[15]. It is likely that hypoxia is affecting LOX production through other signaling pathways involved in the LOX signaling cascade. However, HIF-1α may play an alternative role in tenogenesis, and studies using hypoxia chambers to induce hypoxic environments for longer timepoints may illuminate this role. The chemical inducer of HIF-1α in this study, Cobalt II Chloride ($CoCl_2$), is detrimental to cell health after 3 days of treatment, and therefore unable to be used to study longer timepoints. A hypoxia chamber could address this limitation.

When tendons experience tensile loads, individual tendon cells may experience high levels of shear stress due to the sliding between collagen fibrils[3,10], which is why our study used fluid shear stress *in vitro* as a mechanical stimuli. Using a shaker plate at 1 Hz, we induced shear stress of roughly 25 mPa, but found no significant impact of loading on LOX or HIF-1α production. However, this magnitude of stress may not be appropriate for shear stress found in physiological movement. Human periodontal ligament cells (hPDL) embedded in a collagen gel showed a difference in LOX production at different magnitudes of mechanical stretching. At lower, more physiologically relevant levels of stretching (3%), LOX was found to be increased, as well as the genes for LOX (*LOX*),

collagen type 1 expression (COL1A1), and collagen type 3 expression (COL3A1). At higher stretch rates (10%), LOX was unaffected, but COL1A1 was downregulated and COL3A1 and MMP (a protein that contributes to extracellular matrix degradation) were upregulated[13]. This may suggest that higher levels of loading could encourage ECM degradation rather than production or stabilization. In embryonic chicks, induced hypermotility (excessive loading) did not correspond to a change in LOX levels, although paralysis decreased LOX levels. It is possible any TGFβ2 in the embryonic chick tendon may have overridden the loading impacts[144,146]. Mechanical stimuli can impact LOX production, and therefore tendon mechanical properties, but when attenuation of stresses by tendon is considered, perhaps 25 mPa of shear stress is too high for individual MSCs. A recent study has also explored the role of the PIEZO1, a calcium signaling ion channel, which may be mechanosensitive and upregulate collagen crosslinking[10]. Investigating the role of LOX in relation to PIEZO1 may be worthwhile.

Enzymatic crosslinking is not the only type of crosslinking that impacts tendon mechanics. While LOX-mediated crosslinking occurs in development and contributes to tissue mechanical properties, non-enzymatic crosslinking, or advanced glycation endproducts (AGEs), form over time from free glucose[114,119] in the tissues and have an overall negative impact on tissue mechanical properties[112,113]. This occurs after adolescence and into adulthood, and several factors affect accumulation rate, including diet[125], but most notably, accumulation occurs much more rapidly in those with diabetes[113,122]. While an increase in tendon stiffness and a decrease in maximum force response have been correlated with AGE accumulation[112,121], how exactly these free glucose molecules react to form crosslinks and how these crosslinks affect tendon mechanical properties has been a source of conflict in the field. The original understanding was that AGEs formed between helical regions of collagen molecules, rather than the head-to-tail crosslinking that occurs in enzymatic crosslinking[149]. However, more recent studies have shown that these may occur in similar locations as enzymatic crosslinking occurs, but the crosslinks are inhibited from stabilizing, resulting in physiologically unstable crosslinks that may not be placed appropriately[119]. These inappropriately placed and possibly unstable crosslinks are what have been proposed as the true reason for tissue stiffening. Alternatively, it is important to take into account the collagen degradation that occurs over the course of aging, which still needs to be appropriately accounted considered when studying the role of AGEs on tendon mechanical properties.

Overall, the study of collagen crosslinking continues to expand and fill in the blanks of how and why it can affect tendon mechanical properties. Exciting future work on LOX regulators like

mechanical stimuli, hypoxia, and TGFβ2 will continue to shed light on the process of tendon formation and development. Future studies on LOX-like proteins (LOXL1-4) will contribute to our understanding of the formation of enzymatic collagen crosslinks during development, and investigations into the mechanisms of AGE formation and how exactly AGEs affect tendon mechanical properties will be imperative to understand how crosslinking changes overtime, as tendons age, and how it is affected in diseases, such as diabetes. The more that is discovered about tendon development and formation, the more that can be transferred to clinical applications and may help tendon recovery for athletes, diabetic patients, and perhaps provide insight into the possible effects of microgravity on tendon development and health.

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