HMGB1 INDUCES TENDINOPATHY DEVELOPMENT DUE TO MECHANICAL OVERLOADING

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Tendon is a band of connective tissue that transmits muscular forces to the bone, and as a result, is constantly subjected to large mechanical loads. Over 10 million people suffer from tendon injury and/or chronic tendinopathy in the United States with the cost of treatment exceeding 30 billion dollars each year. While mechanical overloading is considered to be a major risk factor, the molecular mechanism for the development of tendinopathy is largely unknown. The highly conserved nuclear protein, high mobility group box1 (HMGB1), is identified as a potent inflammatory mediator when released to the extracellular matrix from inflammatory or stromal cells in response to stimulation with inflammatory agents or mechanical stress.

Therefore, in this study we hypothesized that HMGB1is responsible for tendinopathy development due to mechanical overloading placed on the tendon. To test the hypothesis, we performed *in vitro* and *in vivo* studies. We found that HMGB1 in tendon cells translocates from nucleus to extracellular matrix when challenged with mechanical overloading *in vitro* and *in vivo*. When implanted in rat patellar tendons *in vivo*, HMGB1 causes cellular and structural changes that mimic the features of tendinopathy. Treatment with glycyrrhizin (GL), a specific inhibitor of HMGB1, blocks HMGB1-induced inflammatory reaction *in vitro* and mechanical overloading-induced tendon inflammation *in vivo*. GL treatment also largely reduces degenerative changes in Achilles tendons of mice subjected to chronic mechanical overloading through treadmill running.

Thus, our study shows that extracellular HMGB1 is a key molecule that plays a vital role in the onset of tendon inflammation, and eventual tendon degeneration in response to mechanical overloading. The findings of this study also indicate that GL may be used to prevent tendinopathy development by blocking HMGB1 signaling in the tendon, particularly in the athletic setting.

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PREFACE

First, I would like to thank Dr. James Wang, for his expertise, mentorship, and guidance in the exploration of my scientific interest. I would also like to thank his patience, understanding, and encouragement that helped me go through all the frustrations and difficulties, in both scientific and personal life. Without his help, I would not have pushed through my limit and presented my work here. I thank all my committee members for their support and valuable feedback on my dissertation project. I also thank all my lab mates, for their kind help, support and all the beautiful days they shared with me.

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1.0 INTRODUCTION

1.1 TENDON BIOLOGY

1.1.1 Tendon structure and composition

Tendon is a band of connective tissue responsible for the function of transmitting forces from muscle to bone and is constantly subjected to large mechanical loads. Tendon has greater mechanical strength per unit area than muscle [1]. The anatomy of tendons reflects this as it mainly consists of well-organized parallel bundles of collagen with rod- or spindle-shaped fibroblast-like cells called tenocytes [2]. The dry mass of human tendon is about 30% of the total tendon mass, water account for the rest 70%. The major component of the extracellular matrix is predominately collagen type I (65%-80% dry mass) [3], whereas collagen type III, V and XII [4], and elastin [5] are also found in tendon tissue. The extracellular matrix also contains proteoglycans, glycosaminoglycan, glycoprotein, and several other small molecules including tenascin-C, fibronectin, and thrombospondin[6, 7], which are crucial in the healing and repair process of the tendon. Collagen in the tendon is organized hierarchically fashion. It begins with the triple helix polypeptide chain, tropocollagen that forms fibrils. Fibrils form fibers and subfascicles that aggregate into fascicles, which then form fiber bundles and finally the entire tendon tissue [8] (**Fig. 1**). The connective tissues in between the fibers are called endotenon and

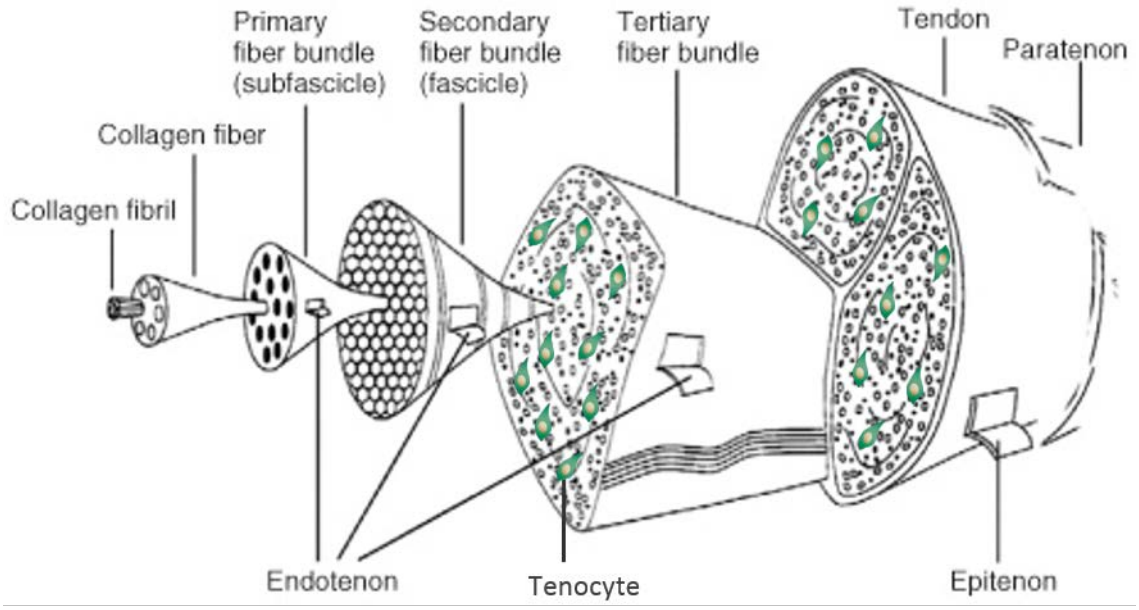

Figure 1. Tendon structure and composition. The figure shows hierarchical structure of tendon tissue. All structure are longitudinally arranged. Tropocollagen secreted by tenocytes united into collagen fibril then form a fiber; fibers are packed up into subfascicle, fascicle and tertiary fiber bundle, Tenocytes are packed in between the dense collagen fibers. Endotenon and epitenon are loose connective tissue hold fibers together; the tendon is surrounded by another layer of connective tissue called paratenon. Those connective tissues are thought to provide microvasculature for the tendon. Some tendon like Flexor tendon are housed within a synovial sheath, but some others like Achilles and patellar tendons are not. Figure source: [9], slightly modified.

epitenon, which hold the fibers together. Tendon is also wrapped in a thin layer of loose connective tissue called paratenon which contains blood vessels and nerves [10]. Some tendons in hands and feet also possess synovial tendon sheath, which ensures efficient lubrication as these areas are subjected to more friction and act like a pulley system [1, 8]. The well-organized and hierarchical structure enables the tendon to withstand significant tensile loads, but not compression forces [11].

1.1.2 Cellular component

Tenogenic cells constitute about 90%-95% of the cell component in the tendon, the other 5%-10% percent of tendon cells are chondrocytes, synovial cells, vascular cells[12]. Recently, a new cell type, tendon stem/progenitor cells (TSCs), was isolated and characterized in human, mouse[13] rat[14] and rabbit[15]tendon tissues. This new type of cells have multi-differentiation potential capable of differentiating into tenocytes (or tendon fibroblasts), chondrocytes, osteocytes and adipocytes as shown by *in vitro* and *in vivo* under different conditions [16-18].TSCs cannot be visualized in the tendon *in situ* because of lack of specific markers[19]; the niche where TSCs reside and developmental origin of TSCs are yet to be discovered[20]. Tendon has a low metabolic rate; the oxygen consumption of tendon is 7.5 times lower than that of skeletal muscles [21]. Tenocytes generate energy through aerobic Krebs cycle, anaerobic glycolysis, and pentose phosphate shunt [3]. This anaerobic metabolic feature allows tendon tissue to sustain long-term mechanical loading with low risk of ischemic damage, but the low metabolic rate, on the other hand, will slow the healing rate after injury[5].

1.1.3 Blood supply

The healthy tendon is relatively avascular. Tendon receives blood supply mainly from three sources, muscle-tendon junction, bone-tendon junction, and extrinsic system through paratenon or tendon sheath [22, 23]. However, the blood vessels originating from junction sites are unlikely to extend to the mid- one-third of the tendon in Achilles tendon. There is a hypovascularity zone that is 2 to 7 cm proximal to the Achilles tendon insertion site [24, 25]. Tendon blood flow is reported to decrease along with aging and mechanical loading [26]. The avascular nature of

tendon is considered to be responsible for the slow and incomplete healing after tendon injury [27].

1.1.4 Mechanobiology of tendon

Tendon tissue possesses high mechanical strength and unique viscoelasticity property to transmit large tensile force while absorbing excessive energy [28]. Tendons respond in different ways when subjected to various magnitudes of mechanical loading. When at rest, the collagen fibers are in a crimped configuration [29], and as the strain reaches 2%, the crimped tendon fibrils are "stretched out". As the strain increases to 4%, which is the physiological upper limit of the tendon, the tendon behaves in an elastic fashion. When strain exceeds 4%, microscopic level tearing of collagen fibers takes place, and when the strain increases to 8%-10%, macroscopic tearing occurs and finally leads to tendon rupture [12, 28, 30](**Fig. 2**). Besides responding mechanically variabilty, physiologically and pathologically also tendon responds differently to different strain levels. Previous studies have shown that proper mechanical loading is beneficial to the tendon regarding inducing the synthesis of matrix collagens and enhancing the tendon strength [31]. It has been reported that four weeks of exercise improved the tensile strength of peroneus breivis tendon in rabbit [32]. But excessive loading could be detrimental resulting in cell shape change and matrix degeneration [33, 34].

In consideration of the uniaxial loading condition of patellar and Achilles tendons, our laboratory has developed an *in vitro* system to mimic the cell alignment and repetitive uniaxial stretching condition *in vivo* [35]. Using this system, we found that with moderate stretching, tendon fibroblasts would increase proliferation and produce more type I collagen[36], but with excessive stretch, there was a significant increase in the inflammatory mediator, PGE_2. This

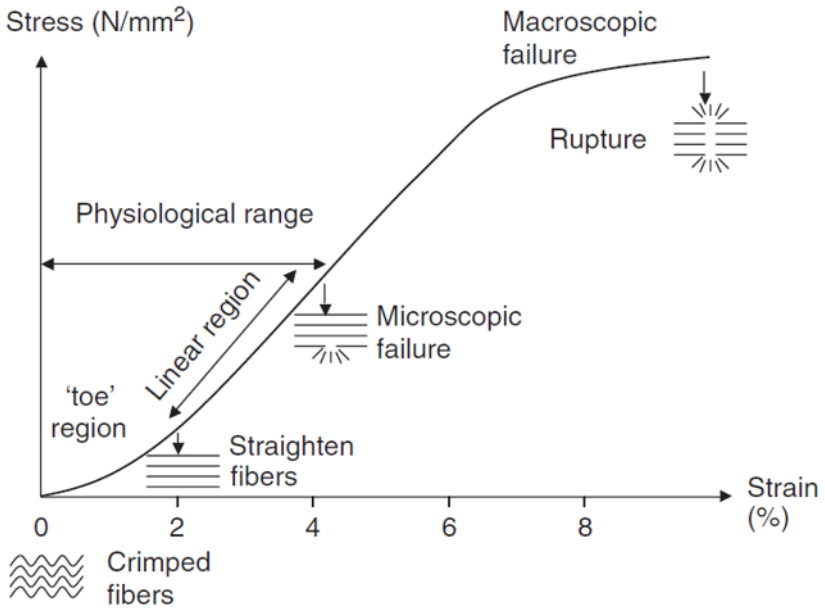

Figure 2. Tendon stress-strain curve. Tendon fibers are crimped when at rest, straightened at 2% strain and physiologically can be stretched to about 4%. If the strain is greater than 4%, microscopic failure of collagen fibers occurs. Finally, if the stain is greater than 8%, macroscopic failure and rupture will take place. Figure source: [30]

increase may lead to degenerative changes of tendon tissue which may be attributed to the decreased collagen production [37] and non-tenogenic differentiation of TSCs [16]. With a mouse treadmill running model, myofibroblasts are found in the tendons of intensive treadmill running mice, which actively participate in tendon repair and remodeling. This finding indicates that treadmill running might result in tendon micro-injury [38, 39].

Insufficient mechanical loading is also detrimental to tendons. Immobilization or disuse of limb leads to significant change in tendon's cellular number and shape; matrix integrity is also adversely affected. These changes would finally result in loss of tendon weight, stiffness and strength that eventually lead to tendon degeneration [40-42]. Mechano-responses of the tendon are mainly due to the mechanical stress (tensile stress, compressive stress, and shear stress)

acting on tendon cells, and the cellular and molecular responses to mechanical loading that may explain the development of tendon disorders such as chronic tendon injury.

1.2 TENDON INJURY AND TENDINOPATHY

Over 10 million people suffer from acute tendon injury or chronic tendinopathy each year in the United States[43] with the cost of treatment that goes over 30 billion dollars [44]. Additionally, the clinical outcome from slow or incomplete healing of tendon injuries has become a growing problem in sports/orthopaedic medicine [45].

Tendon injury can be divided into two categories, acute and chronic. Acute injury like tendon rupture may be spontaneous or caused by direct trauma and/or sudden excessive mechanical loading. On the other hand, chronic tendon injury, or tendinopathy, is manifested with compromised tendon tissue due to the change of tendon cellular and matrix components (tendon degeneration), which will increase the risk of acute injury like rupture or tear[2, 46].

1.2.1 Acute tendon injury

In acute trauma of tendon, the sudden acceleration-deceleration mechanism has been seen in 90% Achilles rupture in sports related field [47]. Histological degenerative changes are very often found in spontaneous tendon ruptures[48], suggesting that acute injury is often not a stand-alone event; rather it is the final result of gradual weakening and degenerating tendon under long-term excessive mechanical loading.

1.2.2 Natural healing of tendon

Most tendon healing studies are performed on acute tendon rupture in human or animal model with transected tendons [12]. The healing of ruptured or transected tendon can be divided into three overlapping stages [49], which is similar to wound healing of skin, i.e., 1) inflammatory stage; 2) proliferative or repair stage, and 3) remodeling stage.

Inflammatory stage typically lasts for a few days to 1 week. The blood clot forms right after the rupture, and the platelets release growth factors and chemoattractants. The fibrin-based clot serves as a temporary scaffold for invading cells such as neutrophils, monocytes and lymphocytes, and tenocytes in tendon and circulating progenitor cells are recruited to the wound site [2]. Approximately two days after the initial injury, the proliferative/repair stage begins. It is characterized by massive proliferation and synthetic activity. The two main cell types that are crucial in this stage are macrophages and tenogenic fibroblasts. Some researchers also have suggested that tendon stem/progenitor cells (TSCs) differentiated into tenocytes are the sources of the fibroblastic cells participating in repair [50]. In this stage, macrophages' role shift from phagocytic to reparative, and begin to secrete growth factors, cytokines, and direct cell recruitment. The intrinsic tenocytes also migrate to the wound site. But a recent research suggested that those "local tenocyte" did not massively participate in the wound repair. Instead, α-SMA -positive progenitor cells from paratenon dominated the wound area in the first few weeks [51]. The level of neutrophils gradually declines in this stage. The tenogenic fibroblasts in this stage deposit a temporary matrix mainly composed of collagen III, as well as GAGs [52]. The remodeling stage begins 1-2 month after the initial injury and can last up to 1 or 2 years. In this stage, the matrix gradually becomes aligned with the direction of tension, collagen I replace collagen III in the wound site, and also the cellularity decreases. The repaired, not regenerative

fibrous tissue becomes scar-like [53] and can reach up to 70% of the mechanical properties compared to intact tendon tissue [54]. In other words, after injury tendon never heals completely such that normal structural and mechanical properties are restored to injured tendons.

1.2.3 Tendinopathy/Chronic tendon injury

Unlike acute tendon injury, tendinopathy is not manifested with macroscopic tearing or rupture of the tendon. Clinically, it is related to pain, focal tenderness, decreased strength and limitation of movement of the affected tendon [55]. Histologically, tendinopathy often has the following features: disorganization of collagen fibrils, increased proteoglycan and GAGs in the extracellular matrix (ECM), hypercellularity, and neo-vascularization [12, 55, 56] (**Fig. 3**). Different types of tendon degeneration changes can be found in tendinopathy, including lipid accumulation typically found in Achilles tendon[57], and fibrocartilaginous metaplasia and calcium deposition in rotator cuff tendinopathy[58].

Tendinopathy is caused by intrinsic and extrinsic factors; excessive repetitive mechanical loading is considered as the main extrinsic factor for Achilles tendinopathy. It is reported that tendon subjected to repetitive overloading above the physiological limit results in inflammation of the sheath, and degeneration in tendon body [59]. Excessive mechanical loading may result in increased and imbalanced cytokine levels, including the release of VEGF, and MMPs [60, 61], which may lead to the degeneration and structural changes in the tendon. These changes could result in further modulation of cell activity, and the cumulative long term effect will finally end up with macro-scale tendon pathological changes that are manifested by clinical symptoms like pain and impaired movement [62].

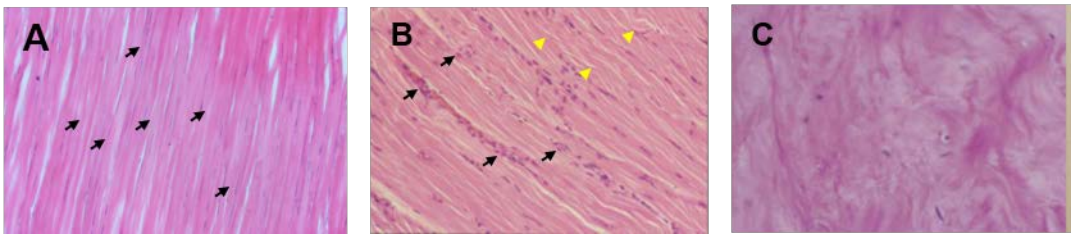

Figure 3. Histological structure in normal and tendinopathic tendon in the human patient. (**A**) Normal tendon has parallel and well-organized collagen fiber structure (pink) and elongated tenocytes (dark blue, arrows). Note that the cell density is relatively low. (**B**) Early tendinopathy with increased cellularity (arrow) and cell morphological change from elongated to round shape. Additionally, tendon matrix becomes slightly disorganized (arrowhead). (**C**) In the late stages of tendinopathy, the tendon is highly degenerated, its matrix organization is totally disrupted, and cell density is greatly decreased.

Tendinopathy displays some common features of the healing process, but more in a disorderly manner. Tendinopathy could be the result of long-term imbalanced cellular activity and matrix integrity from repetitive, excessive mechanical loading. Tendinopathic tendons are susceptible to rupture because of their weakened mechanical strength. The role of inflammation in the development of tendinopathy is not clear, and it has been controversial. Inflammation is not typically reported in tendinopathy in early studies, but recent studies have found evidence of inflammation in human tendinopathic tendons using flow cytometry and immunochemical histology [63]. For example, in a human study in spontaneous ruptured Achilles tendon, immunohistochemical staining confirmed the presence of macrophages (CD68), T cells (CD3) and B cells (CD20) in all 60 ruptured tendon samples [64]. Another study later demonstrated B cells T cells and macrophages are increased in Achilles tendinopathy samples [65]. And many pro-inflammatory agents like prostaglandins and cytokines (i.e.IL-6, IL-1β) have been identified during the tendinopathic process [63, 66]. It is possible that the biopsies from the patients are more likely to represent chronic and cumulative effect rather than early phase phenomenon in the

development of tendinopathy [53]. It has been reported that cyclic mechanical loading increases inflammatory mediator PGE_2 *in vitro* and *in vivo*, and it might be the cause of tendon degeneration due to the non-tenogenic differentiation of TSCs in response to mechanical overloading [16, 37, 45]. Finally, even when inflammation may not usually be present in patients with tendinopathy, it may still play a predominant role in the onset of the disease.

1.2.4 Achilles tendon and Achilles Insertional tendinopathy

The Achilles tendon (also known as the calcaneal tendon) is the strongest and largest tendon in the human body; it attaches the posterior gastrocnemius, and soleus muscles to the calcaneus (heel bone)[67](**Fig. 4A**), the length of Achilles tendon is around 220 - 230mm in adults [68]. Its action is to plantarflex the ankle actively and to resist dorsiflexion. It can withhold a load of about four times a person's body weight during walking. Achilles tendon is vulnerable to injury, especially because of the considerable tension placed on it.

Achilles tendinopathy is one of the most frequently reported overuse injury in sports medicine [69] (**Fig. 4B**). It affects about 9% of recreational runners and causes up to 5% of athletes to end their sports careers [70].One research with 291 elite runners also showed that Achilles tendinopathy is the most common running-associated tendon problem, and 10 years+ runners have a higher risk of development of the disease [71].

Achilles tendinopathy can be divided into insertional and non-insertional tendinopathy (**Figs. 4C, D**). The insertional site of Achilles tendon is located at the tendon-bone junction, and it is divided into four continuous zones, 1) tendon, 2) fibrocartilage,3) mineralized fibrocartilage; and 4) bone[72]. Insertional Achilles tendinopathy often occurs in the tendon matrix proximal to the insertion into the heel bone; it is a degeneration of the tendon fibers and may be associated

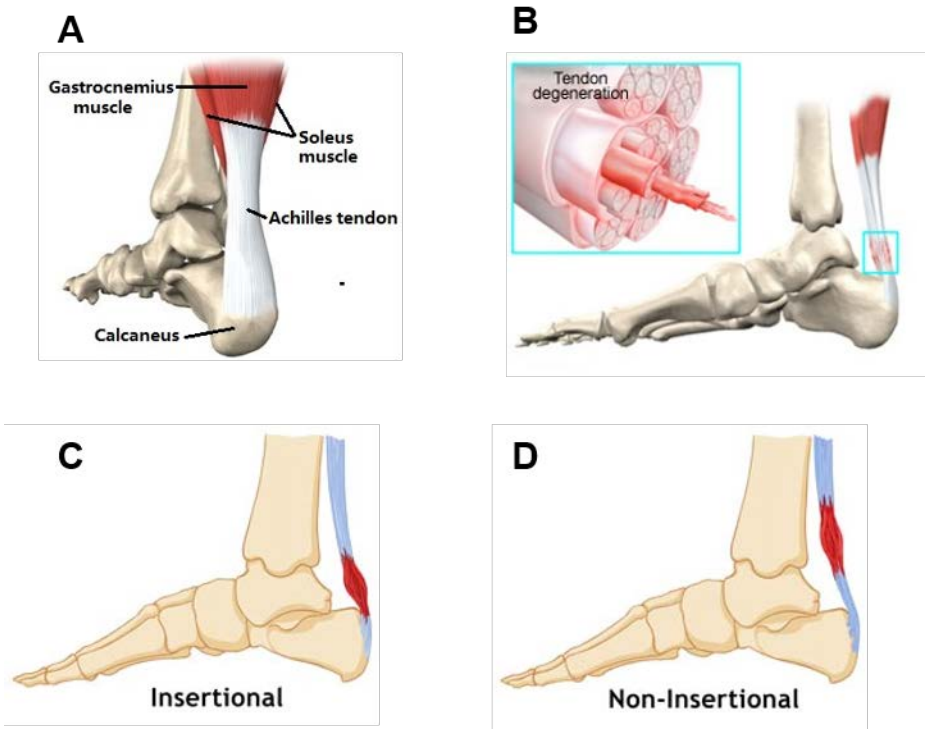

Figure 4. Achilles tendon Anatomy and Insertional Achilles Tendinopathy. Achilles tendon attaches gastrocnemius and soleus muscle to calcaneus bone (**A**). Achilles tendinopathy is usually associated with tendon degeneration. (**B**) Achilles tendinopathy can be divided into insertional (**C**), and non-insertional (**D**). The insertional tendinopathy occurs 2-6cm proximal to the insertion site. Non-insertional tendinopathy occurs in mid-portion of Achilles tendon. Insertional tendinopathy has a higher incidence rate in relatively young and active population such as athletes. Figure Source: Images from Medical Multimedia Group

with an inflammation of the bursa. Histological studies on insertional Achilles tendinopathy show the typical degenerative appearance of tissues, increased number of cells and more GAGs in the matrix as well as disorganized collagen fibers [73]. Insertional tendinopathy accounts for about 20% of all Achilles tendon disorder [74]. The insertional tendinopathy is considered to occur more in young (the average patient age is in the 40s) and in the active population, whereas non-insertional tendinopathy is found more in older, less active and overweight population [75].

Insertional tendinopathy often causes pain, and impaired movement of the ankle and about 10 - 30% patients need surgical intervention [76].

1.2.5 Treatment of Tendon injury and tendinopathy

1.2.5.1 Surgical intervention

Reconstructive surgery is often required for ruptured tendon [77]. The surgery often includes suture and anchor techniques. Severe and reluctant tendinopathy may also need surgical intervention [78], but it comes with the risk of infection.

1.2.5.2 Physical therapy

Active movements and proper mechanical loading are considered beneficial to tendon healing [78]. The eccentric exercise involves lengthening of the (affected) muscle-tendon unit slowly, which strength the muscle. It is reported that eccentric exercise decreases pain in Achilles tendinopathy [79]. Therefore, eccentric exercise is considered to be the most efficient therapy for chronic tendinopathy now and is recommended as first-line treatment [80].

Additionally, many other physical modalities have been used in tendon disorder treatment. For example, extracorporeal shock wave [81], pulsed magnetic fields[82], therapeutic ultrasound[83], laser phototherapy[84], and massage are considered as second-line therapy; however, the efficacy of these treatments is inconsistent [85].

Autologous platelet-rich plasma (PRP) has been considered readily available and safe method for the treatment of tendinopathy. PRP promotes tendon cell proliferation and collagen deposition [43, 86], but the efficacy of PRP treatment in clinical studies has been very controversial. Some researchers claim that the inconsistencies of the treatment outcome of PRP

arise from different preparation conditions of PRP and various pathological conditions of tendinopathic patients [86, 87]. Therefore, the patient conditions should be carefully evaluated before proper PRP preparation is used for treatment.

1.2.5.3 NSAIDs and Steroids

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are broadly used for management of pain and inflammation in tendinopathy [88], but there is little evidence showing that they are effective in treating the chronic tendon injury [89]. Long-term use of NSAIDs to treat tendinopathy may carry a high risk of adverse effects [90]. While steroids like corticosteroids are used in non-inflammatory degenerative tendinopathy, they only provide short-term pain relief [91, 92]. Some studies suggested that steroids might, in fact, weaken tendon tissue, and lead to tendon rupture particularly in weight-bearing tendons like patellar and Achilles tendons [93]. A recent study found that the popular dexamethasone treatment may result in non-tenogenic differentiation of TSCs, which may lead to tendon degeneration [94].

1.2.5.4 Tendon tissue engineering

Tissue engineering approach is also employed for tendon healing. Mesenchymal stem cells (MSCs) [95], adipose-derived stem cells (ADSCs)[96] and tendon stem/progenitor cells (TSCs) [20] have been proposed for augmentation of tendon healing. Among these cells, the TSCs seem to be the most promising, since it will spontaneously differentiate into tenogenic cells while other cells might differentiate into another type of cells i.e. adipocyte, chondrocyte or osteocyte which might lead to tendon degeneration. However, harvesting autologous TSCs for tendon treatment is not possible in the clinical setting at present, so alternative strategies with the use of MSCs and ADSCs are also actively being studied.

The scaffold is another critical element for tendon tissue engineering. These temporary and degradable synthetic/native biomaterials will provide biomechanical support, and physical niche for the cells to move in, reside, proliferate and deposit new matrix. Scaffolds can also be modified or combined with growth factors or stem cells to improve repair/regeneration potential [97].

Various growth factors are reported to participate in tendon development and healing process, and they have been studied widely for their potential to improve tendon healing. They include IGF[98], VEGF[99], TGF-β family[100, 101], PDGF[102], and bFGF[103]. These growth factors promote the proliferation and migration of tendon cells, and cellular production of collagen as well as the deposition of tendon matrix.

1.2.6 Experimental models for tendinopathy

Animal models have been extensively utilized for tendinopathy studies, especially using rodents such as rats and rabbits. Two types of experimental models, chemically-induced and mechanical loading -induced, are popular to induce tendinopathy in animals.

1.2.6.1 Chemically- induced tendinopathy model

Collagenase was first introduced to produce experimental injury to the tendon in animals [104]. It is the most widely used chemical-based tendinopathy model, and it can induce a repeatable lesion with tendon inflammation and degeneration. It has been applied in rat [105], rabbit [106] and horse [107], and experiments were conducted on flexor, Achilles, patellar and supraspinatus tendons [108]. It has been used as a standard model to investigate the effects of all types of treatment intervention.

Carrageenan, a type of vegetable polysaccharide, was reported to induce leukocyte invasion resulting in tendon damage when injected into rat Achilles tendon. This approach also found increased MMP activity and decreased TIMP concentration [109]. This chemical also attracts more macrophages than neutrophils.

Injection of corticosteroid into rat Achilles tendon has been shown to increase cellularity and vascularity, but only 20% of the animals show significant symptoms of tendinopathy [110]. Although corticosteroid injection is not considered to generate a valid experimental model, this study did highlight the potential detrimental effects of corticosteroid injection in athletes.

Prostaglandins were also used to induce tendinopathy since they were seen to be increased in overloading animal models *in vivo* [66] and *in vitro* [35, 66, 111]. The injection of PGE_2 resulted in decreased collagen fibril diameter, and moreover, PGE_2 is also suggested to induce the differentiation of TSCs into non-tenogenic lineage resulting in tendon degeneration [16].

Recently, a strong chondrogenic reagent kartogenin (KGN) was reported to induce tendinopathic changes like the presence of chondrocytes and proteoglycan accumulation in rat Achilles tendon after 5-weeks of implantation [112]. This study also suggested that TSCs are involved in chondrogenic differentiation. Peritendinous elastase was recently utilized to create tendinopathy model by inducing degeneration in rat Achilles tendon [113].

1.2.6.2 Mechanical-loading induced tendinopathy model

The most popular model to induce tendinopathy through mechanical loading is treadmill running of rodents, especially for rat supraspinatus tendinopathy. The model requires rats to run on a 17m/min treadmill track with a 10-degree decline for 1hr/day, 5 days/week [114]. After the treadmill running regimen, the cross-sectional area of supraspinatus tendon increased but with

poor mechanical properties. Histologically, the tendon after treadmill running showed hypercellularity, round chondrocyte-like cells, enhanced vascularity, GAGs deposition and disorganized collagen fibers, the damages in the tendon increased with longer duration of treadmill running [115]. The cartilage marker expression was also found in this treadmill running model [33], indicating a possible fibrocartilaginous change in the tendon that resulted from tendon overuse [34].

Mouse treadmill running protocol (TRP) is similar to that of rat, but current mouse TRP is utilized mainly to explore the pathological change of hinder leg weight bearing tendons like Achilles and patellar tendon, and the speed is lower (12-15m/min) with longer duration each day (3-5hrs), without decline running. It was found that intensive running causes an increase in the production of inflammatory mediator PGE_2, while moderate running does not cannot [66]. It was also reported that treadmill running results in myofibroblast accumulation in mouse patellar tendon [39]. Some other studies also found short-term treadmill running may help to expand the stem cell pool[116] and improve tendon quality on a chemically- induced tendinopathy model [117].

Another category of mechanically- induced model is passive loading. In this model, usually the animal is under anesthesia, and the tendon is passively stretched. One rabbit model has used a kicking machine by inducing active muscle contraction with electrical stimulation and found that the exercised tendon exhibited altered cell nuclei shape and increased fibroblast and vascularity in paratenon with infiltrated inflammatory cells [118]. A passive rat patellar tendon fatigue loading model secured tibia and patella toe to a customized machine to allow loading to patellar tendon [119]. Gene expression levels of collagen I, III were found to be increased with decreased cellularity and disorganized matrix. Additionally, CITED2 and MMP-3 expression

greatly increased with the moderate level of fatigue loading compared to non-loaded tendon tissue [119, 120].

1.3 HMGB1

1.3.1 PAMPs, DAMPs, alarmins and HMGB1

When the mammalian body needs to detect and monitor microorganisms that invade and cause tissue/cell damage, it activates the defense and repair mechanisms with innate and adaptive immune responses. The activation processes often start with recognition of certain molecules or molecular patterns [121]. Pathogen-associated molecular patterns (PAMPs) are a set of microbe –derived molecules such as lipopolysaccharide (LPS), viral RNA and bacterial peptidoglycans. These molecules can be recognized by cells of the innate and acquired immune system, usually through Toll-like receptors(TLRs), and they can activate several signaling pathways including the most distinctive one, NF-κB [122]. Besides PAMPs, there is another set of endogenous molecules that mainly signal tissue and cell damage, called alarmins. Alarmins are equivalent of PAMPs, but they will be released only due to nonprogrammed cell death (necrosis) or cellular injury/stress. The word alarmin was first created in 2006, and at that time, both alarmins and PAMPs were proposed as a subgroup of Damage-associated molecular patterns (DAMPs). However, nowadays, researchers use both alarmins and DAMPs interchangeably, and both refer to the endogenous danger signal molecules that trigger inflammation and tissue repair. The striking features of alarmin/DAMPs molecules are: 1) rapid release from nonprogrammed cell death (necrosis); 2) could be produced and secreted by the immune system; 3) recruit and active

innate immune cells; and 4) help restore homeostasis in damaged tissue [123]. Proposed alarmins/DAMPs include HMGB1, S100 proteins, heat shock proteins (HSPs), IL-1α and more [123]. Among them, HMGB1 is the only molecule that meets all the four criteria, and thus become the most studied alarmin/DAMP.

1.3.2 Discovery of HMG family of proteins

HMG proteins were first discovered in 1973 from calf thymus chromatin and were named "high-mobility group" protein because they migrate rapidly in polyacrylamide gel without aggregation [124]. The two proteins HMG-1 and HMG-2 were identified with at least 10^5 molecules in the nucleus of each cell [125]. Along with other HMG family proteins, HMG-1 and HMG-2 were renamed as HMGB1 and HMGB2, respectively, in 2001 because of its HMG-box functional domain. Other superfamily members were renamed HMGA (formerly HMG-14/17) and HMGN (formerly HMG-1/Y) because of their unique functional motif [126]. HMGB family has totally four members with ~80% amino acid sequence identity.

HMGB1 is the most expressed HMG family member with about 10^6 molecules per mammalian cell. HMGB1 is an extremely evolutionally conserved protein from 525 million years ago [127]. A homolog of mammalian HMGB1 was discovered in yeast, Drosophila, Chironomidae, echinoderms, bacteria, plants, fish, and C. Elegans [128-130]. The protein sequence of HMGB1 is 100% identical between mouse and rat, and 99% identical between rodent and human, only two amino acids on C-terminal are different [131, 132]. HMGB1 knockout mice die shortly after birth due to hypoglycemia [133] suggesting the critical role of HMGB1 as a structural chromosomal protein.

1.3.3 HMGB1 structure and function

HMGB1 consists of 215 amino acid residues and is about 30KDa in molecular weight. It has two DNA binding domains (Box A [9-79aa], Box B [95-163aa]), which are not sequence-specific, and an acidic C-terminal tail (186-215aa), which is the transcription stimulatory domain[134]. There are two nuclear localization signal (NLS) regions, NLS1 (28-44aa), and NLS2c (179-185aa). The "default" location for HMGB1 is in the nucleus, and the change of NLS or nuclear-emigration signals (NES) will result in HMGB1's translocation [135](**Fig. 5A**). Due to its high mobility, HMGB1 can move into the cytosol from the nucleus or other organelles within 1-2s [136, 137]. HMGB1 also has a binding site for RAGE (150-183aa), which is related to cell migration[138], and TLR4 binding site (89-108), which is cytokine activity domain, and p53 transactivation binding domain (7-74aa), which is responsible for regulation of inflammation and downstream gene expression[60]. The C-terminal consists of acidic amino acid residues, and this region was thought to protect Box A and Box B. But, later it was found to be related to DNA binding process of HMGB1 [139]. The secondary structure of two HMG boxes of HMGB1 is very similar with 3 alpha-helices and two loops that form an "L" shape with an 80-degree angle between two arms [140] (**Fig. 5B, C**). The tertiary structure of HMGB1 features 3 cysteines at position 23, 45 and 106, the forming of cys23-cys45 disulfide bond can alter the HMGB1 interaction with receptors [141]. For quaternary structure, HMGB1 is usually in the homodimer and oligomer forms but may vary due to different extraction methods which potentially inactive HMGB1 [142, 143].

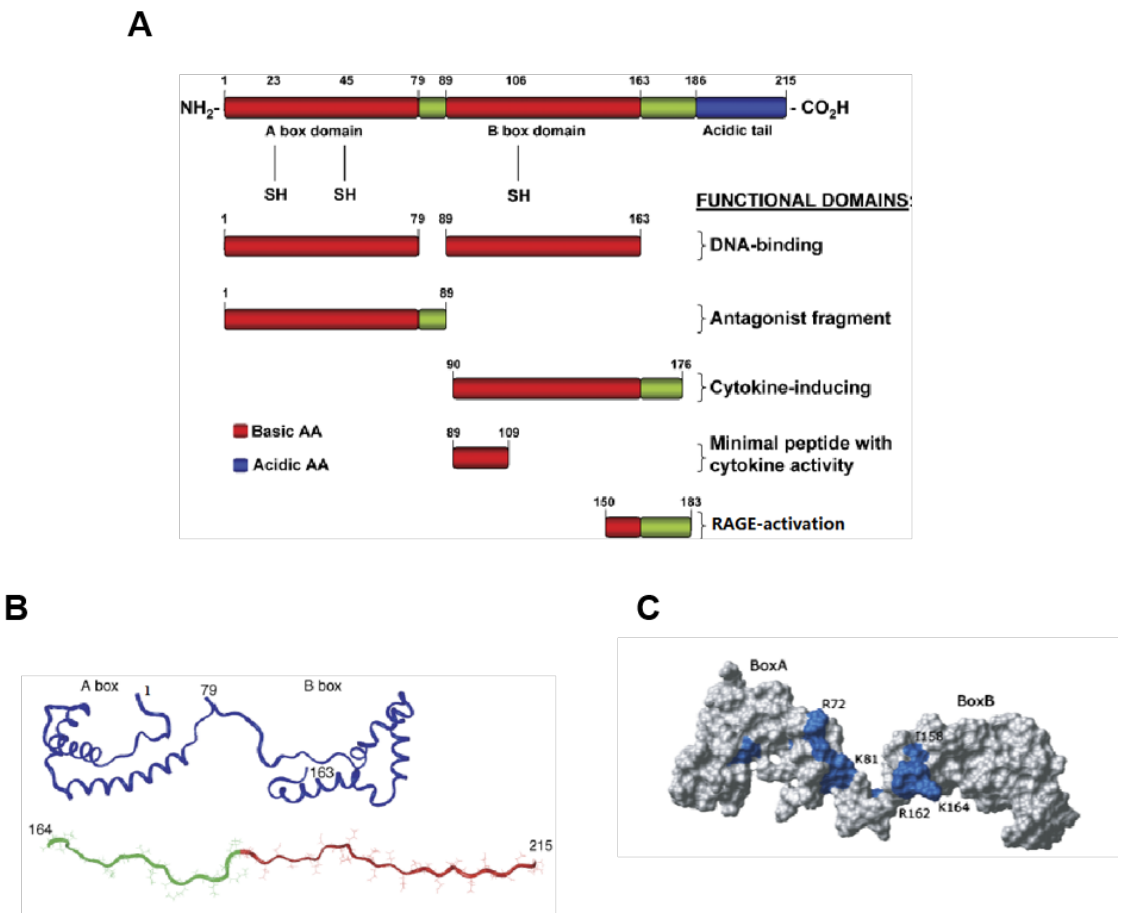

Figure 5. The structure and functional domains of HMGB1. (A) Beginning with N-terminal, HMGB1 protein consists of Box A domain (red, 1-79aa), Box B domain (red,89-163aa), and c-terminal acidic tail (blue, 185-215aa), and connecting segments between them (80-88aa,164-184aa). Box A and Box B are sequence-independent DNA binding domains made up of basic amino acids. Box A (1-89aa), when isolated from the rest of the molecule, acts as a specific antagonist of HMGB1. Box B (90-176aa), when stand-alone, acts as cytokine-inducing molecular. The minimal peptide with HMGB1 cytokine activity is 20 amino acid segment of Box B (89-109aa). The region required for the activation of RAGE is located between residues 150 and 183. (B) Ribbon representation of A box, B box, and the c-terminal acidic tail. (C) Space-filling representation of A box and B box, the two box regions can rotate freely, blue regions show the basic residues that can interact with the acidic tail. Figure source: A: [144], B&C: [145].

1.3.3.1 Nuclear and cytoplasmic HMGB1

HMGB1 has distinct functions depending on its location (**Fig. 6**). Nuclear HMGB1 is a DNA-chaperone and plays a major role in DNA activities like replication, repair, transcription and genomic stability with DNA binding and bending abilities [130]. HMGB1 in the cytoplasm was reported in living fibroblast with a normal ratio (nuclear to cytoplasm) 30:1 [146]. It is known by now that HMGB1 can translocate from nucleus to cytosol when the cells are stimulated with several stressors (cytokines, heat, hypoxia, hydroperoxide, etc.) [147]. A group of researchers has shown that cytoplasmic HMGB1 plays a positive regulatory role of autophagy [148, 149], which is a protective mechanism when the cell is under stress.

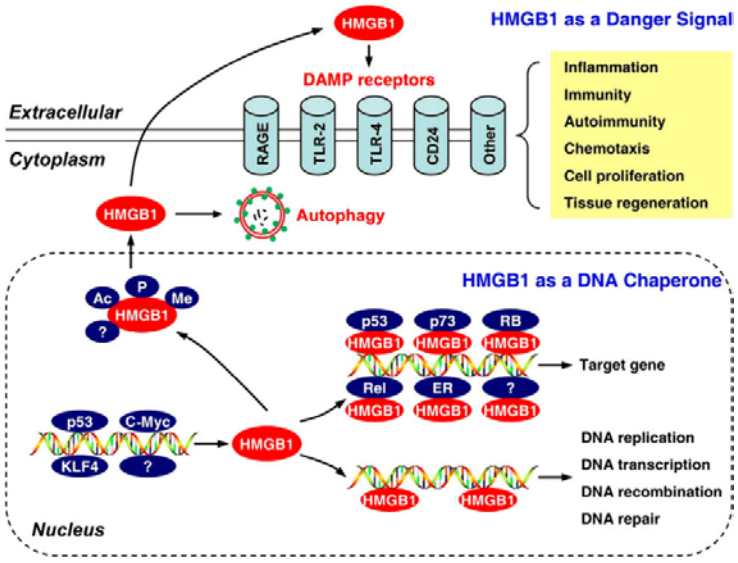

Figure 6. HMGB1 function in nucleus, cytoplasm and extracellular milieu. HMGB1 acts as a DNA chaperone in the nucleus and regulates gene expression and DNA functions. HMGB1 in the cytoplasm is related to cell autophagy. Extracellular HMGB1 is a damage signal, exerting its function through DAMPs receptors like RAGE and TLRs and regulates inflammation and immune responses. HMGB1 also participates in the development of auto-immune disease and tissue regeneration. Figure source: [150].

1.3.3.2 Extracellular HMGB1- release and function

The extracellular role of HMGB1 was uncovered in sepsis in 1999 [151]. Extracellular HMGB1 is either passively released from necrotic, injured, stressed stromal cells, or actively released by immune cells like monocytes and macrophages (**Figs.7A, B**). Once released, extracellular HMGB1 has cytokine and chemokine activities. HMGB1 induces proliferation, cell migration, and survival, as well as development and maintenance of inflammatory response through the induction of its own release from inflammatory cells [152-154].

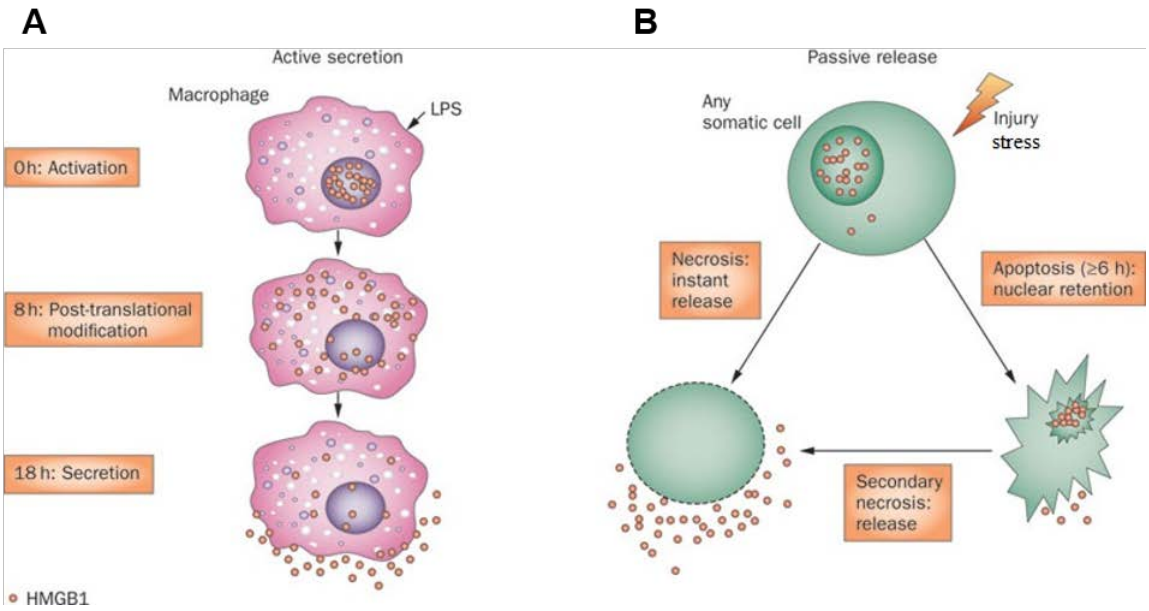

Figure 7. The current theory of HMGB1 extracellular release. (**A**) HMGB1 is released from cells via different pathways. HMGB1 can be actively released when immune cells like macrophages are stimulated by pathogenic agents like LPS. After initial stimulation, modification of HMGB1 occurs to facilitate HMGB1 release later. It takes a few hours for HMGB1 to be released. (**B**) HMGB1 can be passively released from somatic cells subjected to injury or under severe stress. The cells under primary necrosis will release HMGB1 passively and rapidly. Previously found HMGB1 are retained in the nucleus during apoptosis. Recent findings indicate that when those apoptotic cells undergo secondary necrosis, HMGB1 can be released from the apoptotic cells. Figure Source: [155].

HMGB1, through binding to TLR or RAGE, actives macrophages, monocytes, neutrophils, fibroblasts, endothelial cells, and T-cells to produce all kinds of known cytokines like TNF, IL-1β, and IL-6. Also, HMGB1 exerts its pro-inflammatory activity through activation of NF-κb, ERK, and JNK pathways. HMGB1 can also bind to other DAMP or PAMP like LPS, DNA to amplify its pro-inflammatory potential [156, 157]. Inhibition of HMGB1 can significantly decrease the inflammatory response and tissue damage. HMGB1 is no doubt a critical pro-inflammatory cytokine. A key feature of HMGB1 is that it is abundant in cells and can be readily released upon any tissue damage. So HMGB1 was categorized as damage associated molecular pattern (DAMPs) member [158, 159]. It has the potential to "translate" other stress signal, *i.e.* mechanical stress into the biochemical signal, thus initiating inflammatory cascade.

HMGB1 also promotes cell migration in several cell types including smooth muscle cells, stem cells, endothelial cells, monocytes, dendritic cells, and neutrophils [160-162]. These findings suggested that migration initiated by HMGB1 may contribute to the recruitment of immune cells and stem cells to tissue damage repair or wound healing site [163].

Only a few studies reported that HMGB1 promotes angiogenesis by showing that HMGB1 could induce endothelial cell migration *in vitro* [164]. Some studies revealed that HMGB1could induce proliferation of a particular type of mesoangioblasts and cardiac stem cells [161, 165]. In addition to the direct effect of HMGB1 on a certain type of cells on proliferation and angiogenesis, it is highly possible that HMGB1, through the recruitment of immune cells, indirectly promotes cell proliferation and new vessel formation in the injured site.

1.3.4 Redox Status and HMGB1 function

It is suggested that the HMGB1's function largely depends on its redox status [149]. HMGB1 contains three cysteine residues C23, C45, and C106, of which C23 and C45 could form an intramolecular disulfide bond. HMGB1 has three redox status; the first one is fully reduced HMGB1, with all three cysteine residues in reduced status. A few research findings indicate that the fully reduced HMGB1 acts as chemokine and can form heterocomplex with CXCL12 and bind to CXCR4 [153]. The second is disulfide HMGB1 that refers to the HMGB1 with disulfide bond connecting C23 and C45. It was reported to act as a pro-inflammatory cytokine with minimal chemoattractant activity [166]. The third status is fully oxidized HMGB1 that is featured by all cysteine residues oxidized completely. This type of HMGB1 has no activity in cell migration, and cytokine induction [167, 168]. However, some other studies indicate that oxidized HMGB1 can still activate neutrophils, vascular inflammation, and age-associated inflammation [169, 170]. The redox form of HMGB1 can only be detected with mass spectrometry (MS) [171] or nuclear magnetic resonance (NMR) spectroscopy [172].There is no other convenient method available currently.

1.3.5 HMGB1 in diseases

HMGB1 has been extensively studied in several major diseases including cancer, stroke, endotoxemia, and joint disorders [173]. HMGB1 also has a key pathogenic role in diseases of kidney and lung, and arthritis, sepsis because of its pro-inflammatory effect [130, 174-177]. Recently, it was reported that a mechanism for the pathogenic role of HMGB1 in arthritis could be through enhancement of inflammatory and destructive mechanisms induced by other pro-

inflammatory mediators present in the arthritic joint [178]. Injection of HMGB1 into normal joint caused the development of arthritis in 80% of animals in the study (other 20% without obvious arthritis development), whereas development of arthritis could be prevented if HMGB1 was inhibited in this disease model [179]. Another study successfully treated the collagen-induced arthritis by targeting HMGB1 further implicating the crucial role of HMGB1 in the development of this disease [180].

1.3.6 HMGB1 and mechanical loading

There are only a few studies that applied mechanical loading to understand the alterations of HMGB1 expression, translocation, and its regulatory role *in vitro* and *in vivo*. For example, a group of researchers recently demonstrated a basal HMGB1 expression in periodontal ligament (PDL) cell cultures [181], and a significant increase of the mediator when challenged by mechanical loading similar to force levels being applied in orthodontic treatment *in vivo* [182]. They transferred these findings to an *in vivo* microenvironment in an animal model of tooth movement in rats [181]. Their data indicated the potential role of this protein in the regulation of the periodontal remodeling process in the course of orthodontic tooth movement. Later, another study also showed the expression of HMGB1 in the periodontal tissue subjected to orthodontic force application in mice [183].

1.3.7 HMGB1 and tendon injury

Repetitive mechanical loading is considered as a major risk factor for tendinopathy, but to date, no direct mediator has been identified between the mechanical loading and the biochemical

response in tendon tissue. How the "mechanical signal" translate to injury related "molecular signals" to initiate the pathological development of tendinopathy has been unknown. Moreover, the presence of basal level expression of HMGB1 in tendons, its pathogenic role as an inflammatory mediator, and its potential as a therapeutic target in tendinopathy have not been investigated.

In this study, we hypothesized that tendon cells and tissues under mechanical overloading release high levels of HMGB1 extracellularly to initiate an inflammatory cascade. Indeed we observed in this study that mechanical overloading of tendon cells and tissues translocated HMGB1 from nucleus to extracellular matrix. The addition of HMGB1 initiated an inflammatory cascade via enhancement of COX-2 and PGE_2, inflammatory cell migration *in vitro*, and implantation of HMGB1initiated hypercellularity, angiogenesis, and inflammatory cell infiltration *in vivo*. Also, HMGB1 specific inhibitor, GL reversed these inflammatory reactions both *in vitro* and in mechanically overloaded *in vivo* models.

1.4 CURRENT KNOWLEDGE AND GAP IN TENDINOPATHY

1.4.1 Mechanical overloading/overuse is a major risk factor for tendinopathy

For chronic tendon injury, tendinopathy is the term used to describe a complex pathology of the tendon with pain, the decline in function, and reduced exercise tolerance [184]. Histologically, it is often characterized by drastic degenerative changes in tendon such as cell shape change, deposition of GAGs, and disorganized collagen matrix [185].

It is intuitively reasonable that tendinopathy is the result of tendon overuse. Clinical and animal studies also provide some evidence for the intuition. For example, the incidence of tendinopathy is rising in developed countries because of increased participation in sports and physical activities [186]. It was reported that about 30% runners exhibit Achilles tendinopathy, with a 7-9% annual incidence [70]. Compared with age-matched control, long-distance runners have increased the incidence of Achilles tendinopathy [187]. Patellar tendinopathy is common in volleyball, handball, basketball, track and field as well as in soccer [188]. The fact that athletes are more prone to tendinopathy compared to general population suggests that the disease could be a cumulative result of long-term overuse.

In animals, tendinopathy in rat supraspinatus tendon has been successfully created with overuse through treadmill running [33]. The tendon with tendinopathy showed degenerative changes such as increased cellularity and loss of normal collagen fiber organization similar to those in human patients. Another study revealed that uphill treadmill running of rats could also induce the cellular and structural changes typical of tendinopathy degeneration in Achilles tendon [34].

1.4.2 Whether inflammation is involved in tendinopathy has been controversial

Early sports-related tendinopathy studies in human patients have shown that there is neither clinical evidence (redness, tenderness, skin changes or elevated inflammatory markers in blood) nor microscopic evidence (inflammatory cell infiltration) of inflammation in degenerative tendon tissue specimens [189, 190]. Also, anti-inflammatory agents were largely unsuccessful in treating the condition[191]. The opinion that tendinopathy is devoid of inflammatory process was widely

accepted among the clinicians and basic science researchers in early times. So the involvement of inflammation has long been underestimated in the development of tendinopathy.

1.4.3 A continuum pathology model for the development of tendinopathy

One popular theory to explain the pathology of tendinopathy without inflammation was proposed by Dr. Jill Cook in 2009, based on clinical, histological and imaging evidence. She suggested that a progression of tendinopathy consists of three related stages, so-called three-stage continuum model. The excessive mechanical load is considered as a risk factor to move towards the irreversible degenerative tendinopathy. The three stages involved in tendinopathy development are as follows.

1. Reactive tendinopathy, characterized by a non-inflammatory proliferative response in cell and matrix, thickness of tendon, which is considered to result from acute overload or unloaded tendon.

2. The second stage is tendon disrepair. This stage is manifested by increased cell number, mainly as chondrocyte-like cells and myofibroblasts, with increased presence of proteoglycans in the tendon, which results in disorganization of the matrix and may associate with vascular and neuron ingrowth. Moreover, the first two stages, if mechanical loading is properly modified, could be reversed; in other words, the tendinopathic tendons at the two stage could be back to normal tendon.

3. The third and final stage is degenerative tendinopathy with cell death, a vast area of the disordered matrix and a large amount of vessel ingrowth, and the affected tendon has little capacity of reversing its pathological changes[192].

This model was mainly established based on findings from human tendinopathic patients; the model is also used to develop the treatment plan for tendinopathy patients. In this theory, neither is inflammation included nor is it considered as a causative factor in the eventual degenerative development of tendinopathy.

1.4.4 Tendinopathy is related to inflammatory response in recent studies

It has been observed that the incidence of tendinopathy is higher in patients with obesity and decreased insulin sensitivity, as seen in type 1 and type 2 diabetes mellitus (T1/T2DM) patients [193-195]. One common shared risk of these two patient populations is the development of chronic low-grade, systemic inflammation [194, 196]. Inspired by this finding, researchers began to investigate the possible role of inflammation in tendinopathy.

Recent discoveries in tendinopathy that have recognized the lack of acute inflammatory infiltrate in the late stage tendinopathy patients do not exclude the role of inflammation in the pathogenesis of tendinopathy in its early onset. Increasing evidence suggests that inflammatory mechanisms are activated during the early stage of tendinopathy development that probably contributes to dysregulated homeostasis [197]. One study has found the infiltration of macrophages, mast cells, B and T cells in early supraspinatus tendinopathy in human patients [198]. Also, the presence of CD68 and CD206 markers representing the macrophages has been found in both equine [199] and human [200] tendinopathy in the early and late stage of the tendon disease. A systematic review also revealed increased number of macrophages in four studies, mast cell in three studies, T-cells in one study in tendinopathic tissues compared to healthy control tendons [201].

Previous studies conducted in our lab found that inflammation could be connected with tendinopathy through the non-tenogenic differentiation of TSCs, and high levels of PGE_2 can induce TSCs to differentiate into adipocytes, chondrocytes, and osteocytes, which could be responsible for lipid deposition, proteoglycan accumulation, and calcification in tendinopathic tendons [16, 66, 202].

1.4.5 A proposed inflammation oriented tendinopathy theory

The model Dr. Cook developed was widely approved but did not give consideration to the immune system; the model is not in complete agreement with recent findings suggesting a possible role of inflammation in tendinopathy. Another theory was recently proposed by Dr. Neal Miller based on recent findings related to inflammation in tendinopathy. He suggested that at least three compartments are involved in the development of tendinopathy.

The stromal compartment, mostly tenocytes, may respond to DAMPs or PAMPs and shift into proinflammatory phenotype and interact with leukocytes as a similar behavior has been reported in rheumatoid arthritis synovial fibroblasts (RASFs). The previous study showed that activation of the RASFs resulted in accumulation, survival, and retention of leukocytes at the site of disease [203]. The immune-sensing compartment is composed of resident immune cells since mast cells have been found in both normal tendon and tendinopathic tendon [200]. Mast cells have been found to stimulate tenocytes to produce increased level of COX-2 and PGE_2, as well as elevated MMP1 and MMP7 transcription in 3D collagen lattice, reflecting their potential role in homeostatic and tendinopathy development [204].The infiltrating compartment is comprised of influx immune cells, as increased number of macrophage, mast cells, NK cells, and T cells

have been found in tendinopathy biopsy [201, 205], indicating the important role of infiltrating immune cells in the development of tendinopathy.

1.4.6 Tendon overuse induces inflammatory reactions

Animal models have been widely explored to study the relationship between overuse and inflammation in tendon tissue, and much evidence indicates that overuse of tendon may result in an inflammatory phenotype of the tendon in terms of inflammatory cell infiltration and increased expression of inflammatory markers. For example, a repetitive upper extremity overuse rat model study suggested an accumulation of macrophages and mast cells in rat flexor tendon due to long-term (12 weeks) mechanical loading [206]. Another rat overuse model suggested increased mast cell infiltration in the calcaneal tendon with 7-week treadmill running [207].

IL-1β as an important inflammatory regulator of innate and adaptive immunity was found to be increased due to mechanical stretching of tendon cells, and it down-regulated the expression of collagen type I mRNA which may result in imbalanced ECM turnover in vivo [208]. Another study has shown that overuse with treadmill running led to the production of the potent inflammatory mediator PGE_2 in mouse patellar and Achilles tendons at 3-week time point [66]. Also, repeated high dosage intratendinous injection of PGE_2 caused degenerative changes in mouse tendon [45]. Moreover, peritenodinous injection of PGE_1 resulted in the pathologically thickened tendon at both 1-week (1.34 times thicker) and 4-week (1.25 times thicker) time points. Therefore, it is likely that the development of tendinopathy could involve prolonged unresolved chronic inflammation.

1.4.7 Current knowledge gap: what is the contributing mechanism of abnormal mechanical loading in the onset and maintenance of inflammation in overused tendon?

With the knowledge from several studies, a possible relationship among tendinopathy, tendon mechanical overloading(overuse) and inflammation could be deduced. The repetitive tendon overuse results in inflammation that contribute to the early development of tendinopathy potentially by the prolonged deregulated inflammatory response with subsequent degenerative changes (**Fig. 8**).

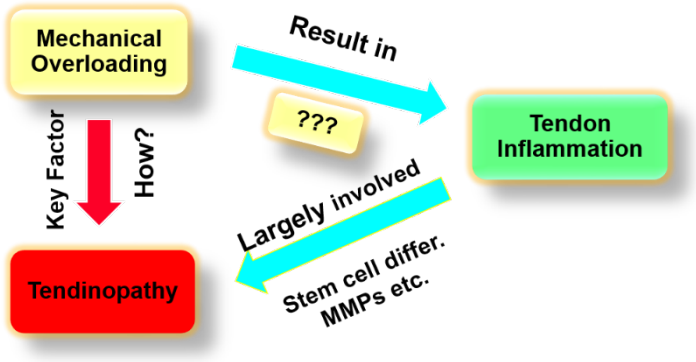

Figure 8. The knowledge gap for the development of tendinopathy. Mechanical overloading of the tendon, or tendon overuse, has long been considered as a risk factor for the development of tendinopathy. It is known that mechanical overloading can result in tendon inflammation. Growing evidence indicates that tendon inflammation is involved in the development of tendinopathy possibly through the non-tenogenic differentiation and breakdown of the matrix with MMPs from inflammation. But how the mechanical overloading as a physical signal triggers the cellular response that invokes tendon inflammation is largely unknown.

However, the mechanism by which the mechanical stress from overuse is converted into the initial inflammatory response is not clear [209]. Literature supported that stromal cells may play a crucial role in the onset and persistence of chronic inflammation [210]. Considering the

lack of such studies in the tendon, another musculoskeletal disease such as rheumatoid arthritis (RA) might be informative in inflammatory responses. RA synovial fibroblasts (RASFs) as a pathological phenotype of synovial fibroblast, could alter ECM synthesis and produce inflammatory cytokines and chemokines such as MCP-4, CCL18, CXCL9, SDF-1, MCP-1 that recruit and maintain leukocytes in the synovial compartment and thus promote chronic inflammation [211].The resident tenocytes in tendon tissue may function similarly, as they are directly subjected to mechanical stress in the overuse scenario, a similar mechanism by which cytokines or inflammatory mediators are released to attract immune cells might operate by tenocytes, thus causing the onset and development of the chronic inflammation in overuse tendon.

Some studies have been performed to fill the knowledge gap. One of them involves heat shock proteins, a member of the DAMPs. HSP72 protein was found to increase in tendon fibroblasts subjected to mechanical stress [212]. HSP 27 and HSP70 were also expressed in the matrix of torn supraspinatus (established pathological model) and matched subscapularis (early tendinopathy model) [213]. However, further studies are needed to determine the role of HSP as an inflammatory mediator in the development of tendinopathy.

Hypoxia is considered as a risk factor of inflammation and apoptosis in early tendinopathy. A recent study found that the expression of hypoxia-inducible factor 1α (HIF-1α) in intact subscapularis tendon (representing early pathology) was elevated compared to matched torn supraspinatus tendon (established pathology) in human patients. Tenocytes under hypoxia showed increased production of proinflammatory cytokines like IL-6, IL-8, MCP-1[214], and another study also suggested that hypoxia contributes to apoptosis of tenocyte that may contribute to the development of tendinopathy [215]. Studies performed in our lab showed that

TSCs might participate in the development of degenerative tendinopathy when exposed to inflammation conditions; specifically, TSCs exposed to high levels of PGE_2, an inflammatory mediator, undergo non-tenocyte differentiation *in vitro*[16, 66].

1.4.8 HMGB1 causes the onset of tendon inflammation due to mechanical overloading

In tendinopathy, external pathogens do not contribute to the development of the disease, because such a tendon injury is usually not associated with an open wound, but it is related to mechanical loading. If the development of tendinopathy is related to inflammation, the signal that invokes inflammation should be internally generated within the tendon.

Damage-associated molecular patterns (DAMPs) are normal intracellular molecules that are released from cells upon cell death or cellular damage [121]. They recruit and activate inflammatory cells in the innate immune system including neutrophils, macrophages, and dendritic cells. Major DAMPs include HMGB1, S100, and heat shock proteins (HSPs) [123], among which HMGB1 is the most widely studied molecule.

HMGB1 is a highly conserved non-histone nuclear protein that binds to DNA and participates in stabilizing nucleosomes and regulates gene expression [130, 170]. It is considered to be present in all types of mammalian cells [130]. HMGB1 possesses unique characteristics; firstly it can be passively released in damaged/necrotic cells (or cell under severe stress) while retained in apoptotic cells. However, S100 and HSPs are released in both cases. The apoptotic cells can modify their chromatin so that HMGB1 binds irreversibly, and therefore it is not released [158]. Secondly, once released from the cytoplasm, HMGB1 participates in triggering inflammatory response through chemotactic activities on immune cells like monocytes, macrophages, neutrophils and dendritic cells [123], as well as on other type of cells that

participate in the wound healing process, including smooth muscle cells [163], and endothelial cells [216], and even certain kind of stem cells [161]. HMGB1 can also be released through active non-classic secretion by immune cells like macrophages. Additionally, HMGB1 is released upon mechanical stress in periodontal ligament tissue and synovial fibroblasts [181, 183]. This particular feature is of great importance as they demonstrated that HMGB1 could be released upon mechanical stress very similar to what tenocytes are subjected to in overused tendon. Extracellular HMGB1 has been studied in kidney, lung, arthritis, sepsis and other diseases for its pro-inflammatory effect [130, 174-177, 217]. HMGB1 has been targeted in multiple preclinical models of sterile diseases, including arthritis, by inhibition of its pro-inflammatory effect [218, 219]. All the features of HMGB1 indicate that it could be an excellent candidate to fill the knowledge gap of how damage signal is generated in response to abnormal mechanical stress and subsequent inflammatory reaction. However, its presence and function have rarely been studied in tendon biology or tendinopathy. To date, there is only a single published report that found higher expression of HMGB1 in tenocyte of the rotator cuff in arthritis patients [220]. However, studies are lacking in terms of the role of HMGB1 as an inflammatory agent in overuse models or clinical tendinopathy.

1.5 GOAL OF THE STUDY AND CENTRAL HYPOTHESIS

The goal of this research is to investigate the origin and inflammatory function of HMGB1 in tendon biology, and its role in overuse tendinopathy model, as well as to explore a therapeutic method to treat or even prevent the development of tendinopathy at the early stage by blocking the effects of HMGB1. The central hypothesis is that extracellular HMGB1 induced by excessive

mechanical loading in overuse tendon model is responsible for the onset of tendon inflammation and early development of degenerative tendinopathy, and inhibition of HMGB1 activity may be beneficial in treatment or prevention of tendinopathy.

To test this central hypothesis, we propose the following three aims:

Aim 1: To demonstrate the presence of HMGB1 in both tendon cells and tissues as well as their ability to release HMGB1 from tendon cells to the extracellular space/matrix after mechanical overloading

Immunofluorescence (IF) staining and Western blotting (WB) were performed to demonstrate the presence of HMGB1 in the tendon. *In vitro* cyclic stretching on tendon cells was conducted to determine HMGB1 release upon mechanical stimuli. An *in vivo* mouse treadmill running model with different intensities was conducted, and HMGB1 released to the tendon extracellular matrix was measured.

Aim 2: To investigate the function of extracellular HMGB1 in triggering the inflammatory reaction in tendon cells *in vitro* and tendon tissue *in vivo*.

The pro-inflammatory *in vitro* effect of HMGB1 on tendon cells was determined by administration of HMGB1 protein to cultured tendon cells. The effects of HMGB1 on cell proliferation and migration were evaluated. Inflammatory factors such as PGE_2, COX-2, and MMP-3 were measured by Western blotting/ELISA.

The *in vivo* inflammatory effect of HMGB1 was tested by administration of recombinant HMGB1 into tendon tissue at various time points. H&E and IHC staining were utilized to

evaluate overall tendon structure and cell density, vessel formation, and inflammatory cell infiltration, as well as COX-2 and MMP-3 presence.

<u>Aim 3:</u> To explore the effect of glycyrrhizin (GL), a direct HMGB1 inhibitor, on preventing of the development of degenerative tendinopathy due to mechanical overloading

In vitro inhibitory effects of GL on HMGB1 were performed with tenocytes and measured for COX-2 and MMP-3 with ELISA and IHC staining.

Mice were subjected to intensive treadmill running for 3-24 weeks with/without treatment with HMGB1 inhibitor glycyrrhizin. ELISA and immunostaining were used to evaluate the short-term (3 weeks) effect of GL in blocking overloading induced the inflammatory effect. The tendon structure and cellular composition were examined by H&E, IHC staining to verify the development of the degenerative model and prevention effect from GL.

In Chapter 2, major finding on the HMGB1 presence in tendon and how HMGB1 release is related to mechanical loading will be presented, as well as studies on the release mechanism of HMGB1 from tendon cells induced by mechanical loading. In chapter 3, the results from Aim 2 will be covered, by analyzing/determining the potential effect of HMGB1 in tendon tissue and cells. In chapter 4, findings from Aim 3 will be presented, and also how GL, a known HMGB1 inhibitor, could reverse the inflammatory and degenerative change of overloaded tendon in short and long term overloading model in mice will be presented. In Chapter 5, extensive discussion of the significance of the findings from this study will be presented with an emphasis on how they fit in with current knowledge in terms of the development of tendinopathy, and how these finding can advance our understanding of tendon biology, pathology, and etiology of degenerative tendinopathy.

2.0 HMGB1 IS PRESENT IN TENDONS AND IS RELEASED UPON OVERLOADING

This chapter will introduce the evidence for the presence of HMGB1 in the tendon, and its release due to mechanical loading. We found that HMGB1 is present in both cells in tendon tissue and cultured tendon cells, while the HMGB1 is not present in the matrix of tendon tissue. HMGB1 is released to extracellular milieu when the tendon cells are being excessively stretched *in vitro* and HMGB1 is released *in vivo* in tendon tissue due to intensive treadmill running in mice. Mechanism of HMGB1 release from tendon cell was studied, and we have shown that mechanical loading -induced HMGB1 acetylation is likely responsible for the translocation of HMGB1 to the cytoplasm and temporary membrane damage may provide the pathway of HMGB1 release into extracellular space from tendon cells.

2.1 HMGB1 IS PRESENT IN NORMAL TENDON TISSUE AND CELLS

2.1.1 Rationale

HMGB1 is a conserved nuclear protein with an important function on genome stability and gene expression, as well as DNA folding. HMGB1 has been reported to be present in most mammalian cell types [130]. It is reported that there are 10^6 HMGB1 molecules in one cell,

which is only an order of magnitude less than the core histone protein[221], which indicates the constructive role of HMGB1 in the cell nucleus. A Previous study reported that HMGB1 is present in the cytoplasm in rat fibroblasts [146]. The normal content of cytoplasm HMGB1 is about 1/30 of that in the nucleus[222], and there is a signaling system that controls the nuclear localization of HMGB1 which involves the modification of two nuclear localization signals on HMGB1 protein[135]. In the normal state, HMGB1 is stored in the nucleus but can translocate to the cytoplasm, or it can be even released from cells when cells are under stress or damaged.

However, neither its presence nor its role has been studied carefully in tendon cells or tissue. Considering HMGB1's fundamental role in maintaining normal cell function, it is highly expected to be present in the tendon cells as well as within tenocytes in tendon tissue.

We first explored the presence of HMGB1 in tendon tissue and cells with Western blotting and immunostaining. **Fig. 9B** shows HMGB1 is present in the total lysate of the patellar tendon (PT) and Achilles tendon (AT), and in both types of tendon cells isolated from the corresponding tendon tissue (**Fig. 9A**). The Western blot bands are generated by an infrared tag with Odyssey Clx infrared imaging system (LI-COR Biosciences, Lincoln, NE). **Fig. 9C** shows that HMGB1 is not present in the normal tendon matrix. The tendon tissue was not processed with detergent to penetrate the cells. So intracellular HMGB1 is not shown in this figure. **Fig. 9D** shows the tendon tissue penetrated with 0.2% Triton X-100 for 30min and stained for HMGB1, which demonstrates that HMGB1 is present in both nucleus and cytoplasm in tenocyte *in vivo*. As we see that no HMGB1 signal was detected in tendon matrix with immunostaining (**Fig. 9C**) but can be found within the tendon cells in the tendon (**Fig. 9D**). This means HMGB1 detected using western blot (**Fig. 9A**) in the tendon tissue lysate come from the tendon cells. At the same time, we found not all tenocytes are positive for HMGB1 *in vivo* (**Fig. 9D**). But in **Figs. 9E, F,**

and **G** show that in normal cultured tendon cells, HMGB1 is almost positive in 100% of the cells and concentrated in the nucleus with minimal staining in the cytoplasm (passage 3, rat Achilles tenocytes). The differential distribution of HMGB1 *in vivo* and *in vitro* will be discussed in detail in chapter 5.

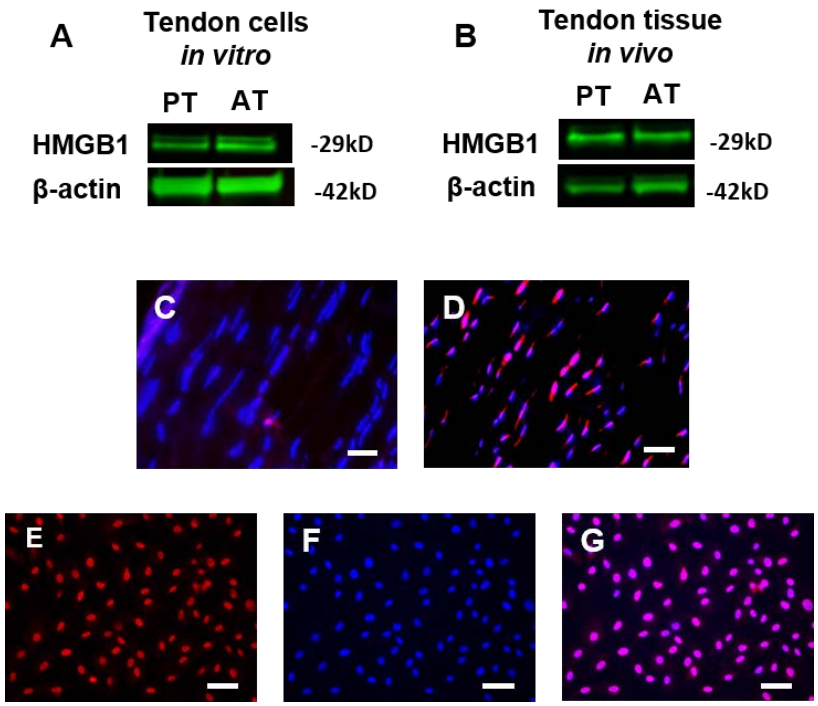

Figure 9. HMGB1 is present in tendon cells in vitro and tendon tissue *in vivo*. (A) Tendon cells isolated from the patellar (PT) and Achilles (AT) tendons show HMGB1 presence *in vitro.* Western blot analysis was performed, and β-actin was used as an internal reference. **(B)** The presence of HMGB1 in both patellar tendon (PT) and Achilles tendon (AT) tissues *in vivo.* **(C)** Tendon tissue stained with HMGB1 without penetration with detergent show that HMGB1 is minimal in tendon matrix. **(D)** HMGB1 staining in the tendon with Triton X-100 penetration shows that HMGB1 is expressed in tendon cell nucleus and cytoplasm. Note that some cells in tendon are negative for HMGB1 staining. (**E**) HMGB1 staining in Achilles tendon cells cultured *in vitro (*red), which overlaps with the Hoechst H33342 stained nuclei (**F**, blue). **(G)** Overlay of both stainings showed the presence of HMGB1 in the nuclei of tendon cells (pink). Bar - 50 µm.

2.2 RELEASE OF HMGB1 FROM TENDON CELLS *IN VITRO*

2.2.1 Rationale

It is regarded that extracellular HMGB1 is not naturally present in normal physiological conditions, but only released from inflammatory or stromal cells through active secretion or passive release under certain stimulation or damage. Active release is mainly found in macrophages and other inflammatory cells upon the stimulation of PAMPs or DAMPs. HMGB1 has known to be passively released from necrotic cells and triggers inflammatory reactions [158]. It can also be released when stromal cells are subjected to severe stress, including hypoxia [223], irradiation, hyperthermia, and hyper pressure [224]. Recently, it has been reported that mechanical loading can induce HMGB1 release in periodontal ligament tissue [181]. Since tendon cells are exposed to highly repetitive mechanical loading conditions *in vivo*, HMGB1 can be stimulated to be released from tendon cells by mechanical loading.

2.2.2 The translocation and release of HMGB1 from nucleus of tendon cells to cytoplasm and extracellular space in vitro in response to excessive mechanical loading

The previous study showed an increased inflammatory mediator PGE_2 release from 8% cyclic stretched tenocytes but not from 4%, compared to non-stretched control [66]. This model was selected for this experimental setting as 8% stretch is considered as excessive loading condition and 4% stretch as moderate loading. To mimic the human activity in the *in vitro* experiment, the stretching frequency was set at 0.5Hz. **Fig. 10A** shows the customized stretching machine used for the *in vitro* stretching, and **Fig. 10B** shows the microgroove surface of the stretching dish

which carries the cells. It was designed to mimic in vivo arrangement of the tendon cells that is displayed in Fig. 10C. Detailed description and discussion of this model and the customized stretching machine can be found in chapter 5.

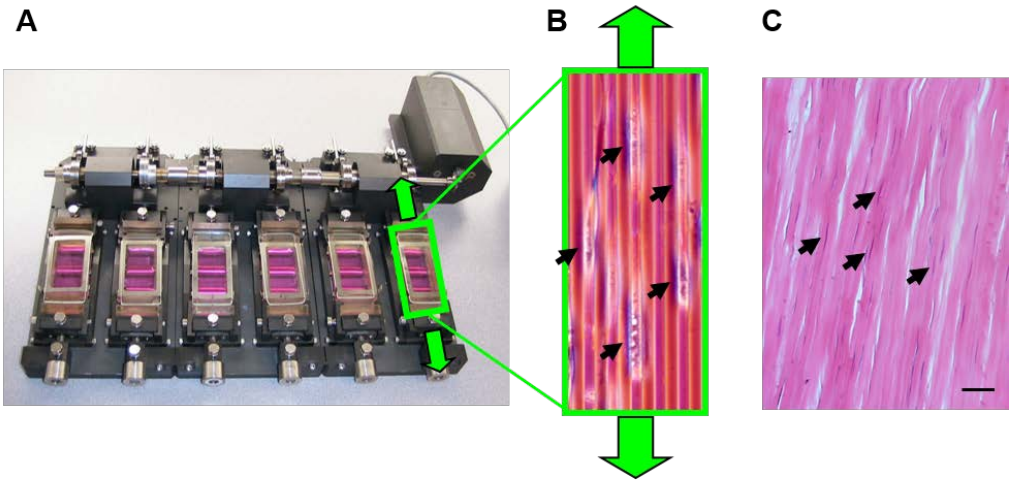

Figure 10. **Custom designed apparatus for cyclical cell stretching *in vitro*.** (**A**) Tendon cells are seeded into microgrooved silicone dishes (green rectangles, **A**) attached to the cyclical stretching machine, with which uniaxial stretching is applied to cultured cells to mimic physiological mechanical loading conditions on tendon tissue. (**B**) Cells are plated and stretched in the center of each silicone dish where the surface strains are uniform. Black arrow points to a tendon cell on the microgrooved culture surface; green arrows show the stretching direction. Tendon cells align along the ridge of the microgrooved surface with the same axis where stretching is applied. (**C**) It is very similar to the physiological condition of the tendon cells *in vivo* shown by black arrows in (**C**) that point to the tendon cells in normal tendon tissue that align along the collagen fibers. Bars - 50 µm

Rat Achilles tendon cells at passage 4 were seeded in the microgrooved dishes. After 72hrs stretching, immunostaining with monoclonal HMGB1 antibody showed that HMGB1 was predominantly expressed in the nuclei of tendon cells in the unstretched control group (**Fig. 11A, D**), as well as in the group subjected to moderate stretching at 4% stretch level for 72hrs (Fig. 11

B, E). In contrast, HMGB1 staining was negative in the nuclei of about 65% tendon cells stretched excessively at 8% strain level for the same duration (**Fig. 11C, F**). This suggests that excessive mechanical stretching triggers the translocation of HMGB1 from the nuclei of tendon cells to the cytoplasm. Semi-quantification of the immunostaining also revealed an ~ 65% decrease in HMGB1 staining in the cell nucleus stretched excessively at 8% strain level (**Fig. 11G**). Of the 65% cells without nuclear HMGB1, about 40% cells showed cytoplasmic staining of HMGB1, and about 25% cells completely lost HMGB1 staining (**Fig. 11H**), which indicated that HMGB1 might have been released to the medium. To test whether HMGB1 is released into the medium from tendon cells, HMGB1 from the supernatants of culture medium in each group was quantified by ELISA. Compared to the control level, HMGB1 level in the 4% stretching group showed no significant change. However, the HMGB1 protein level in the medium of the 8% stretched cells was significantly higher than the control group (2.4 fold higher, $P < 0.05$) (**Fig11. I**). This result confirmed that HMGB1 translocated to the cytoplasm and subsequently released to extracellular space in response to excessive mechanical loading in tendon cells *in vitro*.

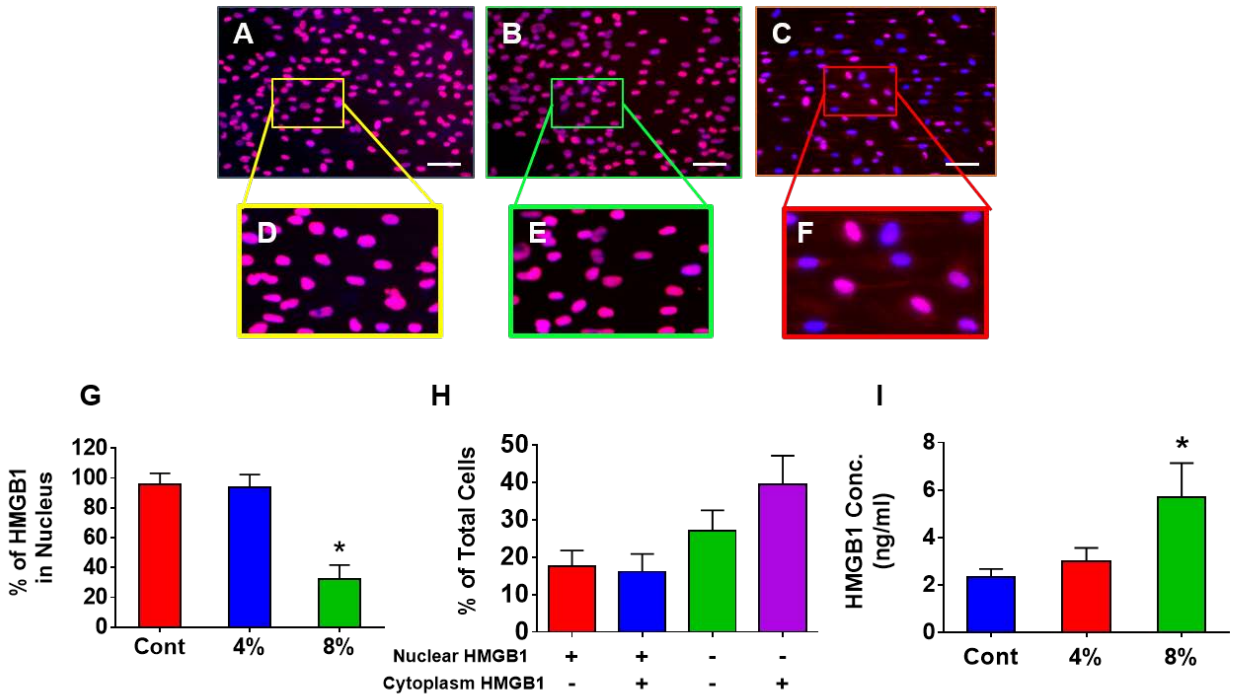

Figure 11. Mechanical overload induces translocation of nuclear HMGB1 to the cytoplasm and extracellular milieu in tendon cells. (A, D) Unstretched control cell nuclei stained positive for HMGB1. (**B, E**) 4% stretched cell nuclei also stained positive for HMGB1. (**C, F**) 8% stretched cell shows sparse positive stain for HMGB1 in the nuclei indicating that it has translocated from the nuclei under excess mechanical load (8%). The majority of cells at 8% stretch show HMGB1 in the cytoplasm, which is not seen in unstretched control or 4% stretch group. **(F)** Cells were immunostained for HMGB1 and counterstained with Hoechst 33342. Data shown are representative of at least two replicate experiments. **(G)** Unstretched control and 4% stretched cells are enriched with HMGB1 positive nuclei (~95%), but in 8% stretched cells, there is ~60% reduction in HMGB1 positive nuclei. **(H)** Distribution of HMGB1 in 8% stretching group. About 17% cells retain HMGB1 in nucleus without HMGB1 in the cytoplasm, and approximately similar percentage of cells are with both nuclear HMGB1 and cytoplasm HMGB1; these cells are considered only slightly affected by the stretching. About 25% cells completely lost HMGB1 in nucleus and cytoplasm, which could have been released to extracellular space. About 40% cells are with cytoplasm staining but not nucleus staining of HMGB1; these cells might be in the process of releasing HMGB1. **(I)** The levels of HMGB1 in unstretched control, 4%, and 8% stretched cells after 3 days of stretching at 0.5Hz. 8% stretch significantly increases HMGB1 levels in culture medium compared to control and 4% stretch when quantified by ELISA. All data are means ± SD. n=6 *****P< 0.05. Bars - 50 µm.

2.3 MECHANISM FOR HMGB1 TRANSLOCATION DURING CYCLIC MECHANICAL LOADING ON TENDON CELLS

2.3.1 Rationale

The mechanism of HMGB1 translocation during excessive mechanical loading was investigated subsequently. Acetylation is a process that when an acetyl functional group is transferred from one molecule to another. In the proteins, it happens to the epsilon-amino group of a lysine residue. Deacetylation is the reverse reaction when the acetyl group is removed from, this two processes are typically enzyme catalyzed in cells, acetylation can be mediated by histone acetyltransferase (HAT), and deacetylation is induced by histone deacetylase(HDAC), even namely histone related, the acetylation targets are not restricted to histones, but also found in transcription factors, other nuclear proteins(i.e. HMGB1), and cytoskeleton, metabolic enzymes and signaling regulators in cytoplasm [154]. Acetylation is considered as a major post-translational modification nowadays, and this process affects protein functions and localizations in cells. [225]

It is reported that acetylation is critical for HMGB1 localization, and hyperacetylation of HMGB1 in monocytes will direct its translocation from nucleus to the cytoplasm [135]. In this study, researchers found that upon external stimulation (i.e. LPS), HMGB1 could be acetylated and will lose the nuclear-localization signal then traffic, and accumulate in the cytoplasm instead of staying in the nucleus in macrophages[135]. When monocytes are stimulated by LPS, HMGB1 is known to translocate to the cytoplasm, then secreted, and deacetylation could block this translocation process through inhibition of HATs [226]. These two studies together suggest that acetylation is involved in HMGB1 translocation in inflammatory cells. We tested this

mechanism in tendon cells and found that HMGB1 translocation is at least partially due to the acetylation induced by the excessive mechanical loading.

During the *in vitro* tendon cell stretching experiment (**Fig. 11**), it is shown that tendon cells that lost HMGB1 in nucleus due to mechanical loading are still firmly attached to the dish surface, unlike necrotic cells which would detach and float in the medium. Based on these results, it is possible that HMGB1 translocated in tendon cells from nucleus to cytoplasm due to the acetylation of HMGB1 that is mediated by excessive mechanical loading and then released to the extracellular milieu.

2.3.2 HMGB1 translocates from nucleus to cytoplasm upon hyper-acetylation

Trichostatin A (TSA), a deacetylase inhibitor, is widely used to inhibit the deacetylation process. As in cells, both the acetylase and deacetylase co-exist and delicately balanced, if one type of enzyme was inhibited, the balance would drastically skew to the other direction, in our experimental setting, the inhibition of deacetylase with TSA will result in hyperacetylation of nuclear and cytoplasm proteins.

The treatment of TSA resulted in HMGB1 translocation in macrophage due to hyperacetylation of HMGB1 in a previous study [135]. We expect that treatment of TSA on tendon cells may lead HMGB1 to translocate to the cytoplasm in normal culture condition.

Rat Achilles tendon cells at passage 4 were treated with 0, 10, and 100 ng/ml TSA for 2hrs, and then immunostained with HMGB1. The results showed a significant increase of HMGB1 staining in the cytoplasm in both 10 and 100 mg/ml TSA treated groups (**Fig. 12B, C**), while HMGB1 was constrained in the nucleus of the non-treated cells (**Fig. 12A**).

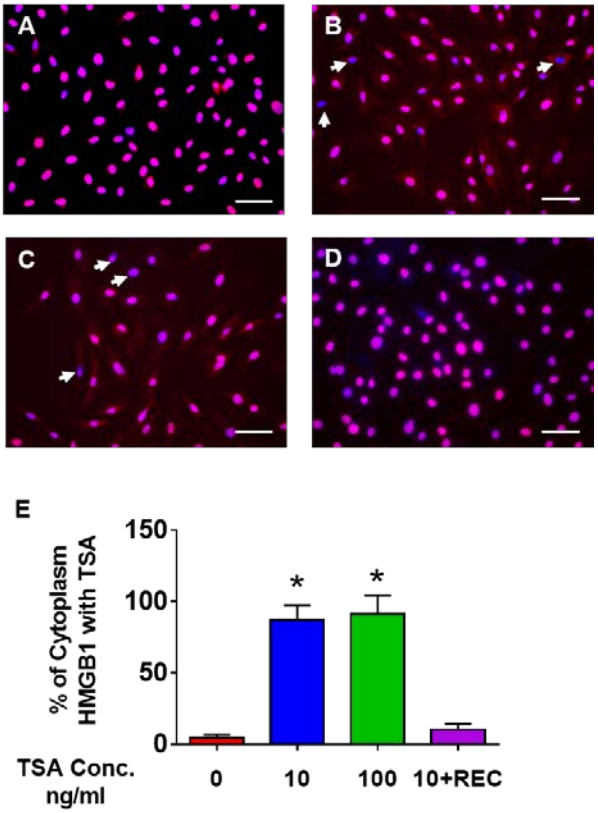

Figure 12. **Inhibition of deacetylation by Trichostatin A (TSA) treatment results in HMGB1 translocation from nucleus to cytoplasm in cultured tendon cells.** Tendon cells (rat AT P4) were treated with 0, 10,100ng/ml TSA for 2hrs and stained for HMGB1. The cells without TSA treatment show HMGB1 signal restricted to the nucleus **(A)** but with 10 ng/ml **(B)** or 100 mg/ml **(C)** TSA treatment show HMGB1 translocation to the cytoplasm in the majority of the cells. When the TSA (10 ng/ml) was removed after 2 hrs treatment and fresh medium was supplied to the cells for 4hrs recovery (10ng/ml+REC), the HMGB1 returns to the nucleus from cytoplasm after 4hrs. This shows that HMGB1 translocation is reversible **(D**). A small percentage of cells (4-5%) with TSA treatment may have completely lost HMGB in the nucleus (arrow in **B, C**). Semi-quantification of cytoplasm HMGB1 positive percentage is from at least 3 replicate wells **(E)**. *P<0.05 compare to control Bars - 50 µm

Interestingly, this effect was reversible when the TSA treated cells were supplied with fresh medium for 4hrs, most HMGB1 translocated back to the nucleus (**Fig. 12C**). However, the

observation that not all HMGB1 translocated from nucleus to cytoplasm indicated that other mechanisms or post-translational modification might be possibly involved.

2.3.3 Mechanical loading-induced HMGB1 translocation is blocked by inhibition of acetylation process

It was reported that LPS mediated HMGB1 translocation in macrophage could be blocked by anacardic acid (AA), which is a histone acetyltransferase activity (HAT) inhibitor [226]. It could block the acetylation process of HMGB1 and result in HMGB1 deacetylation in nucleus and cytoplasm. Now that we found HMGB1 is translocated during the hyperacetylation in tendon cells in section 2.3.2, and excessive mechanical loading can also translocate and release HMGB1, it is reasonable to propose that the translocation of HMGB1 may be mediated by acetylation of HMGB1 in the nucleus. If that is true, blocking the acetylation process during stretching should help to keep HMGB1 in the nucleus of tendon cells.

Similar to the previous stretching experiment settings with the customized stretching machine, the tendon cells were stretched under 8% strain with 0, 5, and 25 μM AA for 72hrs with changes of fresh medium (with the corresponding different concentration of AA) every 24hrs. 100 μM AA caused major cell death. Therefore it was not included in the assay. At the end of the stretching, cells were stained with HMGB1. Immunostaining results show that HMGB1 is expressed in the nucleus in non-stretched cells (**Fig. 13A, E**). , About 55% of tendon cells lost HMGB1 in the nucleus in the stretch only group (**Fig. 13B, F**). HMGB1 was partially retained in the nucleus after stretching with 5 μM and 25 μM AA treatment groups (**Fig. 13 C, D, G, H**). Semi-quantification showed that 70% and 85% cells are with HMGB1 staining in the nucleus with 5 μM or 25 μM AA treatment respectively (**Fig. 13I**).

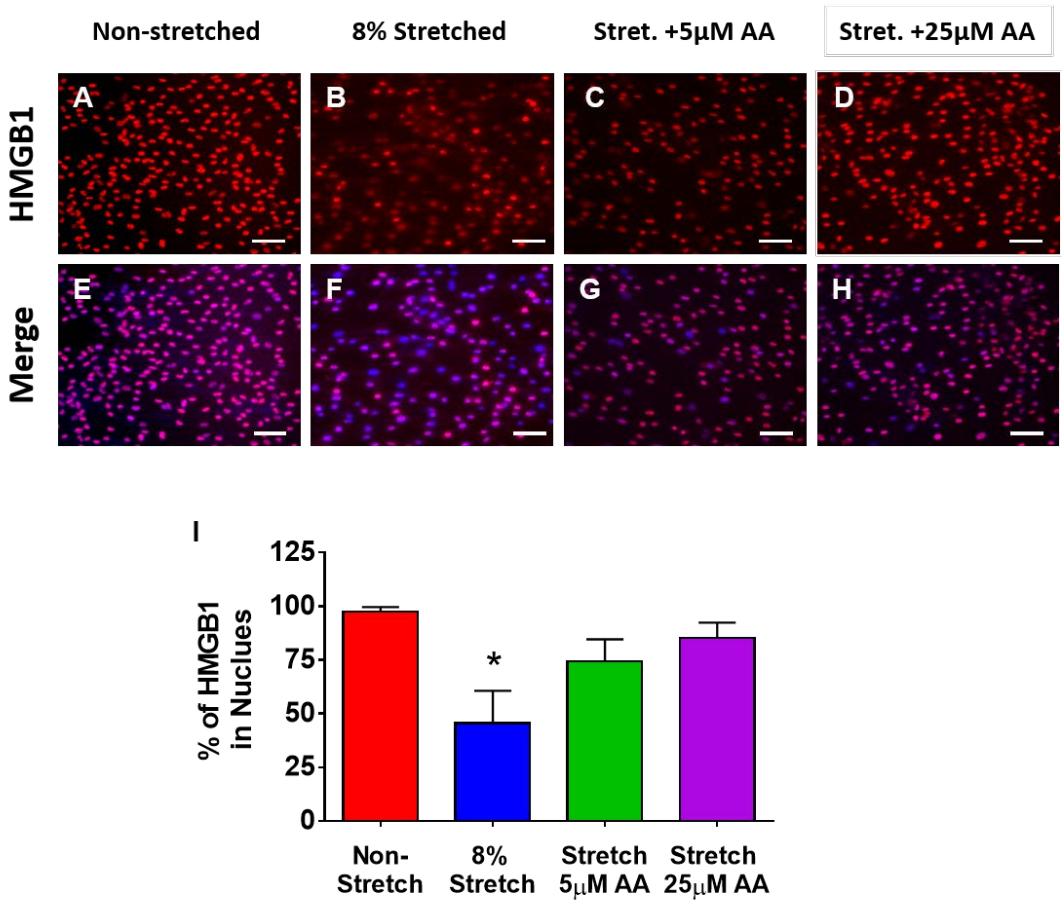

Figure 13. Blocking the acetylation process induced by mechanical loading with Anacardic Acid (AA) prevents the HMGB1 translocation. AA was found to prevent the HMGB1 translocation from macrophage due to LPS treatment. Tendon cells (rat AT P4) were seeded in microgrooved dishes and subjected to 8% stretch for 3 days, and 0, 5, and 25 μm AA were added to culture medium with a change of medium every day. Cells were stained with HMGB1 by the end of day 3. **(A)** In cells without stretching, HMGB1 is located in most nuclei. **(B)** There is a drastic loss of HMGB1 in nuclei after 8% stretching for 3 days. **(C)** AA treatment (5 μm) helps to retain HMGB1 in nuclei in part of the cells after stretching. **(D)** The addition of 25 μm AA prevents more cells from the translocation of HMGB1 induced by stretching than 5 μm. **(E),(F),(G), and (H)** are merged images of **(A),(B),(C), and(D)** with nucleus counter-stained with H33342(sigma). **(I)** Semi-quantification shows HMGB1 positive percentage in cell nucleus in each group (*P<0.05 compared to all other groups). Bars - 100 μm

AA prevents HMGB1 translocation with excessive mechanical loading likely in a dosage-dependent manner. However, some cells are still negative for HMGB1 staining after AA treatment. The result indicated that mechanical loading-induced HMGB1 translocation is mediated by the acetylation of HMGB1 in tendon cells. Blocking the acetylation process may help to retain HMGB1 in the nucleus, and may prevent the release of HMGB1 from the cytoplasm to extracellular space, but the mechanism by which HMGB1 in the cytoplasm is released is not known previously.

2.4 HMGB1 RELEASE MECHANISM FROM TENDON CELLS DURING STRETCHING

2.4.1 Rationale

HMGB1 can be released from cells through active or passive ways; it is widely accepted that active release is restricted to inflammatory cells [227]. HMGB1 is known to be passively released from stromal cells when the cells are damaged or under stress. Tendon cells under mechanical stress translocate and release HMGB1 to extracellular space. In section 2.3, we have discussed the translocation mechanism but how HMGB1 is released after translocated to cytoplasm from the nucleus is still not clear. The two possibilities are that excessive mechanical loading is severe physical stress that might induce temporary damage to the plasma membrane [228], or it could be related to mechanical loading mediated DNA damage-induced apoptosis and secondary necrosis [152, 229]. Or maybe both processes are involved.

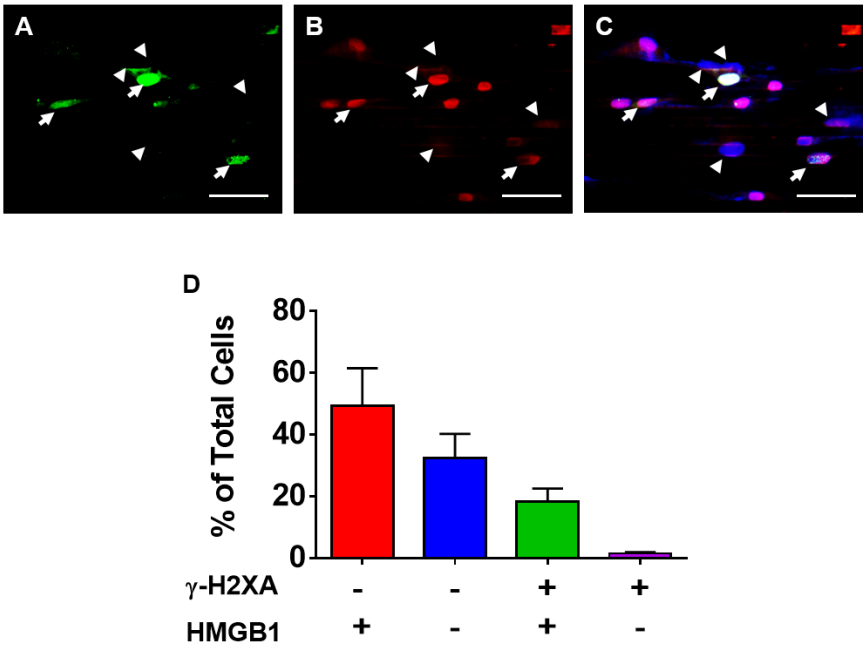

Figure 14. DNA damage is not necessary for HMGB1 translocation from the nucleus. Rat AT tendon cells were stretched for 3 days at 8%, then stained with **(A)** γ-H2AX as a DNA damage marker and **(B)** HMGB1 **(C)** staining images merged with counter-staining of the nucleus with H33342. **(D)** Cells can be divided into 4 different types depending on whether they are positive for γ-H2AX and/or HMGB1 in the nucleus. **a)** γ-H2AX-/HMGB1+(49.3%), which represents the cells in normal status with no DNA damage or HMGB1 translocation, which are not many in this analysis. **b)** γ-H2AX-/HMGB1-(32.3% arrowhead), cells with HMGB1 translocation without DNA damage. A large number of this type of cells indicates HMGB1 translocation without DNA damage. **c)** γ-H2AX+/HMGB1+(18.2%, arrows) cells are with DNA damage but without HMGB1 translocation, which also suggests that DNA damage is not associated with HMGB1 translocation. **d)** γ-H2AX+/HMGB1-(1.5%) type with DNA damage and HMGB1 translocation but is very rare in our experiment. So from the distribution of various cell types, we can conclude that DNA damage is not likely associated with HMGB1 translocation induced by mechanical stretching. Note that cell numbers were counted in 3 different regions of each well, and a total of separate wells were included.

2.4.2 DNA damage is not related to mechanical loading-induced HMGB1 release

To explore the possible DNA damage-related HMGB1 release by mechanical loading, γ-H2AX was utilized as a DNA damage marker. H2AX is a variant of the H2A protein family, which is part of the histone octomer structure, and when DNA damages histones are phosphorylated subsequently, and the newly phosphorylated protein γ-H2AX, is responsible for recruiting and localizing proteins for DNA repair[230]. γ-H2AX was used to visualize DNA damage caused by chemical agents, environmental and physical damage[230]. In this experiment, we employed γ-H2AX as DNA damage marker and tested the possible DNA damage induced by excessive mechanical loading and the relationship between DNA damage and HMGB1 release.

With a similar setting as previously mentioned stretching experiments, tendon cells were stretched 8% for 72hrs, and γ-H2AX and HMGB1 double staining was performed to study the correlation of DNA damage and HMGB1 release. Cells can be divided into four types according to the positive staining of γ-H2AX and HMGB1. The first type of cells that are about 45-50% is the γ-H2AX negative/HMGB1 positive cells. These cells are without HMGB1 release or DNA damage, and this type of cells is considered normal cells. The second type of cells which are about 30-35% is the γ-H2AX negative/HMGB1 negative cells (**Fig. 14 A, B, C** arrowheads). These cells show that HMGB1 could be released without DNA damage. The third type of cells that represented about 15-20% of all cells are γ-H2AX positive / HMGB1 positive (**Fig. 14A, B, C** arrows), which means those cells are with DNA damage, but without HMGB1 release. The fourth type of cells is rare (<3%) with γ-H2AX positive/HMGB1 negative; that should be abundant if DNA damage is associated with HMGB1 release due to mechanical loading. Considering that the cells can release HMGB1 without DNA damage and cells with DNA

damage do not necessarily release HMGB1, we can conclude that DNA damage is not directly related

2.4.3 Temporary plasma membrane damage may contribute to HMGB1 release

It is suggested mechanical loading could introduce temporary disruption of the plasma membrane [228], and this might be the mechanism how the translocated nuclear HMGB1 in tendon cells will be released from cytoplasm to extracellular milieu. To study this possibility, the live/dead cell assay with Propidium Iodide (PI) and Fluorescein Diacetate (FDA) was performed. While FDA is an esterase substrate that can mark the live cells with green fluorescence when hydrolyzed by intracellular esterase, PI is a membrane- impermeable DNA binding agent that generally will not stain live cells. It specifically stains the necrotic cells by binding to its DNA through the compromised membrane. Hence, the special feature of PI is very helpful in our experimental settings as it could go through the plasma membrane if the membrane is temporarily disrupted. So, by using PI and FDA double staining, we can identify live cells that had temporary membrane disruption, which may help explain the HMGB1 release mechanism. It is reasonable to propose that during excessive mechanical stretching, the tendon cell membrane will be temporarily disrupted and will allow diffusion of PI into tendon cells.

Tendon cells are stretched with 8% strain following the previous stretching protocol for 6hrs. 20 μg/ml PI were added at the beginning of the experiment and 10 μg/ml FDA were added at the end of the stretching to make sure cells survived the stretching was measured. After addition of FDA, cells were then kept still for 1hr before counter-staining with H33342 and then pictures were taken.

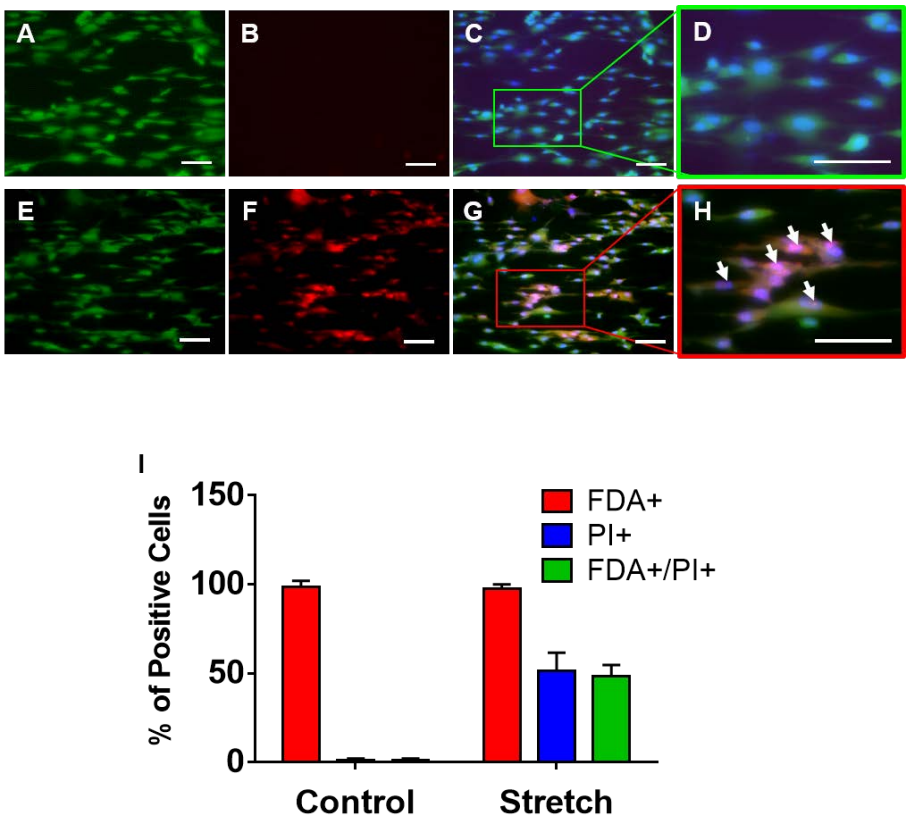

Figure 15. Mechanical loading-induced plasma membrane damage but not cell death may explain HMGB1 release. Tendon cells were seeded in microgrooved silicone dish with or without 8% stretching for 6h, and Fluorescein Diacetate (FDA, 10 µg/ml) and Propodium Iodide (PI, 20 µg/ml) were added to the culture medium at the beginning of the stretching. Cells without stretching **(A)** or with stretching **(E)** are positive for FDA derived green fluorescence signal indicating live cells. Control cells are negative for PI **(B)**, but most cells in the stretch group **(F)** is positive for PI indicating that they may be subjected to temporary plasma membrane damage. **(C, G)** show merged images of FDA derived signal, PI and nuclear counter-staining with H33342. **(D, H)** enlarged figures to show positive staining of PI in stretching group (**H**, arrows) but not in control group **(D). (I)** Semi-quantification shows ~50% cells after stretching are positive for both FDA derived signal and PI. PI detects dead cells with plasma membrane leak and binds to DNA, and the reason for the live cells with PI signal is that the temporary and reversible damage of plasma membrane allow PI to enter through the membrane. HMGB1 may diffuse to extracellular space through the temporary cellular plasma membrane damage. Bars - 50 µm

We found that in the control group without stretching, the green signal (which come from the processed FDA in live cells) was very strong, and almost all cells were positive for green fluorescence signal (**Fig. 15A**), and this also holds true for the stretched cells (**Fig. 15B**). As expected, the red signal of PI in the control cells is minimal (**Fig. 15C**), but is strong in stretched group (**Fig. 15D**), and **Fig. 15H** clearly shows that the PI staining inside the nucleus of green signal positive cells.

Semi-quantification indicates that after stretching, about ~50% cells are positive for both PI and green signal (**Fig. 15I**), which means those were live cells subjected to temporary plasma membrane damage during the stretching. This implied that HMGB1 could be released during the temporary disruption of plasma membrane induced by excessive mechanical loading.

In summary (sections 2.3 and 2.4), *in vitro* HMGB1 was translocated from nucleus to cytoplasm and released from tendon cells due to excessive mechanical loading. This process is regulated by acetylation of HMGB1 and temporary disruption of plasma membrane when tendon cells are subjected to mechanical stress.

2.5 *IN VIVO* HMGB1 RELEASE INDUCED BY MECHANICAL OVERLOADING IN TENDON TISSUE

2.5.1 Rationale

Having established the translocation and release of HMGB1 *in vitro* by mechanical overload and explored the related mechanism, next question would be whether mechanical overloading induces the release of HMGB1 in the tendon *in vivo*. Mouse treadmill running was utilized as an *in vivo* model, since the previous study in our laboratory has found that intensive treadmill running resulted in an inflammatory reaction, indicating that tendon tissue damage might be induced in this model[66].

2.5.2 HMGB1 is released to extracellular matrix after long-term or one-time intensive treadmill running

8-week old C57BL/6 mice were divided into 4 groups, 1) a cage control group without any treadmill running (cage control), 2) a moderate treadmill running group (MTR), 3) an intensive treadmill running group (ITR), and 4) a one-time fatigue treadmill running group (OTR). The running regimens are presented in **Table 1**. All the running mice received 1-week acclimatization training with 15 min running for 5 days before the individual treadmill running regimen started.

Table 1 Treadmill Running Protocol

Group	Description
Cage Control	**Cage Activity**
Moderate Treadmill Running (MTR)	3 weeks(5 days/week) 15m/min for **50min**
Intensive Treadmill Running (ITR)	3 weeks(5 days/week) 15m/min for **3h**
One-time Fatigue Treadmill Running(OTR)	One time treadmill until mouse fatigue Mean running time 5h

Achilles tendon sections of the cage control group showed the presence of tendon cells that stained blue with Hoechst 33342, but the tendon matrix was not stained red indicating the absence of HMGB1 in tendon matrix both in the mid-portion and near insertion site of the tendon (**Fig. 16 A, B, C, D**). The tissue sample was not penetrated with detergent to show the HMGB1 signal only in the matrix. The 2x magnification of **Fig. 16A,** that is **Fig. 16B,** clearly shows the blue staining of cells, but the absence of red staining in the matrix; this also applies to the tendon matrix near the insertion site (**Fig. 16C, D**). Some peripheral areas that appear red are not tendon tissues, but are paratenons (**Fig. 16. A, C, E,** arrows**)**. In the tendon sections from mice on the moderate treadmill running (MTR**)** regimen, HMGB1 staining was absent in the tendon matrix of the mid-portion and near insertion site(**Fig. 16 E, F, G, H**). However, a marked increase in HMGB1 staining was observed in the tendon matrix of mice on the intensive treadmill running (ITR) regimen (**Fig. 16 J, K, L, M**). A 2x magnification of **Fig. 16J** shows clear red staining that is positive for HMGB1 (**Fig. 16K)**.The HMGB1 staining is present outside the tendon cells and in the tendon matrix (**Fig. 16J,** arrowheads).

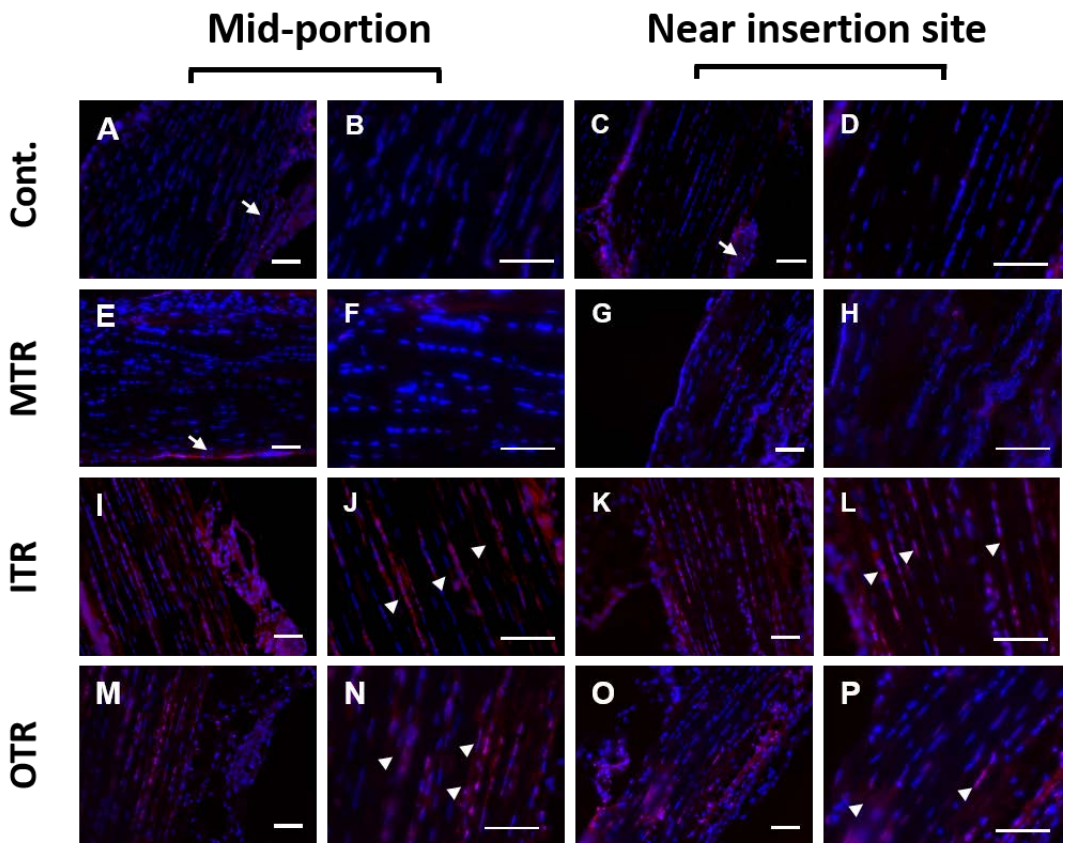

Figure 16. Mechanical overloading increases HMGB1 levels in Achilles tendon ECM in both mid-portion and near the insertional site. Cell nuclei in all figures show negative stain for HMGB1 because sections were not permeabilized with detergent. **(A)** Mid-portion of control Achilles tendon (AT) shows minimal HMGB1 staining in tendon matrix. (**B**) 2x magnification of **A** clearly shows the absence of HMGB1 staining in the matrix. **(C)** Tendon tissue in control AT near insertional site is negative for HMGB1. **(D)** 2x magnification of **C**. (**E**) A representative tendon section from moderate treadmill running (MTR) group showing no positive stain in the matrix. **(F)** 2x magnification of E showing negative for HMGB1. **(G)** HMGB1 near the insertional site of MTR AT (**H)** 2x of **G.** Both **G** and **H** are negative for HMGB1 near the insertional site of MTR AT. (**I**) Tendon matrix shows strong positive stain in the intense treadmill running (ITR) group indicating that HMGB1 has released to the matrix. (**J**) 2x magnification of **I** clearly shows positive stain in the matrix. Arrows point to positive staining. **(K)** HMGB1 is positive near the insertional site. **(L)** 2x of **K** shows HMGB1 positive staining more clearly. (**M**) Similar HMGB1 positive staining in the matrix of tendon mid-portion section from one-time treadmill running (OTR). **(N)** 2x of **M**. **(O)** HMGB1 positive staining near the insertional site. **(P)** 2x of **O**. Arrowhead points at the Positive HMGB1 site. Control mice were allowed cage activity without running, MTR running regimen was 50min/day and ITR 3h/day, 5 days/week for 3 weeks, and OTR was more than 5h until fatigued at a running speed of 15 m/min. The sections were not permeabilized with detergent to avoid the release of HMGB1 from the nuclei. Also, the pink stains observed in the periphery of **A, C,** and **E** (arrows) are not tendon tissues but may be paratenon or adjacent connective tissue. Data are the representatives from two independent experiments (n=6 each group) of AT sections. Bars - 50 µm.

HMGB1 is also expressed in the matrix of the tendon tissue near the insertion site(**Fig. 16L, M**).The same increasing trend for HMGB1 staining was observed in the tendon matrix of mice on the OTR regimen in the mid-portion and near insertion site(**Fig. 16 M, N, O, P,** arrowheads).

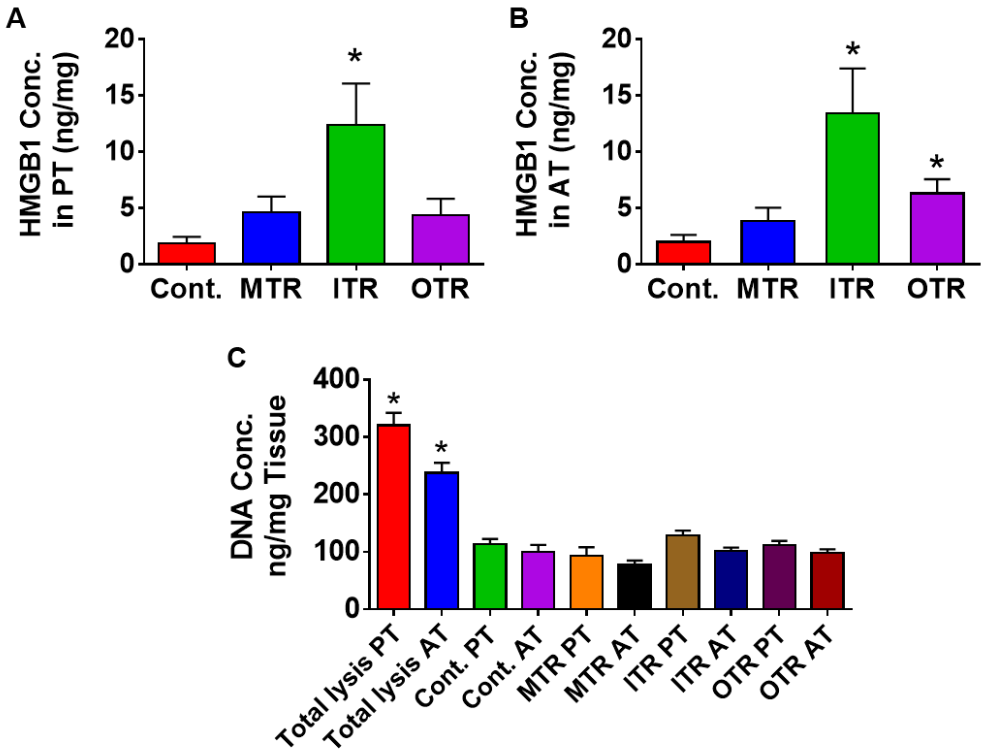

Figure 17. HMGB1 content is increased in tendon matrix in mechanically overloaded tendons. (**A**) Intense and one-time treadmill running (ITR and OTR) significantly increases HMGB1 levels in Achilles tendons (AT) compared to control. There is no significant change in moderate treadmill running (MTR) group. (**B**) ITR significantly increases HMGB1 levels, and OTR and MTR do not significantly alter HMGB1 levels in patellar tendons (PT) compared to control. HMGB1 content is normalized to tissue weight. Data represent mean ± SD. n = 6. *$P < 0.05$. (**C**) DNA content is same in control and treadmill running groups in the homogenized tendon sample indicating that the high levels of HMGB1 in ITR and OTR group are not from the damage of cells since live cells contain HMGB1 in the nuclei.

This finding was also confirmed by ELISA measurement of HMGB1 levels in mice Achilles and patellar tendon matrices (AT & PT respectively) subjected to mechanical loading protocols (**Fig. 17 A, B**). Those tendon samples were mildly processed to avoid destruction of cells which might create a high background for HMGB1 measurement in matrix only.

Compared to the control level, both AT and PT in MTR groups did not show a significant difference in HMGB1 when quantified using ELISA (**Fig17. A, B**) However, in mice that were on ITR regimen, the maximum amounts of HMGB1 were observed in both tendon tissues. Specifically, HMGB1 levels were 6.6 fold higher in Achilles tendon, and 6.8 times higher in patellar tendon of ITR group when compared to the control mice (**Fig. 17 A, B**). HMGB1 levels were also significantly higher in the Achilles tendon tissues of OTR mice (3.2-fold higher; $p < 0.05$) when compared to the control, while patellar tendon tissues showed a 2.32-fold change compared to control without statistical significance (**Fig. 17A, B**). These results indicated that only excessive mechanical loading conditions**,** ITR and OTR triggered the release of HMGB1 from the tendon cell nuclei into the tendon matrix.

2.5.3 Measurement of tendon lysate DNA content shows minimal damage of cells in processing TR tendon samples

The tendon samples for the measurement of HMGB1 concentration using ELISA were prepared in a gentle manner by only mincing and soaking them in PBS. It is still possible that the intracellular HMGB1 in tendon cells may be released during the preparation process and “contaminate” the extracellular HMGB1 that we were measuring. It is known that HMGB1 in the damaged cells releases together with the DNA. To confirm that the higher HMGB1 concentration in ITR and OTR groups is not greatly affected by the “contamination” from

intracellular HMGB1, the total DNA content was measured in all samples as indicators of cell destruction and “contamination” gauge.

It is found that all the samples are with equivalent and significantly lower DNA content than total lysis tendon sample with T-PER lysis buffer and vigorous homogenization using BioMasher(Takara, Japan)(**Fig. 17C).** This result helped to explain the baseline concentration of HMGB1 found in the control and MTR group, and also validate that the increased HMGB1 measurement in ITR and OTR are due to the effect of repetitive mechanical loading-released HMGB1 in the tendon matrix.

2.5.4 Intensive treadmill running results in inflammatory cell infiltration in Achilles tendon

Since extracellular HMGB1 is the cause of sterile inflammation in many organs[231], and it is known to function as a chemoattractant for inflammatory cells, and the tendon proper is largely avascular, long-term repetitive loading may still result in the establishment of HMGB1 level gradient and induce inflammatory cell infiltration in the tendon. Clinically, inflammation is not apparent in degenerative tendinopathy [55, 232]. However, since most patients seek medical intervention usually at their late stage of tendinopathy, inflammation could still be there and play a crucial role during the early development of the disease and could be easily overlooked, and inflammation could take place long before the symptoms appear. Therefore, signs of inflammatory cells in the intensive treadmill running tendons could be helpful to explain the development of tendinopathy in the long run.

The result showed that immunohistochemical staining for the inflammatory cell marker, CD68, was absent in the cage control group (**Fig. 18A, E**) and in both mid-portion and near

insertion site of MTR group (**Fig. 18B, F**). However CD68 signal was clearly visible in the Achilles tendon sections of mice on the ITR regimen, and positive in both mid-portion and near the insertion site (**Fig. 18C, G**). The induction of inflammatory cell infiltration by ITR implies that HMGB1 may invoke the inflammatory reaction in the tendon (arrowheads, **Fig. 18C, G**). CD68 was found negative in OTR regimen **(Fig. 18D, H).** Collectively, these data show that excess mechanical loading induces significant HMGB1 release into the Achilles tendon matrix resulting in inflammatory cell infiltration in the long run.

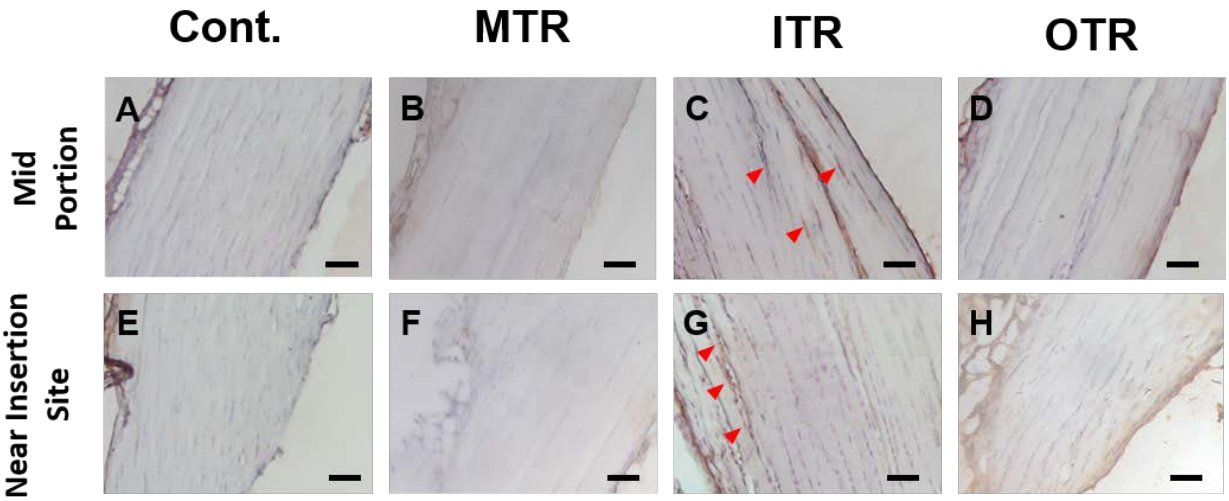

Figure 18. Inflammatory cells infiltrate in long-term mechanical overloaded tendons (ITR) but not in one-bout excessive loading (OTR). Treadmill running mouse ATs were stained with CD68 for inflammatory cells like macrophages and monocytes. In Mouse AT without running, neither the mid-portion of the tendon **(A)** nor the insertional site towards heel bone **(E)** is stained positively with CD68. **(B)** MTR group mice's AT is similar with cage control group showing no CD68 staining within the tendon mid-portion tissue and **(F)** near the insertional site. **(C)** AT from ITR group with several positive spots for CD68 in both the mid-portion and **(G)** near the insertional site. **(D, H)** CD68 is not positive in OTR group in both mid-portion and near the insertion site. Note that the brown at the edge of the tendon in adjacent soft tissue is staining artifacts, possibly because of trapped antibody there.

2.6 MATERIALS AND METHODS

2.6.1 Tendon cell isolation and culture

Tendon cells were isolated from patellar and Achilles tendons of 3-4 months old female Sprague Dowley (SD) rats weighing 200-250 g. Briefly, tendons dissected from rats were minced into small pieces and digested by incubating in phosphate-buffered saline (PBS) containing 3 mg/ml collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) and 4 mg dispase (StemCell Technologies Inc., Vancouver, BC, Canada) at 37°C for 2-6 hrs. The digest was then filtered through a 70 µm filter to remove tissue residue and centrifuged at 700 g for 10 min. The cell pellet was resuspended in 20% Fetal Bovine Serum (FBS, Atlanta Biologicals, Flowery Branch, GA) in Dulbecco's modified Eagle's medium (DMEM, Lonza Group, Basel, Switzerland) with 100 U/ml penicillin and 100 U/ml streptomycin. Finally, the cells were plated in T-25 or T-75 flasks and cultured at 37°C in the presence of 5% CO_2. Cultured tendon cells were split when 80% confluence was reached. For all experiments, cells in passages 3-5 were used.

2.6.2 Immunostaining of tendon cells

Tendon cells ($1x10^5$ cells/well) were seeded in 12-well plates and allowed to grow to 70-80% confluence at 37°C in the presence of 5% CO_2. Then, the culture medium was removed, and the cells were fixed in 4% paraformaldehyde for 10 min and treated with 0.1% Triton-X in PBS to allow antibodies to penetrate the nuclear membrane for an effective immunostaining. The cells were then incubated with rabbit anti-HMGB1 antibody (1 µg/ml,ab18256, Abcam, Cambridge,

UK) at 4°C overnight, followed by a 2hrs incubation at room temperature with goat anti-rabbit secondary antibody conjugated with Cy3 (1 μg/ml, EMD Millipore, Billerica, MA, AP132C). Cell nuclei were counterstained with Hoechst 33342 (10 μg/ml Sigma, St. Louis, MO, B2261) and were visualized with an inverted fluorescence microscope (NIKON, Tokyo, Japan) equipped with a CCD camera. The percentage of cells that stained positive by immunostaining was calculated by semi-quantification. Briefly, five random images were first obtained per well using the SPOT™ imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI). Then, the percentage of positive staining was calculated by dividing the total area viewed through the microscope by the positively stained area. These values were averaged to represent the proportion of cells that stained positive for HMGB1.

Immunostaining for cells in stretching silicone dish is similar as described above, and the staining of γ-H2AX together with HMGB1, monoclonal mouse anti- γ-H2AX antibody was used (1 μg/ml, ab26350, Abcam, Cambridge, UK). 2nd antibody reaction was conducted with goat anti-mouse secondary antibody conjugated with FITC(1 μg/ml, EMD Millipore, Billerica, MA, AP124F) was utilized.

2.6.3 Western blot analysis of HMGB1 in tendon tissue and cells

Total protein was extracted from rat Achilles and patellar tendons using the protein extraction reagent (ThermoFisher, Pittsburgh, PA). After quantification, 20 μg of total protein from each tendon sample was separated on a 10% SDS-PAGE, transferred onto a nylon membrane and incubated with rabbit anti-HMGB1 primary antibody followed by goat anti-rabbit infrared tag conjugated secondary antibody (LI-COR Biosciences, Lincoln, NE) following the

manufacturer's instructions. Positive signals were detected via the Odyssey CLx infrared imaging system (LI-COR Biosciences, Lincoln, NE).

2.6.4 *In vitro* mechanical stretching experiment

Mechanical loading was applied to tendon cells *in vitro* using our customized mechanical loading device (**Fig. 10**), which can apply repetitive uni-axial loading on cells in silicon dishes. The micro-grooved silicon dishes in the loading device were sterilized and coated with 100 µg/ml collagen solution (Stem Cell, Vancouver, Canada, 04902) for 2hrs at room temperature to facilitate cell attachment to the dish surface. The dishes were washed with PBS 3 times; cells were then plated in the silicon dishes at a density of $0.5X10^5$ per dish accounting to about 50% confluence in 20% FBS DMEM medium. Cells were allowed to attach to the dish surface overnight followed by 4% or 8% stretching at 0.5Hz for 3 days according to our previous published protocol. Moreover, these stretching magnitudes represent moderate (4%) and excessive (8%) mechanical stretching on cells [66, 233]. Un-stretched cells served as controls.

2.6.5 Translocation of HMGB1 with TSA treatment in tendon cells

Rat tendon cells were seeded as described above, and after leaving them overnight for attaching, the medium was removed, and 10% FBS DMEM was added. Then 0, 10, 100ng/ml TSA was added to the culture medium for 2hrs. Half of the cells were fixed immediately, and for the other half, the medium with TSA was removed, and fresh medium with 10% FBS was supplied, after 4hrs in fresh medium. All the rest of the cells were fixed with 4% PFA for 10min and proceeded

to perform HMGB1 immunostaining in the same way as mentioned above. Each condition was done in triplicate.

2.6.6 Blocking HMGB1 translocation induced by mechanical loading with anacardic acid in tendon cells

A total of $5X10^4$ rat tendon cells were seeded in 12 microgrooved silicone dishes, and 0, 5 μM, 25 μM, and 100 μM AA were added to the 10% FBS DMEM culture medium of 4 dishes. The groups with AA addition received a change to fresh medium with the corresponding concentration of AA every 24hrs to keep the AA concentration at a stable level, and the stretching was done 8% at 1Hz for 72hrs. Immediately at the end of the stretching, cells were fixed with 4% PFA, and HMGB1 staining was conducted as described above.

2.6.7 *In vivo* mouse treadmill running model

In total, 48 mice were used for the *in vivo* treadmill running experiments and were divided into four groups with 12 mice in each group. The control mice remained in cages and were allowed cage activities. The remaining three groups ran on the treadmill but at different intensities; i) moderate treadmill running (MTR), ii) intensive treadmill running (ITR), and iii) onetime treadmill running (OTR). The running speed for all regimens was 15 meters/min. In the 1st week, mice were trained for 15 min to acclimatize them to the treadmill running protocol and environment. In the following 3 weeks, mice in the MTR group ran for 50 min, and those in the ITR group ran for 3hrs a day, 5 days a week. Mice in the OTR ran for more than 5h until fatigue. The performance of the mice (i.e. running time) was recorded to recommend inclusive/exclusive

criteria. The treadmill running experiment was done twice independently to record variability. Immediately after running, the Achilles and patellar tendon tissues were harvested from the 4 mice groups. Half of the samples were used for ELISA tests, and the remaining half was used for immunostaining.

2.6.8 ELISA assay of HMGB1 in cell culture medium

In this study, cell culture medium was collected after the stretching experiment and centrifuged at 3000g for 10min; the supernatant was collected for HMGB1 level measurement (Shino-Test Corporation, Tokyo, Japan, ST51011) according to manufacturer's instructions. All samples were analyzed in duplicates. The kit used “sandwich” ELISA method.

2.6.9 ELISA assay and immunostaining of HMGB1 in tendon tissue

For tendon tissue ELISA, mice Achilles, and patellar tendon tissues were weighed, minced and soaked in PBS (about 200 µl) for 2hrs in room temperature allow HMGB1 diffuse from the minced tissue to the solution. This is considered gentle homogenization in order to keep the tendon cells intact. This was done to prevent intracellular HMGB1 leaking during the preparation process which may interfere with the detection of extracellular HMGB1. This process allowed precise quantification of HMGB1 that was released to the extracellular space. The samples were then centrifuged at 2,000 g for 30 min at 4°C, and the supernatants were collected to measure HMGB1 concentrations using ELISA kit (Shino-Test Corporation, Tokyo, Japan, ST 51011) according to the manufacturer's instructions. All samples were analyzed in duplicates.

For all HMGB1 immunostaining, Achilles and patellar tendons dissected from the mice were immediately immersed in O.C.T compound (SAKURA FINETEK USA INC, Torrance, CA) in disposable molds and frozen at -80°C. Then, cryostat sectioning was performed at -25˚C to obtain about 8 µm thick tissue sections, which were fixed in 4% paraformaldehyde for 1 min and blocked with universal blocking solution (ThermoFisher Scientific, Pittsburgh, PA, 37515). The sections were then incubated with rabbit anti-mouse HMGB1 antibody (1 µg/ml, Abcam, ab18256) at 4˚C overnight followed by goat anti-rabbit secondary antibody conjugated with Cy3 for 1hr at room temperature (0.5 µg/ml, Millipore, Billerica, MA, AP132C), then counterstained for the nucleus with Hoechst 33342(10 µg/ml.ThermoFisher, Pittsburgh, PA, H3570). Since the purpose of this staining was to evaluate the presence of HMGB1 in the extracellular milieu, the tissue sections were not treated with the penetration reagent-Triton X-100 that permeates the nuclear membrane. HMGB1 levels in each tendon sample were normalized to the corresponding tissue weight.

2.6.10 Measurement of DNA concentration in tendon lysate

Tendon lysate samples from treadmill running mice were collected as described above. The samples were gently processed as mentioned in section 2.6.9 to avoid destruction of the cell membrane. Total lysates from Achilles and patellar tendons were prepared with T-PER tissue protein extraction reagent (ThermoFisher, Pittsburgh, PA, 78510) instead of PBS. The samples were weighed and vigorously homogenized with BioMasher Standard (Takara, Shiga, Japan, 9790A). The DNA content was measured using DNA quantitation kit with bisBenzimide (Sigma, St. Louis, MO, DNAQF) according to the manufacturer's instruction, and the final result was normalized by the corresponding tissue weight.

2.6.11 FDA and PI staining for stretched cells

Cells ($5X10^{5,}$ rat AT P4) were seeded in the microgrooved dish as described previously and stretched at 8% strain level for 6hrs with 20 µg/ml PI in 10% FBS DMEM medium, and 10 µg/ml FDA were added to the culture medium at the end of the stretching and left for 1hr. A non-stretched group of dishes was utilized as the control. The cells were washed with PBS and immediately counter-stained with H33342 10 µg/ml for 5 min without fixation and pictures were taken similar to the procedure for immunostaining.

2.6.12 Statistical Analysis

Student's t-test and One-way ANOVA was used, followed by Fisher's least significant difference (LSD) test for multiple comparisons. When P-values were less than 0.05, the two groups compared were considered to be significantly different.

3.0 INFLAMMATORY EFFECT OF HMGB1 ON TENDON *IN VITRO* AND *IN VIVO*

In the previous chapter, a series of evidence were drawn to show the presence of HMGB1 in tendon cells, as well as its release upon excessive repetitive mechanical loading *in vitro* and *in vivo*. The next step is to explore the physiological and pathological function of HMGB1 in terms of its effects on tendon cells and tendon tissue. HMGB1 is known to be a pro-inflammatory cytokine with chemoattractant properties for inflammatory cells [153, 158], endothelial cells [216, 234, 235] and stromal cells [236, 237]. Previous studies reported that HMGB1 could attract blood-derived stem cells [238] and promote wound healing by activating fibroblasts *in vitro* [236]. Therefore, it is reasonable to propose that HMGB1 can invoke inflammatory reaction by attracting inflammatory cells, induce angiogenesis, and may activate resident tendon cells in tendon tissue. However, tendon proper is highly avascular in nature [27] and constantly subjected to large mechanical loads. Its cellular response to HMGB1 might be different from other types of tissues. The effect of HMGB1 on tendon cells has not been studied yet. This chapter presents the mechano-biological responses of tendon cells *in vitro* by examining proliferation, migration, and the effects of HMGB1 in tendon tissue with *in vivo* HMGB1 implantation method, e.g. angiogenesis, infiltration of inflammatory cells, etc. We found that HMGB1 did not stimulate the proliferation of tendon cells alone *in vitro* and had minimal angiogenesis potential, but promoted tendon cell migration. However, in *vivo* experiments, we found that HMGB1 implantation induced inflammatory cell infiltration, angiogenesis, and cell

proliferation in tendon tissue. The reason behind the differential response *in vivo* compared to the *in vitro* situation could be due to the presence of multiple types of cells like macrophages, and endothelial cells that may be simultaneously acting *in vivo*. Detail discussion about the different effects can be found in chapter 5.

3.1 HMGB1'S EFFECT ON TENDON CELLS AND ENDOTHELIAL CELLS *IN VITRO*

3.1.1 HMGB1 Alone does not promote proliferation of tenocyte *in vitro*

First, we evaluated the proliferative effect of HMGB1 on tendon cells. In clinical settings, some tendinopathic patients show signs of hypercellularity in the affected area which are represented by the abnormally high density of cells [239]. It is possible that the HMGB1 released from mechanical overloading can induce proliferation of tendon cells through cytokines secreted by the recruited of macrophages. The direct effect was tested with *in vitro* tendon cell model.

Briefly, cultured rat tendon cells were exposed to concentrations ranging from 0.1 to 10 μg/ml of HMGB1 in DMEM medium without FBS. Tendon cells exposed to 0% FBS without HMGB1 in the medium was set as the negative control, tendon cells with 2% or 20% FBS supplied medium were used as positive control. After 48hrs, total cell numbers were measured by CCK-8 kit (Sigma), which is based on a similar mechanism as MTT assay as a colorimetric assay for the determination of the number of viable cells. The results showed that HMGB1 did not increase tendon cell number at any of the concentrations tested (0.1, 1, 10 μg/ml) compared to the negative control (**Fig. 19A**).

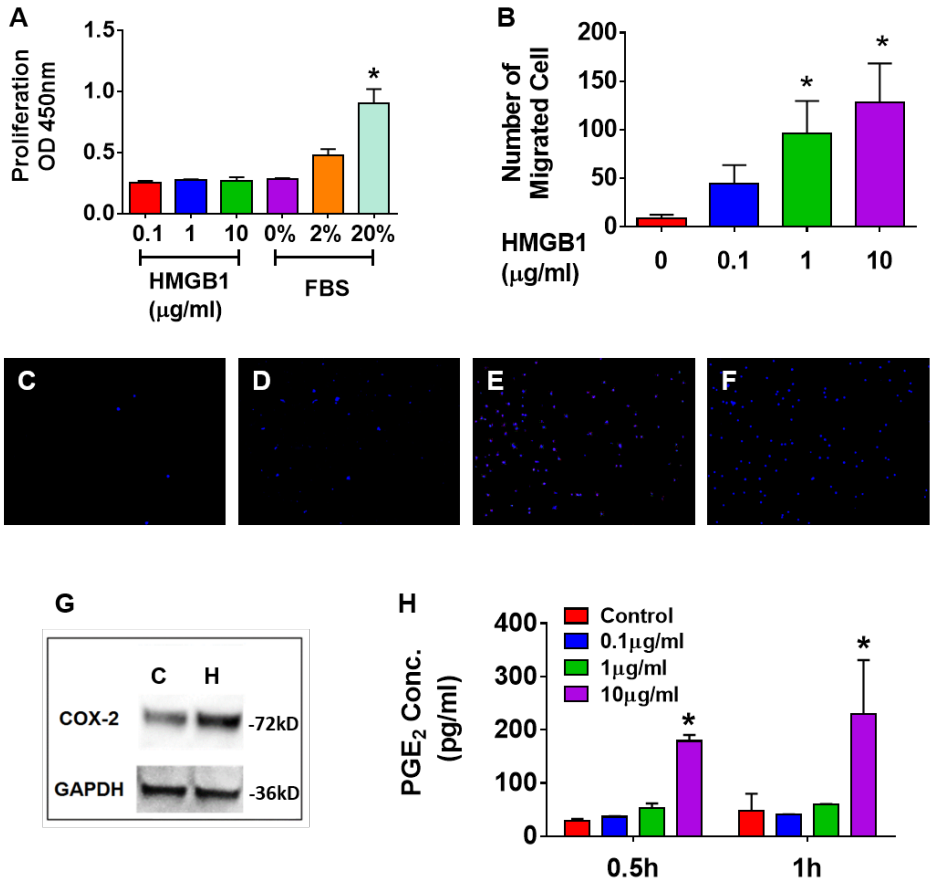

Figure 19. HMGB1 does not promote proliferation but exerts chemoattractant effect and induces COX-2 expression and PGE_2 production in rat Achilles tendon cells *in vitro*. (**A**) HMGB1 does not induce cell proliferation at all the concentrations tested at 48h, while 20% FBS control induces significant cell proliferation. (**B**) HMGB1 (1 and 10 μg/ml) induces significant cell migration after 24h. Growth medium with neither FBS nor HMGB1 served as negative control (first column). **(C)** Representative 40x images of the control transwell membranes stained with H33342 show a small number of cells migrated. **(D-F**) Images of samples treated with 0.1 μg/ml, 1 μg/ml, and 10 g/ml HMGB1 show that tendon cell migration increased with the HMGB1 concentration. Three microscopic fields were counted per transwell membrane, and 4 replicates were used per group. (**G**) HMGB1 (10 μg/ml) induces 3.2-fold COX-2 protein expression compared to control. The band is a representative one from three different experiments. GAPDH shows an equal amount of protein loading. (**H**) PGE_2 significantly increases in response to 10 μg/ml HMGB1 at 0.5 and 1hr. Lower concentrations of HMGB1 do not bring about a significant increase in PGE_2 production.

In contrast, tendon cells cultured in the presence of various concentrations of FBS significantly increased tendon cell proliferation (**Fig. 19A**).

The results showed that HMGB1 alone does not affect tendon cell proliferation, in other words, HMGB1 does not induce tendon cell proliferation directly.

3.1.2 HMGB1 promotes tendon cell migration *in vitro*

Next, we evaluated the effects of various concentrations of exogenously added HMGB1 on tendon cell migration. It is known that HMGB1 functions as a chemoattractant for many types of cells, including macrophages, neutrophils, endothelial cells and fibroblasts through the receptor for the advanced glycation end-products (RAGE). It also has a paramount role in skin wound healing by recruiting various types of cells to the wound site [240]. It is possible HMGB1 produced during mechanical overloading in tendon may also play a similar role.

To determine whether HMGB1 has chemoattractant properties on tendon cells, we then performed an experiment in a transwell system. Briefly, tendon cells suspended in DMEM medium without FBS were planted on a polycarbonate microporous membrane with 8 μm pores mounted on an upper chamber, and the DMEM medium (without FBS) with/without various concentrations of HMGB1 (0.1,1,10 μg/ml) were added to the lower chamber. The cells attracted by HMGB1 in lower chamber would migrate across the pores and attach to the other side of the membrane. The non-migrated cells (cells remained on the upper side of the membrane) were removed, and only cells migrated to the lower chamber were stained with H33342 and counted to reflect the corresponding chemoattractant potential of HMGB1 in the growth medium. The results revealed that in the control group, the lower chamber that contained the culture medium with no HMGB1 showed a minimal number of cells migrated through the microporous membrane, 24hrs after plating the cells in the upper chamber (**Fig. 19C**). Similarly, only a minimal number of cells were present in the group treated with 0.1 μg/ml of HMGB1 (**Fig. 19D**).

However, significantly higher numbers of tendon cells had migrated to lower chamber wells containing 1 and 10 µg/ml HMGB1 (**Fig. 19E, F**). Semi-quantification of the data specifically showed that tendon cell numbers in the group treated with 1 and 10 µg/ml HMGB1 were about 11- and 14-fold higher than the control respectively(**Fig. 19B**). These results indicated that HMGB1 is a chemoattractant for tendon cells *in vitro* at relatively high concentrations and in a dosage-dependent manner.

3.1.3 HMGB1 exerts inflammatory effect on tendon cells *in vitro*

As an inflammatory mediator and Damage Associate Molecular Pattern (DAMPs), HMGB1 has been well studied for its inflammatory effect on macrophages. It is considered as a trigger of sterile inflammation in several diseases, like ischemic damage in the heart, kidney, and lung [241-243], as well as in arthritis [244]. Previously, our group reported that tendon cells release PGE_2 when subjected to intensive mechanical loading [66]. This inflammatory response might be mediated by HMGB1. In order to assess whether HMGB1 mediates PGE_2 production in tenocytes, cultured tendon cells were exposed to different concentrations of HMGB1 (0.1-10 µg/ml) for 0.5 and 1hr. First, using Western blot analysis, we showed that COX-2, an upstream regulator of PGE_2 synthesis, was produced at significantly higher levels in tendon cells treated with 10 µg/ml of HMGB1 for as short as 0.5hr (**Fig. 19E**). There was 3.9- fold increase in COX-2 when compared to control. Next, we determined the effect of various concentrations of HMGB1 on PGE_2 production. PGE_2 levels in the tendon cells treated with 0.1 and 1.0 µg/ml of HMGB1 for 0.5 and 1hr showed no significant increase **(Fig. 19F**). However, PGE_2 levels were significantly increased at the highest concentration of HMGB1 (10 µg/ml) for both treatment and

at 1h, it was 3.82-fold higher than the control. durations (**Fig. 19F**). Specifically, at 0.5hr, PGE_2 levels were 5.28-fold higher than the control,

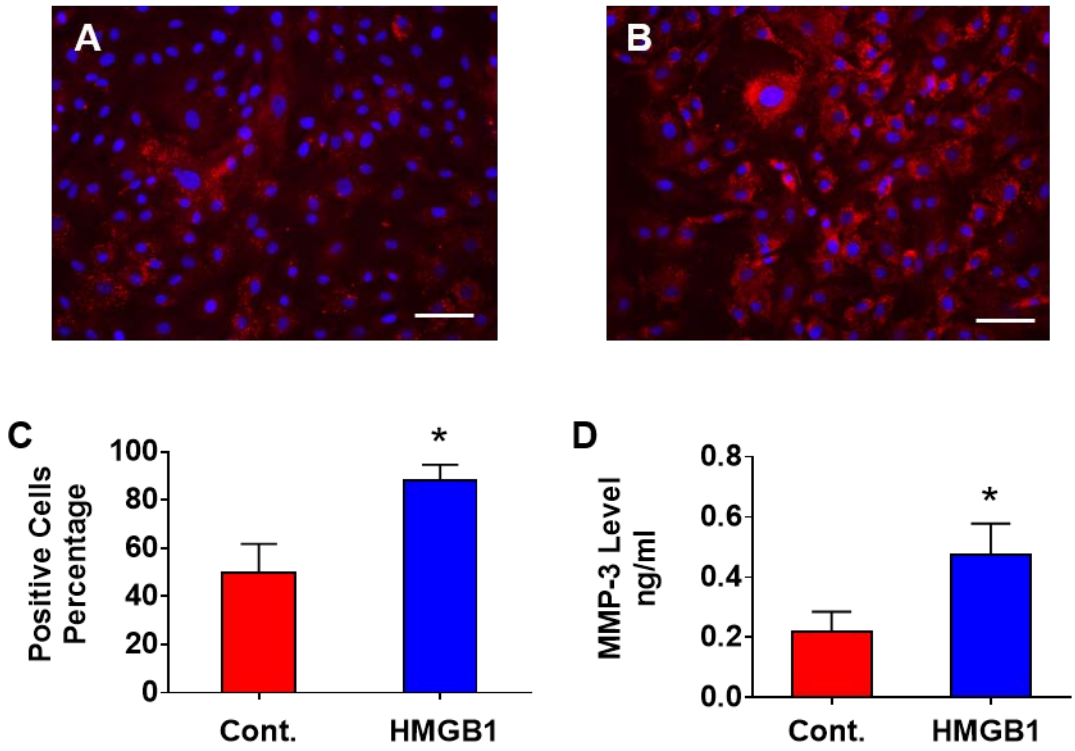

Figure 20. HMGB1 induces MMP-3 expression and release in tendon cells *in vitro*. (**A**) Tendon cells without HMGB1 treatment show some extent of HMGB1 staining in the cytoplasm **(B)** High concentration of HMGB1(10 µg/ml) treatment for 24h significantly increases MMP-3 expression compared to control in tendon cells. **(C)** Semi-quantification shows a significant increase in the proportion of MMP-3 positive cells with 10 µg/ml HMGB1 treatment. (**D**) HMGB1 at 10 µg/ml significantly increases MMP-3 release to culture medium from tendon cells at 24hrs. Data represent mean ± SD from six experiments. *P<0.05 Bars - 50 µm.

HMGB1 has been shown to increase the production of MMPs [178]. In our study, we found that matrix metalloproteinase-3 (MMP-3) is also increased upon HMGB1 stimulation.

Cultured tendon cells were stimulated for 24hrs by a high dosage of HMGB1 10 μg/ml). In the control group without HMGB1 treatment, about 50% cells are positive for MMP-3 in the cytoplasm (**Fig. 20A, C**). The proportion of HMGB1 positive cell increased to about 80% after 24hrs HMGB1 treatment (**Fig. 20B, C**). The MMP-3 expression level assayed using ELISA in the culture medium of the HMGB1 treated cells is much higher than that in the control group (**Fig. 20D**). MMP-3 released to culture medium is 2.1-fold in the HMGB1 stimulated cells compared to that in control group. Collectively, these data show that HMGB1 can induce the release of an inflammatory mediator such as PGE_2 and matrix-degrading enzyme, MMP-3 in tendon cells.

3.1.4 HMGB1 does not induce angiogenesis *in vitro*

HMGB1 was found to promote endothelial cell function and participate in angiogenesis process [245, 246]. It was also suggested that 1 μg/ml HMGB1 could induce angiogenesis *in vitro* in the hypoxic condition in Human dermal microvascular endothelial cells [234]. Therefore, we tested the effect of HMGB1 on angiogenesis process *in vitro*. For that purpose, an endothelial cell tube formation assay was utilized. Briefly, Human Umbilical Vein Endothelial Cells (HUVECs) was seeded on solidified Matrigel with 1 μg/ml or 10 μg/ml HMGB1 for 24hrs. A basal medium without FBS was served as negative control, and 2% FBS was added to the culture medium as a positive control. While there is no tube formation in the negative control (**Fig. 21A**), the endothelial tubes formed successfully in the 2% FBS group (**Fig. 21B).** However, like the negative control group (there is no tube formation in either concentration of HMGB1 in DMEM only medium **(Fig. 21C, D).** These results suggested that HMGB1 alone may not induce angiogenesis *in vitro*.

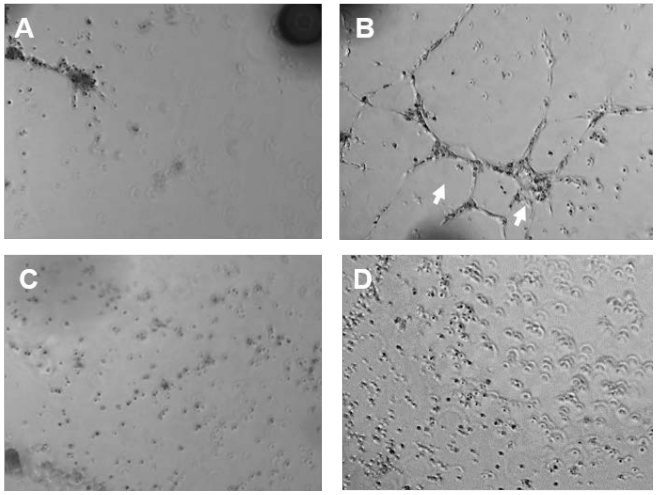

Figure 21. HMGB1 alone does not induce angiogenesis *in vitro*. HUVEC cells (10^5) were seeded on Matrigel in a 24 well plate and then added with 0% FBS, 2% FBS, 1 µg/ml or 10 µg/ml HMGB1 in the basic HUVEC medium, and the cells were allowed to grown in respective conditions for 24h. (**A**) The group without FBS shows no tube formation by the endothelial cells. (**B**) The group with 2% FBS exhibits visible tube formation. (**C**, **D**) Neither 1 µg/ml nor 10 µg/ml HMGB1 induces tube formation by endothelial cells.

3.2 HMGB1 IMPLANTATION RESULTS IN CELL INFILTRATION AND INFLAMMATORY REACTIONS IN TENDON TISSUE IN VIVO

After presenting some evidence about the *in vitro* effect of HMGB1 on tendon cells as a chemoattractant and inflammatory mediator, we proceeded to explore *in vivo* function of HMGB1 in tendon tissue by administration of HMGB1 with alginate beads into rat tendon tissue. We found that HMGB1 implantation results in inflammatory cell infiltration, angiogenesis, hypercellularity, and inflammatory marker COX-2 and matrix degenerative enzyme MMP-3

expression after 2 weeks. Those effects vanish at the 4-week time point, indicating that HMGB1 induces a reversible inflammatory reaction *in vivo*.

3.2.1 Using alginate beads for HMGB1 implantation

We found in our treadmill running experiment that HMGB1 may accumulate in tendon tissue after long-term mechanical loading. We suspected that continuous exposure of HMGB1 in tendon tissue might be the necessary to evoke the inflammatory effect of HMGB1. So, we decided to establish a local and relatively long-term HMGB1delivery system.

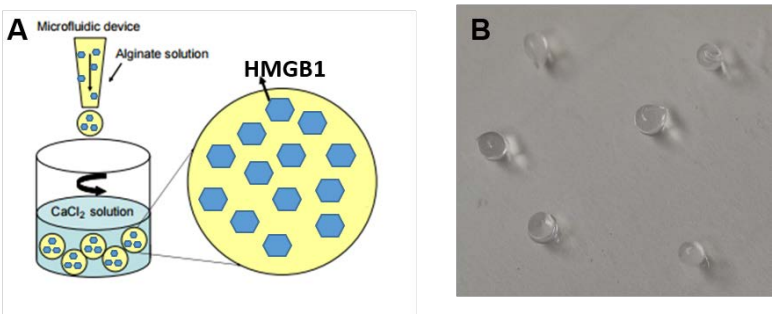

Figure 22. Illustration of the procedures for fabricating alginate gel beads in containing cells and the appearance of prepared beads. (**A**) Alginate solution (2%) with/without HMGB1 was added to 2% CaCl2 solution with a 10 µl pipette; each gel spheres volume is 5ul. Once contacted with the CaCl2 solution, the alginate drop immediately crosslinks and forms a gelled bead. The gel spheres were retrieved from CaCl2 solution with two wash of PBS. (B) Prepared alginate beads, the diameter of the alginate beads with 5ul volume is about 0.3~0.5mm

We chose alginate beads to encapsulate HMGB1 and implant into the tendon. Our laboratory has previous experience using this drug delivery method to successfully create tendinopathy model

[112]. Besides, alginate is a non-toxic natural polysaccharide with excellent biocompatibility and biodegradability [247] with minimal pro-inflammatory effect. It has been widely used in wound healing, cartilage repair, bone regeneration, and drug delivery [248, 249]. Although many other synthetic biomaterials could serve as delivery systems, the denaturing and loss of bioactivity of the carried drug is a big concern as preparation of these materials often involves the use of using organic solvents [250]. Alginate could be easily cross-linked and shaped with metal ions (usually Ca^{2+}) under room temperature and does not require other harsh **chemical processing, which makes it an excellent carrier for HMGB1 protein.**

3.2.2 HMGB1 beads implanted subcutaneously induced cell infiltration and angiogenesis in skin tissue

Before implanting the HMGB1 beads into the tendon, we first validated its effect by implanting the beads subcutaneously. After 1-week implantation, the skin tissue was dissected, and we observed that HMGB1 beads were able to attract a large number of cells to infiltrate into the beads area and also induce angiogenesis around the beads implantation site (**Fig. 23A, C)**, while the blank control beads are almost empty with few cell infiltration (**Fig. 23B, D**). The result indicated that HMGB1 *in vivo* could induce strong “wound healing” effect in skin tissue, and HMGB1 was active and functional through the preparation process of alginate beads.

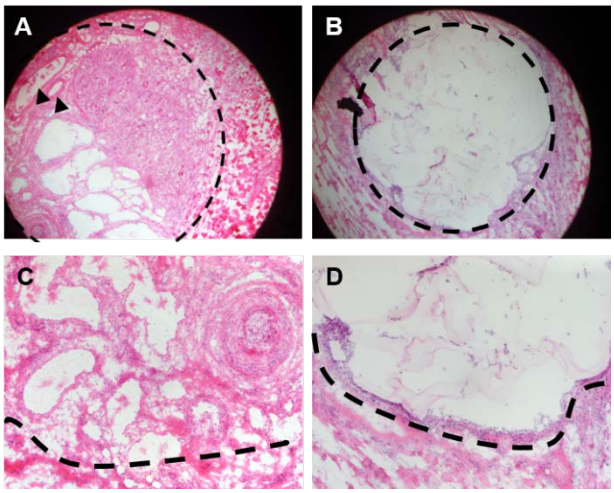

Figure 23. Implantation of HMGB1 beads in subcutaneous tissue. induces tissue formation, angiogenesis, and cell infiltration. Alginate beads with/without HMGB1 were implanted subcutaneously for 1 week. (A) HMGB1 beads induce tissue ingrowth and vessel formation in the implanted site (arrow heads). Dash lines show the rough edge of the bead. (C) Enlarged area of the HMGB1 bead implanted group shows massive cell infiltration and tissue formation. (B) The tissue section with an empty bead implanted. The dash line indicates the actual bead boundary. Cell infiltration can be seen in the bead area but is much less than the HMGB1 group with no obvious vessel formation in or near the implantation site. (D) The enlarged area of the implanted empty bead. Cell accumulation is seen near the edge of the implanted beads but with minimal cell infiltration or tissue ingrowth.

3.2.3 HMGB1 beads implanted in rat patellar tendon induced hypercellularity, angiogenesis, and inflammatory cell infiltration

We conducted the *in vivo* evaluation of the effect of HMGB1 on tendon tissue by implanting HMGB1 protein encapsulated in 2% alginate beads into rat patellar tendons. The reason for using rat patellar tendon is that, rat is relatively larger than mice and easy to operate on, and patellar tendon is generally flat (compared to other most researched tendon Achilles, shape of

which is cylindrical) and easier to implant the alginate beads at same location every time to prevent the migration of the beads.

Each bead was 5 µl in volume and contained 2.5 µg HMGB1. We chose the 2 weeks and 4 week time points instead of 1-week time point to avoid possible confusion with the spontaneous healing reaction of the tendon, since at week 1, the inflammation may result from the surgical injury itself. By week 2, the inflammation may have subsided, and we may be measuring the relatively more actual effect of HMGB1 itself.

We chose a high concentration of HMGB1 (0.5 µg/µl) in the implantation in a measure to compensate the relatively short experimental time to develop the model. Since we know that chronic tendinopathy with tendon structural changes, which might be the result of the HMGB1 effect may take years or decades to develop in human patients, it is nearly impossible to recapitulate the development of the disease in an animal model. So, we assume that the short term high dosage effect, at least to some degree, may reflect the cumulative effect and cellular/structural changes in the development of tendinopathy in patients.

After sterilization and opening the rat knee skin, the soft tissue above tendon was carefully dissected, and the patellar tendon was split along the long axis with microsurgical tweezers to create a spot for bead implantation (**Fig. 24**). This procedure ensures minimal trauma injury to the tendon tissue that may provoke wound healing which may mask the HMGB1 effect.

At 2 and 4 weeks after bead implantation, the overall structure and inflammation signs were evaluated by H&E and immunohistochemical staining Generally, we found that very drastic cellular and structural changes occurred in 2 weeks but not in 4 weeks group with HMGB1 implantation. . At 4 weeks, the major changes might have resolved as HMGB1 may have been depleted by that time as the tendon tissue was subsequently repaired.

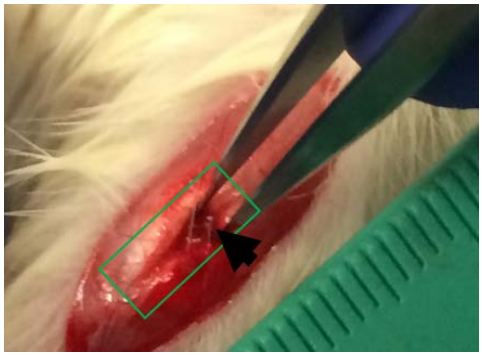

Figure 24. Surgical photograph shows the implantation of the beads into rat patellar tendon. Arrow points to the alginate bead, and the green box indicates the patellar tendon, and the bead was implanted near the mid-portion of the patellar tendon. The spot for implantation was created by splitting the tendon along the long axis of the patellar tendon.

Unlike the control (**Fig. 25A**), the HMGB1-bead implanted tendon region shows hypercellularity after 2 weeks (highlighted in the white box, **Fig. 25B**). Moreover, the 4-week group shows slightly higher cellularity in some area (arrow in **Fig. 25C**) than control but is significantly lower than that in 2 weeks group (**Fig. 25C**). Moreover, significant inflammatory cell infiltration was also observed in the HMGB1 bead implanted tendon in 2 weeks' time point. The HMGB1 bead implanted tendon section is stained positive for CD68 (arrowhead, **Fig. 25E)**, which is a marker for inflammatory cells such as macrophages/monocytes. These cells are not present in the control tendons implanted with only alginate bead (**Fig. 25D**) or the 4 weeks HMGB1 implanted group (**Fig. 25F**). It is also observed that there are a lot of CD68 negative cells surrounding the CD68 positive cells (**Fig. 25E**). These cells might be the migrated tendon cells in response to both by the chemoattractant HMGB1 as well as by the growth factors and cytokines secreted by the recruited CD68 positive inflammatory cells.

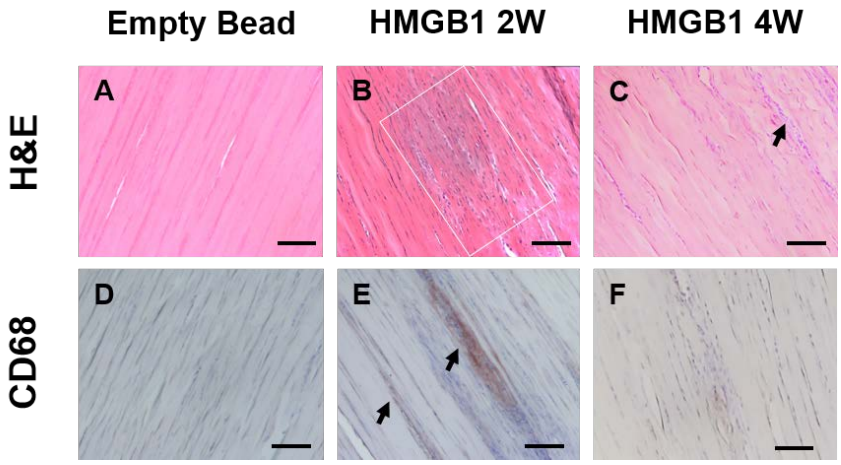

Figure 25. HMGB1 induces hypercellularity and inflammatory cell infiltration in rat tendons at 2-week but not at the 4-week time point. (**A**) H&E staining of control tendon implanted with 5 µl empty beads shows no cell proliferation. (**B**) Tendon section with implanted HMGB1 beads (2.5 µg in 5 µl) shows extensive cell proliferation (highlighted in the white square) at 2 weeks post-implantation. **(C)** The implantation site at 4 weeks; higher number of cells (arrow) compared to control can be seen but is much less compared to the 2 weeks group. (**D**) Control with no positive CD68 staining. (**E)** Positive CD68 staining in HMGB1 implanted sample for 2 weeks (arrows). **(F)** HMGB1 bead implantation group at 4 weeks shows minimal positive staining for CD68. Each image shows representative results from at least 3 samples. Bars - 100 µm.

Moreover, the presence of blood vessel formation is found in 2-week implantation group (arrowheads, **Fig. 26A**), which also stained positive for the endothelial cell marker, CD31, by immunohistochemical staining (arrowhead, **Fig. 26B**). The similar structure was not found in control or the 4-week implantation group.

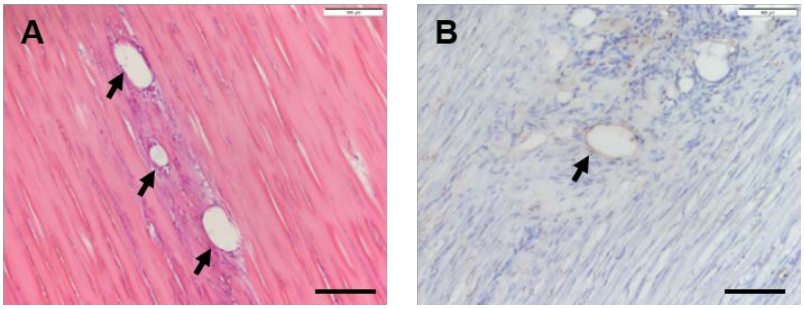

Figure 26. HMGB1 induces angiogenesis in rat tendons at 2 weeks. (**A**) HMGB1 bead group at 2 weeks post-implantation shows vessel-like structures; arrows point to vessels formed in the region. (**B**) Positive staining for CD31 in the tissue sample of the HMGB1 bead group (arrow). No similar staining results were detected in control group with empty beads or in 4 weeks implantation group. Bars – 100 μm

Finally, high expression of COX-2 (**Fig. 27B**) and MMP-3 (**Fig. 27E**) could be detected near the implantation site in the 2-week implantation group, control tendon with empty beads implanted showed negative for both proteins (**Fig. 27A, D**), 4-weeks group show negative for COX-2 staining (**Fig. 27C**), but show some degree of MMP-3 staining in the cell concentrated area **(arrow Fig. 27F).** It is also noteworthy that the expression of COX-2 and MMP-3 is very concentrated in the implanted area and has not spread to adjacent tendon tissue. There was also drastic cell density change in the 2 weeks HMGB1 implanted tendon compared to the control.

Collectively, we demonstrated that HMGB1 exerts inflammatory and chemoattractant function *in vitro* and *in vivo*, especially *in vivo*. The administration of HMGB1 showed several effects including hypercellularity, inflammatory cell infiltration, and angiogenesis that may link to the development of tendinopathy. The release of HMGB1 by mechanical overloading and its subsequent chemoattractant and inflammatory functions is a very promising explanation of the early onset and subsequent development of tendinopathy. In the human patient settings, the long

term overloading of the tendon may result in the continuous production of HMGB1. The cumulative effect of HMGB1 by attracting inflammatory cells may lead to chronic low-level sterile inflammation in tendon tissue, which may gradually compromise the structure of tendon. Given this hypothesis, the next chapter presents the preventive strategies of the chronic effect by blocking HMGB1 function. This approach could lead to the development of early prevention method for the high-risk tendinopathy population like professional athletes.

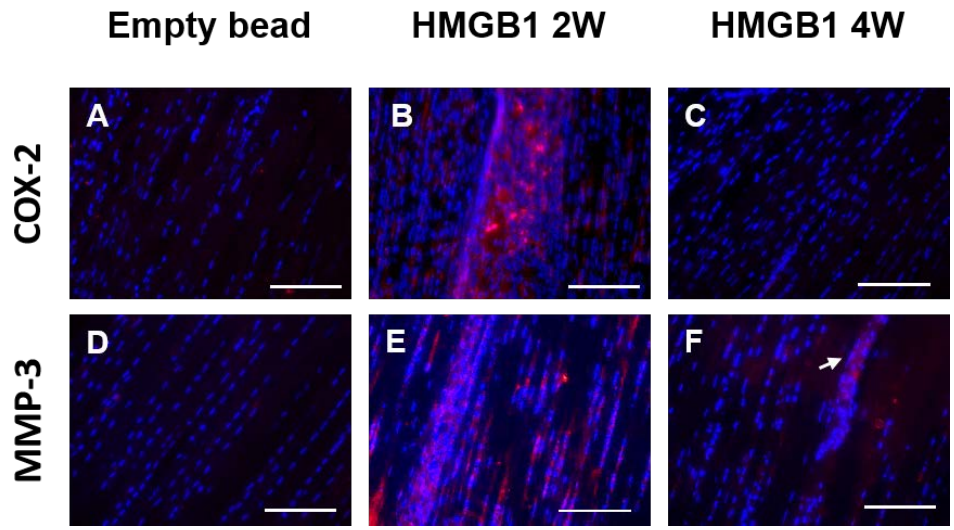

Figure 27. HMGB1 induces COX-2 expression, and PGE_2 and MMP-3 production in rat patellar tendons at 2 weeks. **(A)** Control tendon with empty beads shows no sign of COX-2 signal. **(B)** HMGB1 bead implanted tendon at 2 weeks shows intensive COX-2 staining. **(C, D)** Control tendon shows negative for MMP-3 staining. **(E)** In the HMGB1 bead implanted tendon at 2 weeks, positive staining for MMP-3 of is concentrated in the high cell density area. (**F**) HMGB1 bead implanted tendon at 4 weeks still shows a positive signal for MMP-3 but not as strong as 2 weeks group (arrow). Note the drastic high cell density is only seen in the 2 weeks group, but neither in control group nor in the 4 weeks group. Bars - 100 µm.

3.3 MATERIALS AND METHODS

3.3.1 Quantifying tendon cell proliferation

Tendon cells isolated from rat patellar tendons at passage 3 (~2000 cells) were seeded in 96 well plates and allowed to attach overnight in 10% FBS+DMEM. Next day, the culture medium was replaced with serum-free DMEM containing different concentrations of HMGB1 (0.1-10 µg/ml). After 48 h, tendon cell proliferation was assessed using the CCK-8 kit (Sigma, St. Louis, MO, 96992). This experiment was repeated 6 times.

3.3.2 Tendon cell migration assay

A transwell system (Corning, Corning, NY, 3422) containing a membrane with 8 µm pore size was used to evaluate the chemoattractant effect of HMGB1 on rat patellar tendon cells. P3 cells (~$1X10^5$ cells) were seeded in the upper chamber, and various concentrations of HMGB1(TECAN, Switzerland, REHM114) in DMEM (0, 0.1, 1, 10 µg/ml) were added to the lower chamber of the 24 well transwell system. In the positive control well, 20% FBS was added to DMEM. After 24 h, cells that migrated into the lower chamber through the membrane were counted by visualizing through a microscope (40x) from at least 4 different locations in each sample. This experiment was repeated three times.

3.3.3 Evaluating inflammation in tendon cells *in vitro*

Rat patellar tendon cells at passage 3 were seeded (~2 x10^5 cells/well) in a 6-well plate containing 2% FBS+DMEM with various concentrations of HMGB1 (0, 0.1, 1, and 10 µg/ml). After 0.5 and 1 h, the culture medium was collected to quantify PGE_2 by ELISA.

The cells were also collected separately and used for Western blotting. A 20 µg of total protein extracted from the cells treated with HMGB1was separated on a 10% SDS-PAGE. The proteins were then transferred onto a nylon membrane and incubated with 2 µg/ml rabbit anti-COX-2 antibody (2 µg/ml, Cell Signaling, Danvers, MA, 12282S) at 4° C overnight followed by incubation with 2 µg/ml mouse anti-rabbit secondary antibody conjugated with HRP for 1hr (Abcam. Cambridge, UK, ab6721). HRP substrate was added to the membrane and photos were taken immediately with Biorad ChemiDoc XRS+ imaging system, with an exposure time of 30 s-1 min.

3.3.4 *In vitro* angiogenesis effect of HMGB1

HUVEC cells at passage 3 were used for this experiment. Those cells were cultured and passaged with the vascular cell basal medium (ATCC, Manassas, VA, PCS-100-030) with the addition of endothelial cell growth kit (ATCC, Manassas, VA, PCS-100-040,). 100ul liquid Matrigel (Corning lnc., Corning, NY, 354247) was added in 24-well dish and allowed to solidify at 37°C for 30min. The well was washed with basal medium twice. HUVECs cultured in the flask were harvested and resuspended in basal medium, and 1x 10^5 HUVECs were added to each well. Then, 2% FBS (final concentration) and 1 or 10 µg/ml HMGB1 were added to

corresponding wells; then the plate was incubated at 37°C for 24hrs. The picture was taken with SPOT CCD digital camera.

3.3.5 HMGB1 implantation *in vivo*

Since most sports injuries are caused by repetitive or long-term mechanical loading, we developed a system to deliver HMGB1 into tendons *in vivo* to mimic long-term release of HMGB1 induced by repetitive mechanical loading. Our delivery system consisted of a degradable polymer called alginate that contained HMGB1 to ensure local and continuous delivery of HMGB1 to maximize the effect in a relatively short period.

3.3.6 Preparation of alginate beads

A 2% alginate solution was first prepared by dissolving alginate powder in double distilled water after vigorous vortexing. Then, HMGB1 (TECAN, Switzerland, REHM114) was added to the 2% alginate solution to reach the final concentration of 0.5 mg/ml. With a pipette, about 5 µl of the HMGB1-alginate solution was then added to 2 mM $CaCl_2$ solution in the form of drops, which solidified to form alginate beads. Control alginate beads were prepared without adding HMGB1. The beads were then removed from the $CaCl_2$ solution and allowed to air dry. The final diameter of the beads was around 0.05 mm, which is about 1/6 of the rat patellar tendon width. This protocol was developed in our laboratory with the successful delivery of another bioactive chemical Kartogenin to develop a rat tendinopathy model [112].

3.3.7 HMGB1-alginate bead implantation in rat skin and patellar tendon

Rats (Female, SD, 6 months) were sedated by inhaling 2-3% isoflurane. The skin over the patellar tendon was then shaved, sterilized and a small incision was made on the skin to expose the tendon. HMGB1-alginate beads (2.5 μg in 5 μl) or control beads (5 μl) were implanted subcutaneously on the back of the rats, and the rats were sacrificed after one week, and the skin was used to validate the effect of HMGB1.

After validation with the skin implantation experiment, HMGB1-alginate beads (2.5 μg in 5 μl) or control beads (5 μl) were implanted in the central part of the left and right patellar tendons. After 2 and 4 weeks, 3 rats in each group were used for H&E and immunohistochemical staining to evaluate structural changes in the tendon tissue.

3.3.8 Immunohistochemical staining of HMGB1 implanted tendon

Patellar tendons were harvested from 3 rats that received HMGB1-alginate bead or control bead implantation and processed similarly as in the last chapter under *ELISA and immunostaining of tendon tissue*. Anti-rat CD31 antibody (1 μg/ml, Abcam, Cambridge, UK, ab64543) was used to detect endothelial cells and vessels. Anti-CD68 antibody (2 μg/ml, Abcam, Cambridge, UK, ab125212) was used to detect monocytes/macrophages. Anti MMP-3(1 μg/ml, Abcam Cambridge, UK, ab52915) and Anti-COX-2(1 μg/ml, Cell Signaling, Danvers, MA, 12282S) were used to evaluate corresponding protein expression in implanted tendon tissue. HE staining was used to evaluate overall tendon structure and cell density.

3.3.9 Statistical Analysis

Student’s t-test and One-way ANOVA was used, followed by Fisher's least significant difference (LSD) test for multiple comparisons. When P-values were less than 0.05, the two groups compared were considered to be significantly different.

4.0 INHIBITION OF HMGB1 TO NEGATE THE EFFECTS IN OVERLOADED TENDON

The results from the previous chapter showed that HMGB1 released in response to mechanical overloading could invoke inflammatory reactions *in vivo* and *in vitro*, and suggested that these responses may have an adverse impact on long-term tendon health. Since these results demonstrated that HMGB1 could induce inflammatory responses in tendon cells and tissues, the next aim was to explore the means to mitigate these effects with inhibition of HMGB1 activity.

We first tested the *in vitro* inhibitory effect on HMGB1 with its direct inhibitor Glycyrrhizin (GL). We found that in 3-week treadmill running mice, GL could reduce the inflammatory response induced by HMGB1. Also, longer time running (12 weeks or 24 weeks) induced degenerative changes like increased the presence of chondrocyte-like cells, SOX-9 expression, GAGs deposition and collagen II deposition in tendon tissue near the insertional site of Achilles tendon-bone junction but not in the mid-section of Achilles tendon, or in the patellar tendon. Injection of GL to inhibit HMGB1 effect along with the treadmill running for 24 weeks could attenuate those degenerative changes.

In a broad viewpoint, inflammation is a protective response to injuries and infections in our body and a complex network of cellular and molecular responses leads to resolving the inflammation and/or repair of damaged tissue. However, inflammation is also involved in chronic injury and degeneration [218].

Chronic sterile inflammation is potentially detrimental in tendon tissue, since it may disturb the well-organized tendon structure by introducing inflammatory cells into the tendon causing hypercellularity, angiogenesis, and catabolic phenotype of the tendon tissue. Changes in the well-organized collagen fibers with cells, vessels or disrupted fibers will substantially impair the mechanical properties of the tendon.

Current treatment for chronic tendon injury with steroids can suppress the overall immuno-response, but it is considered harmful for the whole body. Direct anti-inflammatory therapy like administration of non-steroid anti-inflammatory drugs (NSAIDs) is widely used in clinics, however NSAIDS administration is only inhibiting the downstream mediator, not the source itself., for example NSAIDS mainly inhibit COX activity, which is a relatively downstream pathway in the inflammatory cascade. Various other cytokines and MMPs can be produced upon the stimulation of HMGB1 on inflammatory cells possibly through other pathways. Inflammatory cells can induce the catabolic phenotypes of resident tendon cells, which result in the production of MMPs and other inflammatory cytokines such as IL-1β and IL-6. So, it is more important to block the activation and migration of inflammatory cells to the tendon tissue *in vivo* and resolve the inflammation at the very beginning stage.

In most cases, chronic tendon injury or tendinopathy is not an open wound; there are no external inflammatory triggers. HMGB1 is known to be a DAMP molecule that triggers sterile inflammation *in vivo*[231], and it is proposed to play a critical role in the initial recruitment and activation of inflammatory cells[153, 231, 251, 252]. Therefore, inhibition of HMGB1 activity may suppress the migration of inflammatory cells to tendon tissue, and thus might exert a protective effect on tendon tissue.

4.1 INHIBITION OF HMGB1 EFFECT *IN VITRO* WITH GLYCYRRHIZIN

Glycyrrhizin (GL), a glycoconjugate triterpene produced by the licorice plant, Glycyrrhiza glabra (**Fig. 28A**). It has been shown to inhibit the chemoattractant and mitogenic activity of HMGB1 *in vitro* and *in vivo* by directly binding to the HMGB1 functional domain on Box A(**Fig. 28B**) [253].

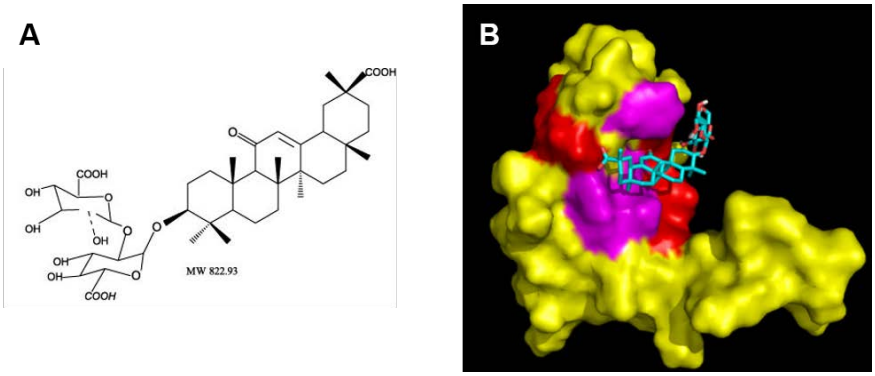

Figure 28. The structure of glycyrrhizin (GL) and its binding site on Box A of HMGB1. (A) The chemical structure of GL. **(B)** Model of GL's binding on the box A of HMGB1. (Sources: Fig. 28A [254], Fig. 28B [255])

It has been administered to patients with hepatitis B and C, and it is considered safe [253]. GL has been widely investigated to inhibit HMGB1 signaling in the treatment of patients and research on disease models. GL was found to sequestration HMGB1 effect in patient with rhinitis [256], GL was utilized to attenuates HMGB1 induced hepatocyte apoptosis in human cell line[257]; GL was also discovered to exert protective effect on focal cerebral ischemia/reperfusion-induced inflammation mediated by HMGB1[258], GL inhibit HMGB1 protect brain injury after diffuse axonal injury model in rat through the anti-inflammatory

effects[259], GL administration showed decreased troponin I-induced myocardial inflammation in mice mediated by HMGB1 [260]. The detailed discussion on HMGB1 inhibitory agents and the logical reason to use GL can be found in chapter 5.

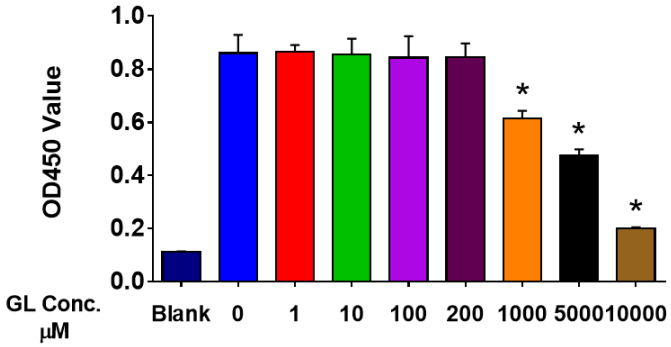

Figure 29. Toxicity of GL on tendon cells in culture. Tendon cells were exposed to various concentrations of GL to test the effect of GL on cell viability using CCK-8 kit. OD450 values represent the live cell numbers in culture. The concentrations of GL range from 1 μm to 10 mM. The results showed that GL does not affect cell viability up to 200 μm. When GL concentration exceeded 1mM, cells viability decreased drastically and in a dosage-dependent manner. Blanks are cells untreated with GL and no CCK-8 added, which represent the baseline of OD450 value. *$p<0.05$ compare to the cells not treated with GL. n = 6.

4.1.1 GL in certain range of concentrations is non-toxic for tendon cells

GL solution is acidic, which is potentially detrimental to cells. To assess the suitable non-toxic working concentration for HMGB1 inhibition, we first tested the effect of GL on cell viability *in vitro*. In previous studies, GL *in vitro* concentration was used up to 2 mM [257]. Briefly, tendon cells were exposed to various concentrations of GL from 1 μM to 10 mM for 72hrs, and cell

viability was tested with CCK-8 kit. We found that GL did not affect cell viability up to 200 μM (**Fig. 29**). However, from 1mM to 10mM, the cell viability decreased drastically in a dosage-dependent manner. To maximize the inhibition potential without adverse effect on cell viability, 200 μM was selected as the *in vitro* GL concentration for all following experiments.

4.1.2 GL negates HMGB1 inflammatory effect and decreases MMP-3 production in tendon cells *in vitro*

In our previous experiment, we have shown that HMGB1 can induce PGE_2 and MMP-3 production in tendon cells. Cultured tendon cells treated with HMGB1 were used to test the inhibitory effect of GL on PGE_2 and MMP-3.

The rat patellar tendon cells were treated with 10 μg/ml HMGB1 for 0.5, 2, and 4hrs and measured the PGE_2 levels. 10 ng/ml IL-1β was added as a positive control. ELISA results showed that there was a significant increase in PGE_2 levels at all-time points ($p<0.05$) **(Fig. 30A).** When GL (200 μM) was added together with 10 μg/ml HMGB1, it could effectively attenuate the PGE_2 production at all-time points. In addition, 200 μM GL has shown the similar inhibitory effect on HMGB1-induced MMP-3 release from tendon cells in 24hrs culture with 10 μg/ml HMGB1 (**Fig. 30B**).

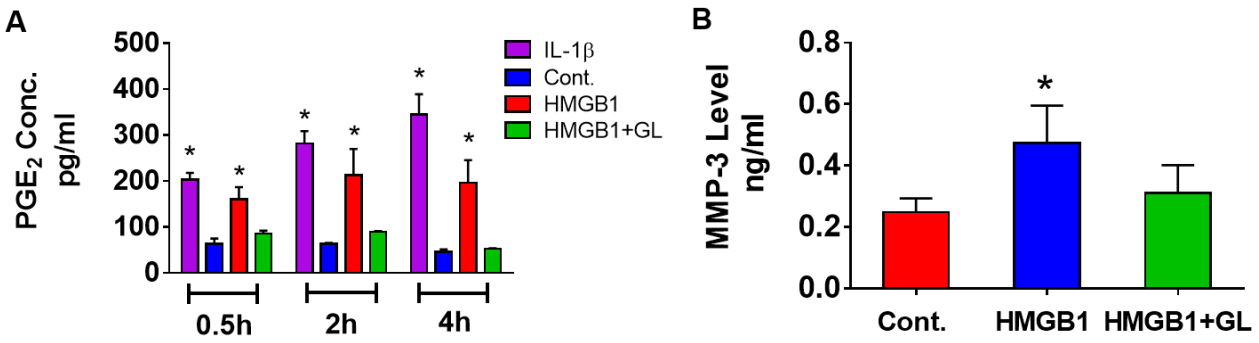

Figure 30. GL treatment blocks HMGB1-induced PGE_2 and MMP-3 production in tendon cells *in vitro*. (**A**) Rat Achilles tendon cells were treated with 10 μg/ml HMGB1 or 10 μg/ml HMGB1+ 200 μm GL for various durations. IL-1β (10ng/ml) was served as a positive control. As seen, PGE_2 levels significantly increase in the HMGB1 treatment group at 0.5, 2, and 4h post-treatment. However, combined treatment with GL blocks the effects of HMGB1. (**B**) HMGB1 treatment significantly increases MMP-3 production in the tendon cells, and GL treatment with HMGB1 blocks the HMGB1 effect and reduces MMP-3 to a similar level as the non-treated control. Data represent mean ± SD. n = 4. * $P < 0.05$.

4.2 GL REVERSES HMGB1-INDUCED *IN VIVO* EARLY INFLAMMATION IN TENDON SUBJECTED TO INTENSIVE TREADMILL RUNNING

In the previous chapter, it was shown that long-term intensive treadmill running could induce the release of HMGB1 into tendon matrix and it could invoke inflammatory reactions *in vivo*. The next aim of the study was to determine whether inhibition of HMGB1 effect could result in the decrease of inflammation in tendon tissue induced by HMGB1 *in vivo*. The mouse treadmill running model was employed in this study. Since GL showed a promising inhibitory effect in *in vitro* study, it was utilized as the HMGB1 inhibitor for *in vivo* studies.

4.2.1 IP injected GL can be transported and maintained in tendon tissue

Whether GL can be transported and maintained in the tendon region after injections in mice were determined first. The dose of GL was selected at 50 mg/kg body weight on the basis of previous animal studies using GL. For the GL *in vivo* administration, the recorded highest dosage was 400 mg/kg [261], minimal was 10 mg/kg in mice [262] and the average mostly under 100 mg/kg. Previous studies showed that 50 mg/kg GL or lower dosage was enough to take effect *in vivo* to treat lung and liver disease models in mice and rats [257, 263, 264]. A dosage of 50 mg/kg was selected and verified in tendon study first, because of the concerns about the high concentration that might result in unexpected overall health deterioration of the mice, and the low concentration that may not be enough to sustain in the rather avascular tendon tissue throughout the time frame of the experiment.

GL (50 mg/kg 400 µl dissolved in PBS) was intraperitoneally (IP) injected into 3 three-month-old C57B/6 female mice. Three separate mice were injected with the same amount of PBS which served as controls. Quantification of GL with thin layer chromatography (TLC) 3hrs after injection showed significantly higher amounts of GL in mice patellar and Achilles tendons compared to the mice injected with PBS. This result demonstrated that GL could remain in tendon tissue in high concentration after IP injection. It can at least last through the treadmill running duration (3hrs) to deactivate HMGB1 which may be produced due to the mechanical overloading process by intensive treadmill running **(Fig. 31).** When comparing the quantity of HMGB1 and GL that could exist in tendon tissue, HMGB1 could be as high as 10~15 ng/mg tissue according to our previous results in Chapter 2 **(Fig. 17A, B**); GL content was about 30~50 µg/mg tissue**(Fig. 31)**. The amount of GL is much higher than HMGB1. So, it is possible that HMGB1 released from intensive treadmill running can be completely inhibited by injected GL.

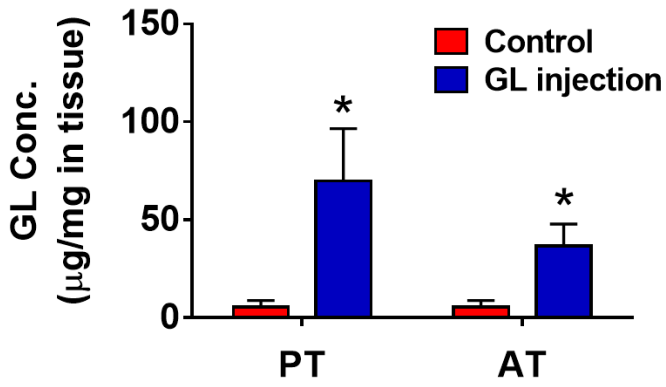

Figure 31. GL is present in mouse tendons after GL injection. After IP injection of GL (50mg/kg) for 3 h, the amount of GL concentration in mouse patellar and Achilles tendons was measured by TLC measurement. Data represent mean ± SD. n = 4. * $P < 0.05$.

4.2.2 GL reverses the HMGB1-mediated early stage inflammation *in vivo* that is induced by intensive treadmill running

The next step was to explore the inhibitory effect of GL on HMGB1 *in vivo* using treadmill running model. Totally 24 mice were divided into 4 groups, and 3-week treadmill running experiment with or without GL injection was performed.

1. **Cage control group (C):** These mice was allowed cage activity, and IP injection of PBS (400 μl) was given 5 days a week
2. **Glycyrrhizin only group (GL):** This group of mice was also allowed cage activity with IP injection of GL (50 mg/kg body weight)

3. **Intensive treadmill running (TR):** In this group, the mice were subjected to treadmill running for 3 weeks, 5 days a week, 3hrs a day, 15 m/min with IP injection of PBS (400 µl) 15min before treadmill running.
4. **Intensive treadmill running with Glycyrrhizin treatment (TR+GL):** The mice in this group were subjected to intensive treadmill running same as in group 3 with IP injection of GL (50 mg/kg body weight) 15min prior to treadmill running.

The glycyrrhizin only group was set up to monitor the effect of GL injection on the overall health of mice. A previous publication has shown that IP injection of GL 50 mg/ml daily for 28 days had no side effects in rats [264]. The body weights of all mice were monitored daily. Overall activity and appearance were monitored to track any possible side effects of GL injection. The body weight changes were tabulated (**Table 2).** Even though the weights of TR and GL+TR mice did not increase as in the controls, these groups of mice showed no significant drop in body weights (**Table 2)**. Mice in GL injected group had similar appearance and activity levels as the control group.

Table 2 Mouse Body Weight after 3 weeks Study

Group	Body weight(g) before study	Body weight(g) after study
Control	19.62 ± 1.45	21.12 ± 1.59
GL only	19.38 ± 1.61	19.80 ± 1.35
TR only	19.39 ± 1.53	19.92 ± 1.47
TR+ GL	20.36 ± 0.62	19.68 ± 0.39

At the end of week 3, after the last treadmill running, all mice were sacrificed, and the patellar and Achilles tendons were harvested and homogenized to measure the inflammatory factors (PGE_2, MMP-3) in tendon tissues. According to a previous model, treadmill running for 3 weeks was still in the early inflammatory stage or tendon injury, so that no obvious structural changes could be detected at this time point. If structural changes should be visualized, it needed at least 12-16 weeks of running according to published papers on similar experiments carried out on rats [34, 265].

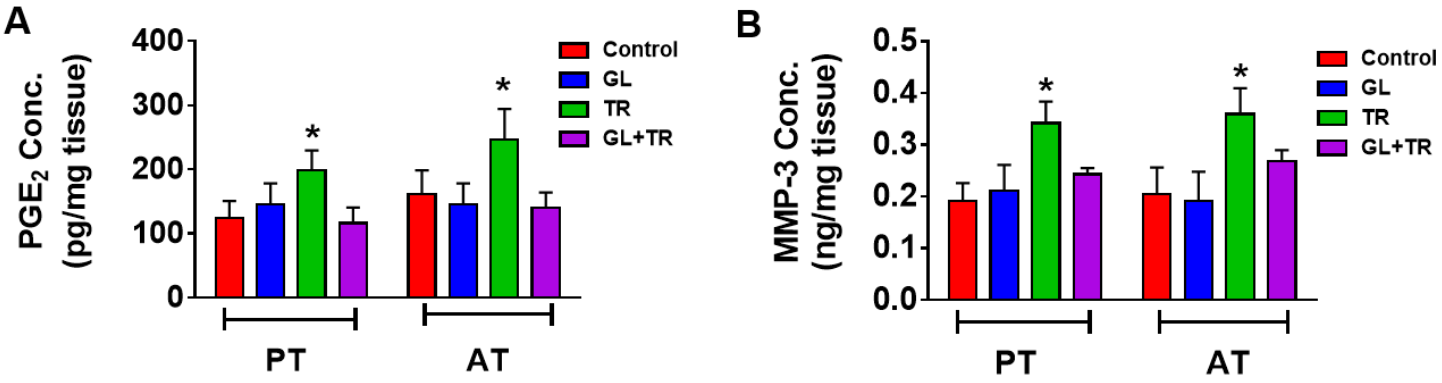

Figure 32. GL injection blocks HMGB1 mediated PGE2 and MMP-3 production due to mechanical overloading *in vivo*. (**A**) PGE_2 concentrations significantly increase in patellar tendon (PT), and Achilles tendon (AT) of intensive treadmill running (ITR) group and GL administration (50 mg/kg body weight, daily IP injection) reverses these enhancements. (**B**) MMP3 levels significantly increase in PT and AT of ITR and GL administration reverses these effects. Data represent mean ± SD. n = 6. * $P < 0.05$.

ELISA assays of the PGE_2 concentration showed that GL injection only mice did not alter the PGE_2 levels in patellar and Achilles tendon when compared to the control mice (**Fig. 32A).** PGE_2 levels were significantly higher in TR mice tendons (1.5 and 1.6 fold increase in AT

and PT respectively compared to control mice after the TR regimen (**Fig. 32A)**. However, GL injection before TR inhibited PGE_2 production by reducing it to a similar level as the control group (**Fig. 32A**). Similar effects were observed with MMP-3 levels in mice tendons after GL injections. MMP3 levels in both PT and AT were significantly elevated in TR group (1.9 and 1.8 fold increase compared to control), but GL negated the enhanced release **(Fig. 32B).** While statistically not significant, the MMP-3 levels in GL+TR group appeared to be higher than the control group, indicating that there might be other mechanisms regulating MMP-3 production (**Fig. 32B).**

4.3 LONG-TERM TREADMILL RUNNING INDUCES DEGENERATIVE CHANGES IN ACHILLES TENDON NEAR TENDON-BONE INSERTION SITE

A rat supraspinatus tendon overuse model (treadmill running) showed that there was a significant increase in gene expression levels such as Col II and SOX-9 of cartilage lineage in tendon[33] indicating the potential degenerative effect of long-term excessive mechanical loading in tendon tissue. Before we explored the inhibitory effect of GL on HMGB1, we developed a degenerative model in Achilles tendon. Previous studies found that long-term treadmill running can induce cellular and structural changes in tendon tissue, including cell rounding and disruption of tendon matrix structure [34, 245, 266]. One degenerative change found in tendinopathy is fibro-cartilaginous[267], which is the degenerative change of tendon tissue with chondrogenesis. Chondrocyte markers were expressed in the clinical samples of calcific insertional Achilles tendinopathy [268]. The chondrocytes are rare in normal tendon tissue, and they have a very unique appearance in histology images, chondrocytes are contained in cavities in the matrix,

called cartilage lacunae, which is drastically different from the tendon cells (**Fig. 34H**). Other markers indicate chondrogenesis in tendon includes SOX-9 expression, GAGs deposition and collagen II in tendon matrix[112].

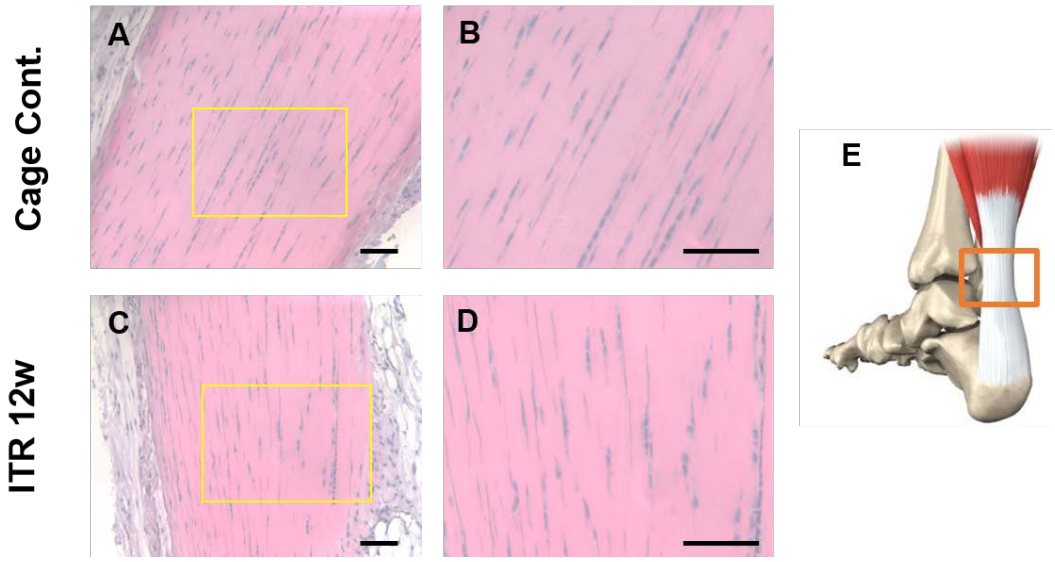

Figure 33. Minimal cellular or structural changes in the mid-portion of mouse Achilles tendon after 12-week ITR. (A) Control tendon with typically organized collagen matrix and spindle shape tenocyte. (B) The enlarged are (yellow box, A) shows normal tendon tissue. (C) Representative Achilles tendon shows no overall degenerative change. (D) The enlarged area (yellow box, C) shows normal cell shape and intact tendon matrix structure. (E) The illustration shows the mid-portion of Achilles tendon. Note that the tendon cells are tightly packed in the collagen fibers. Most cells are spindle-shaped and arranged along the long axis of the tendon with the collagen fibers. H&E staining Bars - 100 µm.

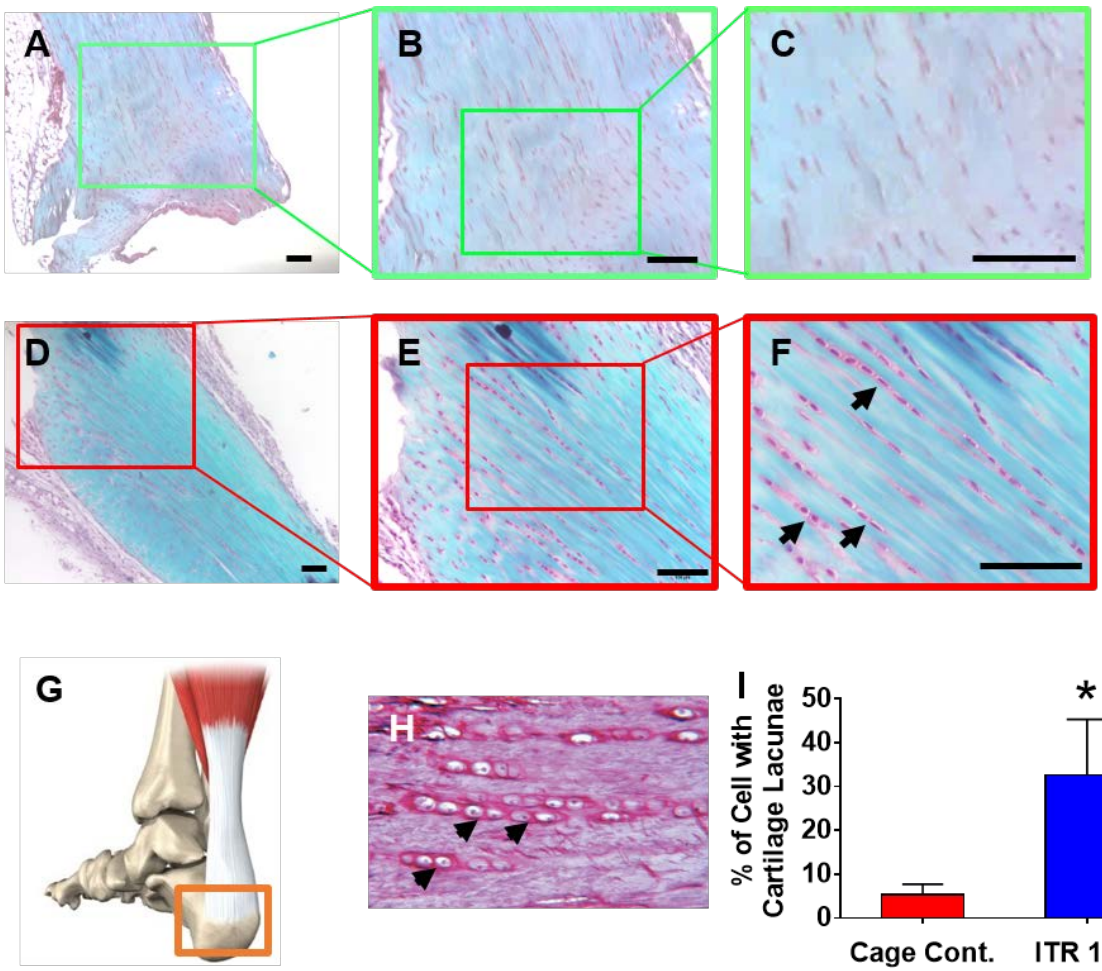

Figure 34. Tendon tissue near Achilles-bone insertion site shows cell morphology change after 12-week intensive treadmill running. (A, B, C) Control tendons near insertion site show regular tendon matrix structure and spindle cell morphology. Tenocyte is tightly packed amongst collagen fibers with little space between the cell body, and the matrix **(D, E, F)** show chondrocyte-like "round" cells with cavities called cartilage lacunae in ITR tendon (**F, black arrow**), with obvious "blank" area between cells and extracellular matrix. **(G)** The anatomy region near the insertional site of the tendon-bone junction where the tendon tissue is presenting. **(H)** fibrocartilage histology image shows the chondrocytes and the cartilage lacunae (Fig. 34 H source: Mescher AL: Junqueira's Basic Histology: Text and Atlas 12th edition) **(I)** semi-quantification of the percentage of round cells with cavities in a 20x field on the end site of Achilles tendon shows around 30% round cells with cartilage lacunae. Bars - 100 μm.

A total of 6 mice were divided into two groups, the control group and the treadmill running group. The control group was allowed cage activity, and the treadmill running group was

subjected to 12 weeks treadmill running similar to our intensive treadmill running protocol in previous chapters (3hrs a day, 5 days a week at 13m/min). After 12 weeks of intensive treadmill running, no obvious structural changes were found in the mid 1/3 section in Achilles tendon of TR (**Fig. 33**). But the histological analysis at the tendon tissue near the bone insertion site of the Achilles tendon showed significant cell shape changes in the TR group mice **(Fig. 34 D, E, F**) compared to the control group (**Fig. 34 A, B, C)**. Part of the cells in TR group showed round shape with cavities around the cells which are a very typical chondrocyte appearance (**Fig. 34F**, arrows). Typical chondrocyte morphology in cartilage tissue can be seen in **Fig. 34 H** which is very similar to the arrow pointed cells in TR tendon (**Fig. 34 F**), which is largely different from

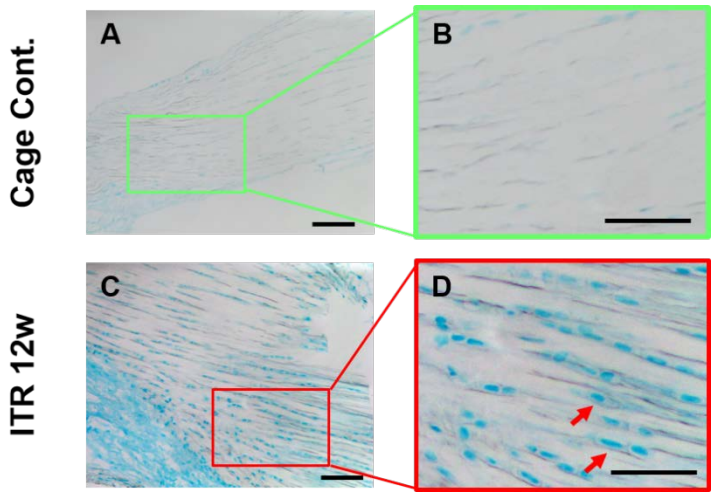

Figure 35. GAGs deposition in Achilles tendon from 12-week ITR mice shows early degenerative changes of the overuse tendon *in vivo*. Achilles tendon stained with Alcian blue near the insertion site. **(A)** Control group shows minimal GAGs deposition near the insertion site. **(B**) Enlarge figure of **(A)**. **(C)** Achilles tendon from ITR group shows a high amount of GAGs deposited in cells and the only slight amount in tendon matrix near the insertion site. **(D)** Enlarged **(C)**, shows Alcian blue is positive in cells, but with weak matrix staining signal. The arrow in D points to the cells with cartilage lacunae in the ITR group. Bars - 100 µm.

normal tendon cells in control group that are tightly packed in the collagen tissue without lacuna and with spindle shape (**Fig. 33B**).

Alcian blue staining was used to identify possible GAGs deposition in the TR mice Achilles tendon. While the control group showed minimal staining signal which indicated GAGs are not largely present in tendon cells or matrix (**Fig. 35A, B**), GAG was produced by the cells that were in round shape near the insertion site and only a minor amount was deposited to the tendon matrix in the treadmill running group (**Fig. 35C, D**). Some of those round shaped cells in TR tendon were also positive for SOX-9 (**Fig. 36C, D**), while most of the cells in normal tendon were negative for SOX-9 (**Fig. 36 A, B**).

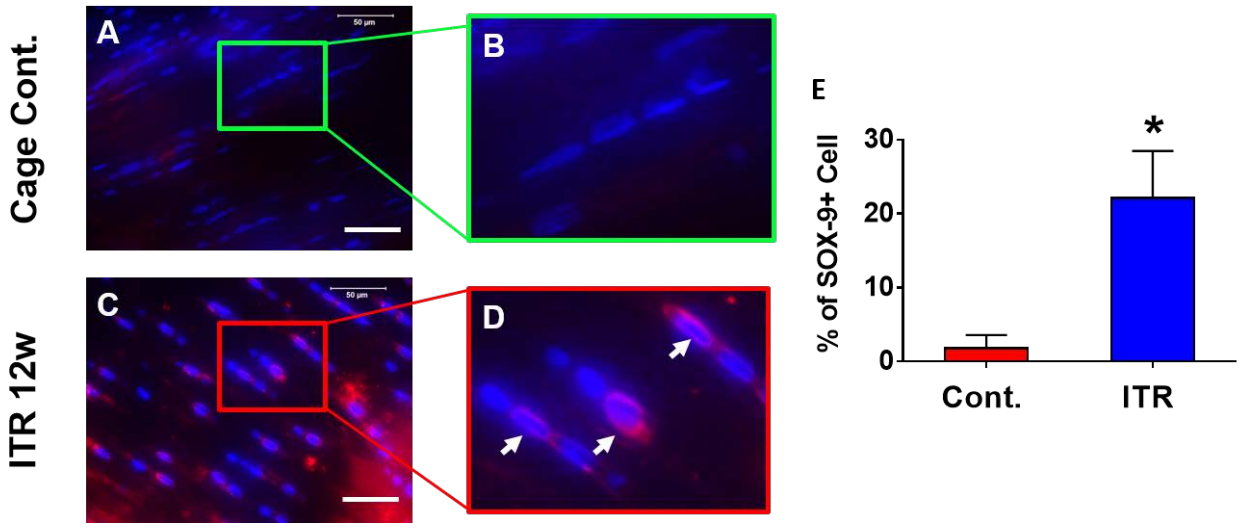

Figure 36. Cartilage lineage marker SOX-9 is expressed in ITR tendon near insertion site but not in control tendon *in vivo*. (A, B) Achilles tendons from control group show minimal staining for SOX-9. (**C, D**) Achilles tendons from ITR group show Sox-9 staining in tendon area near insertion site (white arrow). (**E**) Semi-quantification shows about 20% cells are SOX-9 positive near the insertion site while nearly no cells in control group express SOX-9. Data represent mean ± SD. n = 4. * $P < 0.05$. Bars - 50 µm

Semi-quantification revealed that about 20% cells in 12 weeks TR group tendons near insertion site were positive for SOX-9 (**Fig. 36E**). The structure of the tendon fiber was still well-organized. All these evidence implied that the mice on 12 weeks of TR could develop early symptoms that could represent the insertional tendinopathy similar to that in human patient settings. This will be a valuable model to evaluate possible treatment or prevention method for insertional tendinopathy. Unlike the chemically-induced tendinopathy model such as the collagenase model, or physical injury-induced tendinopathy, the injury was induced purely by overuse of the tendon itself in this model. Therefore, it may better represent the pathological development of the disease and thus may provide insights into the etiology and treatment of tendinopathy.

These results prompted to re-evaluate the model and focus the analysis on the insertion site of Achilles where the insertional tendinopathy in humans likely occur.

The insertional site of Achilles tendon is located at the tendon-bone junction, and it is divided into four continuous zones, 1) tendon zone, 2) fibrocartilage,3) mineralized fibrocartilage and 4) bone [72]. The degeneration often takes place in the tendon zone, and in human patients, a histological study showed typical degenerative tendinopathy appearance, increased number of cells, and more GAG in the matrix as well as disorganized collagen fiber in insertional Achilles tendinopathy [73]. It is of great value to use this model to evaluate the effect of GL in preventing the degenerative change in tendon due to mechanical overloading in this model.

4.4 GL PREVENTS DEGENERATIVE CHANGES NEAR INSERTION SITE IN ACHILLES TENDON SUBJECTED TO LONG TERM INTENSIVE TREADMILL RUNNING

As we established an early tendinopathy model with 12-week intensive mice treadmill running, next aim was to prolong treadmill running period to 24 weeks to maximize the effect while testing the inhibitory effect of GL on HMGB1 in preventing the degenerative changes.

The groupings for the mice were same as in the 3 weeks running groups. A total of 28 mice were used in this study with 24 being analyzed. 4 mice were excluded from the experiment either because of accidental death during treadmill running (2 mice) or due to failure to carry out the treadmill running treatment (2 mice) for the entire duration. The mice were divided into 4 groups: Cage control, GL, TR, and TR+GL as previously described. The treadmill running speed was 13 m/min but total running time remained the same, 3hrs per day, 5 days a week. The lower speed compared to 3 weeks treadmill running (15 m/min) was set to avoid possible acute injury, and sudden death during the long-term treadmill running that may significantly decrease the available mice at the end of the experiment.

Table 3 Body weight of mice after 24 weeks TR study

Group	Body weight(g) before study	Body weight(g) after study
Control	19.88 ± 1.49	25.80 ± 2.04
GL only	19.64 ± 1.51	24.65 ± 1.33
TR only	19.28 ± 1.57	21.75 ± 1.54*
TR+ GL	19.75 ± 1.61	21.23 ± 1.64*

*p<0.05 compare to control

In the first 12 weeks, the treadmill was set flat for the running, but for the next 12weeks of treadmill running, the 5° uphill was set up to increase the load on Achilles tendon. The body weight and activity level, as well as the gross appearance of all the GL, injected mice were monitored. It was found that 24 weeks GL injection did not result in an obvious change in body weight **(Table 3, Fig. 37A)** or gross appearance and the body composition (percentage of fat, fat/lean ratio, measured by Echo MRI whole body composition analyzer echo medical systems Houston ,Texas)**(Fig. 37B, C)** was also very similar to control group. The treadmill running mice were thinner with lower body weight, fat percentage, and low fat: lean ratio compared to the control mice **(Fig. 37 A, B, C)**. This finding indicates that long-term treadmill running reduces weight and body fat.

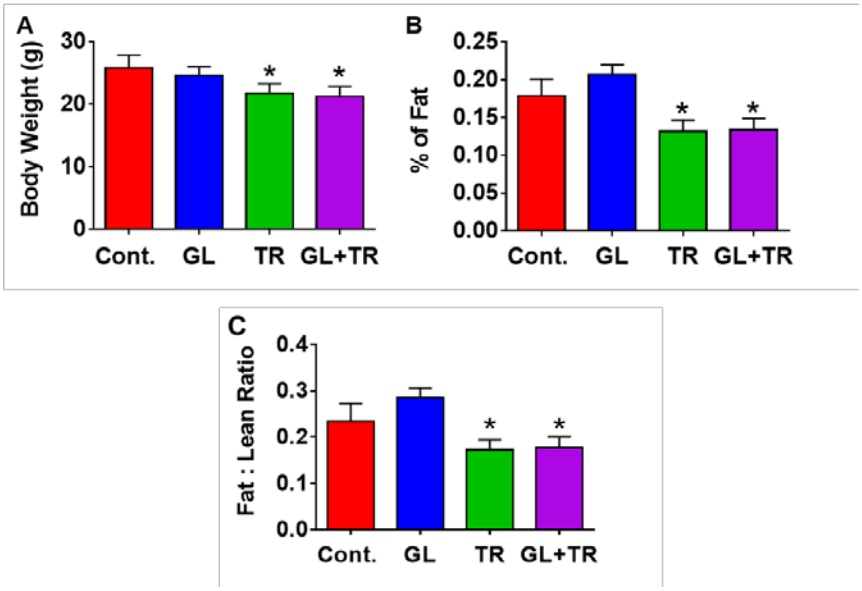

Figure 37. Body composition of mice changes after 24 weeks treadmill running regimen. All group of mice started with similar average body weight. **(A)** After 24 weeks treadmill running, both the TR and TR +GL injection group's body weight is significantly less than control, and GL only injected groups. No significant difference in body weight was found between control and GL only injected group. **(B)** Percentage of fat and **(C)** fat to lean ratio are significantly lower in TR and TR+GL group. Data represent mean ± SD. n = 6. * $P < 0.05$.

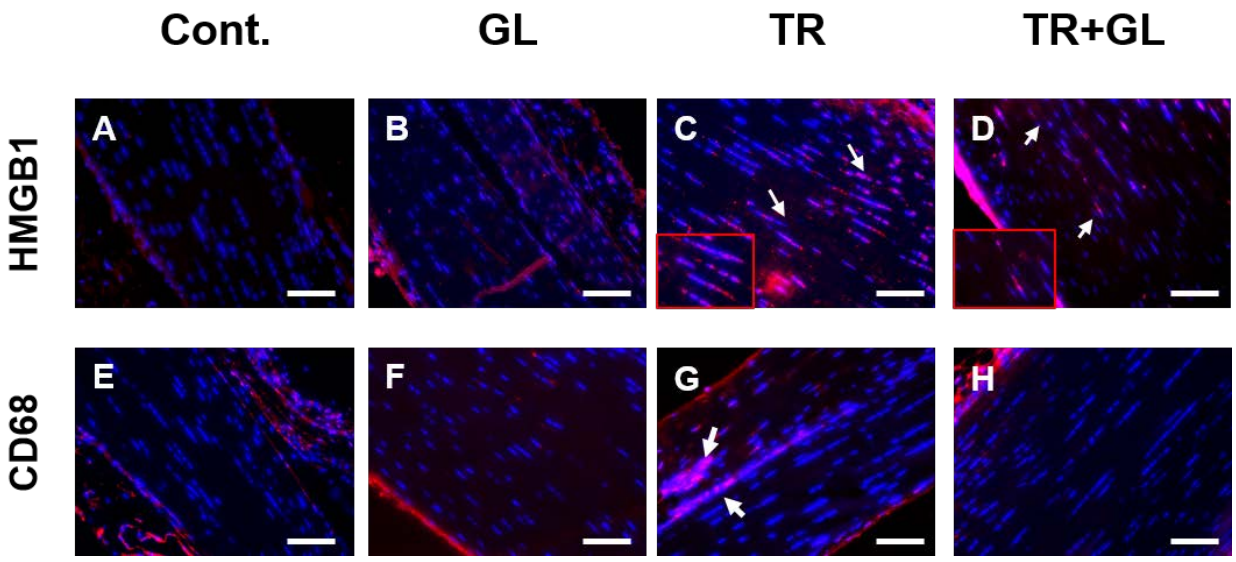

Figure 38. HMGB1 (in the matrix) and CD68 expression are positive in 24-week treadmill running mice Achilles tendon near the insertional site. (**A, B**) The HMGB1 signal is minimal in the tendon matrix near the insertion site of the mice Achilles tendon. **(C)** HMGB1 is expressed in tendon matrix in the treadmill running group (arrows). **(D)** HMGB1 can also be detected in the matrix of GL+TR group. **(E, F)** CD68 is negative in cage control and GL injection only group. **(G)** CD68 is positive in TR sample (arrows) and gathered in a clustered form. **(H)** No positive CD68 signal in the GL-treated TR tendon tissue. Bars - 50 µm

At the end of the 24 weeks, the Achilles tendons were harvested for tissue section and staining. We first checked the HMGB1 in the tendon matrix near the insertional site in all groups. HMGB1 was not present in the control or GL group as expected (**Fig. 38A, B**). HMGB1 was still expressed in the matrix of the TR group mice after 24 weeks running **(Fig. 38C**). In the TR+GL group mice tendon (**Fig. 38D**), HMGB1 was positive in the matrix but was relatively weak compared to the TR group. For CD68 staining, we can clearly see that CD68 was only positive in the TR group (**Fig. 38G)** but not in control, GL or TR+GL group (**Fig. 38E, F, H)**. This indicates that GL treatment did block the migration of CD68 positive cells into tendon matrix after treadmill running for 24 weeks. The weaker HMGB1 expression in GL+TR group also indicated that cells may still be stressed and may have released HMGB1.

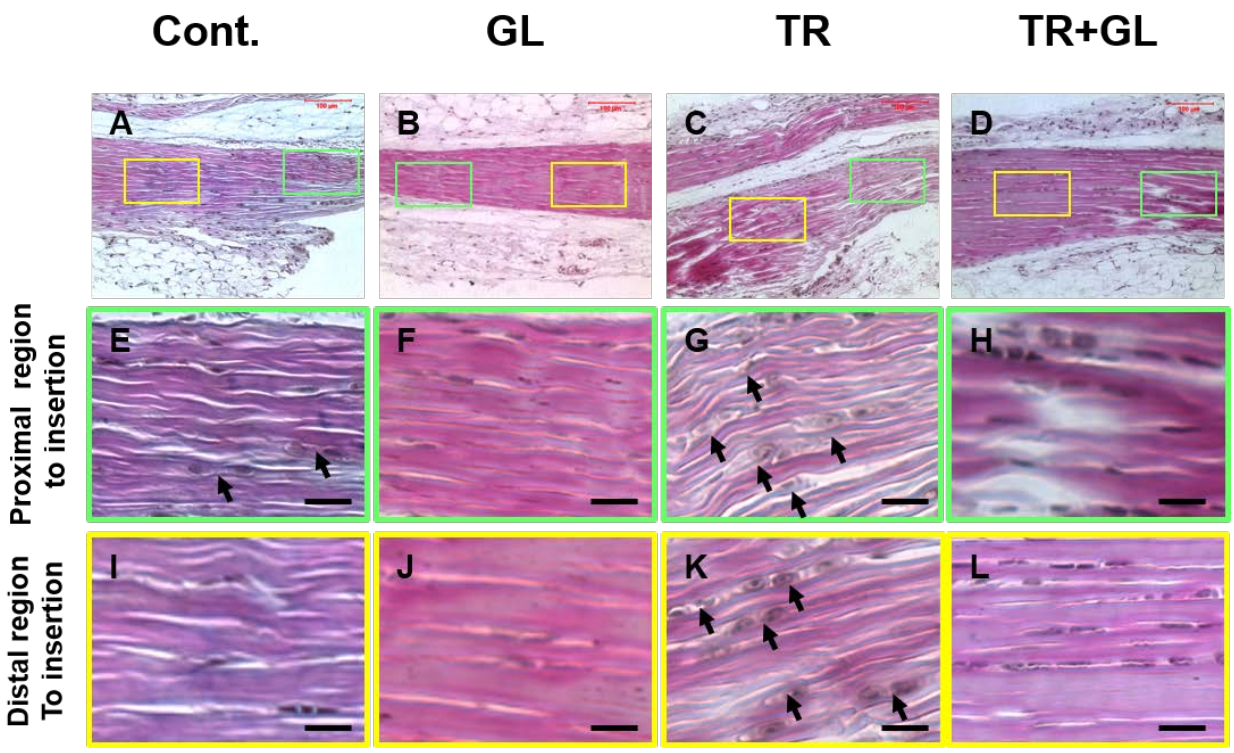

Figure 39. GL attenuates cell shape change in 24-week intensive treadmill running mice Achilles tendon near the insertional site. Achilles tendon near the insertion site in **(A)** Cage control. **(B)** GL injected only. **(C)** Treadmill running. **(D)** Treadmill running +GL injection groups. To better evaluate the TR's effect and GL treatment effect, we roughly divided tendon tissue near insertion site to proximal region (to insertion), which is the tissue relative close to the tendon-bone junction, and the distal region (to insertion) which is relatively away from the junction. (**E)** The proximal region of cage control tendon. Because this site is very close to the junction, it may contain some round shaped cells with cavities (with space between cell and matrix, these cells are likely chondrocytes). **(F)** The proximal region of GL injection only tendon shows a few round shaped cells. **(G)** The proximal region of TR Achilles tendon shows lots of cells with cavities (arrows). **(H)** The TR+GL group tendon shows some round shaped cells without cavities. **(I, J)** Cage control and GL only group do not show round cells with cavities, meaning no cartilage cells exist in this area at the distal region. (K) Compared to the distal region of GL only tendon, TR tendon shows a high amount of chondrocyte-like cells with cavities. **(L)** GL treatment along with TR shows minimal chondrocyte-like cells in the distal region, indicating that GL treatment attenuates the cell type change near insertion site induced by TR. Pictures are representatives from 6 mice. Bars - 25 μm.

Then we closely examined the cellular and structural changes in the tendon tissue near the insertion site. In order to better evaluate the treadmill running effect and GL inhibitory effect, we divided the Achilles tendon near the insertion site into two parts, the proximal region of the tendon-bone insertion, which is very near the end of the tendon tissue that could be considered as the transitional zone between tendon and bone, and the distal region to the tendon-bone insertion, which is considered normal tendon tissue. We found that a small number of chondrocyte- like cells might exist in the proximal region in control group (**Fig. 39E**). So we focused on the distal region since it better represents tendon tissue degenerative change site rather than the region of possible pre-existing cells.

H&E staining showed that the change in the morphology of tenocytes to round shaped cells with lacuna predominated at both the proximal and distal regions of TR tendon (**Fig. 39 G, K**), but not in the distal region of control, GL injected or TR+GL injected Achilles tendon (**Fig. 39I, J, L).**

Alcian blue staining for GAG deposition showed that the TR group had very strong blue staining in the tendon matrix at the proximal and distal regions (**Fig. 40G, K**). while control GL only and GL+TR group showed weak staining signal only at the proximal region **(Fig. 40E, F**) but not the distal region (**Fig. 40 I, J**). In the GL+ TR tendon, GAGs deposition could be identified weakly in the proximal part (**Fig. 40H)** and negative in the distal region (**Fig. 40L).** The round-shaped cells with lacuna were visible near the strong staining site with chondrocyte-like morphology (**Fig. 40K)**.

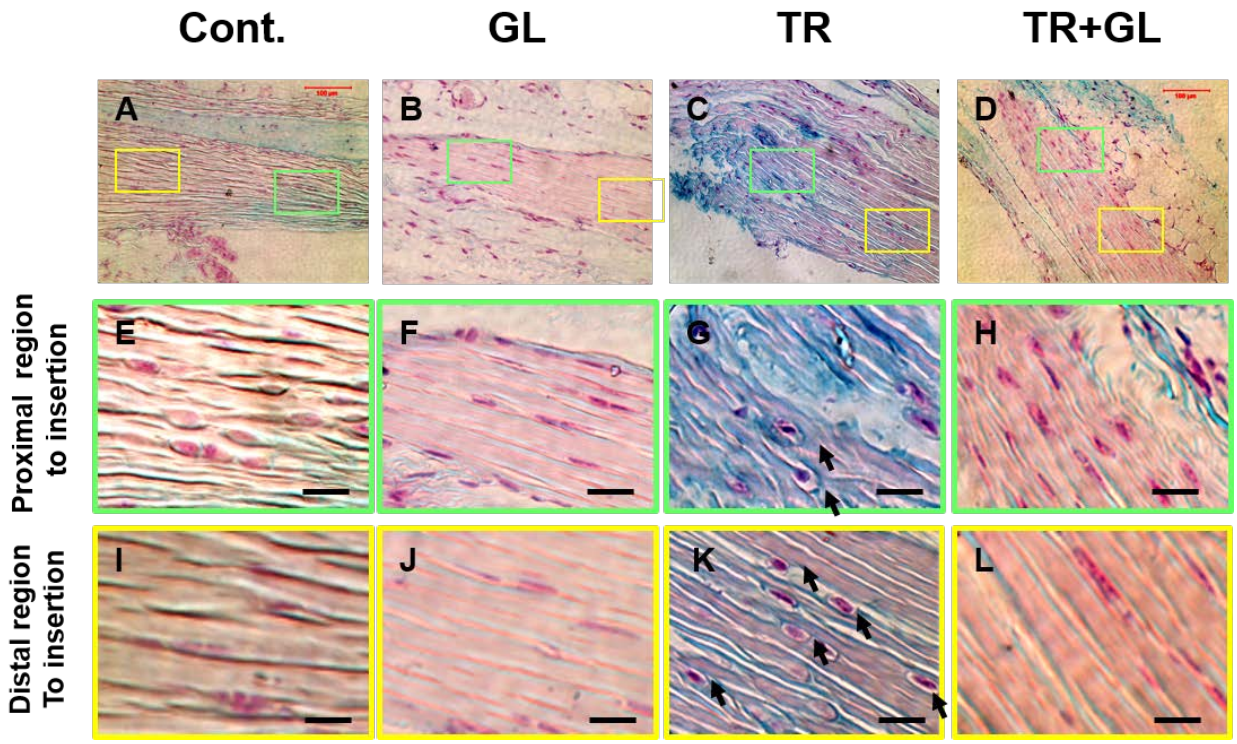

Figure 40. GL treatment prevents the GAGs deposition induced by 24-week treadmill running near the insertional site of mice Achilles tendon.

Alcian Blue staining shows the overall GAGs deposition in **(A)** Control. **(B)** GL injected only. **(C)** Treadmill running. (**D)** Treadmill running +GL injection groups near the insertional site. **(E, F**) Cage control and GL only groups show slight staining in the proximal region (closer to the junction). **(G)** 24-week treadmill running group shows very strong staining of GAGs with cartilage-like cells (arrows). **(H)** GL-treated group shows weak staining in the proximal region. **(I, J)** Minimal staining of GAGS is present in the distal region (relatively away from the insertion) of the tendon tissue. **(K)** The distal region of TR group still shows strong staining of GAGs in the matrix with chondrocytes like cells (with very clear cavities around the cells called cartilage lacunae). **(L)** Minimal staining of GAGs at the distal region of the GL-treated TR tendon tissue. Bars - 25 µm.

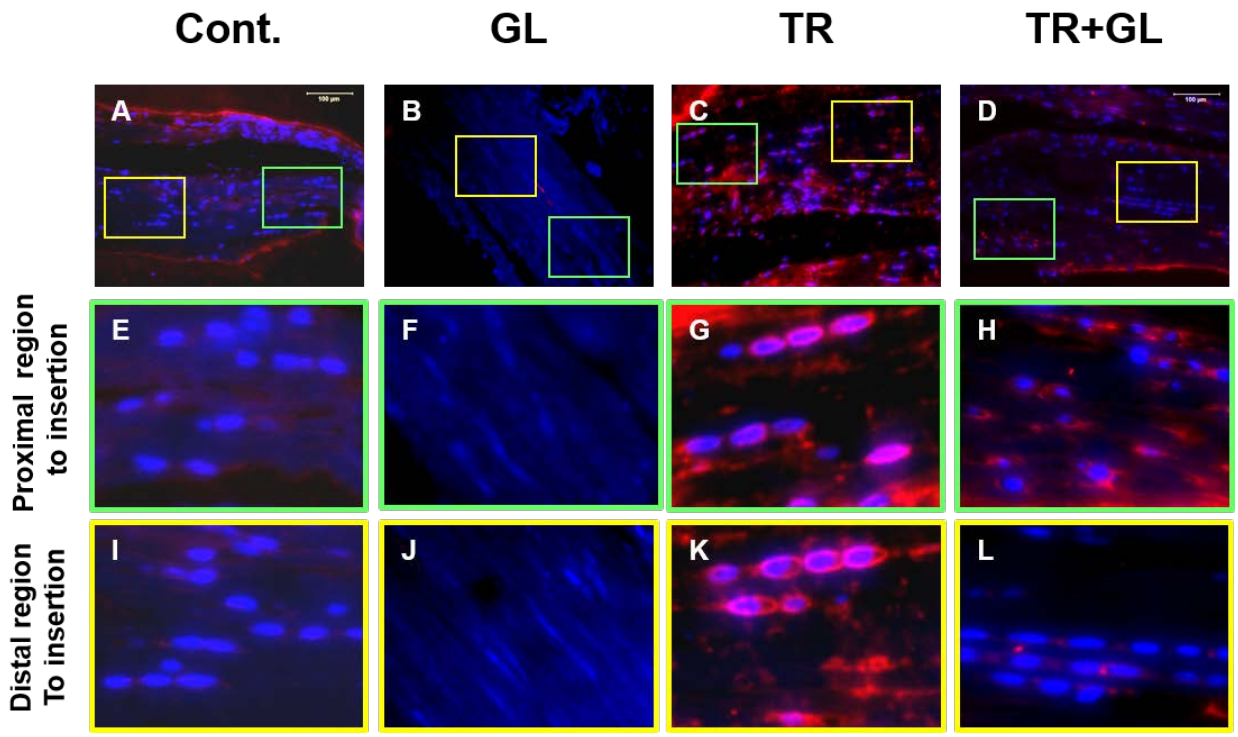

Figure 41. GL treatment reduces the expression of SOX-9 induced by 24-week treadmill running near the insertional site of mice Achilles tendon Overview of SOX-9 staining. **(A)** Control. **(B)** GL injected only. **(C)** Treadmill running. **(D)** Treadmill running +GL injection groups near the insertional site. **(E, I)** Proximal and distal regions of the control group with round cells but without SOX-9 signal**. (F, J)** No signal of SOX-9 in GL only group at the proximal or distal region. **(G, K)** Strong SOX-9 staining at the proximal and distal region of 24-week TR tendon along with the round shaped cells. **(H)** SOX-9 is positive for some cells in the proximal region of the TR+GL group Achilles tendon, **(L)** but SOX-9 signal in the distal region was minimal in the TR+GL group, indicating that GL treatment can decrease but may not completely suppress the expression of SOX-9 induced by treadmill running. Bars - 25 µm.

The SOX-9 staining is used as a marker for the chondrogenic cells. The results showed that in the control group, even some round-shaped cells exist in the proximal region that is not positive for SOX-9 (**Fig. 41E**), that hold true for the distal part **(Fig. 41I**). The GL injection only group also stained negative for SOX-9 at both sites (**Fig. 41F, J**). TR group showed very strong staining of SOX-9 in and around the nucleus of the round-shaped cells at both site (**Fig. 41G, K),**

further confirming that those cells are chondrogenic cells which are induced by long-term treadmill running.

For the collagen II staining, in control tendon and GL only tendon, collagen II can be lightly detected at the proximal region (**Fig. 42E, F)** but not at the distal region (**Fig. 42I, J),** but collagen II was expressed strongly in the TR group at both sites (**Fig. 42 G, K**), which confirmed the structural chondrogenic change in tendon tissue induced by treadmill running. However, in the TR+GL group this effect was suppressed with much less collagen II staining at the proximal region (**Fig. 42H**) and minimal at distal region (**Fig. 42L**)

These results indicated that the 24-week long-term TR induced a very strong chondrogenic degenerative change in the Achilles tendon tissue near the insertion site shown by increased chondrocytes cells, elevated GAGs deposition, SOX-9 expression in cells in the tendon, and collagen II deposition. That effect could be contributed to the chronic inflammation induced by inflammatory cells migrated to tendon tissue that was attracted by HMGB1. The GL injection along with the treadmill could very effectively reduce the CD68 cells and attenuate that degenerative effect, and as a direct inhibitor of HMGB1, the inhibitory effect from GL is very likely though inhibition of HMGB1 effect. Therefore, HMGB1 could be a therapeutic target for over-use induced insertional tendinopathy in Achilles tendon.

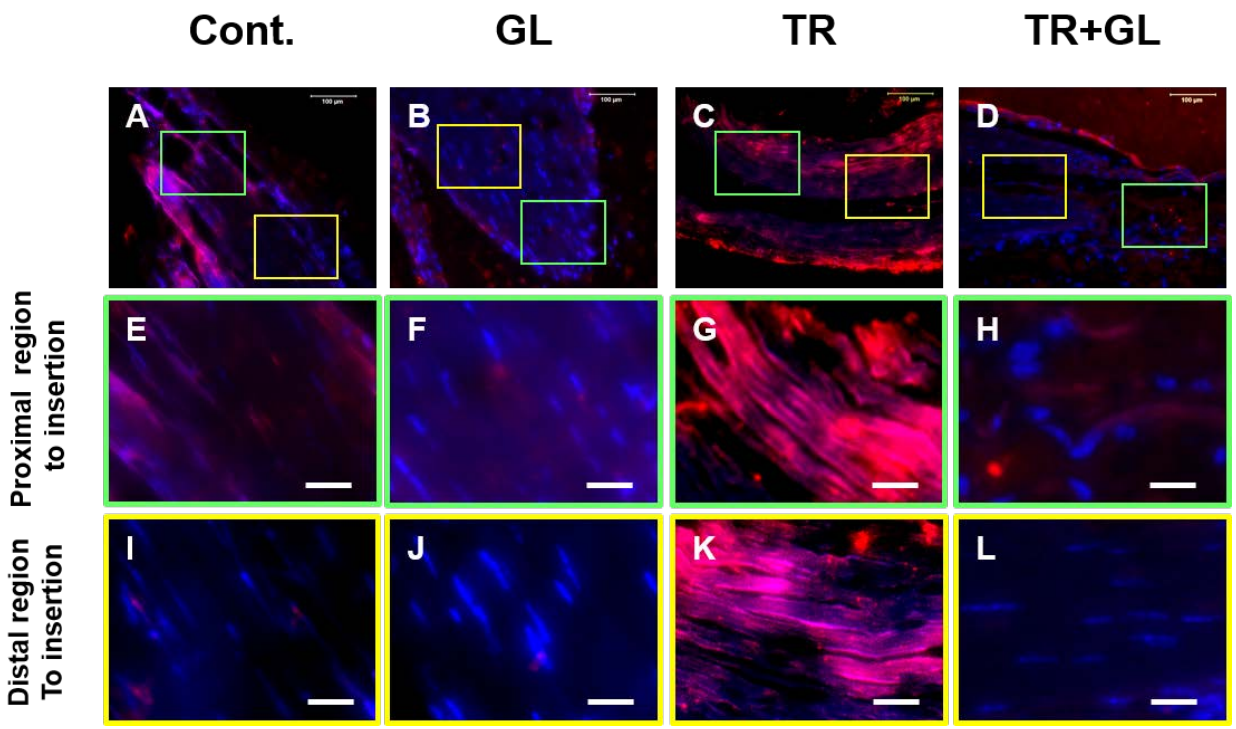

Figure 42. GL treatment reduces the deposition of Collagen II induced by 24-week treadmill running near the insertional site of mice Achilles tendon. Overview of collagen II staining in (A) Control. (B) GL injected only. (C) Treadmill running. (D) Treadmill running +GL injection groups near the insertional site. (E) Collagen II presence in the proximal region of the cage control tendon. (F) Collagen II staining very weak in the GL injection only group. (G) Collagen II is strongly expressed in the matrix of the TR group in the proximal region. (H) Light staining of Collagen II in the matrix of the proximal region tendon tissue in the TR+ GL group. (I, J, L) Collagen II is mostly negative for the control, GL, and TR+GL group at the distal region, but the expression of collagen II is still very strong at the distal region of the TR group tendon tissue, which implies that 24-week running induced collagen II deposition in matrix can be attenuated by GL treatment at the distal region. Bars - 25µm.

4.5 METHODS USED IN THIS CHAPTER

4.5.1 GL toxicity on tendon cells

2000 rat Achilles tendon cells (P4) were plated in 10% FBS +DMEM into each well of 96 well plate and allowed to attach overnight. The medium was changed to 10% FBS+DMEM with different concentrations of GL (0, 1, 10, 100, 200, 1000, 5000, and 10000 μM) with condition repeated 6 times. The cells are then cultured for 72hrs and 10 μL CCK-8 solution was added to each 96 well. 6 wells with no CCK-8 addition and no GL treatment were served as a blank group. The whole plate was then cultured for 2hrs and then OD value under 450nm was measured as an indicator of the cell viability.

4.5.2 Safranin O and Fast Green staining

Safranin O staining was conducted by first putting slides with tissue section on into iron Hematoxylin for 5 min then rinsed with double distilled water. The slides were dipped in 1% acidic alcohol for 10s followed by rinsing with double distilled water. The slides were then dipped into 0.02% Fast Green for 1min, and then in 1% acetic acid for the 30s followed by staining with 1% Safranin O for 30min. The slides were finally rinsed in 95% EtOH and then dehydrated with 2 changes of 95% EtOH and 2 changes of 100% EtOH each for 1min. Finally, slides were washed 3 times with Xylene and covered with coverslips. Pictures were taken under histology microscope.

4.5.3 Alcian blue staining

Alcian blue staining kit was purchased from Abcam, (Cambridge, UK, ab15066) and followed the manufacturer's protocol. Briefly, glass slides with tissue on it were hydrated first and incubated in acetic acid for 3 min. The slides were incubated in Alcian blue (pH 1.0) solution for 30min at room temperature and then rinsed with acetic acid. They were then rinsed with running tap water for 2 min, followed by washing with two changes of distilled water. The slides were stained with Alcian blue solution for 5min, followed by rinsing with running tap water and two changes of distilled water. The slides were dehydrated with graded alcohols, washed in with Xylene and covered with coverslips. The pictures were taken with histology microscope.

4.5.4 ELISA and Immunostaining of tendon tissue

For PGE_2 and MMP-3 ELISA, the samples were placed in 200 µl T-PER tissue protein extraction reagent (ThermoFisher, Pittsburgh, PA, 78510) instead of PBS. The samples were vigorously homogenized with BioMasher Standard (Takara, Shiga, Japan, 9790A), centrifuged at 2,000 g for 30 min at 4°C, and the supernatants were collected for ELISA.

For immunostaining, Achilles tendons samples were dissected from the mice immediately at the end of treadmill running and fixed with 4% paraformaldehyde for 2hrs then embedded in paraffin, and sectioning was performed to obtain about 5 µm thick tissue sections. The sections were deparaffined with Xylene and were treated with EtOH, with concentrations decreased for each treatment (100%, 80%, 50%, and 30%). For CD68, Sox-9 and Collagen II staining, the tissue sections were treated with 0.2% Triton X-100 in PBS for 1hr at RT to penetrate cell membrane before the blocking with universal blocking solution at RT for 1hr

(ThermoFisher Scientific, Pittsburgh, PA, 37515). The tendon tissue samples were stained with rabbit anti CD68 (2 µg/ml, Abcam, Cambridge, UK, ab125212), goat anti SOX-9 (1 µg/ml, Santa Cruz, Dallas,TX, Sc-17340) or rabbit anti collagen-2(1 µg/ml, Abcam, Cambridge, UK, ab116242) at 4˚C overnight followed by corresponding secondary antibody conjugated with Cy3 for 1hr at room temperature (0.5 µg/ml, Millipore, Billerica, MA, AP132C, AP305P). All antibodies were prepared in the blocking solution. Then they were counterstained for the nucleus with 10 µg/ml Hoechst 33342 for 5min. Pictures were taken with fluorescence camera same as mentioned in chapter 2. The HMGB1 was stained similarly with rabbit anti HMGB1 (2 µg/ml, Abcam, Cambridge, UK, ab18256), and goat anti-rabbit secondary antibody conjugated with Cy3 (0.5 µg/ml, Millipore, Billerica, MA, AP132C), but without Triton X-100 treatment.

4.5.5 Determining the presence of GL in mice tendons after GL injection

Tendon tissues from the control and treadmill running mice (see above) were weighed, minced and homogenized in PBS (1 mg tissue/0.5 ml PBS) at 4˚C. Then, the samples were centrifuged at 10,000g for 30 min at 4˚C to collect the supernatant. Simultaneously, a 1 mg/ml GL stock solution in methanol was prepared and diluted in double distilled water to prepare various concentrations of GL solution (0-1000 µg/ml) to get a standard curve for GL quantification. Then, 0.1 ml of a GL standard (0-1000 µg/ml) was mixed with 0.1 ml of the supernatant from the GL injected (GL group) mice tendons (see above for details). The control received 0.1 ml PBS (0.1 ml). Nucleic acids and proteins in all samples were removed by passing 0.2 ml of each sample through individual spin columns (Qiagen, Valenia, CA) followed by centrifugation at 8000 rpm for 2 min. The GL concentrations in the resulting supernatants were determined at 252 nm using a spectrophotometer (Molecular Device, Sunnyvale, CA) according to a previous

publication [269] and calculated based on the equation OD=0.0015X + 0.2041 (R^2=0.9931) obtained by the standard curve.

4.5.6 GL inhibition of HMGB1 effect *in vitro*

To test whether GL can inhibit HMGB1's inflammatory effect on tendon cells *in vitro,* the following experiment was performed. Tendon cells isolated from rat patellar tendons in passage 3 (~2 x 10^5 cells) were seeded in 6 well plates and allowed to attach overnight in 10% FBS+DMEM. Next day, the culture medium was replaced with 2% FBS DMEM. Then, the control group received PBS while the treated group was supplied with HMGB1 such that the final concentration was 10 µg/ml. HMGB1+GL group received a mixture of GL (200 µM, Sigma, St. Louis, MO, 50531) + HMGB1(10 µg/ml). The culture medium was collected at 0.5, 2 and 4hrs to quantify PGE_2 production by ELISA.

4.5.7 3-weeks Treadmill running and GL inhibition of HMGB1 effect *in vivo*

In these experiments, a total of 24 female C57B6/L mice (3 months old) were used with 6 mice in each of the 4 groups; i) Cage control group (C) where mice received intraperitoneal(IP) injection of 400 µL PBS 5 days a week and allowed cage activity served as control group, ii) Intensive treadmill running (TR) group where mice ran on the ITR regimen (see *in vivo mouse treadmill running model* in chapter 2 for details) with daily injection of PBS, iii) Glycyrrhizin injection only (GL) where mice received daily (IP) injection of GL (50 mg/kg body weight), and iv) Intensive treadmill running with Glycyrrhizin injection (TR+GL) group where mice received daily IP injection of GL (50 mg/kg body weight) and ran on the ITR regimen. After treadmill

running, patellar and Achilles tendons were dissected out, and the right and left side of each tendon from a single mouse were homogenized in T-PER buffer (Themofisher, Pittsburgh, PA) and the supernatants were used for ELISA to measure PGE_2 and MMP-3.

4.5.8 24 weeks treadmill running and GL inhibition

The treadmill running protocol was the very similar to the 3-week running protocol, totally 24 mice were divided into 4 groups, the only difference was that the TR mice and GL+TR mice ran a horizontal treadmill in the first 12 weeks, and then ran a 5° uphill treadmill to increase the load on Achilles tendon to maximize the treadmill running effect. At the end of week 24, all mice were sacrificed, and the Achilles tendons were dissected and used for histology and IHC analysis.

4.5.9 Statistical Analysis

Student’s t-test and One-way ANOVA was used, followed by Fisher's least significant difference (LSD) test for multiple comparisons. When P-values were less than 0.05, the two groups compared were considered to be significantly different.

5.0 DISCUSSION

This study demonstrated that mechanical overloading-induced HMGB1 release from the nucleus into the extracellular milieu both in tendon cells and in tendons. Also, we showed that addition of HMGB1 promoted tendon cell migration and inflammatory reactions by enhancing the levels of COX-2 and PGE_2 *in vitro.* Furthermore, high dose HMGB1 implantation into rat patellar tendon resulted in hypercellularity, angiogenesis, and inflammatory cell infiltration, characteristics of the early inflammatory stage of tendinopathy in tendon tissue *in vivo.* Interestingly, GL reversed the HMGB1-induced PGE_2 and MMP-3 release *in vitro*, and mechanical overload-induced PGE_2 and MMP-3 in an *in vivo* model. We also established an insertional tendinopathy model induced by long-term treadmill running(up to 24 weeks). The exercised tendon showed cell shape change, Sox-9 expression, GAGs and collagen II depositions near the insertion site of Achilles tendon. Additionally, we showed that daily GL injection effectively reversed the overloading-induced degenerative changes in Achilles tendon.

These results provide the first evidence for the role of HMGB1 in the onset of the inflammatory cascade in tendon pathology which leads to the development of degenerative tendinopathy. Therefore, HMGB1 could serve as a potential therapeutic target to curb chronic inflammation associated with the pathology of tendinopathy. The reversal of inflammatory reactions in tendons by GL also suggests the putative therapeutic potential of this natural triterpine.

GL treatment could be suitable for the athletes who have to participate in overload training, and provides a protective effect against the HMGB1 generated during these overloading on the tendon, thus block the sustained inflammation in tendon and allow the tendon to heal without being stuck in the inflammation phase due to the constant HMGB1 release. The treatment could also be helpful to the athletes who have to participate in the overexercise for a while, as we found in our study that administration of HMGB1 could induce hypercellularity and vessel ingrowth in 2 weeks. But by the end of 4 weeks, there is a great reduction of all these effects which may be due to the depletion of HMGB1, suggesting that HMGB1 induced change could be reversible in the early stage.

GL treatment may not be able to treat established degenerative tendinopathy directly, but it might be helpful to act as a post-surgical treatment. Surgical removal of the degenerative tissue is sometimes conducted in late stage tendinopathy to attenuate the inflammation induced by HMGB1 released from the damaged and necrotic cells during the trauma of the surgical process.

One important issue is about the safety and cost of GL administration. GL is the major bioactive component in licorice and can be extracted and purified without relatively low cost. Licorice is a widely used Chinese medicine [270], and it is also a popular sweetener found in many soft drinks, food products, snacks and herbal medicines [271]. It is approved by Food and Drug Administration. Health products like licorice-flavored cough mixtures, throat pearls, licorice tea, licorice-flavored diet gum, laxatives are known to contain licorice [271]. There are known side effects of overdose licorice. The side effect of GL is mainly from its metabolic product glycyrrhetic acid after oral ingestion. Glycyrrhizin or glycyrrhizic acid(**Fig. 43A**), can be hydrolyzed to glycyrrhetic acid(**Fig. 43B**) by intestinal bacteria through a specialized β-glucuronidase [272], both glycyrrhizic acid and glycyrrhetic acid can inhibit 11-β-hydroxysteroid

dehydrogenase(11- β-HSD), which will result in increased sodium reabsorption, causing pseudo-hyperaldosteronism[273], usually characterized by hypertension, kaliuresis and reduced plasma renin [274]. However, the metabolic product glycyrrhetic acid is 200-1000 times more potent inhibitor of 11- β-HSD, so the side effect is more relevant after oral ingestion of licorice [271]. A dosage of 380 and 814 mg/day but not 108 or 217 mg/day GL ingestion was able to induce side effect in healthy volunteers in 4 weeks [275]. A case study reported a 78-year old male patient who ingested GL (280 mg/day) for 7 years was hospitalized for damaged skeletal and cardiac muscles and acute kidney failure with other symptoms like hyporeninemic hypoaldosteronism which are closely related to GL side effects [276]. Those studies suggested that the side effect of GL is largely dosage and time dependent.

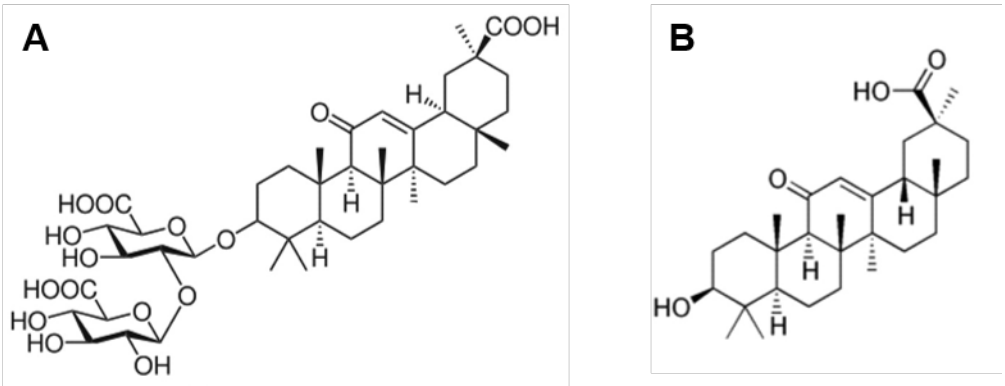

Figure 43. The structure of Glycyrrhizin and glycyrrhetinic acid. (A) Chemical structure of Glycyrrhizin **(B)** Structure of hydrolyzed product of glycyrrhizin: Glycyrrhetinic acid. (Source: [277])

Intravenous GL has already been in clinical use to treat chronic hepatitis patients [278], but overall, the non-oral administration of GL was not extensively studied in human patients. However, according to our animal study, 50 mg/kg IP administration of GL for up to 24 weeks

(5 days a week) did not induce significant body weight or body composition change in young mice, and the mice received GL injection ran treadmill as good as the control group without GL. This finding is also in agreement with several other GL studies which used similar dosage injection [263, 264].Overall, non-oral GL administration seems to be a safe way, but still, needs to be extensively tested clinically.

Another concern about GL is its off-target effect. It was reported that GL administration has anti-inflammatory effects. One study found that GL treatment can attenuate sepsis-induced kidney injury through inhibition of NF-κb pathway [279]. This attenuation effect in this study could be highly related to the inhibition effect of GL on HMGB1 since, in the mice model, the HMGB1 antibody is sufficient to attenuate sepsis-related mortality [151]. It is known HMGB1 binding to its receptor can subsequently activate NF- κb pathway [280, 281]. Another study found that GL treatment could decrease COX-2 expression in macrophage from parasites-infected mice *in vitro* [282]. COX-2 is also regulated by NF-κb pathway [283-285].None of these studies mentioned or excluded the possibility of GL's inhibition of HMGB1 effect. It is highly possible that that "off-target" effect of GL is still through the inhibition of HMGB1 directly. However, clinically speaking, even if the GL off-target effect on NF-κb and COX-2 is independent of HMGB1 inhibition effect. It can still work synergistically with its principal effect on blocking HMGB1 on the attenuation of the inflammatory reactions during the development of tendinopathy, those off target effects are not considered detrimental.

5.1 HMGB1 RELEASE TO EXTRACELLULAR MILIEU INDUCED BY REPETITIVE OVERLOADING

5.1.1 HMGB1 is new to tendon biology

Our study identified the presence of HMGB1 for the first time in patellar and Achilles tendon cells and tissues of rodents. The identification of the presence of HMGB1 in patellar and Achilles tendon in this study is the first confirmatory study of HMGB1 in the tendon. This finding is of importance as the role of inflammation in the development of tendinopathy is gaining attention, based on the premise that alarmins including HMGB1, HSPs, S100 may participate in the development of tendinopathy [174, 286]. The putative role of HSP-70 and HIF-1 in inflammatory tendinopathy has been reported, but the role of HMGB1 is not investigated yet. These findings will reinforce the notion that as an alarmin, HMGB1 functions as an inflammatory mediator in tendon biology and pathology.

To date, there are no in-depth studies on the role of HMGB1 in tendon physiology and pathology. However, as researchers are focusing on the relationship between tendon inflammation and development of tendinopathy, there is a strong trend now to explore the effect of HMGB1 and other DAMPs in tendon biology and tendinopathy [174]. A recent study has identified significantly elevated levels of HMGB1 in the tenocyte of the rotator cuff in arthritis patients. However, it lacked additional research about the role of HMGB1 in such disease settings [220].

It was found that extracellular HMGB1 play important role to promote inflammation when cells were subjected to accidental damage[158].The role of HMGB1 as an inflammatory mediator was not investigated in muscular-skeletal disorders until its presence was identified in

synovial tissues and fluids of rheumatoid arthritis patients [287]. Moreover, inhibition of HMGB1 release and activity could prevent the development of arthritis in experimental animals [288, 289]. It is very worthwhile to explore the potential function and pathological significance of HMGB1 in tendon research field.

5.1.2 The different expression pattern of HMGB1 in tendon cells *in vitro* and *in vivo*

HMGB1 is widely-expressed in various tissues [290]. Its expression and localization may change depend on cell and tissue types [130]. It is very interesting that in our study we found that *in vitro* cultured tendon cells are almost 100% positive for HMGB1 and most of the signal is concentrated in the nucleus (**Fig. 9E**), while the *in vivo* staining of HMGB1 in tendon tissue suggested that HMGB1 expression pattern is different from that of *in vitro*. HMGB1 is expressed in nucleus and cytoplasm in tenocytes *in vivo,* and some cells are even negative for HMGB1. One possible explaining is that the HMGB1 negative cells are aged or inactive cells, and HMGB1 positive cells (especially cells with HMGB1 in the nucleus) are young and active cells. When the cells are extracted and cultured *in vitro*, those "young" and active cells will overgrow those inactive cells and dominate in the culture.

HMGB1 is considered involved in the aging process. HMGB1 is down-regulated in aged neurons in the brain [291] and also down-regulated in the liver in old rat [290]. Our preliminary data also suggested that the location of HMGB1 in high passage cells (>passage 15, considered "aged" cells) was different from the low passage cell (passage<4, considered "young" cells) in the tendon. HMGB1 in "aged" tenocytes are translocated to the cytoplasm or even lost in cells (**Fig.44B**) while most HMGB1 in normal "young" **Fig.44A** tenocytes from mice reside in the nuclei(**Fig. 44A**). Please note that there is an enlarged cell (**Fig.44A**, arrow) in the "young"

group morphologically as a senescent cell, lost HMGB1 staining in the nucleus and cytoplasm. The HMGB1 expression maybe largely depends on the physical status of the cell.

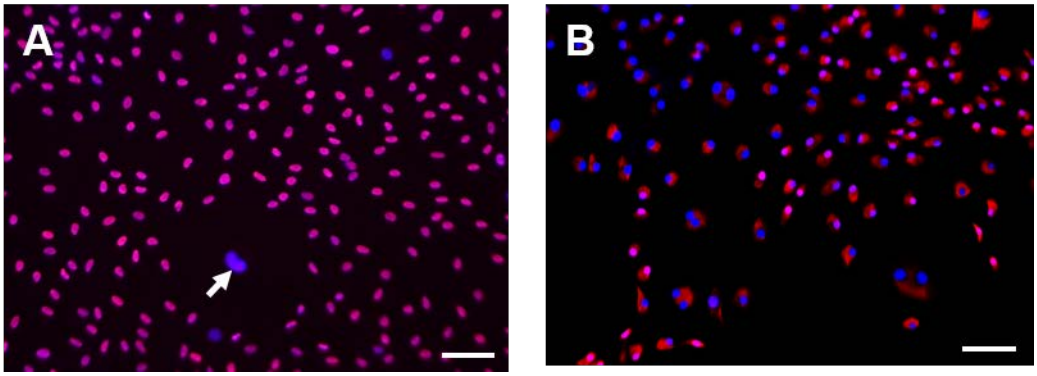

Figure 44. HMGB1 location in low and high passage cultured rat Achilles tendon cells. (**A**) HMGB1 is located in the nucleus of most cells in low passage "young" cells (P3). (**B**) HMGB1 is located in the cytoplasm or lost in the majority of high passage "old" cells (P15), please note in (**A**) there is a giant cell (arrow) with minimal HMGB1 staining, it is possibly a senescent cell. Bars - 50 µm

Another possible explanation for the lack of HMGB1 staining in some cells is from the technique side; tenocytes are highly packed in collagen fibers, it is possible that the antibody was not easily accessed to all cells in a tissue section slides. Thus result in the different staining pattern of *in vivo* tenocytes.

5.1.3 HMGB1 is released to extracellular milieu in tendon cells and tendons in response to excessive mechanical loading

Our result showed that uniaxial repetitive stretching of the tendon cells *in vitro* at 8% but not 4% strain for three days caused translocation of HMGB1 from the nucleus, and there was also a remarkable increase in HMGB1 in the cell culture supernatants, suggesting HMGB1 is released to extracellular space due to mechanical overloading. When *in vitro* findings were transferred to *in vivo* treadmill running model in mice to examine the physiological relevance, the results were in agreement with the *in vitro* findings for the intense treadmill running regimen. We found that that intensive treadmill running but not moderate treadmill running for three weeks resulted in increased expression of HMGB1 in tendon matrix, confirmed by ELISA and immunostaining.

The finding of our study is consistent with those in previous studies. Mechanical loading also has a similar stimulatory effect that is demonstrated in periodontal ligament (PDL) cells and tissues previously [182]. In this study, PDL under mechanical load showed that the compression and tension force induced translocation of HMGB1 from the nucleus to the cytoplasm *in vivo*, and increased the amounts of HMGB1 protein expression in response to the induction of mechanical loading following orthodontic tooth movement as early as at day 3 and day 6 in rat model. As tooth movement continued over 9 and 12 days, HMGB1 immunoreactivity decreased suggesting a role of HMGB1 acting as an alarmin indicating tissue damage and functioning as an early mediator in the periodontal remodeling process [182].

The finding that only mechanical overloading and intensive running induced HMGB1 release suggested that tendon disorder might be loading-magnitude dependent that is, overexercised but not moderately exercised tendons results in inflammation. This finding is in agreement with the previous finding in our lab that only excessive loading *in vivo* induces

significantly higher production of PGE_2 in the mouse treadmill running model[66], also in another study, moderate exercise could enhance the tendon stem cell pool and increase collagen production[116]. Together these findings provide practical suggestions to the athletes and the recreational population that proper exercise is beneficial for tendon, but over-exercise can be detrimental, especially in long term practice. Interestingly several studies found that in humans , both the collagen synthesis and degradation are increased in tendon tissue after acute exercise, but the first 24-36hrs the response in total is a net loss of collagen, and it is followed by a net synthesis 36-72hrs after exercise[292-294](**Fig. 45**). These results suggested that repeated training with short rest period may lead to net loss of collagen resulting in overuse tendon injury [295]. As our finding indicate that HMGB1 is already present in tendon matrix after one-bout treadmill running, its inflammatory invoking potential is considerable. It is highly possible that tendon tissue turned into a pro-inflammatory phenotype and lasted for 24-36hrs, the inflammatory response may gradually get resolved after the first day or two. But if the exercise is repeated with short rest time (i.e. daily treadmill running in our experiment), the inflammation may sustain to cause more collagen degeneration, which could lead to tendinopathy in the long-term running.

.Another interesting finding is that, while both ITR and OTR groups were positive for HMGB1 in tendon matrix(**Fig. 16**). ITR Achilles tendon tissue was positive for CD68 while OTR was negative, macrophages are known to actively secrete HMGB1 under inflammatory status, it is hard to tell if the HMGB1 in the ITR groups is from the release of the tenocyte due to overloading or from the recruited macrophage possibly through other pathways. The negative result of CD68 indicated that there is minimal macrophage infiltration in tendon matrix during

the one time 5hrs running, which implied the HMGB1 released during OTR is mainly released from resident tenocyte induced by overloading

We also found that HMGB1 and CD68 are both positive in the mid-portion and near the insertional site of the Achilles tendon, as we found later that long-term treadmill running induced degenerative change was only occurring in the near the insertional site of the Achilles tendon indicate there might be other risk factors involved in the development of degenerative tendinopathy, HMGB1 might be necessary but not sufficient for the development of tendinopathy *in vivo*.

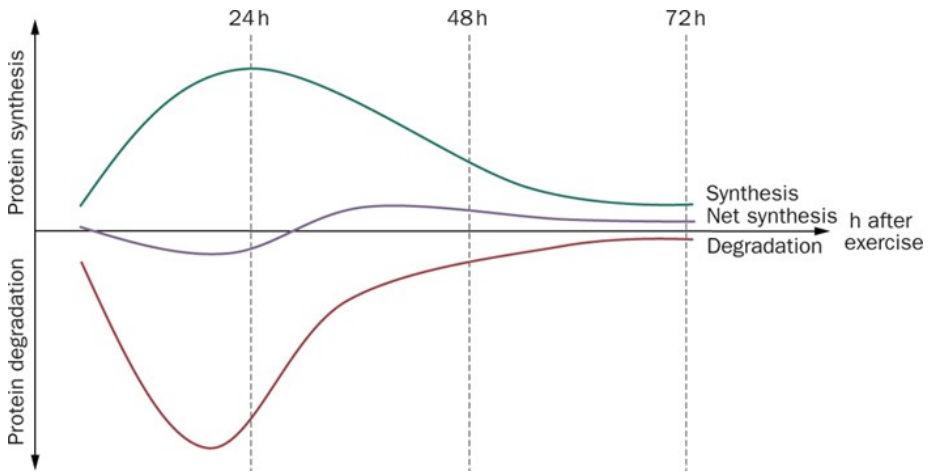

Figure 45. Schematic graph show collagen synthesis and degradation followed by an acute exercise in humans. In the first 24-36hrs, both synthesis and degradation are increased but result in a net loss of collagen; then both effects decreased gradually during the 36-72hrs time after exercise. So repeated the intensive exercise with short rest time can result in a net degradation of tendon matrix and lead to overuse injury. (Source: [295])

5.1.4 *In vitro* mechanical loading model

This study focused on load-bearing tendons like Achilles and patellar tendon. This type of tendon only bears uni-axial tension but not compression *in vivo*. The translocation of HMGB1 was tested in tenocytes with a customized uniaxial stretching device which was designed to mimic the physiological condition where tenocytes are subjected to mechanical loading *in vivo.*

There is a commercially available *in vitro* cell stretching system from flexcell International Corporation including early BioFlex model and new UniFlex system, and this system utilizes vacuum to create mechanical loading to stretch the soft substrate where cells reside. However, the commercialized *in vitro* cell stretching systems apply stretch in arbitrary directions, which is not suitable for our study.

The customized uni-axial stretching device developed earlier in our laboratory had been successfully used in several studies [35, 111, 296, 297]. With a different configuration, it could apply 3 different magnitudes of tension regarding percentage stretch or strain-4%, 8% and 12%. Both 4% and 8% are the most applicable magnitudes, 12% stretch is far beyond the physiological deformation of the tendon. A biomechanical study on tendon suggested that a 4% stretch represents the physiological limit of tendon deformation, and 8% stretch in tendon tissue results in micro-level damage that is considered detrimental. When the stretch exceeds 8%, macroscopic rupture of the tendon occurs [30]. Any magnitude over 8% does not reflect the actual *in vivo* tendon stretching condition, and therefore, it was not considered for this study.

The previous study showed increased inflammatory mediator PGE_2 production in human tenocytes with 8% cyclic stretch but not with 4%, compared to non-stretched control [35]. The human tenocytes were stretched in micro-grooved silicone dish for 24hrs at 4% and 8%

stretching magnitudes, and only 8% stretch resulted in a 1.7-fold increase of PGE_2. These results indicated that 4% stretch was relatively mild and did not invoke a strong inflammatory response.

Based on these results, non-stretch, 4% stretch, and 8% stretch groups were selected to explore the effect of cyclic stretching on HMGB1 release from rat Achilles tendon cells in this study. Besides the stretching device, the stretch container is also specifically designed. An earlier study in our laboratory found that if cells were stretched on a smooth surface such as silicone, initially, the orientation of cells will be arbitrary after seeding, but during the cyclic stretching, the cells will change their orientation to minimize the stress they are subjected to [298]. Therefore, the magnitude of stretch applied on the cells will be reduced from the initial design; cells also will not be stimulated in a uniform manner which may potentially result in inconsistent results. A micro-grooved dish was then designed to resolve the problem. The surface of the silicon dish where cells were seeded was micro-patterned with horizontal grooves (10 μm wide and 3 μm deep, optimized for cell attachment) parallel to the stretching axis. The description of the microgrooves silicone dish fabrication is detailed previously [299]. This design ensures that the cells only align along the stretch axis without any change in orientation during the stretch, which very closely mimics the circumstances the tendon cells are subjected to *in vivo* tendon stretching situation(**Fig. 10B**).

For the stretching experiment, the cyclic frequency should be set at a reasonable value, favorably mimicking the frequency of human activity. The previous studies in our laboratory used 0.5Hz[37] so as to mimic the frequency during normal walking activity. In this study, the frequency was set at 1Hz with increased intensity to increase the odds of detecting changes of HMGB1 in tendon cells. A frequency of 1Hz is still physiologically valid since it is similar to the stretching frequency during running.

The *in vitro* stretching model has certain limitations. With this setup, the stretching machine is customized and only available in our laboratory, and even with the commercially available system, it might be difficult to directly use the parameter in this study and trying to replicate the well-established model.

5.1.5 *In vivo* tendon mechanical loading model

The *in vivo* experiment model utilized in this study for assessment of HMGB1 release in tendon matrix is mouse treadmill running.

Study of the pathogenesis of tendinopathy in human is difficult since tissue biopsy could only be obtained from individuals with advanced tendinopathy. Comparative normal tendon samples are rare [300]. Furthermore, the samples from early stage tendinopathy are almost impossible to obtain since there are no symptoms for early diagnosis. By using the animal model, the development of tendinopathy can be studied more thoroughly especially the early stage, which may lead to novel treatment and prevention options for patients.

Several different overuse models are employed in tendon biology and pathology studies. One method was developed in rabbits by stimulating the muscle to produce contractions with a certain frequency that results in cyclic passive loading of the tendon[118, 301]. By stimulating the flexor Digitorum muscle, with 10 reps/min and 60 reps/min stimulation increased micro-tears in the tendon [301], and, stimulated triceps surae muscle of rabbit resulted in increased capillaries and inflammatory cells[118]. However, even the unexercised leg also had tendinopathic changes [302], so there might be other mechanisms involved. The use of relatively high-frequency electronic stimulation would potentially affect the direct assessment between

HMGB1 release and mechanical loading. Therefore, treadmill running method was considered for the *in vivo* mechanical loading model in this study.

The most developed and used *in vivo* tendinopathy model is rat downhill treadmill running. The rats ran on a 17m/min, a 10-degree decline treadmill for 1hr a day, 5 days a week for 4,12, and 16 weeks. Increased cell number and change of cell shape were detected as early as 4 weeks in supraspinatus tendon, and mechanical property including maximum stress and tissue modulus were decreased in 12 and 16 weeks [114]. The reason for using rat model over a mouse one is that the supraspinatus tendon of rat is anatomically very similar to its counterpart in human. Several studies that used this model found increased growth factors, inflammatory mediators, GAG accumulation, etc.[300]. However, despite all the changes found in supraspinatus tendon, the similar downhill running protocol had no effect regarding mechanical properties of Achilles tendon up to 16 weeks[303]. These results indicate that tendons respond to mechanical loading in a variety of ways. But while the downhill running increases the loading intensity on supraspinatus tendon, Achilles tendon may experience relatively decreased loading; this might explain the difference in effects between two types of the tendon.

A 10° uphill treadmill running effect on rat Achilles tendon found disorganized collagen fibers, hypercellularity, and neovascularization [34]. In contrast, no pathological changes were identified with same treadmill running protocol up to 12 weeks with even increased running speed (20m/min compared to 17m/min), and it even improved the mechanical properties of Achilles tendon[304]. While using a similar running protocol, no significant changes was found in Achilles tendon up to 9 weeks [305]. The effect of uphill treadmill running is still in controversy. One possible explanation is the variability in the running duration that is relatively short during each day. All the uphill rat treadmill running were done with 1hr running per day,

the age of the rats was between 2-6 months, generally considered young, all but one study[305] used male SD-rat. For this study, a strain of HCR rat was used, HCR rat was selectively bred with high running capacity[305], which may help explain the reason for not developing tendinopathic changes. Other studies are also not in agreement with each other. Short term running was found beneficial in our previous studies[116]. In the mouse treadmill running conducted earlier in our lab found that only 3 hrs but not 50 min treadmill running resulted in increased PGE_2 production in mouse patellar and Achilles tendon after 3 weeks running[66]. This study also found that HMGB1 was released in the 3 hrs running group but not the 50min group. These findings indicated the running duration is a critical factor in generating the positive or adverse effect on the tendon.

Another reason for the controversial results is about the total running duration.; It was found that rat supraspinatus tendon change cell shape as early as 4-weeks and tendinopathy change after 12 weeks treadmill running. But it is not necessary that Achilles tendon respond in the same way. In fact, Achilles tendon is subjected to ~1400 N(2 times body weight) during slow running[306] and >2000 N(3 times body weight) during jumping[307]. It is reasonable to assume that Achilles tendon could better resist the stress during running and need longer time to develop a stable degenerative change.

Mouse treadmill running was studied to explore its effect on cardiac and skeletal muscle [308] and later introduced in tendon as a counterpart of rat treadmill running. Mouse treadmill running now is focused on the weight bearing tendon on the hinder leg. For example, for patellar tendon and Achilles tendon, the running protocol of mice and rats are similar but different in running speed (12-15 m/min v.s. 17-20 m/min in rat), and with longer running duration each day (3-5h v.s. 1hr in rat). As mentioned above, the running duration is critical in mice treadmill

running. 3-5h running was referred as intensive running(ITR) and known to render detrimental effect on mice Achilles tendon as a result of inflammatory mediator PGE_2 accumulation[66]. In this study, we found that HMGB1 increased PGE_2 production in tendon cells which may at least partially account for the elevated levels of PGE_2 generated during intensive running. Moreover, PGE_2 induced a degenerative change in tendon tissue[309] potentially through the induction of non-tenogenic differentiation of resident Tendon Derived Stem/progenitor cells(TSCs)[66]. Short term (50min) referred as moderate treadmill running (MTR) was beneficial to tendon quality by expanding stem cell pool in tendon [116]. Mouse treadmill running model is suitable for HMGB1 study as the distinct effects of intensive and moderate running protocols may induce different cellular responses and that we could expect to see a differential response of HMGB1 under different loading regimens.

The setup of the One-time fatigue Treadmill running (OTR) group was to validate the model and explore the presence of HMGB1 in tendon matrix (**Fig.16**). As HMGB1 exists in tendon cells, it is possible that the increased HMGB1 seen in ITR groups may be due to the cell proliferative effect since exercise could activate tendon stem cells with higher proliferative potential[116]. To exclude that HMGB1 content from proliferative cells in 3-week time point, OTR group was set up where the mice run 4-7 hrs until final fatigue(average 5hrs). While testing to see if HMGB1 can be released with one-bolt running without inference of proliferative effect, we found that OTR group had increased HMGB1 release but significantly lower than that in ITR group afterr 3 weeks running. This result also confirmed the cumulative effect of HMGB1 on the repetitive loading condition, if tendon was repeatedly exposed to overloading condition, the HMGB1 level might be elevated and sustained which may result in chronic inflammation. Since macrophage could actively secrete HMGB1 in the presence of other inflammatory mediators like

IL-1β, INF-γ and TNF-α[310, 311], it is very hard to distinguish whether HMGB1 in the matrix is derived from tendon cells or infiltrated inflammatory cells at the 3-week time point as we identified inflammatory cells in tendon tissue. It is more likely that both cells are contributing to the accumulation of HMGB1 in longer duration runs. However, in the OTR group, minimal inflammatory cells were identified in the matrix after 5hrs running while with an increase of HMGB1 release in tendon matrix, this finding suggested that the tendon cells are mainly responsible for the initial release of HMGB1 due to mechanical loading.

It is widely accepted that only extracellular HMGB1 acts as damage signal and invokes inflammatory reactions [312]. Since most cells possess intracellular HMGB1, it is possible the intracellular HMGB1is released during the tissue processing. It will create a general concern when interpreting the results, especially in this study. To avoid the interference from the intracellular HMGB1, the tendon samples are not processed in a total lysis buffer but chopped up and soaked in PBS. Also, HMGB1 in the matrix to diffuse into the solution, the DNA content in the solution as an indicator of cell destruction was measured and found consistently low in all samples compare to the total lysis sample. The leakage of DNA is not avoidable and may contribute to the background measurement value. That explains the reason why we still found HMGB1 in the control sample during the ELISA measurement.

It is well known that mechanical loading well within the physiological range does not harm the tendon but reinforces it. This biochemical adaptation of loading is characterized by the release of inflammatory mediators and growth factors both in the circulation and locally in tendons, well known among them is IL-1β that can upregulate COX-2 and MMPs. [37, 208, 313]. HMGB1 also seems to have a similar function in pathological progress in tendon as evidenced by our studies in which we applied moderate level stretching or moderate treadmill

running regimen. Appropriate loading range may keep HMGB1 levels appropriate for the tendon cellular regulation, function and remodeling; however, under excessive mechanical loading, abnormal release from the nucleus and subsequent extracellular placement will render HMGB1 as a danger signal that initiates inflammatory reactions which may finally lead to chronic tendinopathy.

There are limitations of the mouse treadmill running model. Most mouse TR models focused on the load-bearing tendons like patellar and Achilles tendons instead of other types of mostly motion-transmitting tendons such as flexor tendons. Therefore, the findings of HMGB1 in this model may not be directly translated to those tendon tissues and related diseases like flexor tendinopathy, but still, provide possible research target in future studies of the different type of tendon.

5.1.6 The mechanism of HMGB1 release induced by mechanical loading in tenocytes

The intracellular HMGB1 could go through active or passive release. Active release is usually induced by exogenous microbial products like endotoxin, or endogenous stimuli inflammatory cells like macrophages and monocytes [151]. The passive release was first described in necrotic cells[158], then in apoptosis, and in autophagic cell death[130]. The HMGB1 release also happens during cellular injury following chemical or physical stimulations such as chemotherapy, irradiation, hypoxia, hyperpressure[224]. As in passive release, cell structure integrity is often lost, which happens both in immune cells and stromal cells like fibroblasts. HMGB1 can be secreted from human fibroblast upon the stimulation of microbial products without necrosis[314]. When human gingival fibroblast was exposed to lipopolysaccharides (LPS) and other microbe products, HMGB1 was translocated from the nucleus to cytoplasm as

early as 6hrs and was also detected in the cell culture supernatant after 48hrs stimulation. HMGB1 was also released from epithelial cells with stimulation of 100 μg/ml LPS at 48 or 72hrs time point but not as early as 24hrs [315]. Tenocytes are regarded as fibroblast cells implying that there could be the underlying mechanism for HMGB1 release in stromal cells. In our *in vitro* mechanical loading experiment, HMGB1 was translocated to the cytoplasm and discharged into the supernatant (confirmed by ELISA) after intensive (8%) stretching. Cell density was not significantly decreased, indicating that no major cell death occurred during mechanical loading at this magnitude. DNA damage, which is often associated cell necrosis, was minimal in tenocyte subjected to intensive mechanical loading *in vitro* and the coincidence of HMGB1 translocation and DNA damage was also minimal. These results indicated that HMGB1 translocation and release in tenocytes subjected to mechanical stress was not regulated by necrosis or cell death.

Acetylation regulates the cytoplasmic translocation and secretion of HMGB1 in mouse monocytes and fibroblast[135]. Deacetylase inhibitors(TSA) and mutation of six lysines to glutamine to mimic the acetylated lysine in the nuclear location signal(NLS) sequence resulted in relocalization of HMGB1 to the cytoplasm [135]. Also, LPS induced HMGB1 translocation was inhibited by acetylase inhibitor anacardic acid[226]. In this study, since TSA treatment resulted in HMGB1 translocation in tenocyte from the nucleus to the cytoplasm, and *in vitro* stretching model also induced HMGB1 translocation, it is possible that mechanical loading mediated HMGB1 translocation is through the regulation of acetylation process. Mechanical stimulation and acetylase inhibitor showed that treatment with AA blocked the majority of HMGB1 translocation induced by 8% mechanical stretch.

Mechanical stress could result in temporarily and reversible plasma membrane damage [228], and mechanically active tissue cells like skeletal muscle cells, cardiac myocyte,

endothelial cells frequently and normally experience plasma membrane disruption[316], with outflux of cytosolic constituents and influx extracellular contents. bFGF was released from a cytosolic storage site through the plasma membrane disruption in endothelial cells[317]. Similar to skeletal muscle, load-bearing tendons like Achilles and patellar tendons are constantly under cyclic mechanical stress. In tendon tissue, tenocytes could also suffer from plasma membrane disruption under intensive and long term stretching. In this study, the live/dead cell assay was utilized with propidium Iodide (PI) and Fluorescein Diacetate (FDA). FDA marks the live cells with green fluorescence, and PI is a membrane impermeable DNA binding reagent that stains the necrotic cells by binding to its DNA through the compromised membrane. A special feature of PI is that it would go through the plasma membrane when the membrane is temporarily disrupted. Tenocytes subjected to mechanical stress at the magnitude of the possible release of HMGB1, double staining with PI and FDA was regarded as cells with temporary membrane disruption. FDA processed intracellularly will generate green fluorescence signal but not in dead cells, Over 95% cells were positive for green fluorescence (means cells are alive), indicating no major cell death which was in agreement with our finding during the *in vitro* stretching (Fig. 16). Furthermore, we found that around 50% of the cells were PI positive and green fluorescence positive, which was a strong indication of the occurrence of reversible plasma membrane damage. These findings explain the possible HMGB1 release to extracellular milieu once it was translocated to cytoplasm due to repetitive and intensive mechanical stress.

Based on these findings, we propose a plausible theory to explain the HMGB1 release in tenocytes under mechanical stress. First, the magnitude of mechanical stress should be sufficient to induce HMGB1 translocation through the regulation of acetylation of HMGB1, the acetylated HMGB1 lost nuclear localization signal (NLS) and was fast trafficked to the cytoplasm. At the

same time, the mechanical stress results in temporary plasma membrane damage, that results in an outflux of HMGB1 from the cytoplasm. The plasma membrane disruption is repairable, and may not lead to major cell death or necrosis during the mechanical stretching.

5.2 HMGB1 EXERTS INFLAMMATION EFFECT ON TENDON

5.2.1 Physiological and pathological effects of HMGB1 in tendon

We demonstrated that addition of HMGB1 promoted tendon cell migration and inflammatory reactions by enhancing the levels of COX-2 and PGE_2 *in vitro.* HMGB1 did not induce tendon cell proliferation but induced MMP3 production *in vitro*. Furthermore, high dose HMGB1 implantation resulted in hypercellularity, angiogenesis, and inflammatory cell infiltration characteristics of the early inflammatory stage of tendinopathy in tendon tissue *in vivo.* In agreement with *in vitro* finding, COX-2 and MMP-3 were also increased at the HMGB1 implanted site in the tendon.

Tendinopathy may not progress through a classic pathogen-induced inflammatory pathway, but may rather involve a local sterile inflammation initiated by damaged cells that could produce and release molecules functioning as danger signals like HMGB1. In tendons under mechanical loading, this release is initiated by resident cells. Previously we reported mechanical loading associated inflammatory reactions via upregulation of COX-2 and PGE_2 both *in vitro* and *in vivo* [35]. The mechanism for the pathogenic role of HMGB1 could be through enhancement of inflammatory and destructive mechanisms induced by other inflammatory mediators. Also, in synovial fibroblasts of RA and OA patients when stimulated with HMGB1

alone or in combination with IL-1β, it was shown that HMGB1 in complex with IL-1β increased MMP production and PGE_2 compared to treatment with HMGB1 or IL-1β alone [178, 318]. HMGB1 also acts as a chemoattractant in various cell types [152]. Our study also demonstrates this well-known property of HMGB1 in tendons.

The concept of inflammation in tendinopathy is a hotly debated issue and controversial with conflicting results. Some previous studies have reported the absence of clinical signs of inflammation and invasion of inflammatory cells [189, 319, 320]. However, this may not be true since previous studies did not closely check the presence of inflammatory cells in injured tendons. The availability of tissues for analysis at different times after injury, small sample size, and late presentation of human patients when the inflammation may have resolved may be some of the contributing factors for such findings.

In the implantation experiment, we found hypercellularity at the implantation site. It is highly possible that after the first few tendon micro-injuries, stressed or injured tendon cells release the initial wave of HMGB1 which triggers the sterile inflammation process. HMGB1 by its chemoattractant property may recruit neutrophils, monocytes, and macrophages to sites of injury. While exploring the HMGB1 effect *in vitro*, we found that this mediator did promote cell migration and inflammatory reactions, but did not induce cell proliferation. Interestingly, in the HMGB1 implantation experiment, HMGB1 induced hypercellularity in tendon tissues and among those cells, only a partial population of the cells was stained with CD68 indicating themselves as inflammatory cells. Other cells displayed elongated shape that appeared at the outer skirt around the CD68 positive cells. It is highly possible that they are tenogenic cells. It is most likely that HMGB1 exerts its function by recruiting inflammatory cells to the “injury site,”

and then initiates the release of cytokines and growth factors that result in the proliferation of the recruited tendon cells *in vivo*.

As reported in previous studies, HMGB1 was found to activate and migrate endothelial cells[216], attract other pro-angiogenic cells like mesoangioblasts *in vivo*[238], and macrophages attracted by HMGB1 may secrete pro-angiogenic cytokines and growth factors[246, 321] thus promote angiogenesis process. In our study, we found HMGB1 did not induce HUVECs tube formation *in vitro* (**Fig. 21**), but induced blood vessel formation in the skin implantation and tendon implantation experiment, this finding indicate that HMGB1 angiogenesis effect is potentially through the cytokines and growth factors recruited inflammatory cells. At the same time, as we found in our *in vitro* study that HMGB1 treatment induced tenocyte migration in a dosage-dependent manner (**Fig. 19**) it is also possible that HMGB1 released in tendon matrix makes resident tendon cells to switch to pro-inflammatory phenotype by secretion of the inflammatory mediator such as PGE_2 as well as a matrix-degenerative enzyme like MMP-3. Indeed our *in vitro* study showed that HMGB1 could induce PGE_2 and MMP-3 production.

Our implantation experiment showed that a high dose HMGB1 implantation evokes inflammatory reactions such as inflammatory cell infiltration, angiogenesis, and hypercellularity that took place at the 2-week time point, but not at the 4-week time point. This observation indicates that there might be a pathway *in vivo* to counteract HMGB1 effect and can resolve inflammation-related tendon tissue damages if HMGB1 is not continuously present in tendon matrix. This result might explain why tendinopathy is more often found in athletes who are subjected to long term, repetitive joint loading. After tendon gets overloaded and micro-injured for a short period, only a small amount HMGB1 may be released causing relatively low and repairable change in tendon structure; then tendon tissue could be able to recover from the injury

and restore normal structure by itself without any intervention. However, in persistent overloading, continuous release of HMGB1 into tendon matrix may result in chronic sterile inflammation. HMGB1 can form a complex with another molecule like IL-1β to exert stronger inflammation effect in synovial fibroblast [318], and cytokines produced during inflammation may also contribute to the persistence of inflammation, which is harder to resolve. Chronic sterile inflammation is potentially detrimental to tendon tissue since it may disturb the well-organized tendon structure by introducing inflammatory cells into the tendon while causing hypercellularity, angiogenesis, and catabolic phenotype of the tendon tissue with increased production of MMPs. Replacing the well-organized collagen fibers with cells, vessels or disrupted fibers will substantially impair the mechanical properties of the tendon which makes them vulnerable to rupture or macro-scale injury when subjected to even regular mechanical loading.

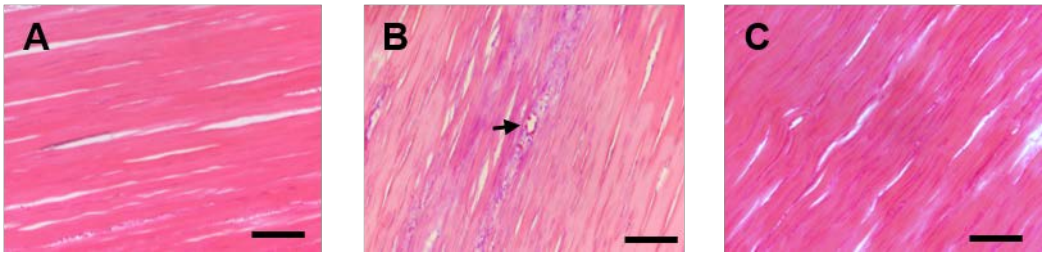

Figure 46. HMGB+GL implantation attenuates the structural and cellular change induced by HMGB1 in rat patellar tendon in 2 weeks. (A) Implantation site of control tendon with empty bead, show normal tendon structure after 2 weeks implantation. (B) HMGB1-encapsulated bead (2.5 µg in 5 µl) induce hypercellularity and vessel formation (arrow) 2 weeks after implantation. (C) HMGB1+GL encapsulated bead (2.5 µg in 5 µl HMGB1+ 1mM GL) showed little change at the implantation site. Bars - 100 µm

To further confirm that the inflammation effect is from HMGB1 itself rather than just response to large amout of foreign protein, we did an additional experiment on the implantation, we prepared the alginate beads with 1mM GL and 2.5 μg HMGB1 and implanted them into the SD-rat's patellar tendon as described in chapter 2 for 2 weeks. And found the very little structural change in the implanted tendon tissue without any vessel found near the implantation site (**Fig. 46C**) similar to the control tendon implanted with empty beads(**Fig. 46A**), the HMGB1 bead implanted tendon show vessel formation and hypocellularity(**Fig. 46B**).

The result indicated that HMGB1 is responsible for the structural change in the implantation experiment. Also,this finding indicated that inhibition of HMGB1 with GL could attenuate the structural changes induced by HMGB1.

5.2.2 *In vivo* implantation model with alginate beads

In our *in vivo* experiment, to explore potential effect HMGB1 on tendon tissue, we used one-time alginate bead implantation of high concentration of HMGB1 instead of direct injection for the following reasons.

Firstly, development of tendinopathy or visible tendon structure change by the natural cumulative effect of long-term micro-injury is likely with lower but sustained HMGB1 presence. The gradual degradation of alginate beads provided a relatively slower and localized release pattern of HMGB1 in tendon tissue to maximize its *in vivo* effect. Also, we have previous experience using alginate beads with KGN implantation into patellar tendon to create a tendinopathy model successfully [112].

Secondly, as considering the alternative method with the one-time injection of HMGB1, patellar tendon is relatively flat, and tendon tissue is usually very densely packed, thus not

capable of “holding” liquid. Moreover, the majority of the injection may diffuse to adjacent tissue quickly may not be able to retain in tendon long enough to cause any substantial structural change. However, if multiple injections were to be applied, it may be very hard to ensure that the injection was directed to the same location every time. Also, multiple injections may create trauma in the tendon, and also the surrounding tissue may mask the effect from HMGB1 as the trauma is enough to invoke inflammatory reactions free from HMGB1.

Moreover, Alginate is a natural polysaccharide with excellent biocompatibility and biodegradability [247], and it is non-toxic, with minimal pro-inflammatory effect [322]. It has been widely used in wound healing, cartilage repair, bone regeneration and drug delivery[248, 249]. Although many other synthetic biomaterials could serve as a delivery system, the denaturing and loss of bioactivity of the carried drug is a big concern as preparation of these materials often involves using organic solvents[250]. Alginate could be easily cross-linked and shaped with metal ions (usually Ca^{2+}) under room temperature and does not require other harsh chemical processing to retain protein activity. The preliminary result showed that 2% alginate beads remained *in vivo* in tendon without much degradation for at least 2 weeks.

In our early trials, we also tried to implant the beads into Achilles tendon but found as Achilles tendon is macroscopically cylindrically shaped; it is extremely hard to keep the beads in a fixed position for 2 weeks. Although it is easy to implant and keep beads in position in patellar tendon, after the beads implantation; some beads may migrate out of tendon and move away from its original position. This phenomenon may be due to the movement of rat joint that “squeezed” the beads out and resulted in certain failure rate in inducing a structural change in the tendon. The problem was solved by suturing the connective membrane above tendon tissue, and

beneath skin tissue, which acts as a barrier to keep the beads in the tendon area, thus we can obtain a more stable result from this animal model.

Before we implanted HMGB1 alginate beads into the tendon, we first tested its effect within the skin, skin is a more vascularized area compared to the tendon. The beads were implanted subcutaneously for one week and found inflammatory change near the beads (beads not yet degraded) including significant vessel formation (**Fig. 23**). By 2 weeks HMGB1 could invoke major structural changes in tendon and another 2 weeks allowed the changes to resolve(**Fig. 25**). It is possible that tendon inflammation takes longer to induce structural change and takes long to resolve which makes tendon more vulnerable to repetitive loading for an extended period without enough rest, the repetitive loading constantly induce the release of HMGB1 thus maintain a chronic inflammatory environment which continuously damages the tendon tissue.

5.3 INHIBITION OF HMGB1 EFFECT WITH GL

5.3.1 The logical reasons for the selecting GL as HMGB1 inhibitor *in vivo*

Several HMGB1 inhibitors are available, and major inhibitors widely used in HMGB1 related studies are an HMGB1 neutralizing antibody, recombinant Box A, and Glycyrrhizin. A few other chemically synthesized compounds (sivelestat, atorvastatin, simvastatin, gabexate mesilate) were also explored in HMGB1 inhibition studies [100, 323-326].

The HMGB1 neutralizing antibody is designed to target the functional area of HMGB1 sequences to prevent HMGB1 from binding to its receptors; the HMGB1 antibody was mainly

utilized to demonstrate the HMGB1 involvement in disease conditions due to its high specificity. Administration of HMGB1 antibody attenuated endotoxin lethality in mice [151], and polyclonal HMGB1 antibody applied systemically successfully suppressed inflammation and tissue damage in collage-induced arthritis in mice and rat model [180]. The HMGB1 antibody was also used in hemorrhagic shock and resuscitation (HS/R)-induced gut barrier dysfunction, with an improved survival rate in mice model [327]. In these experimental models, the administration of HMGB1 antibody was relatively short, but as we are developing a long-term exercise model, the usage of HMGB1 antibody will be huge and extremely costly. Moreover, since tendon is relatively avascular; one may need even higher amount to ensure effectiveness. Host immune reaction is another concern when antibody as a foreign protein, may invoke adaptive and innate immune response.

Box A is the functional domain of HMGB1 peptide; it is the 9-79 amino acid of HMGB1. Box A could act as an antagonist for HMGB1 by binding to its receptor RAGE but not activate it since it lacks the inflammatory signal from Box B [281]. Recombinant Box A was found to protect endotoxin and sepsis lethality [328], as well as protect joint tissue and inhibit inflammation in collagen-induced arthritis model [180]. Its behavior has been shown very similar to HMGB1 antibody, but Box A directly binds to RAGE by competing with HMGB1. One concern about using Box A is that HMGB1 may signal through other receptors like TLR2, TLR4. However, Box A, in some scenarios may only partially block HMGB1 functions.

Glycyrrhizin (GL), a glycoconjugate triterpene produced by the licorice plant, Glycyrrhiza glabra, has anti-inflammatory and antiviral properties. GL binds to both Box A and Box B of HMGB1 ($K_D \approx 150$ μM), and it inhibits the chemoattractant and mitogenic activity of HMGB1 *in vitro*[253]. GL has been administered to patients with hepatitis B and C and

considered safe to use[253]. It is the most studied small-molecule inhibitor of HMGB1 and has been demonstrated numerous times that GL successfully inhibits extracellular HMGB1 cytokine activity and shows a protective effect on spinal cord, liver, brain, renal tissue against HMGB1 mediated ischemia-reperfusion injury in animal models[323]. Especially in collagen-induced arthritis model, GL was equally effective as HMGB1 antibody and Box A[253]. It is also used in other preclinical investigation to treat other diseases by inhibiting HMGB1 signaling [256].

Gabexate mesilate (GM) and sivelestat sodium hydrate(sivelestat) are synthetic molecules found to at least partially inhibit HMGB1 effect through indirect ways. GM was found to inhibit HMGB1 through blockage of HMGB1 release after LPS treatment [324]. Sivelestat,an inhibitor of neutrophil elastase, which is important in acute lung injury, inhibited NF-kB activity then reduced the release of HMGB1 from neutrophils[326]. The two statin molecules, atorvastatin, and simvastatin were found to downregulate HMGB1-RAGE axis. Atorvastatin was found to protect rat brain from ischemic injury through attenuation of over-expressed HMGB1[325], and simvastatin administration to $ApoE^{-/-}$ mice was found to attenuate vascular inflammation and atherosclerotic lesion and decreased HMGB1 expression in aortic tissue[94]

In this study, GL was selected as the inhibitor for the *in vivo* model for the following reasons.

Firstly, it is undoubted that the neutralizing antibody has the most precise and direct inhibition of HMGB1, but it may not be suitable for our long term *in vivo* study. It is extremely expensive if it has to be injected into mice every day for 3 weeks or even longer since it is still an extraneous protein that might invoke immune- reaction in the long run, which may also interfere with the effect directly come from the treadmill running.

Secondly, the inhibition potential of GL is similar to neutralizing antibody and Box A. GL has a similar inhibitory effect on HMGB1 as recombinant box A peptide in a mouse liver disease model by reducing the inflammatory cells recruitment to the liver[329], which indicates the GL is not an inferior choice as HMGB1's inhibitor *in vivo*. The small synthetic molecules mentioned above are usually targeting the inflammatory cells' release of HMGB1 or other indirect ways; most of them are only tested in one certain model. GL is the only small molecule that is known for sure to directly bind to HMGB1 and inhibit its chemoattractant and cytokine activity. GL has been used in multiple disease models and shows significant attenuation of HMGB1 effect[180, 253, 258, 330]. So GL is a better choice over the small synthetic molecules.

Thirdly, GL could be prepared in relatively high dosage and could be administrated through IP injection instead of local injection into the tendon. Local injection into tendon tissue, in the long run, creates trauma that may very much interfere with the experimental outcomes since the inflammation effect from the procedure could very possibly mask that from the treadmill running. Moreover, if we would use antibody or Box A to inhibit HMGB1, the likely choice will be the local injection method due to the relatively small amount of the preparation.

Fourthly, GL administration in an animal model has been proven safe; previous studies show that 50 mg/kg body weight GL injection daily for up to 6 weeks without adverse effect in a lung disease rat model[264]. Overall, there was no negative response up to 80 mg/kg body weight GL administration in mice for consecutive 20 days[263]. GL's safety is also proven by our finding that GL-injected mice for up to 24 weeks had similar average body weight and body composition compared to the cage control group. Before conducting the *in vivo* experiment, we also showed *in vitro* that GL is safe by demonstrating that GL does not decrease tendon cell

proliferation at the concentration of 100uM. But it does decrease cell number at 1mM, which may be due to the change in pH of the culture medium instead of GL toxicity itself.

There is also some concern about using GL *in vivo*. As a naturally existing small molecule, there are some potential off-target effects of GL including inhibition of NF-kB activity[331]. It is possible that some conjunction effect in the study exerted by GL could not be attributed to HMGB1 inhibition.

Another concern is that whether GL would affect the normal function of the intracellular HMGB1. The binding site of GL has been identified on HMGB1 with NMR chemical shift difference mapping. Moreover, surprisingly, even the binding site of GL on HMGB1 partially overlap with HMGB1's DNA binding site intracellularly, and GL only mildly interferes with HMGB1 binding to DNA in living cells with slightly decreased affinity to DNA[253]. This finding implied that administration of GL would not greatly interfere with HMGB1's normal function as DNA chaperone. By considering all the pros and cons, GL is still the most suitable inhibitor in our study.

5.3.2 GL reverses the inflammatory effect of HMGB1

Our studies using this inhibitor demonstrated that GL is very effective in reversing the inflammatory effects caused by HMGB1 *in vitro* and *in vivo*. HMGB1 could upregulate COX-2, PGE_2, and MMP3 *in vitro* and *in vivo*, and GL could inhibit these effects. We found in our implantation experiment that HMGB1 can cause hypercellularity, vessel ingrowth, and inflammatory cell infiltration when administrated to rat patellar tendon in alginate beads, and if GL is mixed with HMGB1 in the alginate beads, it can effectively attenuate the effect mentioned above. Therefore, it is conceivable that HMGB1may act as an upstream mediator in tendinopathy

associated inflammatory cascade. Currently, NSAIDs administration and corticosteroid injections to suppress PGE_2 via COX inhibition dominate the treatment strategies to manage tendinopathic pain and inflammation [92, 332]. However, they have limited success in offering long-term relief without unwanted detrimental side effects [85]. Unfortunately, conservative treatments like rest, drugs (NSAIDS, corticosteroids), stretching and strength training can lead to surgical intervention in up to 45% of cases [333, 334]. Moreover, non-steroidal anti-inflammatory drugs and steroids may be beneficial for pain and function in the early phases of the disease but are usually ineffective later [335-337]. Furthermore, COX activity is a relatively downstream player in the inflammatory cascade of tendinopathy. Therefore, NSAIDs are useful only for the inhibition of late mediators not for the source itself. In clinical settings, current treatment with steroids on chronic tendon injury can suppress the overall immune response, but it is considered harmful with side effects. It is also possible that suppression of PGE_2 may have reduced the extent of degenerative injuries, but the regular matrix remodeling would also be affected, which may contribute to the failed tendon healing. Therefore, HMGB1 as an upstream mediator in the inflammatory cascade may serve as a better therapeutic target than PGE_2, especially for early intervention.

MMPs have been studied in tendon pathology with human samples, and one human study compared the MMPs in ruptured and normal supraspinatus tendon and found increased MMP-1 but decreased MMP-2 and MMP-3 activity[61]. Another study on chronic Achilles tendinosis with 5 female patients(mean age around 60) found that compared to clinically normal tissue, the biopsy from tendinosis sites showed higher mRNA level of MMP-2 but lower MMP-3 in the same patient[60]. Moreover, in various studies of cultured tendon fibroblasts, mechanical stretching increased MMP-1 and -3 expressions, with no change in MMP-2 or -9[313, 338].

HMGB1 was found to increase MMP-3 expression in rheumatoid synovial fibroblasts with the presence of LPS, but in relatively low concentration (100ng/ml). Another study also indicated that HMGB1 could induce MMP-3 expression with IL-1β, at low concentration (40ng/ml). We found that MMP-3 was elevated with 10 μg/ml HMGB1 treatment both intracellularly and in cell culture medium; also found increased in the tendon of 3-week intensive treadmill running and was suppressed by GL intervention. This finding may suggest that MMP-3 induced by HMGB1 in early stage could have a different function compared to that of the late stage of tendinosis without inflammation. It is possible that MMP-3 may contribute to the development of early stage tendinopathy. It is hard to conclude the role of MMP-3 in the context of our model since the degeneration of tendon collagen matrix is regulated together with other MMPs as well as TIMPs. Therefore their behavior should be extensively studied in overuse models in future.

5.3.3 Establishment of novel experimental insertional Achilles tendinopathy model

By conducting long-term (12-24weeks) treadmill running, we first successfully developed an overuse insertional tendinopathy model in mouse Achilles tendon with degenerative changes, like the appearance of round-shape chondrocyte-like cells, deposition of GAG in matrix and cells, expression of chondrogenic marker Sox-9 and collagen type II in tendon matrix. We found that the round-shaped cells with the Sox-9 expression as well as GAG deposition in cells appear as early as 12 weeks. Moreover, by the end of 24 weeks, GAGs deposition was found extensively in tendon matrix as well as collagen II began to appear near the insertion site. We also found that daily injection of GL to the treadmill running mice could effectively decrease the

GAG deposition with minimal collagen II in tendon matrix, also with less round shaped cells compared to the treadmill running the only group.

Clinically, the insertional tendinopathy is more frequent in young (the average patient age in the 40s [75, 339]) and more active population, whereas non-insertional tendinopathy is found more in older, less active and overweight population [75]. These findings suggest that all types of tendinopathy do not develop in the same way while insertional tendinopathy may largely relate to recreational or athletic activities. Insertional tendinopathy shows a typical degenerative change in tendon matrix with increased number of cells and more GAG in the matrix and disorganized collagen fiber [73]. We found that the degenerative change is occurring near the insertion site of Achilles tendon towards the calcaneus bone. In our model, the mice started running when they were around 8-9 weeks old and ended up around 32 weeks. Since the average lifespan of the C57BL/6 mice is around 600-700 days[340], 32 weeks old mice can be considered young and equivalent to about 30-40 years old human. Our model is in agreement and recapitulates the major degenerative change found in real patients. This similarity emphasizes the significance of this model, and the model may be of great value in exploring the underlying mechanism of insertional tendinopathy development induced by overloading, and to help develop possible treatment or prevention method in the management of insertional tendinopathy. The findings from this model could be highly translational to clinical practice.

Several models of tendinopathy have been created with various chemicals like Carrageenan, corticosteroid, prostaglandins, as well as collagenase[105]. Although in these models, the degenerative change could be found as evidence of the successful development of the model, and they provided useful information in post-tendinopathy treatment, they could not provide much information on the risk factors that initially trigger the pathological change. A few

treadmill running models have also been developed, among them, the rat supraspinatus tendinopathy model induced by downhill treadmill running was widely accepted [114]. However, the development of Achilles tendinopathy through treadmill running results are controversial with some reports that did not find any effect of treadmill running in terms of inducing tendinopathic changes [34, 305]. One possible reason is that the running protocol was not intense enough to induce damage to Achilles tendon. Moreover, all the rat treadmill running protocols were derived from the initial supraspinatus tendon study with 1hr running per day and 5 days a week, for up to 16 weeks. Based on this study and previous finding in our lab that 50min running every day for 3 weeks result in an expansion of the stem cells pool in tendon [116] and also did not induce PGE_2 production in tendon[66], we consider 1hr of running per day as beneficial and may not invoke an inflammatory reaction. We showed that 1hr treadmill running for 3-week did not significantly increase HMGB1 release in tendon matrix. Another reason is that the researchers may focus on the mid-section of the tendon instead of closely checking throughout the whole tendon piece. It is very hard to retain the appropriate structure on the end of tendon tissue during the dissection and tissue section, but it is relatively easy to do so in the mid-section. Our findings also suggest that future development of tendinopathy models should check the tendon tissue more closely for any potential structural and cellular changes.

5.3.4 GL prevents degenerative changes in long-term treadmill running model

We found that GL intervention could effectively block or delay the development of tendinopathy by decreasing GAGs deposition and prevent collagen II in tendon matrix in our 24-week long-term treadmill running model. This finding indicates that GL blocked the pathway which is directly related to the degenerative changes. As we previously found in the 3-week running

model, GL was able to decrease the production of inflammatory mediator PGE_2 induced by overloading *in vivo*. HMGB1 also attracted inflammatory cells and resulted in an increase of COX-2 and PGE_2 production in tendon tissue which could lead to the non-tenocyte differentiation of TSCs as our previous study indicated[16, 66]. GL is known as the direct inhibitor of HMGB1, and even with possible off-target effect, we think that the inhibitory effect of GL in the development of tendinopathy may be mainly through the inhibition of HMGB1 released as a result from overloaded tendon cells.

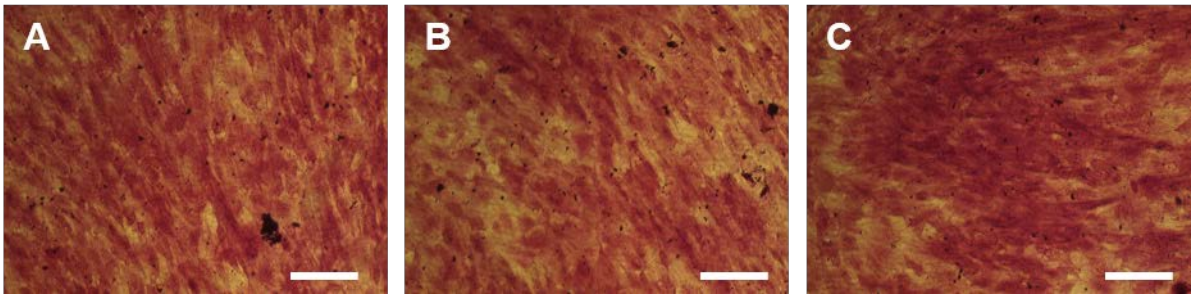

Figure 47. GL did not prevent Chondrogenic differentiation of Tendon stem cells (TSCs) in 3 weeks. TSCs are treated with chondrogenic differentiation medium with 0, 50, 200 μM GL for 3 weeks, the medium was changed every 3 days. (A) Control group without GL treatment show strong Safranin O staining indicate strong GAGs deposition (B, C) Chondrogenic differentiation of TSCs with 50 μM (B) and 200 μM (C) GL treatment show similar staining to control. (C) Chondrogenic differentiation of TSCs with GL treatment. Bars - 100 μm

One may argue that GL may potentially block the differentiation process directly without interacting with HMGB1, As in the model, we found most degenerative change is related to chondrogenesis, we conducted an additional experiment that to test out the prevention effect of GL in the differentiation of tendon stem cell *in vitro*. Chondrogenesis induction medium was prepared according to our previous study [15]. We found that neither low (50 μM, **Fig. 47B**) or

high (200 μM, **Fig. 47C**) GL affected the differentiation of TSCs into chondrogenic lineage compare to the control group without GL treatment, all groups are stained mostly positive for GAG-rich matrix with Safranin O assay (**Fig. 47A, B, C**). This result indirectly supported that the GL prevention effect in tendinopathy is through the inhibition of HMGB1.

5.3.5 Proposed pathological model for Achilles insertional tendinopathy

The pathological model we proposed from all findings in this research could be summarized as below (**Fig.48**). Repetitive loading and overloading of tendon result in micro-tears and release of HMGB1 from stressed or injured tendon cells. The extracellularly released HMGB1 attracts inflammatory cells and resident tendon cells to the injury site, and in response to the damage signal from HMGB1, inflammatory cells release cytokines and growth factors. The resident tendon cells are also activated and shift to pro-inflammatory phenotype. The proliferation of tenocytes, ingrowth of blood vessels and destruction of well-organized collagen matrix result in compromised mechanical property of the tendon that is vulnerable to even normal mechanical loading. Like the persistence of the overloading, the inflammation status was not resolved but further enhanced, and result in chronic sterile inflammation in tendon tissue, which leads to degenerative changes that finally develop tendinopathy. The previous study on the PGE_2 treatment induced non-tenogenic differentiation of tendon stem cells into adipocyte, chondrocyte, and osteocytes both *in vivo* and *in vitro*[16, 66], and this study helped to explain how chronic inflammation may result in a chondrogenic phenotype change in our treadmill running model.

There are a few limitations to our study. First, our research focuses on the load-bearing tendons like patellar and Achilles tendons instead of other types of mostly motion-transmitting

tendon such as flexor tendons. Therefore, the inflammatory reactions elicited by HMGB1 may not be directly translated to those tissues and their related diseases. Second, this model takes very long time to develop before inducing its effects, and may only suitable for exploring the insertional tendinopathy in Achilles. We are not sure about what will happen to the tendon if longer running time is permitted (i.e. 36 weeks), as the tendon structure has been compromised, its mechanical property may also decrease and may result in more damage and the degenerative change may spread to the middle portion of the tendon, or even result in tendon rupture.

Also, even when we detected inflammation in patellar tendon, patellar tendinopathy did not develop through this model even after 24 weeks running. Patellar tendon also has a unique structure and may have an even higher tolerance of inflammation than Achilles and supraspinatus tendons before developing into tendinopathy. Overloading patellar tendinopathy models have not been developed yet, which implies that the development of patellar tendinopathy may have other important risk factors involved such as nutrition and individual genetic variance.

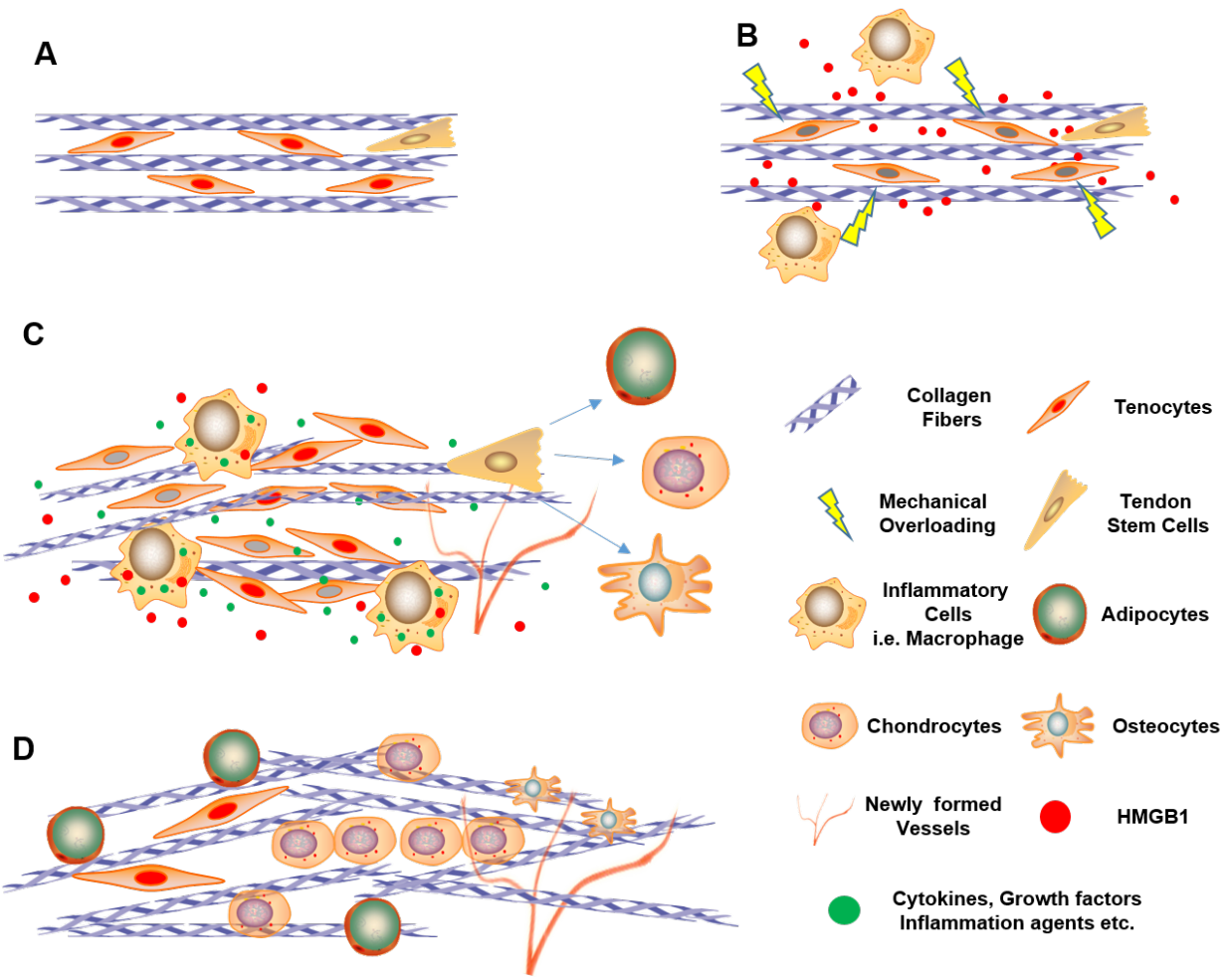

Figure 48. The proposed model of tendinopathy development in bear-loading tendon caused by HMGB1 due to mechanical overloading. **(A)** Shows the tenocyte a tendon stem cell resident in between collagen fibers in the tendon, red in nucleus indicate the HMGB1 location in normal tenocytes. **(B)** When under mechanical overloading, tenocyte passively release HMGB1 (red dots) in tendon matrix, inflammatory cells like macrophages were attracted to tendon tissue. **(C)** In responding to HMGB1 signaling, inflammatory cells begin to release cytokines, growth factors and inflammatory agents that trigger the sterile inflammation in tendon, prolonged inflammation may cause the hypercellularity, vessel ingrowth and breakdown of matrix, tendon stem cells under inflammatory condition tend to differentiate into non-tenogenic lineage cells like adipocyte, chondrocytes, and osteocytes. **(D)** If this pathological condition persists, the tendon will develop into chronic tendinopathy with degenerative changes like lipid deposition, chondrogenic change and even calcification which drastically compromise the mechanical properties of tendon tissue, and may possess a high risk of tendon injury and rupture.

5.4 CONCLUSION

In conclusion, our study helps to understand better how excessive mechanical loading-induced inflammation is developed into degenerative tendinopathy within the tendon through the release of the pro-inflammatory factor HMGB1. This study will also contribute to identifying a novel therapeutic target – HMGB1 signaling – in the management of tendinopathy. Our finding of GL as a direct inhibitor of HMGB1-induced inflammation in tendons will serve as a potential lead to develop this compound as a novel therapeutic agent to protect the tendon from the long-term structural change which impairs the tendon and make it vulnerable to injury or rupture. These findings may have a great impact in clinical trials of tendinopathy and may encourage further studies on how to target early tendon injury symptoms rather than focusing on late treatments after the injury. Future studies may focus on detailed molecular mechanisms of tendon inflammation and involvement of the MMPs regarding their role in the early and late stage of tendinopathy. This finding might help develop better treatment plans for athletes who have to constantly undergo high training burden especially those involved in excessive repetitive motion. Also, this study may inspire the research of other non-bear loading tendon diseases to uncover potentially new mechanisms and development of new and improved treatment strategies.

APPENDIX A

LIST OF ABBREVIATIONS

Abbreviation	Full term
AA	Anacardic Acid
AT	Achilles tendon
CCK-8	Cell Counting Kit-8
CCL18	Chemokine (C-C motif) ligand 18
CD68	Cluster of Differentiation 68
CITED2	Cbp/P300 Interacting Transactivator With Glu/Asp Rich
Col II	Collagen type II
COX-2	Cyclooxygenase-2
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
CY3	Cyanine 3
DAMPs	Damage-associated molecular patterns
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
ELISA	Enzyme-linked immunosorbent assay

Abbreviation	Full term
ERK	Extracellular signal-regulated kinases
FBS	Fetal Bovine Serum
FDA	Fluorescein Diacetate
FITC	Fluorescein isothiocyanate
GAGs	Glycosaminoglycans
H&E	Hematoxylin and eosin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIF-1α	hypoxia-inducible factor 1α
HMGB1	High mobility group box 1
HSP	heat shock proteins
IHC	Immunohistochemistry
IL-1b	Interleukin 1 beta
IP injection	Intraperitoneal injection
ITR	Intensive treadmillrunning
JNK	c-Jun N-terminal kinases
KGN	Kartogenin
LPS	Lipopolysaccharides
MCP-4	Monocyte chemotactic protein-4
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MTR	Moderate treadmill running
NES	Nuclear-emigration signals
NLS	Nuclear localization signals
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroid anti-inflammatory drugs

Abbreviation	Full term
OTR	One-time fatigue treadmill running
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PI	Propidium Iodide
PT	patellar tendon
RA	rheumatoid arthritis
RAGE	the receptor for advanced glycation endproducts
RASFs	rheumatoid arthritis synovial fibroblasts
SDF-1	Stromal cell-derived factor 1
SD-rat	Sprague Dawley rat
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOX-9	SRY-box 9
TSCs	Tendon-derived stem/progenitor cells
TIMPs	tissue inhibitors of metalloproteinases
TLC	with thin layer chromatography
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSA	Trichostatin A
α-SMA	Alpha-smooth muscle actin
γ-H2AX	Gamma-histone H2A member X

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