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“Progressing Towards Better Translatability in In Vitro Tendinopathy Models”

PhD thesis submitted for the fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (PhD)

University of Veterinary Medicine Vienna

submitted by

Ir. Michele Fenu

Vienna, October, 2023

This cumulative thesis is based on the following two publications:

“A novel magnet-based scratch method for standardisation of wound-healing assays.” Published September 2019 in Scientific Reports, Impact Factor 3,998

“Species variations in tenocytes’ response to inflammation require careful selection of animal models for tendon research.” Published in June 2021 in Scientific Reports, Impact Factor: 4,996

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Acknowledgments

I would like to extend my sincere gratitude to Prof. Florien Jenner, the head of the department and my supervisor, for her unwavering support throughout my PhD journey. Her guidance, patience, and extensive knowledge have been instrumental in making this project a reality.

Special regards go to my direct supervisor, Dr. Iris Gerner, with whom I have shared the majority of my Ph.D. work. Her mentorship, encouragement, and collaborative spirit have been invaluable during the entire research and thesis-writing process.

I would also like to express my heartfelt appreciation to Prof. Christopher Gerner and his analytical chemistry lab team. They warmly welcomed me and provided invaluable support throughout the research work. Their expertise and camaraderie have significantly contributed to the success of this project.

I also wish to thank Prof. Gerjo van Osch and her lab team members for her guidance and support during my time in the Rotterdam labs and beyond.

I cannot overlook the great time spent with Gil Lola Oreff, first a dear friend and a fantastic colleague. Our collaboration and strong connection have greatly enriched this Ph.D. journey.

I extend my gratitude to the entire Veterm Lab team, with particular mention of Sinan, with whom I spent several meaningful years. Their collective support has been crucial to the project's success.

Davide Secci's continuous presence, day and night, and our shared experiences have been invaluable. You were there in all and every moment! Thanks a lot!

I am deeply appreciative of Joao Pedro, whose support and friendship have been a constant source of strength.

Last but not least, I want to express my heartfelt gratitude to my family, who stood by me with unwavering support and unconditional love, giving me the strength to pursue this journey.

And, I must not forget to thank myself for never giving up. This journey was filled with challenges, but my determination and resilience were key to its successful completion.

Your support, encouragement, and self-determination have been vital to my success, and I am profoundly thankful for the role each of you has played in my Ph.D.

Contributions

Publication 1

A novel magnet-based scratch method for standardisation of wound-healing assays

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Publication 2

Species variations in tenocytes' response to inflammation require careful selection of animal models for tendon research

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DECLARATION

I hereby confirm that I followed the rules of Good Scientific Practice in all aspects to the best of my knowledge.

Vienna, October 2023

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Summary

Tendinopathies are debilitating overuse injuries and the most common musculoskeletal complaints leading patients to seek medical attention. Despite substantial research efforts, no disease-modifying therapy has made its way to the clinics, serving as a prominent example of the translational challenges in research and underscoring the difficulties in bridging the gap between findings in vitro studies, animal models and their applicability to human clinical treatments. The translation hurdles arise from many factors, with the lack of fit-for-purpose animal models prime among them. The choice of animal models is often based on availability, practicality, and cost rather than the models' ability to mimic disease pathogenesis accurately. Moreover, often these animal models lack comprehensive validation further limiting their applicability. In order to mimic the complexity of tendinopathies models should replicate the pathomechanical mechanisms, inflammatory response, immune reaction, and impaired biomechanics, which represents a significant challenge. Addressing these issues requires the development of more refined and accurate in vitro models that can better capture the multifaceted nature of these diseases.

Therefore, this thesis aims to establish novel technologies and comparative frameworks for in vitro modeling of wound healing and tendinopathies. The overarching goal is to create a more accurate representation of the *in vivo* situation, aligning with the 3R principle (Replacement, Reduction, and Refinement) to minimize the use of animals in research.

To this end, the research presented in this thesis pursues two primary objectives:

The first objective was to develop a standardized, reproducible method to replicate wound healing in vitro. The novel magnetic scratch method promises improved repeatability, reproducibility, and precise geometric control, surpassing existing techniques such as pipette scratch assays. Simulating a wound-healing environment with damaged and dead cells while maintaining high standardization of the gap facilitates fundamental biology studies and drug screening.

The second objective was to explore species-specific variations in musculoskeletal animal models, specifically comparing the inflammatory responses of tenocytes from various model species to humans. This research provides insights into the potential impact of these differences on translational outcomes. While no single animal model perfectly mirrors the human cells, this work contributes to our understanding of musculoskeletal pathologies and species-specific differences.

This work holds promise for investigating various biological phenomena, enhancing our understanding of mechanotransduction pathways, cellular responses to mechanical cues, tissue engineering, and related fields. While challenges persist, this research is part of a broader effort to enhance translational potential, scientific rigor, and ethical considerations in biomedical investigations.

Zusammenfassung

Tendinopathien sind überlastungsbedingte Verletzungen und die häufigsten muskuloskelettalen Beschwerden, die Patienten dazu veranlassen, einen Arzt aufzusuchen. Trotz erheblicher Forschungsanstrengungen hat noch keine krankheitsmodifizierende Therapie den Weg in die Kliniken gefunden. Dies ist ein eindrückliches Beispiel für die translationalen Herausforderungen in der Forschung und unterstreicht die Schwierigkeiten bei der Überbrückung der Kluft zwischen den Ergebnissen von In-vitro-Studien, Tiermodellen und ihrer Anwendbarkeit auf klinische Behandlungen beim Menschen. Die Hürden für die Umsetzung ergeben sich aus vielen Faktoren, wobei der Mangel an zweckmäßigen Tiermodellen an erster Stelle zu nennen ist. Die Auswahl von Tiermodellen basiert häufig auf Verfügbarkeit, Praktikabilität und Kosten und nicht auf der Fähigkeit der Modelle, die Pathogenese von Krankheiten genau zu reproduzieren. Die Komplexität menschlicher Krankheiten, die pathomechanische Mechanismen, Entzündungsreaktionen, Immunreaktionen und Biomechanik umfasst, stellt jedoch eine große Herausforderung dar, und häufig fehlt es an validierten Tiermodellen. Um diese Probleme zu lösen, müssen angepasste und genauere In-vitro-Modelle entwickelt werden, die die Vielschichtigkeit dieser Krankheiten besser erfassen können.

In dieser Arbeit sollen daher neue Technologien und vergleichende Rahmenbedingungen für die In-vitro-Modellierung von Wundheilung und Tendinopathien entwickelt werden. Das übergeordnete Ziel besteht darin, eine genauere Darstellung der In-vivo-Situation zu schaffen und dabei das 3R-Prinzip (Replacement, Reduction und Refinement) zu befolgen, um den Einsatz von Tieren in der Forschung zu minimieren.

Zu diesem Zweck verfolgt die in dieser Arbeit vorgestellte Forschung zwei Hauptziele:

Das erste Ziel war die Entwicklung einer standardisierten, reproduzierbaren Methode zur Immitation der Wundheilung in vitro. Die neuartige magnetische Scratch-Methode verspricht eine verbesserte Wiederholbarkeit, Reproduzierbarkeit und präzise geometrische Kontrolle und übertrifft damit bestehende Techniken wie Pipetten-Scratch-Assays. Die Simulation einer Wundheilungsumgebung mit geschädigten und abgestorbenen Zellen unter Beibehaltung einer hohen Standardisierung des Spalts verbessert biologische Grundlagenstudien und das Screening von Medikamenten.

Das zweite Ziel war die Erforschung artspezifischer Variationen in muskuloskelettalen Tiermodellen, insbesondere der Vergleich der Entzündungsreaktionen von Tenozyten verschiedener Modellarten mit denen des Menschen. Diese Forschung gibt Aufschluss über die potenziellen Auswirkungen dieser Unterschiede auf die Ergebnisse von Translationsstudien. Obwohl kein einzelnes Tiermodell die menschlichen Zellen perfekt widerspiegelt, trägt diese Arbeit zu unserem Verständnis muskuloskelettaler Pathologien und artspezifischer Unterschiede bei.

Diese Arbeit ist vielversprechend für die zukünftige Untersuchung verschiedener biologischer Phänomene u.a. zum besseren Verständnis der Mechanotransduktionswege, der zellulären Reaktionen auf mechanische Reize, des Tissue Engineering und verwandter Bereiche. Da es noch viele Herausforderungen gibt, ist diese Forschung als Teil einer breiteren Anstrengung zu sehen, das Translationspotenzial, die wissenschaftliche Strenge und ethische Überlegungen bei biomedizinischen Untersuchungen zu verbessern.

Introduction

General introduction

Musculoskeletal diseases pose a significant burden on individuals, healthcare systems, and society at large, affecting millions worldwide and impeding mobility, function, and overall quality of life. Tendinopathies are a major challenge in both human and veterinary medicine. After injury, tendon repair often leads to the formation of scar tissue, resulting in impaired biomechanical properties. These compromised tissue biomechanics ultimately contribute to the development of chronic tendinopathies. These conditions not only cause reduced function and impaired quality of life but can also result in permanent disability. As a result, there is a pressing need for effective treatments that focus on tissue regeneration rather than scar formation. However, the underlying mechanisms of tendon repair and the pathophysiology of tendinopathies are still poorly understood, which hampers the development of therapeutic interventions to reverse the progression of the disease. To gain insights into the mechanisms of tendon repair and the development of tendinopathies, as well as to test new treatment strategies, the availability of suitable models is crucial. Animal models have traditionally been the standard for studying diseases and evaluating new therapies. However, they come with limitations, including ethical concerns, limited translatability to human clinical applications, and high costs. *In vitro* models offer a promising alternative, as they can replicate the cellular and tissue-level complexities of *in vivo* systems while providing greater control over experimental conditions. These models range from traditional 2D human or animal-derived cell cultures to more advanced co-culture or 3D cell models and ex vivo tissue cultures. They enable the study of wound healing mechanisms and the testing of therapeutic approaches aimed at promoting tissue regeneration instead of scar repair. Recent advancements have even allowed the incorporation of biochemical and biomechanical stimuli, such as inflammatory factors or cyclic loading, making these models more physiologically relevant. However, there is still a need for further optimization to bridge the gap between *in vitro* and *in vivo* models, improving high throughput, precision, replicability, and cost-effectiveness to enhance the translatability of current models. The purpose of this work was to develop *in vitro* models focused on wound healing and tendons, comparing multiple animal species, to enhance the translatability potential for studying the pathophysiology and aetiopathogenesis of tendinopathies, as well as potentially serving as a platform for testing new therapeutic approaches.

Tendinopathies

Relevance of tendinopathies

Tendinopathy is a common disorder that results in pain, swelling, focal tenderness of the tendon and reduced mobility. It is estimated that up to 30% of all consultations for musculoskeletal pain and up to 50% of sports injuries are related to tendinopathies (Kaux et al. 2011; Scott and Ashe 2006). In previous classifications, acute tendon injuries accompanied by inflammation were named tendinitis while the conditions going along with degeneration of the tendon structure were named tendinosis. Recent review of nomenclature classifies all the clinical conditions with pain and reduced movement of tendons as tendinopathy (Docheva et al. 2015; Kaux et al. 2011). The aetiopathogenesis of this disorder is multifactorial and leads to disruption of the normal tissue micro-structure and mechanical properties resulting in increased risk of injury(Docheva et al. 2015). In addition to physical activity, risk factors for tendon injury include age, obesity, disease (ie. diabetes) and genetic predisposition (Nourissat, Berenbaum, and Duprez 2015). Injuries to the tendon impact quality of life and result in morbidity and reduced mobility (Nourissat, Berenbaum, and Duprez 2015). Spontaneous tissue repair in tendons is limited by intrinsic factors and often new tissue is characterized by suboptimal mechanical properties and is prone to re-injury (Docheva et al. 2015). Current treatments are limited to pain management and symptom relief with surgical intervention upon tissue rupture. Hence, tendinopathies represent a major clinical problem and given the aging population and increasing prevalence of tendinopathy, the socioeconomic impact of this disorder is expected to grow(Hopkins et al. 2016). For these reasons, there is an urgent need to increase research efforts ranging from the elucidation of underlying cellular mechanisms to the development of efficient/alternative treatment.

Tendon in health and disease

Tendons are soft connective tissue structures located between muscles and bones which transmit forces from the muscle to the bone, resulting in joint movement. Tendon tissue is composed of 60% to 80 % water (% wet weight), the other 20-40% consist of up to 86% collagens, mainly type I, 1 to 5% proteoglycans, 2% elastin and other small molecules in smaller percentages (Aicale, Tarantino, and Maffulli 2017; Frank and Hart 1990). In tendons, collagen is organized in a complex hierarchical structure: tropocollagen, triple helix polypeptide chains which unite to fibrils, fascicles and finally the tendon itself (Kastelic et al. 1978; Kuhn 1969; Ricard-blum 2011). The majority of the collagen fibers are longitudinally oriented and are surrounded by a network composed of proteoglycans,

glycosaminoglycans and glycoproteins which attract water into the tissue and provide functional stability to the collagen structure (Thorpe et al. 2013). Type I collagen structures can sustain high tensile load with a certain degree of elasticity, thereby supporting the force transmission between the muscle and the bone (Aicale, Tarantino, and Maffulli 2017; Thorpe et al. 2013). The tendon is enclosed in a sheet of connective tissue named epitenon, which contains blood vessels, lymphatic vessels and nerves, and extends within the tendon as endotenon, which envelops the collagen fiber bundles[30]. Tendons are either surrounded by an alveolar connective tissue, mainly composed of type I and III collagen, named paratenon, or by a synovial tendon sheath. These structures provide isolation from the surrounding tissue supporting frictionless movement during load transmission (Aicale, Tarantino, and Maffulli 2017).

The aetiopathogenesis of tendinopathies is not yet clear due to the high heterogeneity of several factors which appear to be associated with the onset of the disorder and/or might trigger degenerative changes (Steinmann et al. 2020). Among others, age, sex, body weight, habits (i.e. alcohol consumption, smoking), pre-existing pathological conditions such as genetic disorders or type II diabetes and physical activity contribute to the onset of tendinopathies (Steinmann et al. 2020). However, the pathophysiology of tendinopathies first published by JL Cook *et al* in 2009 and then revisited in 2016 is the currently the most accepted notion of the onset of tendinopathies induced by load (Cook et al. 2016; Cook and Purdam 2009). Following acute tendon injury, the tissue undergoes repair in 3 phases(Cook and Purdam 2009). The first phase is an early response to acute injury or trauma characterized by formation of a hematoma and influx of immune cells which last 1-2 days. Cells and blood vessels in the wound area are ruptured, causing blood to leak out into the surrounding tissue and to consequently form a clot triggered by the action of thrombocytes (Gonzalez et al. 2016). The blood clot helps restore tissue hemostasis, temporarily improves tissue function, and provides a matrix for cell migration. As a key feature of the inflammatory phase, leukocytes are recruited to the site of injury within the first 24 hours post injury(Gonzalez et al. 2016). Complement activation and platelet degranulation attract neutrophils to the site of injury(Grose and Werner 2004). Through pro-inflammatory cytokines such as Tumor Necrosis Factors (TNF) and interleukins (IL) these cells are activated to secrete pro-adhesive molecules, namely integrins, which facilitate cell adhesion and interact with somatic cells (Gonzalez et al. 2016). Within the first 2-3 days after injury, monocytes migrate to the site of injury, infiltrate the lesion site and differentiate into macrophages which are responsible for both phagocytosis of debris and secretion of signalling molecules (Das et al. 2015; Gurtner et al. 2008). Among these molecules, Nitric Oxide (NO) and prostaglandins (PGE) play a crucial role by activating the cells surrounding the lesion resulting in a global tissue response. Finally, macrophages play a major role during the transition of the inflammatory to the proliferative phase

(Singer and Clark 1999). During the second phase, resident cells proliferate and abundantly secrete ECM components while sprouting of new blood vessels supports the newly formed tissue (Docheva et al. 2015). The proliferative phase takes place between 2 and 10 days after injury. This phase is characterized by proliferation and migration of different cells including resident cells and fibroblast to the site of injury. Macrophages and fibroblasts transform the fibrin clot into granulation tissue accompanied by a concomitant sprouting of blood vessels. The granulation tissue promotes further migration of resident cells which are responsible for secreting ECM components, such as collagens, which build the bulk of new tissue (Werner, Krieg, and Smola 2007). The remodelling phase of wound healing begins after about 2-3 weeks and lasts up to 12 months or longer. During this phase, inflammatory processes decrease and cease while macrophages and myofibroblasts undergo apoptosis. The granulation tissue is remodelled in an attempt to restore the function of the injured tissue. Hence, this stage is characterized by secretion, degradation and reorganization of ECM components (Grinnell 1984; Nourissat, Berenbaum, and Duprez 2015).

While several factors can contribute to insufficient healing including persistent inflammation or unbalanced chemokine secretion, the outcomes of insufficient healing are typically chronic inflammation, loss of function and biomechanical stability and, consequently, an increased risk of re-injury (Sabine A. Eming, Krieg, and Davidson 2007; Riley 2008). In tissues that are poorly vascularized as tendons, the limited blood supply together with the tight structural organization of the native ECM often results in failure of the wound healing process. Therefore, tendons have limited self-repair capabilities and are capable *at best* only to fibrous repair(Riley 2008; Steinmann et al. 2020). To overcome these problems, current research efforts aim to achieve scarless repair of tendon, which restores the function of the injured tissue and leads to physiological homeostasis. However, scarless regeneration is only reported in fetuses, while in adults the repair process often leads to the formation of fibrotic scar tissue characterized by varying degrees of functional impairment compared to native tissue (El Ayadi, Jay, and Prasai 2020; Kishi et al. 2012; Ribitsch et al. 2018). Although the role of inflammation in tendon healing is still poorly understood, it has been proven that it is a necessary trigger to activate tissue response and the use of early and prolonged anti-inflammatory drugs results in ineffective tendon healing (Dakin, Dudhia, and Smith 2014).

Tenocytes in health and disease

The cells interposed between the collagen fibrils in tendon tissue are 90-95% rod-like spindle-shaped fibroblasts, named tenocytes (Aicale, Tarantino, and Maffulli 2017). The remaining 5-10% are tendon stem/progenitor cells (TSPCs), which can proliferate and differentiate into different

mesenchymal lineages and are involved in tissue repair(Bi et al. 2007). TSPCs are immature cells characterized by a high number of organelles and high metabolic activity(Bi et al. 2007). Upon maturation, TSPCs differentiate into tenocytes, become more elongated, and have a lower nucleus to cytoplasm ratio (Bi et al. 2007; Dakin, Dudhia, and Smith 2014). Tenocytes are active in energy production and are responsible for the production of all ECM components of tendons with a metabolism shifting from aerobic to anaerobic during aging (Kannus, Paavola, and Józsa 2005). Tendons have the capability to adapt to mechanical stimuli, and it is nowadays widely accepted that the interaction between cells and their surrounding environment, plays a major role in the adaptation of the tissue (Nourissat, Berenbaum, and Duprez 2015). Specifically, tenocytes play a crucial role in regulating the synthesis and secretion of collagen subtypes and other fibrous proteins into the extracellular environment (Aicale, Tarantino, and Maffulli 2017). They also modulate the secretion of enzymes responsible for degrading the extracellular matrix, such as Matrix MetalloProteinases (MMPs), as well as their inhibitors, known as Tissue Inhibitors of MetalloProteinases (TIMPs), in order to facilitate the remodelling and adaptation of the extracellular matrix(Del Buono et al. 2013).

The early response to microdamage or traumatic injury results in the development of acute inflammation in and around the injured anatomical location (Docheva et al. 2015; Stauber, Blache, and Snedeker 2020). Endogenous cells respond to pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 with increased proliferation and upregulation of ECM-related genes (col1a, col2a, col3) with a shift from type I to type III collagen production as compared to healthy tissue (Maffulli et al. 2000). Moreover, cytokines released in the inflammatory milieu can promote the migration of tendon stem/progenitor cells to the site of injury, thus enhancing the repair process. One of the mechanisms by which cells influence the surrounding matrix is through the production and secretion in the extracellular environment of matrix components, matrix-degrading enzymes, and their inhibitors Collagens and MMPs are enzymes that hold a central role in the turnover of the tendon's extracellular matrix (ECM), and their dysregulation has been linked to tendon pathologies (Sorsa et al., 2016).As a consequence, during the wound healing process there is an imbalanced ECM deposition and degradation which often results in in the formation of fibrotic scar tissue rather than regeneration of native tissue. Scar tissue compromises the biomechanical properties of tendon tissue, causing endogenous cells to respond by secreting increased amounts of remodelling enzymes in an attempt to restore their native environment (Del Buono et al. 2013; Jarvinen et al. 2000). The recurrence of this cascade of events ultimately results in a degenerative process involving the whole tissue and leading to the development of tendinopathies (Cook et al. 2016).

Animal models

General advantages and disadvantages

The use of animal models in biomedical and medical research dates back to the 6th century BCE, and it continues to be a valuable tool in contemporary research (Ericsson, Crim, and Franklin 2013). Initially, our understanding of anatomy, physiology, and disease pathophysiology relied heavily on studies conducted with animals (Ericsson, Crim, and Franklin 2013). Currently, animal models play an indispensable role in scientific and medical research when it is unethical or unpractical to perform studies on the target species (Robinson et al. 2019). As a consequence, several treatments, vaccines and surgical techniques were developed using animal models (Ericsson, Crim, and Franklin 2013; Robinson et al. 2019). Indeed, animal models offer the complexity of the whole living organism as well as the heterogeneity of donor-to-donor variability, which *in vitro* models cannot yet supply. Therefore, animal models represent a compulsory and important component of translational research requested by both, the US Food and Drugs Association (FDA) and the European Medicines Agency (EMA) (And 2015; European Medicines Agency n.d.). In addition, animals spontaneously develop pathologies and disorders equivalent to humans, leading to the development of the concept of "One Health One Medicine". This concept presumes that animal and human disorders have similar pathophysiology hence they both can benefit from similar therapeutic approaches. Today, more than 20 million animals are used in biomedical and medical research with a large number of them represented by small animals as rats and mice (Robinson et al. 2019).

However, there are raising awareness and concerns both in the public and scientific environment regarding the use of sentient animals in research. The controversy is mostly due to a misbalance between the achieved beneficial outcomes and the potential risk and harm to the animals, but also concerning appropriate selection of the model, study design, inclusion of control animals, etc.... In an attempt to optimize and standardize studies involving animals, the 3Rs principle (to reduce, refine and replace animal models) was introduced in 1959 and was only recently (2010) followed by a publication of guidelines on Animal Research: Report *In Vivo* Experiments (ARRIVE) (Burden et al. 2015; Kilkenny et al. 2010). This document defines the minimum essential information that needs to be reported in studies using animal models with the goal of improving the information obtained from and reducing the numbers of studies using animal models (Kilkenny et al. 2010).

Even though fundamental knowledge was gained from animal models, clinical translatability of treatments tested on animals fails in up to 90% of the cases (Harrison 2016; Van Der Worp et al. 2010). To overcome this hurdle, additional efforts were expended in avoiding methodological

shortcomings in animal and clinical trials, publication bias and overoptimistic conclusions concerning efficacy of the treatment (Van Der Worp et al. 2010). However, often the aspects concerning the generalizability of the animal model received little to no attention. These include both species specific differences of disease pathophysiology and response to dosing or medication (Van Der Worp et al. 2010). Indeed, no animal model can perfectly emulate the human organism and current evidence strongly supports fit for purpose model validation and selection of the most relevant species (Denayer, Stöhr, and Roy 2014; Van Der Worp et al. 2010).

Hence, the EMA and FDA recently published new guidelines stipulating the need for proof of the relevance of animal models. This includes cross-species comparison of target expression, distribution, structural homology, pharmacodynamics, signal transduction and metabolic pathways (And 2015; European Medicines Agency n.d.). While cross-species comparison for stroke or schizophrenia has been performed, studies aiming at evaluating species differences between the various musculoskeletal models are yet to be performed.

Animal models for tendinopathies

Animal models are essential for studying the pathophysiology of tendinopathies and testing potential therapeutic interventions. *In vivo* animal models have been developed in a variety of species, including rats, mice, rabbits, dogs, goats, sheep, and horses (Prabhakar 2012; Zuskov and Soslowsky 2014). These models can be categorized as either spontaneous or induced (Dirks and Warden 2011). Spontaneous models arise from either naturally occurring tendinopathy or genetic modification, as knockdown of specific genes which results in the onset of tendinopathy. However, the appearance of lesions and the progression of the disease greatly vary between individuals (Lui et al. 2011). Induced models, on the other hand, lead to a faster and more consistent onset and development of tendinopathy. Commonly used methods for inducing tendinopathy in animals include physical overload or stress, repeated microtrauma, and injection of irritants or enzymes (Dirks and Warden 2011).

Surgical models involve creating tendinopathies via surgical intervention. For example, the Achilles tendon transection model involves a surgical incision to induce tendon rupture (Dirks and Warden 2011; Zuskov and Soslowsky 2014). The advantages of surgical models include the ability to precisely control the location and severity of the injury. However, surgical models can be invasive, leading to unwanted side effects and a longer recovery period.

Chemical models use substances such as collagenase or corticosteroids to induce inflammation and degeneration of the tendon (Perucca Orfei et al. 2016). The advantages of chemical models include the ability to induce tendinopathy with minimal invasiveness and with a high degree of control (Dirks and Warden 2011; Perucca Orfei et al. 2016). However, the use of chemicals can lead to unwanted side effects and the resulting tendinopathy may not fully reflect the natural pathophysiology of tendinopathy in humans (Ueda et al. 2019).

Physical models involve the use of excessive exercise or loading of the tendon to induce tendinopathy. Examples of physical models include the treadmill running model and the overload model (Nakama et al. 2005). The advantages of physical models include the ability to mimic the mechanical overload that is thought to be a major cause of tendinopathy in humans (Nakama et al. 2005). However, physical models may not fully replicate the underlying biological mechanisms of tendinopathy and may require specialized equipment to induce the appropriate level of stress or strain.

Small animals are an attractive tool to perform research *in vivo* augmented by the possibility to produce humanized or genetically modified organism, which can be tailored for specific research questions (Lui et al. 2011; Zuskov and Soslowsky 2014)[74,76]. In addition, they are cheap in purchase, housing and feeding costs and they are easy to handle (Xing et al. 2016). As a consequence of the extensive use of mice and rats in research, a large number of tools for research as antibodies or molecular markers has been specifically developed for these species (Xing et al. 2016). Furthermore, the availability of large animal cohorts allows to obtain comprehensive datasets and frameworks using omics techniques (Canzler et al. 2020). However, findings deriving from small animal models often do not translate into clinics(Prabhakar 2012). The limitations of small animal models in tendinopathy research predominantly stem from inherent distinctions in several critical domains. Firstly, the variances in inflammatory responses in small animals can result in deviations from the temporal, intensity, and duration aspects of inflammation observed in human(Frazier et al. 2013). These differences may be attributed to variations in the kinetics of pro-inflammatory and anti-inflammatory cytokines(Smith et al. 2019; Wong et al. 2020). Furthermore, the immune system in small animals exhibits disparities, encompassing distinct immune cell populations(Smith et al. 2019). Additionally, differences in body size, limb dynamics, and gait patterns, challenge the accurate replication of the biomechanical aspects of human tendinopathy(Dirks and Warden 2011; Zuskov and Soslowsky 2014). Moreover, small animals do not typically develop tendinopathy spontaneously, which stands in contrast to the natural occurrence of the condition in humans, necessitating the artificial induction of tendinopathy in animal models(Dirks and Warden 2011; Lui et al. 2011). This artificial induction may not comprehensively capture the multifaceted disease progression observed in humans. These

disparities underscore the limits of small animal models in mimicking the pathophysiology of tendinopathy in humans. Diseases affecting the musculoskeletal system often results from unbalanced biomechanics or traumatic injuries, whose force intensities are not well represented by small animals (Wang, Iosifidis, and Fu 2006). In contrast, large animal models as horses and sheep more closely match the human anatomy and physiological condition, with respect to loads and forces exerted on the musculoskeletal system (Lui et al. 2011; Madden et al. 2012). Moreover, they naturally develop musculoskeletal disorders, as tendinopathies, with a pathophysiology and etiopathogenesis equivalent to humans (Lui et al. 2011; Zuskov and Soslowsky 2014). The relatively bigger size of these animals allows for higher sample numbers to be taken and repeated sampling, which in turn contributes to a reduction of the number of animals required per study (Madden et al. 2012; Zhang et al. 2022). However, studies including large animal species go along with higher prices for animal purchase, feeding and housing compared to small animal models (Madden et al. 2012; Zhang et al. 2022). Additionally, there is a lack in specific molecular tools and comprehensive omics frameworks, which impair research performed with these animals. Finally, given the status of some large animals as companion animals, there are considerable ethical concerns using these species.

While the *in vivo* animal models currently used in tendinopathy research are unquestionably useful for research, they usually have poor validation when compared to how the illness develops naturally in humans. One of the critical issues lies in the lack of comprehensive understanding regarding the pathogenetic mechanisms of tendinopathy. Presently, it remains uncertain whether these models faithfully represent the complexities of human tendinopathy. To be considered truly useful, animal models must meet specific criteria that closely align with the pathogenetic mechanisms observed in human patients. However, the existing models often fail to fulfil these criteria, leading to significant gaps in our understanding. To bridge these knowledge gaps and ensure the relevance of animal models for tendinopathy research, rigorous validation against specific pathogenetic mechanisms is imperative. Such validation should encompass a range of factors, including the inflammatory response, immune system involvement, biomechanical forces, and other relevant aspects. Furthermore, it is crucial to recognize that a one-size-fits-all approach does not apply in tendinopathy research. Different scientific questions require specific models that accurately represent the aspects of the disease relevant to the inquiry. For example, while a particular model may be suitable for studying inflammation, it may not effectively mimic the biomechanical aspects of tendinopathy. Therefore, researchers must select the most appropriate model for each research question and rigorously validate its suitability against the specific pathogenetic mechanisms of interest. Yet, it is crucial to acknowledge the limitations inherent in current models; they cannot comprehensively cover every aspect of tendinopathy pathology. Consequently, the scientific

community must invest in targeted research efforts to bridge these gaps, refining existing models and developing novel ones that more accurately mirror the human disease's complexity and pathogenetic mechanisms. In summary, our current understanding of tendinopathy pathogenesis is incomplete, and existing animal models often lack the necessary validation against specific pathogenetic mechanisms. To advance tendinopathy research effectively, we must address these knowledge gaps by developing and validating models that closely mimic the multifaceted aspects of the disease seen in humans. To address these challenges, the use of a more standardized research framework, for instance supported by in vitro models, can help in elucidating the complexities of translational research.

In vitro models

In vitro models offer a systematic and standardized research environment to investigate biological processes under controlled conditions. These processes can include physiological signalling pathways, pathological disease-associated changes, as well as cell toxicity and pharmacological tests. *In vitro* models have several advantages over *in vivo* models as they are easier to perform and replicate, offer a high-throughput, are usually cheap and go well together with the 3Rs principle (Madden et al. 2012). Advantages of *in vitro* models include their ability to provide a rapid and accessible platform for studying various cellular processes, such as DNA synthesis, mitosis, gene expression, and cell differentiation.

Wound healing models

The understanding of wound healing processes is of paramount importance in the field of regenerative medicine (Martin 1997). *In vitro* wound healing models provide valuable tools for investigating the intricate cellular and molecular mechanisms that drive tissue repair (Jonkman et al. 2014). These models offer controlled experimental conditions and enable researchers to focus on specific aspects of wound healing, shedding light on critical factors that influence the repair process (Friedl, Hegerfeldt, and Tusch 2004). By utilizing *in vitro* models, researchers can gain a deeper understanding of cell migration, and the effects of various factors on wound closure kinetics (Hulkower and Herber 2011). Models of wound healing generally involve the creation of a cell free gap in a confluent layer of cells by mechanical, chemical, thermal or electrical removal of cells or physical exclusion in a determined area (Jonkman et al. 2014). Successively, micrographs of the cell free area are taken and analysed (Jonkman et al. 2014; Nyegaard, Christensen, and Trige 2016). Finally, cell

movement into the cell-free area is quantified through analyses of the gap size reduction over time (Jonkman et al. 2014). A commonly used method is physical exclusion of cells in a specific area using a commercially available insert. This technique consists on placing a (silicone) insert in the culture dish before cell seeding which is removed upon cell adhesion, creating a cell free gap (Jonkman et al. 2014). This technique offers high reproducibility with low technical variability; however, it lacks cell injury limiting the applicability to investigate migration in wound healing environment. Although several commercially available techniques are on the market, the 2D scratch assay using a pipette tip to create the cell free area still represents the gold standard to evaluate cell migration in a wound environment (Hulkower and Herber 2011; Jonkman et al. 2014). This is a relatively cheap and easy-to-perform laboratory technique to remove the cells by mechanical force. Additionally, this procedure results in physical injury and disruption of cells at the border of the gap (Ammann et al. 2015; Jonkman et al. 2014). These events trigger a cascade of molecular signalling, leading to the secretion of cytokines and chemokines that simulate the wound environment's characteristics (Ammann et al. 2015). However, reproducibility is hampered by inter- and intra-individual replicability of the gap size and shape as well as the extent of injury. This is due to the fact, that the pressure exerted on the pipette tip as well as the angle and speed at which the tool is used depend on the operator (Jonkman et al. 2014; Nyegaard, Christensen, and Trige 2016). The resulting variation in the gap size and cell injury extent might confound the obtained results (Ammann et al. 2015; Friedl, Hegerfeldt, and Tusch 2004; Hulkower and Herber 2011; Jonkman et al. 2014). Therefore, there is a pressing need for the development of a highly standardized, user-friendly wound healing assay to study cell migration, aiming to overcome the limitations and variability associated with existing methods and to provide researchers with a reliable tool for advancing our understanding of wound healing processes in regenerative medicine.

In vitro models of tendinopathies (inflammation)

With respect to tendon research, it is relatively easy and inexpensive to isolate and expand cells from these tissues through enzymatic digestion (Wagenhäuser et al. 2012). The obtained cells can be used to perform studies in monolayer culture, co-culture, and tissue engineered constructs with increasing levels of complexity (Dirks and Warden 2011). Monolayer cultures are an excellent tool to evaluate a single mechanism of action of physiological processes, pathophysiology of specific diseases and potential treatments (Dirks and Warden 2011; Edmondson et al. 2014). Furthermore, they can be combined with mechanical stimulation as cyclical stretching or the use of pre- or co-treatments with specific substances or cytokines. For instance, by including factors like TNF-α and IL-1β in the growth media, researchers can induce/model inflammatory responses in the isolated cells and investigate the effects of these additional stimuli on tendinopathy development (Dirks and

Warden 2011). However, these models lack cell-matrix interactions and aspects of multicellularity. Furthermore, cells in monolayer might undergo dedifferentiation which results in a change in phenotype and gene expression patterns (Edmondson et al. 2014). Co-culture systems allow for the simultaneous *in vitro* culture of different cell types. These systems are specifically designed to investigate interaction between different cell types either directly or indirectly, using e.g. a transwell system. They are particularly interesting to evaluate the influence of different cell types involved in diseases such as tendinopathies, i.e. the cells of the immune system with tenocytes (Kraus et al. 2013). Using a 3D environment to culture tissue engineered constructs is preferred as it helps prevent loss of phenotype and closely mimics the *in vivo* situation by facilitating cell-matrix interactions. This is typically achieved through techniques such as seeding cells on top of or encapsulating them in 3D materials (Edmondson et al. 2014). Over the past years, several biocompatible materials have been used for this purpose including collagen, hyaluronic acid, fibrin, silk, etc…, which support cell infiltration, survival, proliferation and differentiation, as well as the retention of newly formed ECM components (Edmondson et al. 2014; O'Brien 2011). However, to date no natural or artificial material is truly comparable to the native tissue´s mechanical properties or structural organization (O'Brien 2011). Cells in the musculoskeletal system are known to respond to the mechanics of the extracellular environment, therefore mechanical stimuli which do not appropriately mimic the native tissues may activate different mechanotransduction pathways and result in altered metabolic activity, cell proliferation, differentiation and ECM turnover rates (Muntz et al. 2022; Stauber, Blache, and Snedeker 2020). Furthermore, 3D systems depend on osmotic diffusion of oxygen and nutrients which limits the constructs size to a maximum of 200 µm depth from the surface otherwise it might lead to cell necrosis (O'Brien 2011; Rouwkema et al. 2013). Ex vivo explant culture is a laboratory technique that involves the isolation of tissue biopsies, or explants, from a living organism, which are subsequently cultured under controlled laboratory conditions to maintain viability and enable cellular growth and differentiation (Gomez-Florit et al. 2022). This technique provides a valuable tool for investigating the behaviour of cells and tissues *in vitro*, while still preserving their native architecture and extracellular environment, and thus allowing for studies that more accurately reflect *in vivo* conditions (Costa-Almeida et al. 2018).

One of the major drawbacks of *in vitro* these models is scarcity of available healthy human material for research purposes (Prabhakar 2012). Therefore, the majority of studies performed with cells or tissue either from various animal species or aged and degenerated tissues. In the existing literature, numerous cell sources from tendons derived from different anatomical locations, as well as a variety of species, have been extensively utilized (European Medicines Agency n.d.; Theodossiou and Schiele 2019). This highlights the importance of investigating the cross-conserved pathways and

molecules within these diverse models to assess their translatability and relevance for the target species (European Medicines Agency n.d.). Despite these limitations, *in vitro* models provide valuable insights into the cellular mechanisms underlying tendinopathy and the response to inflammatory stimuli (Theodossiou and Schiele 2019; Wunderli, Blache, and Snedeker 2020). By carefully designing experiments and considering the advantages and limitations of *in vitro* models, researchers can enhance our understanding of tendinopathy pathogenesis and explore potential treatment strategies. However, it is worth noting that the selection of cell sources and models for tendinopathy research is often driven by availability rather than their suitability for the specific research purpose, leading to a reliance on various animal species or aged and degenerated tissues without adequate cross-species comparisons or validation for translatability.

Consideration for an optimal *in vitro* model for tendinopathies

To effectively model tendinopathies *in vitro* and enhance their translational relevance, several key factors must be considered. Tendinopathies are complex musculoskeletal disorders characterized by a range of pathological features, including chronic inflammation, tissue microdamage, altered matrix composition, abnormal cell behavior, and cell damage/death (Arvind and Huang 2021; Dirks and Warden 2011; Docheva et al. 2015). Therefore, an ideal *in vitro* model should include these critical aspects to provide a realistic emulation of the disease. Firstly, the inclusion of tenocytes is paramount as they are the primary cell type involved in tendinopathy (Cook et al. 2016; Docheva et al. 2015). These cells play a pivotal role in maintaining tendon homeostasis, responding to mechanical forces, and mediating inflammation (Dirks and Warden 2011; Docheva et al. 2015). Consequently, the choice of appropriate cell sources and their behavior in response to various stimuli must be carefully considered (Theodossiou and Schiele 2019). However, the current lack of data concerning the species-specific response of tenocytes in *in vitro* models underscores a significant gap in our understanding of this aspect within the field. Tendons are force-bearing structures, and mechanical loading is a fundamental aspect of their physiology and pathology (Nourissat, Berenbaum, and Duprez 2015). To mimic the *in vivo* conditions, *in vitro* models should incorporate mechanical stress, which can help elucidate the mechanisms underlying tendon degeneration and regeneration (Dirks and Warden 2011). Mechanical loading can influence cell behavior, matrix remodeling, inflammation, and even trigger microdamage, making it a crucial component of a comprehensive model (Stauber, Blache, and Snedeker 2020). Microdamage, often associated with repetitive overuse, is a key contributor to

tendinopathy development (Cook et al. 2016; Nakama et al. 2005). Therefore, the model should replicate the formation of microdamage and the subsequent cellular responses, including cell damage and death, which are integral to the wound healing process. This could involve the induction of controlled microtears or disruptions in the extracellular matrix to mimic the initial stages of tendinopathy (Snedeker and Foolen 2017; Stauber, Blache, and Snedeker 2020). Inflammation is a hallmark of tendinopathies, and *in vitro* models should encompass both acute inflammation and the recovery phase from inflammation as well a chronic inflammation (Cook et al. 2016). This includes the presence of pro-inflammatory cytokines and immune cells during acute inflammation, as well as the transition to the successful/non successful resolution during recovery, which is characterized by anti-inflammatory signals and tissue repair processes (Cook et al. 2016; Docheva et al. 2015; Steinmann et al. 2020). Understanding the dynamic interplay between inflammation and tissue repair is crucial for developing effective therapeutic strategies (Riley 2008; Schoenenberger et al. 2018). However, information regarding species-specific responses of tenocytes to inflammation is still lacking. Therefore, it is essential to investigate these responses to determine the suitability of animal models in mimicking tendon injuries and diseases. Cell migration is another critical aspect to consider, as it underlies tissue repair processes in tendinopathies (Docheva et al. 2015; Schulze-Tanzil et al. 2022). *In vitro* models should enable the study of cell migration within a wound healing environment, replicating the complex interplay of cells during tendon repair (Riley 2008; Schulze-Tanzil et al. 2022). Three-dimensional (3D) models hold promise in better mimicking the native tendon microenvironment (Gomez-Florit et al. 2022). They can provide a more realistic spatial arrangement of cells and matrix components, allowing for improved studies of cell-cell and cell-matrix interactions, including those related to cell damage and death (Blache et al. 2022; Muntz et al. 2022). In summary, to create an optimal *in vitro* model for tendinopathies, it is essential to incorporate tendon cells, mechanical loading, microdamage, inflammation (both acute and chronic), cell migration, 3D culture systems, and cell damage and death. These components collectively contribute to a comprehensive understanding of tendinopathy pathogenesis and progression and can facilitate the development of novel therapeutic strategies.

Aims

This thesis therefore aims to improve the reliability and applicability of tendinopathy research by developing novel technologies and comparative frameworks for tendinopathy modelling .

The first objective was to develop a standardized, reproducible method to replicate wound healing in vitro.

This research was developed to test the following hypotheses:

i) Using a novel magnetic scratch method achieves greater repeatability, reproducibility and geometric control than the current golden standard (pipette scratch assays).

ii) It is necessary to induce cellular damage within a cell monolayer to model the wound healing environment.

The second objective was to explore species-specific variations in musculoskeletal animal models, specifically comparing the inflammatory responses of tenocytes from various model species to humans.

This research was developed to test the following hypotheses:

i) The addition of inflammatory cytokines to tendon cells *in vitro* mimics *in vivo* tendinopathy.

ii) The *in vitro* inflammatory response of tenocytes of commonly used animal models mimics the inflammatory response observed in human tenocytes.

The overall aim of this work is to enhance our understanding of wound healing processes, musculoskeletal pathologies, and potential species-specific variations. This thesis sets out to create a more faithful representation of the *in vivo* situation and therefore reduce the overall use of animals in research in line with the 3R principle.

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SCIENTIFIC REPORTS
nature research

A novel magnet-based scratch method for standardisation of wound-healing assays

Received: 13 May 2019
Accepted: 13 August 2019
Published online: 02 September 2019

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A novel magnetic scratch method achieves repeatability, reproducibility and geometric control greater than pipette scratch assays and closely approximating the precision of cell exclusion assays while inducing the cell injury inherently necessary for wound healing assays. The magnetic scratch is affordable, easily implemented and standardisable and thus may contribute toward better comparability of data generated in different studies and laboratories.

Cell migration is fundamental to establishing and maintaining the proper organization of multicellular organisms and plays a pivotal role in numerous physiological and pathological processes including tissue morphogenesis and homeostasis, wound healing, immune surveillance, inflammation and cancer[1,2]. Due to its relevance to health and disease, methods to simulate and explore critical mechanisms of action and to investigate therapeutics under well-defined conditions are of broad interest, and a variety of *in vitro* models have been developed, of which the scratch assay is the most widely used[3,4]. The scratch assay, also known as wound healing assay, is implemented by creating a cell-free area (gap, wound) within a confluent monolayer either by removing the cells post adherence via mechanical, electrical, chemical, optical or thermal means, or through physical exclusion of cells during seeding[1–3,5–11]. Subsequently serial high-resolution images are captured and analysed to quantify the dynamics of cell migration into the gap[3]. However, as the scratch is most commonly created manually using a pipette tip, the extent of the cellular injury and the geometry of the scratch is influenced not only by the scraping tool utilized, but also the level of manual control, the pressure exerted, the angle of the instrument and the velocity of scraping[3,4,6,7]. As a result, repeatability and reproducibility of the assays is limited and dependent on the manual dexterity of the researchers. Variation during wounding may confound interpretation and quantification of the wound healing process and impede comparison of results amongst different researchers and laboratories[1,2,4,6,7].

Therefore, we aimed to develop and establish a novel scratch method allowing creation of a consistent, reproducible scratch with relatively smooth edges and little cellular debris within the gap, that is easy to standardize across different operators, studies and even laboratories.

The novel scratch assay employs magnetic discs of standardised size, which are placed in the cell culture wells. The well-plate is then placed on a matching well-plate lid with affixed magnetic spherical counterparts. The magnetic force between the magnetic spheres and discs provides a standardized pressure and keeps the magnetic discs stationary in the wells while the plate is moved horizontally to create scratches of uniform size in all wells simultaneously. The new magnetic scratching technique is easy to adapt to well-plates of different sizes and shapes and can be handcrafted in every lab using standard cell culture well-plates and commercially available magnets.

Results and Discussion

To validate the novel magnet scratching technique, scratch assays were carried out in technical duplicates by 2 separate operators on confluent monolayers of primary equine tenocytes (n = 3 donors) and chondrocytes (n = 3 donors) employing one of four techniques: (1) the novel magnetic scratch method using 1.5 mm diameter magnetic discs (Fig. 1), (2) a commercially available cell culture insert for physical exclusion of cells in a defined area, (3) a scratch assay using a pipette tip (1250 µl) with 50 g and 4) with 150 g manual pressure (Fig. 2). The different techniques were compared using serial micrographs taken until full closure of the gap to evaluate the target

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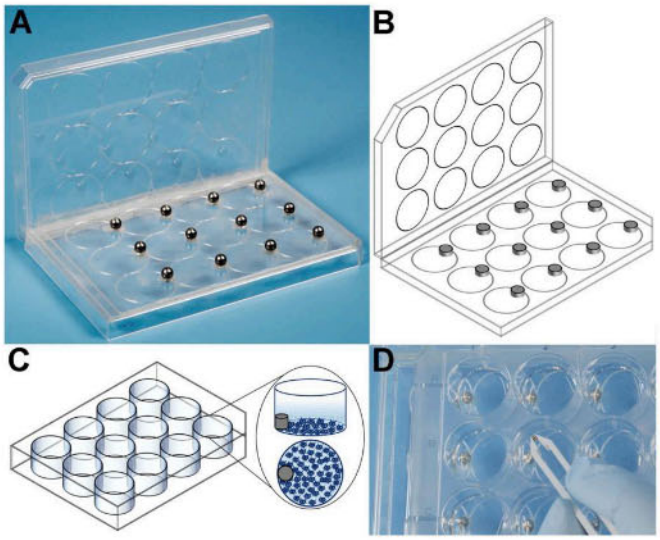

Figure 1. Figures (**A**) (photograph) and (**B**) (schematic) demonstrate the lid with the glued-on magnets on the right side of each etched-in well and the guide rail of the novel magnet scratch method, while figures (**C**) (schematic) and D (photograph) show the well-plate with the magnet being placed in the left side of each well.

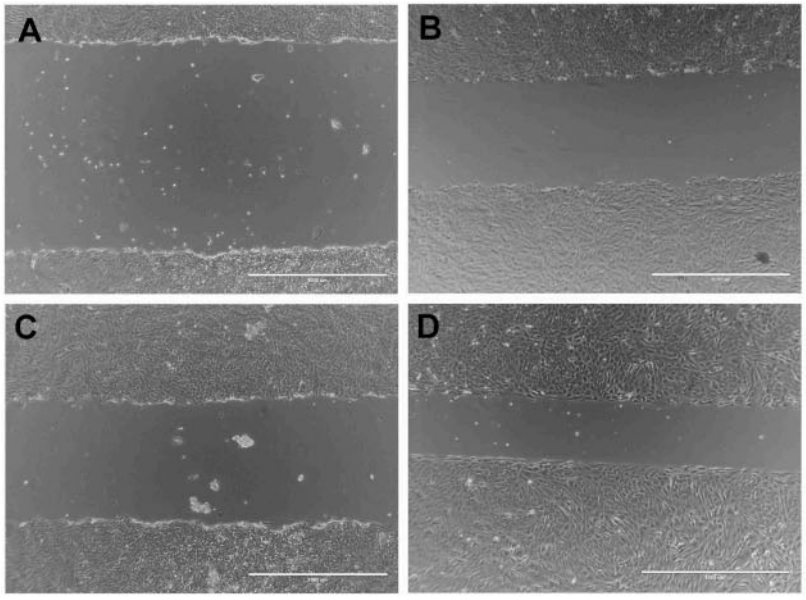

Figure 2. Phase contrast images of representative gaps created in confluent chondrocyte cultures using the (**A**) novel magnetic scratch method, (**B**) pipette tip (1250 µl) with 50 g manual pressure, (**C**) pipette tip (1250 µl) with 150 g manual pressure and (**D**) commercially available cell culture insert, at 0 h (scale bar = 1000 µm). Note differences in gap width homogeneity and gap border straightness between techniques.

	Gap Width											
	pipette 50 g			pipette 150 g			magnet			Insert		
	OP1	OP2	both OPs	OP1	OP2	both OPs	OP1	OP2	both OPs	OP1	OP2	both OPs
Mean	654	800.7	728	784	828.2	798.1	1545	1533	1539	442.7	472	457.6
Std. Deviation	113.8	63.19	58.5	71.16	59.62	35.05	28.72	33.18	23.69	12.2	24.85	13.27
Std. Error of Mean	17.99	9.99	9.25	11.25	9.427	5.409	4.542	5.246	3.746	1.93	3.929	2.098
Lower 95% CI	617.6	780.5	709.3	761.2	809.1	787.2	1536	1523	1532	438.8	464	453.3
Upper 95% CI	690.4	821	746.7	806.8	847.2	809	1554	1544	1547	446.6	479.9	461.8
Coeff. of var.	17.4%	7.89%	8.04%	9.08%	7.20%	4.39%	1.86%	2.16%	1.54%	2.76%	5.27%	2.90%
	Line Straightness											
	pipette 50 g			pipette 150 g			magnet			Insert		
	OP1	OP2	both OPs	OP1	OP2	both OPs	OP1	OP2	both OPs	OP1	OP2	both OPs
Mean	1.25	1.25	1.24	1.22	1.20	1.21	1.14	1.14	1.13	1.07	1.07	1.07
Std. Deviation	0.08	0.06	0.06	0.07	0.04	0.06	0.07	0.06	0.05	0.04	0.04	0.04
Std. Error of Mean	0.02	0.02	0.01	0.02	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01
Lower 95% CI	1.20	1.21	1.21	1.17	1.18	1.19	1.10	1.10	1.11	1.05	1.04	1.05
Upper 95% CI	1.30	1.28	1.27	1.27	1.23	1.24	1.19	1.17	1.15	1.09	1.09	1.09
Coeff. of var.	6.65%	4.51%	4.89%	5.85%	3.31%	4.77%	5.86%	4.95%	4.06%	3.40%	3.95%	3.61%

Table 1. Descriptive statistics of the gap width and the straightness of the gap margins achieved with the 4 scratch methods (pipette 50 g, pipette 150 g, magnet, cell exclusion insert) by the 2 operators as well as overall.

variables I) standard deviation (SD) and coefficient of variation (CV) of the gap width measured at ten points along the gap, II); straightness of the gap margins (ratio of the measured length of the gap margins to the length of an ideal straight line) and III) gap closure rate (slope of the regression of gap area in mm^2 depending on time) (Suppl. Figs 1 and 2).

Reproducibility and repeatability of the assays was determined based on the differences in gap width standard deviation and coefficient of variation observed between and within the 2 different operators (Table 1). The pipette 50 g technique had the worst repeatability and reproducibility (SD 113.8 vs. 63.19, CV 17.4% vs.7.89%), followed by the pipette 150 g (SD 71.16 vs. 59.62, CV 9.08% vs. 7.2%). The magnet (SD 28.72 vs. 33.18, CV 1.86% vs. 2.16%), and the insert (SD 12.2 vs. 24.85, CV 2.76% vs. 5.27%) had the best repeatability and reproducibility.

Scientific discovery is an iterative process and requires reproducible data, which allow multiple data sets to be combined to generate knowledge. Therefore, the need for scientific community-wide implementation of standardized assays to ensure that the data produced has high intra-study consistency and can be replicated and compared successfully across multiple laboratories is increasingly recognized. However, this can only be achieved with cost-effective, platform-independent, easy-to-use techniques, which can be implemented in any laboratory without technical prerequisites, such as the novel magnetic scratch or cell exclusion assays.

Gap width standard deviation varied significantly ($p < 0.001$) between methods (Table 1, Suppl. Table 1, Suppl. Fig. 3A). The insert (SD 13.27), followed closely by the magnet (SD 23.69), had the lowest standard deviation of the gap width, and the manual methods had the highest (50 g > 150 g, SD 58.5 resp. 35.05). The coefficient of variation of the gap width was smallest for the magnet with 1.54%, followed by the cell culture insert with 2.9%, the pipette 150 g with 4.39%, and the pipette 50 g with 8.04% (Fig. 3A, Suppl. Fig. 3B). Correspondingly, straightness of the gap margins varied significantly ($p < 0.001$) between the cell exclusion insert and the other three methods as well as between the magnet and the other three methods (Fig. 3B, Table 1, Suppl. Table 1, Suppl. Fig. 3C). Cell exclusion inserts led to the straightest margins (mean deviation from the ideal straight line = 6.9%, CV = 3.61%), the magnet was intermediate (mean deviation from the ideal straight line = 13.2%, CV = 4.06%), and the pipette methods (50 g > 150 g) showed the largest difference (mean deviation from the ideal straight line = 23.9% resp. 21.1%, CV = 4.89% resp. 4.77%) between the measured and an ideal margin (of a perfect straight line) (Table 1, Suppl. Table 1).

These results highlight the significantly higher geometrical control and precision of the cell culture insert and the magnet in comparison with the manual techniques.

Two cell types (chondrocytes and tenocytes) were included in the validation study of the magnetic scratch to determine whether cell type would, due to differences in cell-cell adhesion, polarity or cytoskeletal organisation, influence gap linearity, gap width SD and CV and gap closure rate. In contrast to chondrocytes, tenocytes have long cell processes and adherens and gap junction-based cell-to-cell contacts forming complex interconnected networks and cell sheets[12,13]. The gap junctions are essential for cell-cell communication and represent a functional network allowing coordination of mechanical and synthetic activity[12,13]. However, cell type showed a significant influence only on gap closure rate ($p < 0.001$) but not on any indicator of gap geometry, accuracy or precision.

Gap closure rate also differed significantly ($p < 0.001$) among methods (Fig. 3C, Table 1, Suppl. Table 2, Suppl. Fig. 3D) and was significantly slower ($p < 0.001$) with the cell exclusion than the injury inducing scratch methods for both cell types as well as overall. This is consistent with previous studies showing differences in cell migration and signalling between cell exclusion and cell injury wound healing assays.

In this study, Fluoresceindiacetat (FDA, C-7521, Sigma-Aldrich) - Propidiumiodid (PI, P4170, Sigma-Aldrich) fluorescent staining confirmed the accumulation of dead cells alongside the scratch gap border for all techniques

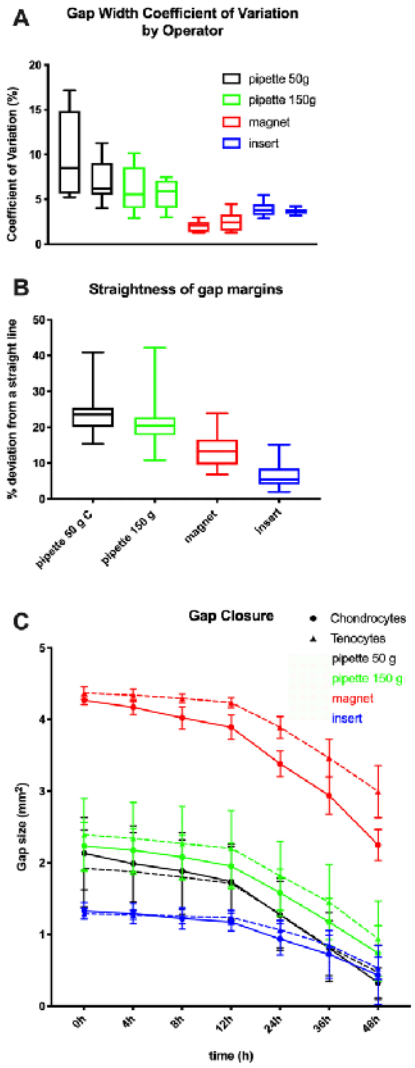

Figure 3. Comparison of the gap width coefficient of variation by operator, the straightness of the wound margins and the gap closure rate between the 4 scratch techniques. (**A**) Box and Whiskers plot of the gap width's coefficient of variation in monolayer cultures. The left plot of each equally coloured pair corresponds to operator 1, the right to operator 2. The box extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers indicate the minimum and maximum. Gap width varied least with the insert followed by the magnet, then the pipette 150 g and last the pipette 50 g. (**B**) Box and Whiskers plot of the straightness of the gap margins achieved in monolayer cultures of chondrocyte and tenocyte. The box extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers indicate the minimum and maximum. Gap margins were straightest with the insert followed by the magnet, then the pipette 150 g and last the pipette 50 g. (**C**) The gap closure rate of chondrocytes and tenocytes from 0–48 hours, the time period of linear gap closure rate in both cell types before the gap closed in any sample, showing the mean remaining gap size ± SD for each time point. Cell type (chondrocyte versus tenocyte) had a significant influence only on gap closure rate ($p < 0.001$). With the scratch methods, which cause cell injury (magnet, pipettes), gap closure speed was faster than with the insert.

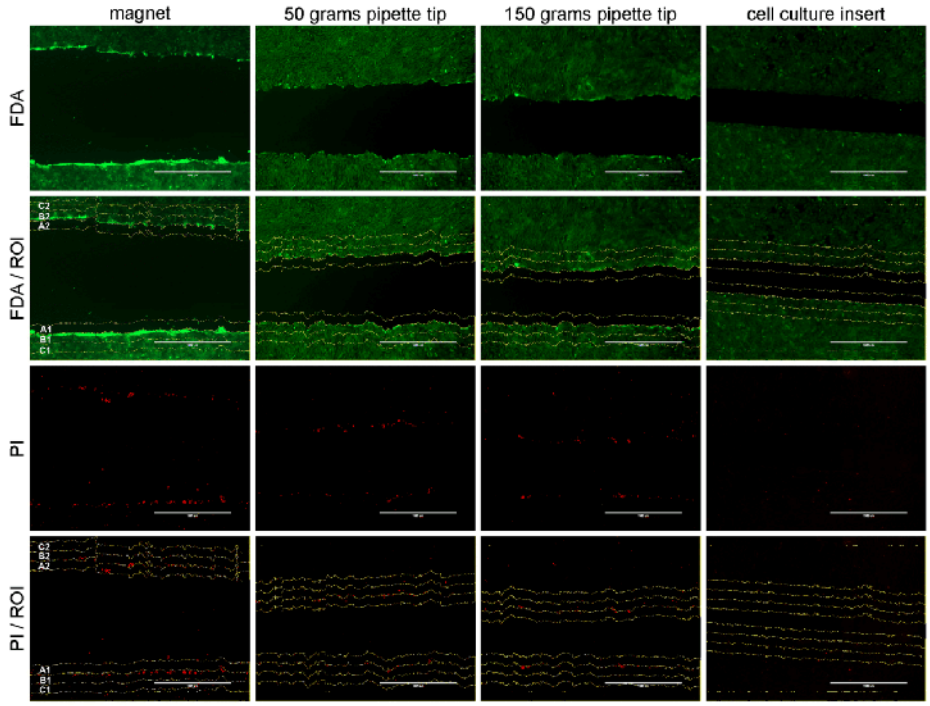

Figure 4. Definition of regions of interest (ROI) along the scratch margins. ROI (**A**) extends 25 µm from the scratch margin into the cell free area of the scratch; ROI (**B**) extends 25 µm from the scratch margin into the remaining cell monolayer (region of main interest: contains cells which died in direct consequence to wounding); ROI (**C**) extends from 25 µm to 50 µm into the remaining cell monolayer (scale bar = 1000 µm).

except the cell culture insert (Fig. 4). The difference in the number of dead cells between all three injury-inducing techniques (magnet, pipette 150 g pipette 50 g) and the cell culture insert was statistically significant ($p < 0.001$, Suppl. Table 3) at all three time points of analysis (0 h, 8 h and 24 h after wounding) independent of cell type (chondrocytes versus tenocytes, Suppl. Table 4, Suppl. Fig. 4). While the injury free release of contact inhibition is sufficient to induce cell migration in cell exclusion assays[3,6,9,14], the injury inflicted upon the cells in scratch wound healing models with the associated disruption of cell adhesions and release of intracellular contents and signalling molecules has been shown to alter the microenvironment in the cell culture and to provide a critical input required for cell-cell coordination in the spreading of the wounded monolayer[1,3,4,6,7,9]. Thus, while cell exclusion assays provide repeatability and geometric control, they fail to induce an injury and the insert used in this study correspondingly led to slower gap closure kinetics compared to the three assays that inflicted injury upon the cell layer.

Gap closure rate typically shows a nonlinear decline with decreasing gap size[15], which we could confirm for all techniques used in this study (Suppl. Fig. 3D). For small gaps this limits the time period in which linear closure rates allow comparative quantification of cell migration to 48 hours. As the scratch assay is predominantly used to study wound healing and cancer cell migration and for corresponding drug discovery screening[7], the creation of a larger standardized gap, as introduced in this study (mean gap width 1539 µm (magnet) versus 457.6 µm (cell culture insert), Table 1), provides a longer observation period to study, e.g., the tumour cell aggressiveness or the efficacy and cytotoxicity of the screened molecules and compounds.

As damage to the plate surface incurred during scratching may affect migration rates[1,8], the surface relief of the culture dish was measured using atomic force microscopy (Suppl. Fig. 5). The unscratched surface was characterized by topographical variation within 40 nm in height. Pipette tip scratches with 150-gram pressure increased topographical variation to 160 nm, and the magnetic scratch method to 250 nm, both with grooves parallel to the orientation of the scratch. However, this level of variation in surface topography appears to be no impediment to cell migration since the gap closure rates of the three different injury-inducing scratch methods were comparable and faster than the cell exclusion assay.

In conclusion, the novel magnetic scratch method combines operational ease with precision and geometric control, induces the cell injury inherently necessary for a wound healing assay and provides excellent repeatability, reproducibility and easy standardization across different observers (Table 2). Thus, the proposed method may

	Manual scratching		Cell culture insert		Magnet	
	Advantage	Disadvantage	Advantage	Disadvantage	Advantage	Disadvantage
Margin straight-ness		Varies within and between operators	Very good		Good	
Gap width		Varies within and between operators	Standardized	Narrow	Smallest coefficient of variation; Wider than other techniques: Longer time span until gap closure = longer observation period. Assays of longer duration might more accurately reflect the combined effects of migration and proliferation[16]	
Homo-geneity		Varies within and between operators	Standardized		Good	
Repeat-ability/Reproduc-ibility		Variation within and between operators	Very good		Good	
Damage to the plate surface	Little		None			Possible
Adapt-ability to plate size	Good			Only one size available	Good	
Simult. scratching of more than one well		Not possible	N.A.	N.A.	Possible	
Price	Lowest costs			Expensive	Very low costs	
Avail-ability	Always and immediately			Needs to be ordered, available in packages of limited numbers.	All parts to tailor scratching device (but the magnets) are standard laboratory equipment.	Magnets need to be ordered. Requires some time to tailor the device.
Other challenges		Steady hand required upon scratching				Steady hand required upon scratching.
Sum	4	6	5	4	8	4

Table 2. Summary of advantages and disadvantages of different wounding methods.

contribute to the generation of data, which are better comparable between different studies and laboratories, and may lead to more efficacy and quality control in research.

Methods

Production and handling of the magnetic scratching tool. The novel magnetic scratching tool can be handcrafted easily by using standard cell culture well-plates and commercially available neodymium magnets. It can be tailored for different types of well-plates and used to scratch all wells of a plate simultaneously. First, the lids of two well-plates were glued together in a 90° angle along their long sides (Fig. 1) with a commercially available standard super glue, such that one lid serves as bottom plate and one as guiding rail. Then magnetic spheres (neodymium magnets, diameter 5 mm +/− 0,1 mm, type K-05-C, Supermagnete, Germany) were glued to the lid serving as bottom plate, which should match the well-plate to be scratched and have the size and shape of the wells engraved as template to facilitate correct placement. The magnetic spheres were glued centred to the far-right end of each engraved well with vertically oriented magnetic force (Fig. 1) while using additional magnetic spheres as counter-parts to enable magnetic alignment.

The magnets used as scratching tools were disc-shaped (Supermagnete, S-1.5-0.5-N) with a diameter of 1,5 mm and height of 0,5 mm (+/−0,1 mm tolerance). Our pilot experiments determined four stacked discs to produce the optimal weight and magnetic force to create homogenous scratches over the full length of the wells. Prior to use, the discs were sterilized in 70% ethanol for 10 minutes followed by two washing steps with PBS for 5 minutes each. To position the scraping magnets, the cell culture well-plate was placed on top of the bottom plate offset to the right, such that the left rims of the wells overlapped the right rims of the engraved well shapes with the magnetic spheres on the bottom plate. The scratching magnets (stack of 4 discs) were dropped into the wells above the magnetic spheres using plastic tweezers. Due to the magnetic attraction they fell into place at the left rim of the wells directed toward the magnetic sphere on the bottom plate. When all magnets were in place the well-plate was shifted to the left, using the rail plate to facilitate creation of straight scratches, until it superimposed the bottom plate. Finally, the magnetic discs were removed from all wells using a sterile spherical magnet attached to a metal bar (Suppl. Video).

Validation of the novel scratching tool. To validate the newly developed scratching tool, it was compared to the current gold standard (manual scratching with a pipette tip) and a cell culture insert for physical exclusion of cells in a defined area (80209, Ibidi™). The validation study was carried out in 12-well cell culture plates and performed with equine chondrocytes and tenocytes (3 biological replicates each) by two independent operators to study repeatability and reproducibility of the different scratch assay methods.

The cells had been obtained from three horses, which were euthanized for reasons unrelated to this study. Tissue collection to obtain these cells had been performed according to the "Good Scientific Practice and Ethics in Science and Research" regulation implemented at the University of Veterinary Medicine Vienna. The animal owner's consent to collect and analyse the samples and to publish resulting data was obtained according to the standard procedure and approved by the ethics and animal welfare committee of the University of Veterinary Medicine Vienna. Cells were cultured at routine cell culture conditions using Dulbecco's modified eagle medium (DMEM, LONBE12-707F, Lonza) supplemented with 10% fetal bovine serum (FBS, F7524, Sigma-Aldrich), 1%

L-glutamine (K0302, Biochrom), 1% penicillin-streptomycin (P4333-100ML, Sigma Aldrich), and 1% amphotericin (A2612, Biochrom) prior to seeding them onto 12 well-plates for the scratch assays in passage 4–5. Medium was changed twice weekly.

For the three scratching tools (magnet, pipette 50 g and pipette 100 g) 100.000 cells were seeded per well. 24 hours later the confluent cell layers were washed twice with PBS and the wounding of the cell layer was performed:

For manual scratching a 1250 µl pipette tip was used applying two different pressures (50 g and 150 g). Application of the correct pressure was monitored by performing the scratching procedure on a precision lab scale. To achieve straight scratches a ruler was used as guiding device.

Scratching with the magnet device was performed as described above.

Linear cell culture inserts (80209, Ibidi™) for physical exclusion of cells at a defined distance (500 µm +/−100 µm) were used according to the manufacturer´s instructions. In brief, the inserts were placed in the centre of each well. 15.000–20.000 cells were seeded into each of the two insert chambers to achieve confluence the following day. To create the gap, the insert was removed using a sterile forceps.

Independent of the scratching technique the cells were washed twice with PBS following gap creation prior to adding new culture medium.

Comparison of the cell free gaps (scratches). The cell free gaps were imaged in phase contrast using the EVOS FL Auto imaging system with a 4x fluorite objective (ThermoFisher Scientific, AMEP4680). To ensure that pictures were always taken at the same position, the "reuse settings" function was applied, which allows resuming of previously used coordinates. Pictures were taken at 0, 4, 8, 12 hours and then every 12 hours until full gap closure (Suppl. Fig. 2). The size of the gaps was measured at all time points using the MRI Wound healing tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool) in ImageJ (https://imagej.nih.gov/ij/, version 2.0.0-rc-43/1.50e). The micrographs taken at 0 hours were additionally analysed for straightness of the wound margins and for homogeneity of the gap width (Suppl. Fig. 1).

Homogeneity of the gap width. Gap homogeneity was evaluated by the mean, standard deviation (SD), and coefficient of variation (CV = SD/mean) of 10 measurements of the gap width performed at equal distances of 128 pixels (Suppl. Fig. 1) using ImageJ (version 2.0.0-rc—43/1.50e, Straight Hand Line Tool, NIH, USA) to measure the distance between the upper and lower gap margins.

Straightness of the gap margins. The margins of the gaps were evaluated relative to an ideal (straight) line, parallel to the horizontal axis of the picture, with a length of 1280 pixel (Suppl. Fig. 1). The length of the gap was analysed with the Free Hand Line Tool on ImageJ version 2.0.0-rc—43/1.50e. Straightness of the gap margins, defined as the length of the edge of the gap divided by the length of a straight line was calculated as the average of the ratio between the length of each gap margin (upper and lower) and the ideal line (Straightness = (length of upper gap margin + length of bottom gap margin)/ (2*length of ideal line)).

Gap closure rate. Gap area was measured at successive time-points (0, 4, 8, 12, 24, 36, 48, 60, 72, 84 hours) using the MRI Wound healing Tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool) on ImageJ version 2.0.0-rc—43/1.50e. The gap closure rate, defined as the slope of the regression of the gap area [mm^2] depending on time, was recorded during the period of linear closure between 24 hours (after the initial lag period) and 48 hours (before the gap closed in any of the samples, Suppl. Fig. 1).

Life-dead staining and analysis. To distinguish between life and dead cells a vital fluorescent double staining was performed using Fluoresceindiacetat (FDA, C-7521, Sigma-Aldrich) and Propidiumiodid (PI, P4170, Sigma-Aldrich) following manufacturer's instructions. Based on the Esterase dependent transformation of non-fluorescent FDA into its fluorescent metabolite fluorescein, cells stained in green indicate life cells. PI was used as a counterstaining, to stain cell nuclei of dead cells. Subsequently, to evaluate the direct influence of the wounding method (scratching versus cell exclusion method) on the number of dead cells, FDA/PI stained cultures (n = 279, thereof n = 140 chondrocytes and 139 tenocytes) were imaged using the EVOS FL Auto imaging system (GFP EVOS LED light cube,AMEP4651, Emission 510/42 nm, Excitation 470/22 nm and Texas Red EVOS LED light cube, AMEP4655, Emission 628/32 nm, Excitation 585/29 nm) at 0 h, 8 h and 24 h after "scratching"and analysed using ImageJ. To distinguish between the overall occurrence of dead cells in the culture and cells, which died in direct consequence to wounding, three regions of interest (ROI) were defined along each scratch margin (lower and upper margin) for each micrograph (Fig. 4): (1) ROI (A) extends 25 µm from the scratch margin into the cell free area of the scratch; (2) ROI (B) extends 25 µm from the scratch margin into the remaining cell monolayer and is the region of main interest as it contains cells which died in direct consequence to scratching; and (3) ROI (C) extends from 25 µm to 50 µm into the remaining cell monolayer. The number of dead cells per ROI was counted manually and the dead cells per ROI of the lower (A1, B1, C1) and upper (A2, B2, C2) scratch margins were added to a total count per ROI. To interpret the influence of the wounding method on cell death and calculate the true amount of "scratch" related cell death per picture (relative number of dead cells in ROI(B)) the following formula was used $\frac{(B1+B2)-(C1+C2)}{(A1+A2)}$. Negative values in the raw data resulting from a higher number of dead cells in ROI(C) than ROI(B) were set to zero.

AFM. In order to assess potential damage to the plate surface due to scratching, the surface reliefs of wells scratched with the magnet scratch method and the pipette with 150-gram pressure were measured using atomic force microscopy and compared to a non-scratched well and a positive control (scratch performed using a #3

scalpel handle with 50 g pressure). Scratching was performed in wells containing PBS. After scratching, the wells were washed twice with ultrapure water to remove any precipitates of the PBS. The surface morphology was evaluated by Atomic Force Microscopy, using a Nanowizard 4 system from JPK (JPK Instruments AG, Berlin, Germany). The AC mode was used, with PPP-NCHAuD cantilevers (330 kHz nominal resonance frequency, 42 N/m force constant and 125microns length). Scan areas of $8 \times 100\ \mu m^2$ were imaged at 26×256 pixel resolution with the scanning direction perpendicular to the scratch direction. Consecutive scans were performed to achieve a scanned area with a final dimension of $8 \times 2000\ \mu m^2$. Three areas per scratch method were imaged. Images were analysed with JPK software and 3D plot using the 3D surface plot in ImageJ.

Statistical analysis. Target variables are: straightness of the gap margins, defined as the length of the edge of the gap divided by the length of an ideal straight line; mean and standard deviation of the gap width measured at ten points along the gap; and the gap area in mm^2. Gap area was measured at successive time-points and the gap closure rate (slope of the regression of gap area in mm^2 depending on time) was recorded at the period of linear closure between 24 hours (after the initial lag period) and 48 hours (before the gap closed in any of the samples). Descriptive statistics, means, standard deviations and coefficients of variation were calculated for the target variables (straightness of the gap margins, homogeneity of the gap width, gap closure rate). With all these target variables, unifactorial ANOVAs were calculated separately with method as factor for each cell type. Both cell types were also combined in analyses, where only for the target variable "slope" cell-type was entered as an additional factor, since growth of chondrocytes and tenocytes differs. We additionally performed more complicated multifactorial ANOVAs with factors: method, operator, biological replicate, and method*operator, for chondrocytes and tenocytes separately. Both cell types were also combined in analyses, where cell-type was entered as an additional factor. With the more complicated ANOVA models, qualitatively and quantitatively similar results were obtained.

Ethical approval and informed consent. Tissue collection to obtain the cells used in this study has been performed according to the "Good Scientific Practice and Ethics in Science and Research" regulation implemented at the University of Veterinary Medicine Vienna. The animal owner's consent to collect and analyse the samples and to publish resulting data was obtained according to the standard procedure and approved by the ethics and animal welfare committee of the University of Veterinary Medicine Vienna.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Acknowledgements

We would like to acknowledge Dr. Simone Gabner and Dr. Alexander Tichy for their support.

Author Contributions

M.F.: study design, data acquisition and interpretation, manuscript preparation. T.B.: data acquisition and interpretation. C.V.: data analysis and interpretation, manuscript preparation. N.D.-M.: data acquisition and interpretation. J.S.: data acquisition. F.J.: study conception and design, data analysis and interpretation, manuscript preparation. I.R.: study conception and design, data acquisition and interpretation, manuscript preparation.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-48930-7.

Competing Interests: The authors declare no competing interests.

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Species variations in tenocytes' response to inflammation require careful selection of animal models for tendon research

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For research on tendon injury, many different animal models are utilized; however, the extent to which these species simulate the clinical condition and disease pathophysiology has not yet been critically evaluated. Considering the importance of inflammation in tendon disease, this study compared the cellular and molecular features of inflammation in tenocytes of humans and four common model species (mouse, rat, sheep, and horse). While mouse and rat tenocytes most closely equalled human tenocytes' low proliferation capacity and the negligible effect of inflammation on proliferation, the wound closure speed of humans was best approximated by rats and horses. The overall gene expression of human tenocytes was most similar to mice under healthy, to horses under transient and to sheep under constant inflammatory conditions. Humans were best matched by mice and horses in their tendon marker and collagen expression, by horses in extracellular matrix remodelling genes, and by rats in inflammatory mediators. As no single animal model perfectly replicates the clinical condition and sufficiently emulates human tenocytes, fit-for-purpose selection of the model species for each specific research question and combination of data from multiple species will be essential to optimize translational predictive validity.

Animal models are cornerstones of biomedical and translational medicine research. They are used when it is unethical or impractical to study the target species to explore basic pathophysiological mechanisms, to evaluate safety and efficacy of new treatment approaches, and to decide whether novel therapeutic candidates warrant the economic and moral costs of clinical development[1–7]. For 90% of new treatment strategies, however, translation from basic science to the clinic fails, mainly because clinical trials show them to be inefficient (52%) or unsafe (24%) during phases II and III[4,5,8]. Such translational failures cost animal lives, strain clinical trial volunteers, and burden biomedical research, the pharmaceutical industry and health care systems. So far, attempts to optimize translational success have mainly focused on internal validity flaws such as methodological shortcomings in animal and clinical trials, publication bias, or overoptimistic conclusions about efficacy. Yet another key factor, the external validity, or generalizability, of animal models has received little attention[4,5,8–13]. Common problems of external validity include species differences in disease pathophysiology, common confounding comorbidities and the selection of outcome measures[5]. An animal model should sufficiently emulate aetiology, pathophysiology, symptomatology and response to therapeutic interventions of the target species to allow extrapolation[5,11]. As no single animal model perfectly recapitulates the clinical realm, fit-for-purpose validation and selection of the most appropriate model species is essential[10–13]. Unfortunately, for musculoskeletal disorders, such as tendinopathy, in depth validation studies of animal models beyond structural and biomechanical similarities are largely lacking.

Tendinopathy, a disabling overuse injury, is the most common musculoskeletal complaint for which patients seek medical attention[14]. It is prevalent in both occupational and athletic settings, afflicting 25% of the adult population, and accounting for 30–50% of all sport injuries[15–18]. Major tendons experiencing high loads are most commonly affected, especially the weight-bearing and energy-storing Achilles tendon, which routinely experiences loads of up to 12.5 times the weight of the individual[6,19]. Many intrinsic and extrinsic factors, including age, body weight and physical loading, influence the aetiopathogenesis of tendinopathy. Overload and repetitive strain lead to accumulation of microdamage and concurrent inflammatory, dysregulated reparative

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and degenerative processes, causing clinical symptoms, e.g., activity-related pain, focal tendon tenderness and swelling, and functional limitations. Overt clinical symptoms such as pain are preceded by tendinous matrix remodelling, an inflammatory cellular process mediated in part by metalloproteinase enzymes[20,21]. Due to its low cellularity, vascularity and metabolic rate, a tendon's response to injury is inefficient, requiring lengthy periods of recuperation and often resulting in a fibrovascular scar. Scar tissue has significantly inferior biomechanical properties than the original tendon tissue and is prone to re-injury[16,22–24]. Current treatment options are mostly palliative and fail to restore the functional properties of injured tendons[16,22–24]. Tendinopathy thus has a significant adverse impact on quality of life and costs individuals and the society an estimated annual expense of $30 billion[25,26]. This is driving research efforts into unravelling the molecular mechanisms of tendinopathy and developing targeted regenerative therapies. Of particular interest in this context are the cellular and molecular processes orchestrating inflammation in tendinopathy and the mechanisms governing the development of chronic inflammation that fails to resolve in persistently symptomatic patients[27].

Tendon injury induces a local inflammatory response, characterized by immune cell infiltration and the expression of pro-inflammatory mediators, which in turn reduce collagen production and induce vasodilation, angiogenesis, and matrix metalloproteinase (MMPs) expression[28–31]. Furthermore, the inflammatory milieu can modify tenocyte physiology by increasing metabolic activity and inducing an activated, proinflammatory phenotype with inflammation memory and the capacity for endogenous production of cytokines such as TNF-α, IL-1β, IL-6, IL-10, VEGF and TGF-β[29,30,32]. While the initial inflammatory response is essential to start the healing process, sustained inflammatory conditions contribute to dysregulated matrix remodelling and fibrovascular scarring during healing[18,31]. Chronic inflammation thus drives tendon degeneration before tearing or any other clinical signs of tendinopathy, impairs healing after injury and promotes the development of tendinopathy[14].

While human tendon tissue can typically be procured only from individuals with advanced pathology, animal models provide the opportunity to obtain tissue during all stages of tendinopathy to study organ, cellular and molecular changes over the entire course of the disease. In animal models, consistent and repeatable injuries can be induced, evaluated and treated, while controlling for potential confounding influences[7,33,34]. Since no species has been established as the gold standard for tendinopathy research, many induced and spontaneous animal models ranging from small rodents (mice, rats) to large animals (sheep, horses) are utilized[19,35–43]. While the biomechanical properties of the various species are well established, their ability to simulate the pathophysiology of human tendon disease, including the molecular behaviour of key genes and pathways, has not been critically evaluated yet and detailed analyses of species- specific differences in cytokine expression and regulation as well as of tenocytes susceptibility to cytokines are still lacking[44].

Considering the importance of inflammation in tendon disease[29,33,45,46], this study compares the cellular and molecular features of inflammation in tenocytes of humans and four common model species (mouse, rat, sheep, and horse) to aid in the evidence-based selection of fit-for-purpose translational animal models for tendon research. Mice and rats are included due to their prevalent use as laboratory animals and availability of species-specific molecular tools[6,47]. Larger animals are used increasingly as translational models due to their more comparable tendon dimensions and biomechanics[23,48–50]. Horses present an attractive model of human tendinopathy since their superficial digital flexor tendon is a weight-bearing and energy-storing tendon analogous to the human Achilles tendon, which is similarly prone to naturally occuring tendon disease with high recurrence rates[9,51,52]. Furthermore equine ageing proceeds similarly to humans[39,51,52]. Sheep are included because features of clinical tendinopathy of horses could be emulated also in ovine induced models[51,53–57]. In particular, the ovine intra-synovial tendon lesion model mimics the clinical intra-synovial tendon disease of humans and horses more accurately than small animal extra-synovial models, e.g., with respect to histology and gene expression, to similarities in the biomechanical environment and to failure of lesions to heal[51,53–57].

Results

Morphology. Tenocytes from all five species shared common characteristics with a fusiform appearance, adherence to the flask and similar dimensions (Fig. 1): human tenocytes measured 177.8 ± 40.1 µm (mean ± s.d.) in length and 20.2 ± 4.4 µm in width, mouse 163.7 µm (± 23.6) × 19.4 µm (± 3.3), rat 182.5 (± 26.6) × 24.2 (± 8.0), sheep 206.3 (± 45.5) × 28.9 (± 8.5) and horse 193.2 (± 8.4) × 15.4 (± 1.4). In high confluency, tenocytes from human, sheep and horse showed similar morphology, creating cell bundles arranged in a storiform pattern (Fig. 1A), while tenocytes from mouse and rat had a more scattered appearance with a random orientation.

Proliferation assay. Proliferation, migration and gene expression of tenocytes of all five species were compared under standard culture conditions (healthy control) as well as under transient (24 h) and constant exposure to inflammatory stimuli (Fig. 2). Under healthy conditions (Fig. 3, Table 1), equine tenocytes had the highest proliferation rates while murine cells had the lowest. Human tenocytes exhibited the second lowest proliferation capacity with an inability to double the cell amount over the 48 h observation period. Sheep and rat tenocytes were in the middle. From Fig. 3, it can be seen that slopes are quite variable among species, but within species variation is low. Therefore, the slopes of tendon cells of all four model species are significantly different from those of humans ($p < 0.001$; to correct for multiple testing using Bonferroni with four comparisons, the nominal significance levels, 0.05, 0.01, and 0.001, are set to the corrected levels: 0.0125, 0.002, 0.0002, respectively), even for only three biological replicates per species. Under constant exposure to inflammatory stimulation (10 ng/ml IL1β and 10 ng/ml TNFα), the proliferation of sheep tenocytes decreased significantly and fell to about human levels, i.e., the difference in the proliferation slopes between healthy and constantly inflamed sheep decreased significantly ($p < 0.01$). All other differences in slopes between healthy, constantly and transiently (only 24 h inflammatory stimulation) inflamed conditions were not significant.

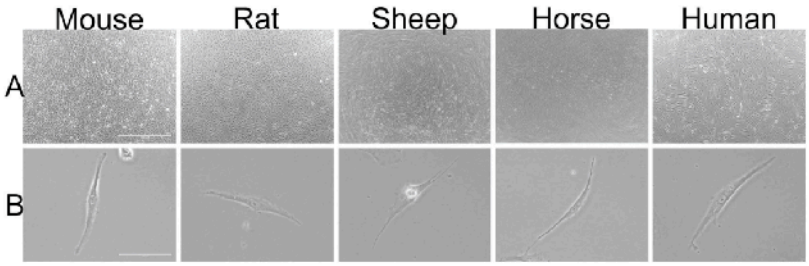

Figure 1. Micrographs of tenocytes derived from the Achilles tendon in the mouse, rat, sheep and human or the superficial digital flexor tendon in the horse. (**A**) shows the tenocytes at a magnification of 40x (scale bar: 1000 µm), while (**B**) was taken at a 400× magnification (scale bar: 100 µm).

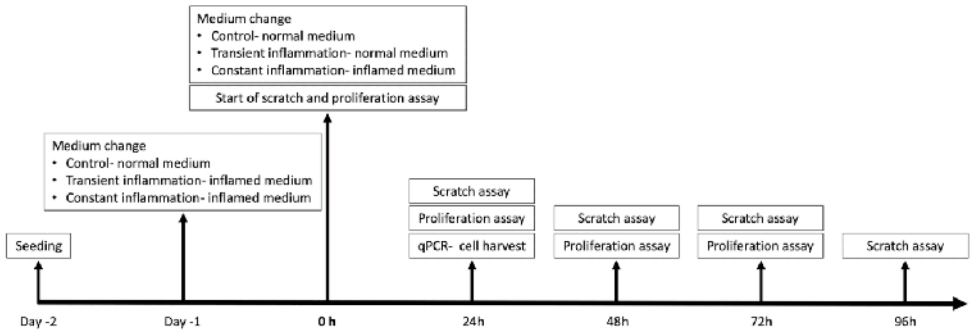

Figure 2. Study timeline detailing the experimental protocol. Gene expression, proliferation and migration of tenocytes of all five species (human, mouse, rat, sheep, horse) were compared under standard culture conditions (healthy control) as well as under transient (24 h) and constant exposure to inflammatory stimuli (10 ng/ml IL1β and 10 ng/ml TNFα). After 24-h exposure to inflammation, the transient inflammation group received fresh culture medium, while for the constant inflammation group fresh medium was again supplemented with inflammatory factors.

Wound healing (scratch) assay. Gap closure was significantly different (in all cases $p < 0.001$) between all conditions, fastest for healthy and slowest for constantly inflamed cells of all species (Fig. 3, Table 1). Murine tenocytes showed the slowest wound healing for all conditions. There was no statistically significant difference in migration speed between healthy and transient inflammatory conditions in tenocytes of any species and between healthy and continuously inflamed conditions only for mouse and rat cells ($p < 0.05$).

Under healthy conditions, gap closure of tenocytes from all species except rats was significantly different (in all cases $p < 0.003$) from humans with cells from rats showing the fastest wound healing (gap closure at 48 h mean 90.08% ± 8.01% s.d.), closely followed by humans (gap closure at 48 h mean 86.25% ± 2.47% s.d.) and cells from mice the slowest (gap closure at 48 h mean 51.49% ± 10.23% s.d.). Under transient inflammation rat tenocytes again were fastest (gap closure at 48 h mean 64.81% ± 7.84% s.d.), with ovine (gap closure at 48 h mean 58% ± 11.17% s.d.), human (gap closure at 48 h mean 56.06% ± 25.27% s.d.) and equine tenocytes (gap closure at 48 h mean 55.71% ± 9.6% s.d.) following with similar wound healing rates, while murine tendon cells again were slowest (gap closure at 48 h mean 29.46% ± 4.36% s.d.). Under constant inflammation, equine tenocytes were fastest (gap closure at 48 h mean 35.23% ± 8.98% s.d.) followed closely by human tendon cells (gap closure at 48 h mean 34.99% ± 19.12% s.d.). The change in migration speed compared to healthy was significantly different from human tenocytes for ovine tendon cells under transient and equine and murine tenocytes under constant inflammation (Table 1).

Quantitative PCR. The species show variable approximations of human expression levels among functional gene groups and conditions (Tables 2 and 3, Figs. 4 and 5, suppl. Figure 1 and 2, suppl. table 1)[58]. A univariate Analysis of Variance (ANOVA) demonstrated significant differences between each species and humans in many genes relevant for tendon function and inflammatory response (Table 2). Remarkably, healthy tenocytes of all four species show significant differences to humans in their expression of Col1, the main tendon matrix

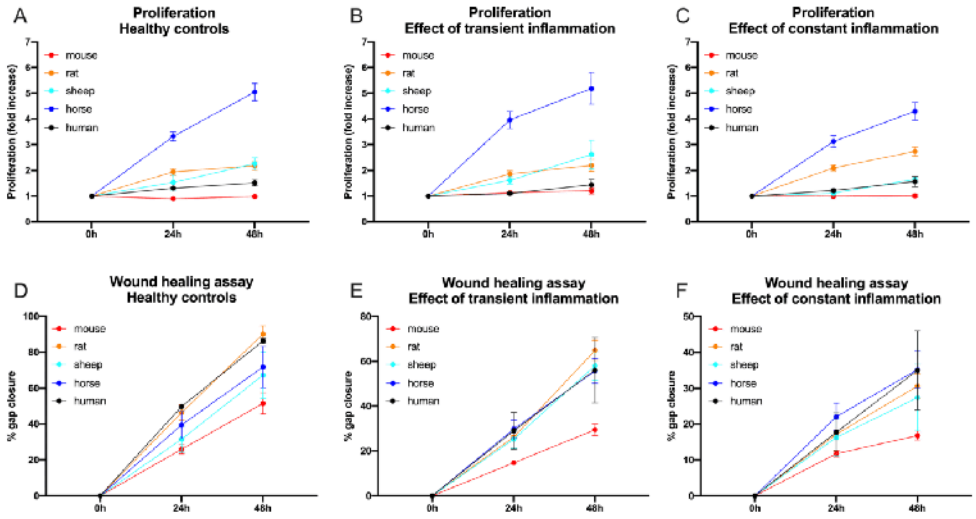

Figure 3. The proliferation capacity (**A–C**) of tenocytes from 5 different mammalian species (mouse, rat, sheep, horse, and human) under healthy (Ctr/A), transient (TI/B) and constant (CI/C) inflammatory condition is illustrated as fold increase over the course of 2 days (indicated as mean + SEM calculated from three biological replicates). For pairwise comparisons and significance values see Table 1. A wound healing assay (**D–F**) was used to determine the migratory capacity of tenocytes from five different mammalian species under healthy (**D**), transient (**E**) and constant (**F**) inflammatory conditions (indicated as mean ± SEM calculated from three biological replicates). For pairwise comparisons and significance values see Table 1.

		Healthy			Transient inflammation			Constant inflammation		
Species		***Prolif. slope***	***Std. error***	***p-value***	***slope: diff. to healthy***	***Std. error***	***p-value***	***slope: diff. to healthy***	***Std. error***	***p-value***
Proliferation	Human	0.1027	0.0168	1.75e-09 ***	−0.0167	0.0237	0.4801	0.0089	0.0237	0.7074
		Slope: diff. to human	***St. error***	***p-value***	***slope: diff. to human***	***St. error***	***p-value***	***slope: diff. to human***	***St. error***	***p-value***
	Mouse	−0.1250	0.0237	1.95e-07 ***	0.0508	0.0335	0.1301	−0.0301	0.0335	0.3691
	Rat	0.0933	0.0237	9.35e-05 ***	−0.0038	0.0335	0.9103	0.0420	0.0335	0.2103
	Sheep	0.1169	0.0237	1.10e-06 ***	0.0585	0.0335	0.0815	−0.0878	0.0335	0.00900 *
	Horse	0.3000	0.0237	<2e-16 ***	0.0233	0.0335	0.4868	−0.0640	0.0335	0.0568
		Migration slope	***Std. Error***	***p-value***	***slope: diff. to healthy***	***St. error***	***p-value***	***slope: diff. to healthy***	***St. error***	***p-value***
Migration	Human	−0.0792257	0.0032	<2e-16 ***	0.0277	0.0045	2.76e-09 ***	0.0475	0.0045	<2e-16 ***
		Slope: diff. to human	***Std. Error***	***p-value***	***slope: diff. to human***	***St. error***	***p-value***	***slope: diff. to human***	***St. error***	***p-value***
	Mouse	0.0311503	0.0045	3.07e-11 ***	−0.0075	0.0064	0.2452	−0.0156	0.0061	0.0151
	Rat	0.0001854	0.0046	0.9155	−0.0063	0.0064	0.3287	0.0035	0.0065	0.5938
	Sheep	0.0185550	0.0045	5.46e−05 ***	−0.0188	0.0064	0.00374 *	−0.0112	0.0064	0.0811
	Horse	0.0132563	0.0045	0.00375 **	−0.0123	0.0064	0.0567	−0.0140	0.0064	0.0302

Table 1. The first column corresponds to the healthy situation, the second and third to transiently and constantly inflamed, respectively. First rows (human) correspond to tests for non-zero slopes of the proliferation and migration curves in humans, i.e., either non-zero proliferation or non-zero gap closure in humans. Second to fifth rows (different animal species) correspond to tests of differences of the animal models to humans in the slopes of the proliferation and migration curves of tenocytes. Calculations used an ANCOVA. Means, standard errors and p-values are reported. To correct for multiple testing with four comparisons using Bonferroni, the nominal significance levels (0.05, 0.01, and 0.001) are set to the corrected levels (0.0125, 0.002, 0.0002, respectively). P-values are marked with stars from * (significant) to *** (highly significant) using this correction.

			SCX	TNC	TNM	COL1	COL3	COL5	MMP1	MMP3	MMP13	IL6	COX2	NFKB	P53	ALP	EAK
Healthy	Mouse	Diff	2.65	−0.74	−1.05	**−3.03**	2.54	−2.17	−9.34	9.45	**8.50**	**4.65**	**9.40**	−0.20	−0.30	**3.14**	−0.25
		Adj p	0.230	0.811	0.951	**0.038**	0.122	0.608	0.064	**0.002**	**0.000**	**0.005**	**0.000**	0.999	0.961	**0.032**	0.995
	Rat	Diff	**5.28**	**2.84**	−1.37	**3.95**	**3.80**	4.62	−2.97	**10.24**	**5.24**	0.54	**11.30**	3.00	**4.47**	1.85	**4.72**
		Adj p	**0.008**	**0.014**	0.882	**0.008**	**0.015**	0.067	0.851	**0.001**	**0.013**	0.978	**0.000**	0.053	**0.000**	0.287	**0.000**
	Sheep	Diff	2.39	**3.79**	−3.52	**−7.91**	−0.50	0.41	−4.71	**9.12**	**9.47**	−0.16	**13.61**	−0.72	−0.36	−0.14	**3.95**
		Adj p	0.313	**0.002**	0.201	**0.000**	0.981	0.998	0.539	**0.003**	**0.000**	1.000	**0.000**	0.931	0.927	1.000	**0.001**
	Horse	Diff	1.49	−0.24	−3.11	**−4.72**	0.41	1.71	−2.93	1.94	**5.37**	**−3.67**	**15.65**	**3.79**	**3.52**	−1.11	2.11
		Adj p	0.711	0.996	0.296	**0.002**	0.991	0.780	0.857	0.793	**0.012**	**0.022**	**0.000**	**0.014**	**0.000**	0.720	0.055
Transient inflammation	Mouse	Diff	−1.22	−0.69	**−2.59**	−2.62	**−3.06**	**−3.07**	1.23	**−5.08**	**−4.96**	**−4.02**	**−4.15**	−1.24	−0.59	0.46	−0.46
		Adj p	0.515	0.870	**0.048**	0.177	**0.017**	**0.010**	0.984	**0.011**	0.001	**0.001**	**0.000**	0.180	0.803	0.990	0.963
	Rat	Diff	**−2.94**	−1.42	**−2.85**	−2.93	**−4.43**	**−3.57**	−0.66	−3.22	−1.56	−0.98	**−4.84**	−0.01	−0.34	−1.54	−1.48
		Adj p	**0.019**	0.342	**0.029**	0.114	**0.001**	**0.004**	0.999	0.124	0.412	0.537	**0.000**	1.000	0.967	0.563	0.299
	Sheep	Diff	−0.59	−0.20	0.59	−0.12	−1.84	−1.49	0.39	**−6.54**	**−4.19**	**−2.83**	**−6.20**	−0.52	0.16	1.38	**1.31**
		Adj p	0.930	0.998	0.937	1.000	0.189	0.291	1.000	**0.002**	**0.004**	**0.007**	**0.000**	0.841	0.998	0.651	0.404
	Horse	Diff	**−0.77**	1.44	−0.34	1.40	−0.36	−0.95	3.97	−0.32	0.15	**3.25**	**−6.63**	−0.16	1.38	2.22	0.98
		Adj p	0.840	0.330	0.992	0.691	0.989	0.671	0.498	0.999	1.000	**0.003**	**0.000**	0.997	0.152	0.249	0.658
Constant inflammation	Mouse	Diff	−1.23	−0.51	−3.33	−1.89	**−3.30**	−2.52	1.64	**−4.82**	−3.41	**−4.54**	−3.96	−1.33	−0.09	2.83	0.22
		Adj p	0.911	0.949	0.166	0.361	**0.003**	0.501	0.977	**0.014**	0.209	**0.021**	0.094	0.586	1.000	0.093	0.998
	Rat	Diff	−3.27	−1.76	**−4.86**	**−3.80**	**−7.16**	**−5.29**	4.75	−1.96	3.26	**1.13**	−1.71	0.13	0.18	−1.09	−1.36
		Adj p	0.238	0.177	**0.028**	**0.020**	**0.000**	**0.039**	0.509	0.490	0.241	0.866	0.727	**1.000**	0.999	0.797	**0.412**
	Sheep	Diff	**−2.01**	−0.63	**−1.41**	0.56	−1.81	−2.94	1.51	**−6.73**	−2.93	−0.42	**−7.68**	−0.18	0.19	1.24	**1.48**
		Adj p	0.653	0.900	0.822	0.975	0.111	0.366	0.983	**0.001**	0.328	0.996	**0.002**	1.000	0.999	0.719	0.333
	Horse	Diff	3.10	2.28	**1.74**	**1.37**	0.39	−2.04	3.59	0.24	1.49	**5.53**	**−8.36**	0.63	0.95	3.18	0.58
		Adj p	0.280	0.059	0.693	0.637	0.972	0.679	0.733	1.000	0.839	**0.006**	**0.001**	0.949	0.678	0.054	0.932

Table 2. Mean difference to human and p-values of the gene expression calculated with ANOVA of healthy, transiently inflamed and constantly inflamed tenocytes of the four animal model species. Significant p-values (Tukey HSD correction, $p<0.05$) and the matching mean differences in gene expression to humans are indicated in bold.

	Mouse	Rat	Sheep	Horse
Mahalanobis distances all genes per condition				
Healthy	20.01	27.44	24.78	23.05
Transient Inflammation	13.49	18.66	10.62	9.59
Constant Inflammation	10.50	17.45	8.94	12.97
Mahalanobis distances all conditions per group of genes				
SCX, TNC, TNMD	5.45	12.21	10.54	6.88
COL1, COL3, COL5	8.28	13.73	12.88	8.45
MMP1, MMP3, MMP13	8.53	11.27	8.60	4.65
COX2, IL6,	15.82	14.23	18.51	17.77

Table 3. Mahalanobis distances of the four model species to humans for all genes combined under healthy, transiently and constantly inflamed conditions as well as for the different functional gene groups: tenogenic markers, collagens, MMPS and inflammatory mediators.

component, of collagenase MMP13 and of the key inflammatory mediator COX2. Similarly, significant differences from humans in the COX2 expression of transiently inflamed tenocytes are evident for all four species and in IL6 expression for all species except rats. In contrast, no significant differences to humans were seen in MMP1 expression for any species and the osteogenic marker ALP was only significantly different in healthy murine tenocytes. NFkB expression exhibited a significant difference only in healthy horse tendon cells and p53 in healthy horse and rat tenocytes.

To condense the information from the univariate ANOVA results to overall measures of similarity between the different animals and humans we calculated the multivariate Mahalanobis distances of the four species to humans. The Mahalanobis distance is a non-dimensional measure of dissimilarity, where between group distances are weighted by the inverses of within group variability, much like the test statistic of a t-test for one variable. Similar to its use in graphically detecting outliers in multiple dimensions, we use it to show multivariate dissimilarity in gene expression among species. In supplementary Fig. 1, we summarize conditions within a gene.

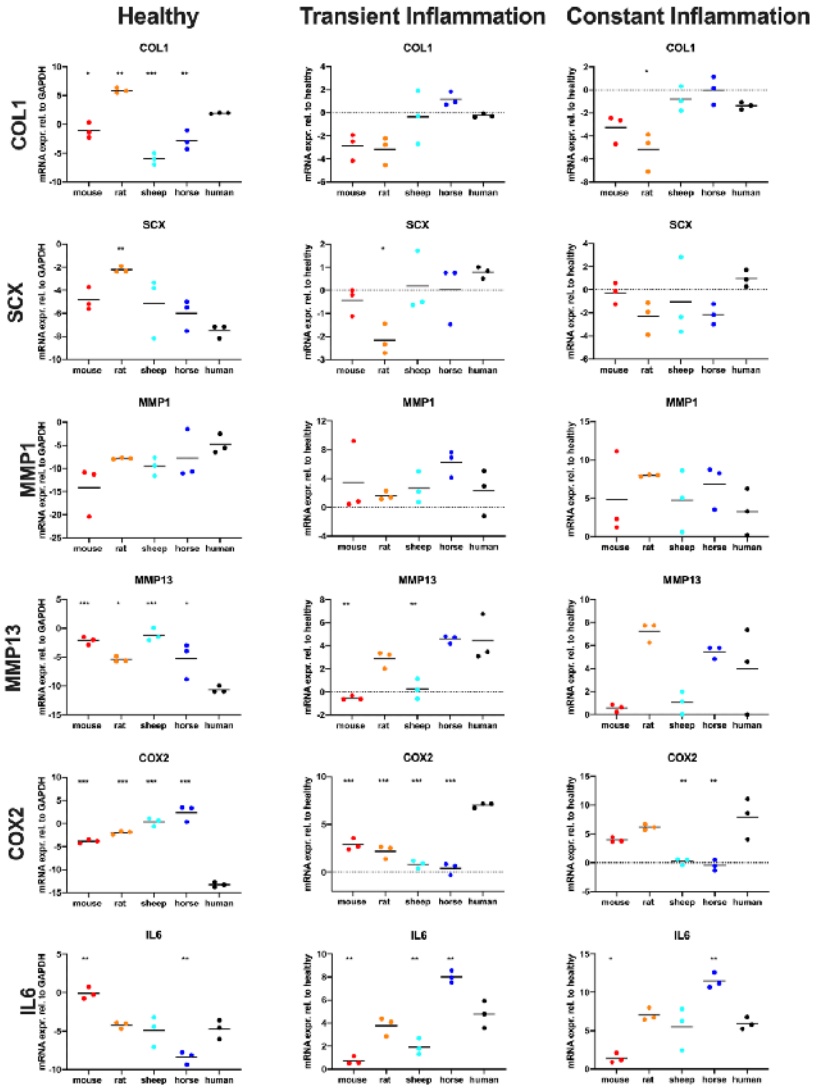

Figure 4. Scatter dot plots showing COL1, SCX, MMP1, MMP13, COX2 and IL6 gene expression (presented as log2) of healthy tenocytes and tenocytes exposed to inflammatory stimuli for 24 h (transient inflammation) or continuously (constant inflammation) in different species (the black lines indicate the respective means). Gene expression in the inflammatory conditions is shown relative to the healthy tenocytes. Each dot represents a different biological replicate. Differences were evaluated using ANOVA with Tukey HSD test, $*p<0.05$; $**p<0.01$; $***p<0.001$.

From Fig. 4, it is evident that Cox2 is rather variable among species (especially human healthy cells differ from all animals, least so from mice) while showing relatively little variation within species under all conditions. Hence the Mahalanobis distances to humans are comparatively large with mice being closest to humans (suppl. Figure 1).

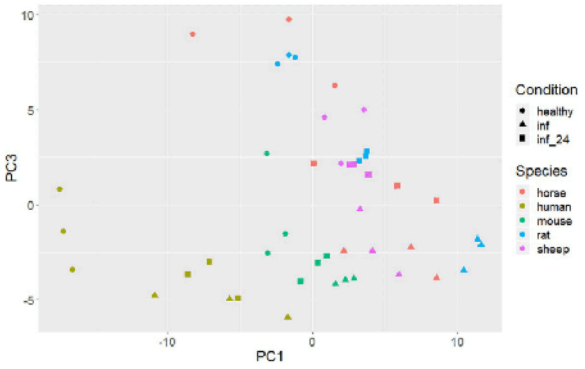

Figure 5. Plot of PC1 (explaining 36% of the variance) vs. PC3 (explaining 14% of the variance) of a Principal Component Analysis of gene expression values. Species are colour coded, conditions (healthy, transiently inflamed, and continuously inflamed) are differentiated by symbols. Each dot represents a different biological replicate.

The second immune gene, Il6, shows also relatively little within species variation (Fig. 4), but comparatively less between species variation; especially rats and sheep are similar to humans, while mice and horses are slightly further away (Fig. 4, suppl. Figure 1). The Mahalanobis distances (Table 3) of the immune genes are relatively large compared to the other gene combinations due to low within species variability. The overall distance of the immune genes is a compromise between the two genes, such that rats are slightly more similar to humans than mice, with sheep and horses further away. When different functional groups of genes are analysed, variable Mahalanobis distances of the four model species from humans are found (Table 3). While mice and horses are closest to humans in their tendon marker and collagen expression, horses appear to be by far the best model for MMPs (Table 3). For the overall gene expression of healthy tenocytes, Mahalanobis distances from human tenocytes are large and similar among species, with mouse tendon cells appearing least and rat tenocytes most distant from human. For transient inflammation, Mahalanobis distances to humans are generally lower, but the spread of differences widens, with horses, sheep and mice relatively close and rats clearly furthest. For constant inflammation, the pattern is qualitatively similar to that under transient inflammation. Overall, the pattern of multivariate dissimilarity of species varies widely and unpredictably among species pairs with no single species most similar to humans.

Principal Component Analysis (PCA) is an exploratory technique for reducing the complexity of data. We used expression data from all genes and plot the results for the different species x condition combinations (Fig. 5, suppl. Figure 2). The plot of PC1 vs. PC3 is easier to interpret than of other combinations of PCs. (Fig. 5). With the exception of humans and mice, species are generally not well-separated. Within all species, the different conditions are spread along an oblique line with healthy to the upper left and inflamed to the lower right. Within mice, healthy is also separated from inflamed, while in sheep the pattern is less clear. Pairwise plots of other PCs show similar but less clear patterns (suppl. Figure 2).

Discussion

Choice of the most appropriate animal model is the most essential and challenging element of animal-based research, and also an important aspect of the 3Rs (i.e., replace, reduce, refine) to ensure the best use of animals[59–61]. Unfortunately, the choice of animal models frequently is based more on convention, financial and practical considerations, such as housing and husbandry requirements or the availability of reagents and biochemical tests, than compelling scientific evidence of the fit to human diseases and clinical contexts[4,5,7,62–64]. The lack of formal requirements for animal models is due to the traditional assumption that genetic homology derived from a common evolutionary origin also implies functional similarities of gene regulation, signalling pathways and developmental systems between species (the "unity in diversity" concept)[7]. Species may, however, differ in critical aspects and rarely have assumed similarities been empirically demonstrated[7]. The diversity of human patients and symptoms is thus unlikely fully represented in highly inbred rodents[65,66]. Even humanized models, which have contributed significantly to research by facilitating functional studies in vivo, cannot replicate the complexity of human disease[67].

Both the European Medicines Agency (EMA) and the USA Federal Food and Drug Administration require the use of fit-for-purpose animal models to evaluate efficacy, durability, dose–response, degradation and safety of new therapeutics for market approval. Recently, these regulatory authorities published guidelines identifying requirements to demonstrate the relevance of animal models for investigational new product testing by cross-species comparison of the structural homology of the target, its distribution, signal transduction pathways and pharmacodynamics[68,69]. Furthermore, several voluntary initiatives have established criteria to encourage the

evidence-based selection of animal models for stroke and schizophrenia[11,70,71]. To this end, both the model species and disease-induction protocols, need to be validated by comparing the animal model with the gold standard or the target species[11]. As no gold standard for tendon research is available, this study compared tenocyte morphology, proliferation rate, wound healing speed, and gene expression of two small animals (mouse and rat) and two large animals (sheep and horse) to human under healthy as well as "diseased" (transiently and continuously inflamed) conditions to determine similarities and differences among species. It can serve as the foundation for a rational, evidence-based choice of optimal animal models for specific aspects of human tendinopathy.

Tendon injury induces a local inflammatory response, which initiates the healing process. Tendon healing occurs in three chronologic phases: inflammation (0–7 days), proliferation (1–6 weeks), and remodeling (6 weeks – 6 months). While these stages overlap, they are characterized by temporally and functionally distinct cytokine profiles and cellular processes[72]. The initial inflammatory phase is characterized by influx of inflammatory cells, which release chemotactic and proinflammatory cytokines and growth factors that lead to recruitment and proliferation of macrophages and resident tendon fibroblasts[44,72–83]. In addition, tenocytes produce also several endogenous cytokines and growth factors which contribute to the healing process in an auto- and paracrine manner[44,75]. During the proliferative stage tenocytes proliferate and produce an immature neomatrix with a predominance of type III rather than type I collagen[44,72,80–82,84]. Lastly, in the course of the remodeling phase, the cellularity decreases, matrix synthesis is reduced and collagen fibrils and tenocytes align linearly with the direction of tension[44,72–79,81–83]. However, in both man and horse suffering from naturally occurring tendon disease, the normal architecture, composition and function of the tendon are never completely restored, predisposing them to recurring injury and tendinopathy[44,72–79,81–85].

Given the importance of inflammation in tendon injury and repair, with pro-inflammatory cytokines acting as a regulatory link between several catabolic and anabolic systems and as a double-edged sword both promoting and impeding tendon repair[44,83,85–88], this study focused on the comparative response to inflammation.

We used IL-1β and TNF-α, two hallmark cytokines of inflammation in tendons, which are associated with tendon injury and tendinopathy in vivo and in vitro, to induce disease-relevant inflammation[78,30,33,45,89–97]. IL-1 activates the NF-κB pathway in tenocytes, induces the production of inflammatory mediators including COX2 and IL6, and matrix remodelling factors such as MMP1, MMP3 and MMP13[28,30,89–91]. It can even cause loss of the tenocyte phenotype, which is associated with decreased expression of tendon-related genes, e.g. COL1, SCX and TNMD[28,30,89–91]. Similarly TNF-α can strongly activate tenocytes, stimulating them to produce more cytokines, including IL-1β, TNF-α, IL-6[28,30,92–94,96]. Accordingly, we used tendon-specific markers (SCX, TNC, TNMD, COL1, COL3, COL5), matrix remodelling proteinases (MMP1, MMP3, MMP13) and inflammatory factors (COX2, IL6, NF-κB, p53) in addition to proliferation and wound healing speed as read-outs to evaluate the response to IL-1β/ TNF-α induced inflammation.

Interestingly, while mouse and rat tenocytes most closely matched human tenocytes' low proliferation capacity and minimal effect of inflammation on proliferation, the human wound closure speed was best approximated by rats and horses. Tenocyte migration to the injured tissue and proliferation are essential processes in tendon healing[88,98]. Accordingly, inflammatory stimulation, e.g. with IL-1β, has been shown to increase tenocyte migration and proliferation, the capacity for which decreases with age[80,83,87,88,100–106]. In this study, we observed a decrease in tenocyte migration and proliferation following inflammatory stimulation in all species (statistically significant for sheep tenocyte proliferation as well as rat and mouse tendon cell migration under constant inflammation) except rats (non-significant trend toward increased proliferation), which may be due to our use of tenocytes from individuals in disease-relevant age groups.

The overall gene expression of human tenocytes was most similar to murine under healthy, equine under transient and ovine under constant inflammatory conditions. The species difference between human and the four animal models was particularly evident in the expression of the main tendon matrix component COL1. Healthy tenocytes of all four model species exhibited significant differences to human in their expression of COL1. Col1 typically amounts to appr. 95% of total tendon collagen or 50–80% of tendon dry weight[107], but cytokines, such as IL-1β and TNF-α, suppress COL1 synthesis, which leads to reduced stiffness[28,44,84,108]. In this study the decrease in COL1 synthesis following inflammatory stimulation could be observed in all species and was most pronounced in rat and mice, least in sheep and most similar to humans in horses.

The expression of the transcription factor SCX, a specific marker of the tendon/ligament lineage[109], while low in all species under healthy conditions, only increased in humans upon constant inflammation. SCX is a transcription factor that regulates tendon genes, including Col1 and Tnmd, and is required for normal tendon development[110,111] and adult tendon repair in mice[112,113]. An increase in its expression is likely to result in changes in the expression of its downstream genes and to be beneficial to tendon healing post injury[112]. The essential contribution of SCX was also shown in SCX-null mice, which fail to convert from producing primarily COL3 to synthesizing mainly COL1 during tendon repair, supporting the hypothesis that the transcriptional control of collagen type I is mediated by SCX[113]. Overall for the six tenogenic factors, rat tenocytes showed the largest difference to humans in the Mahalanobis distance, while tendon cells from mice and horses most closely equaled humans, indicating that these species might be most suitable for studies evaluating ECM production and tendon healing.

For matrix remodelling proteinases, the species differences were most prevalent for healthy tenocytes: tendon cells of all model species differed from humans for MMP13 and all but horses for MMP3. MMPs are key players in physiological and pathological tendon ECM remodeling, contributing to the degradation of tendon ECM and hence the loss of the biomechanical resistance and durability of tendon[44,114–116]. An increase in MMP expression has also been implicated in the pathogenesis of tendinopathy[44,116]. MMP13 specifically was upregulated in rotator cuff tendon tears and flexor tendon injury[117–119]. In this study, inflammatory stimulation increased MMP13 expression in tenocytes of all species, only minimally in mice and horses but 4–eightfold in rats, sheep and humans. In contrast, all species showed similarly increased MMP1 expression following inflammatory

Cell culture. The culture medium was identical for all species: minimal essential medium (α-MEM, Sigma-Aldrich, Vienna, Austria) supplemented with 10% fetal bovine serum (FBS-12A, Capricon, Ebsdorfergrund, Germany), 1% L-Glutamine (L-Alanyl L-Glutamine 200 Mm, Biochrom), 100 units mL^{-1}penicillin and 0.1 mg mL^{-1}streptomycin (P/S, Sigma-Aldrich, Vienna, Austria).

Cells were cultured at 37 °C, 5% CO2 until the desired passage and number of cells was obtained. Experiments were performed with cells either in passage 3 or 4.

Morphology. Cells were imaged both at low and high confluency using the EVOS FI. Auto imaging system in phase contrast with a 40×and 400×objective (ThermoFisher Scientific, AMEP4680). Cell phenotypes and cell sheet patterns were characterised for all species and compared to human cells. Tenocyte dimensions (length and width) were measured for each of the five species.

Inflammatory stimulation. Gene expression, proliferation and migration of tenocytes of all five species were compared under standard culture conditions (healthy control) as well as under transient (24 h) and constant exposure to inflammatory stimuli (10 ng/ml IL1β (Immuno Tools, Friesoythe, Germany) and 10 ng/ml TNFα (Immuno Tools, Friesoythe, Germany)) as previously described[28,89,90,93,115,129]. Successful induction of inflammation was confirmed by upregulation of inflammatory markers (COX2, IL6, see results). After 24-h exposure to inflammation, the transient inflammation group received fresh culture medium, while for the constant inflammation group fresh medium was again supplemented with inflammatory factors (Fig. 2).

Proliferation assay. Tenocytes were plated in 96-well plates (3000 cells/well in technical triplicates) and cultured under control (healthy), transient and constant inflammatory conditions. The cell number per plate was quantified via DNA fluorescence using the CyQuant assay (Invitrogen) according to the manufacturer's recommendations on day 0, 1, 2, and 3 (Fig. 2). As cell proliferation sets in after a lag time of about 24 h and relative proliferation rates decrease steadily, we used log(cell nr) as the target variable and log(time in hours minus 23) as regression variable for the parametric statistical analysis.

Wound healing (scratch) assay. Migration of tenocytes was evaluated in a wound healing model using a magnetic scratch device to create standardized cell-free gaps of 1.5 mm width in confluent sheets of tenocytes[130]. Cells were seeded in 12-well plates (100,000 cells/well in technical triplicates) and left to adhere overnight. Inflammatory stimuli were added to the transient and constant inflammation groups and scratches were created 48 h after seeding under control (healthy), transient and constant inflammatory conditions (Fig. 2). The cell-free area was imaged at 24 h intervals (0, 24, 48, 72, 96 h, Fig. 2) in phase contrast using the EVOS FL Auto imaging system with a 4×fluorite objective using coordinate recovery function. The gap size was measured using the MRI Wound healing Tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool) in ImageJ (https://imagej.nih.gov/ij/, version 2.0.0-rc-43/1.50e). As gap closure approached 100% in the fastest group, healthy rat tenocytes, at 48 h, this time point was chosen as cut-off for slope calculations and comparison of conditions. For the parametric analysis, we used the untransformed gap area [mm^2] as target variable and the untransformed time between 0 and 48 h (before the gap closed in any of the samples) as regression variable.

Quantitative PCR. Tenocytes were seeded in 12-well plates (100,000 cells/well in technical triplicates) and cultured under control (healthy), transient and constant inflammatory conditions. Cells were harvested for RNA isolation using RNA isolation reagent (Trizol, ThermoFisher Scientific, MA, USA) 48 h after initiation of inflammation, as previously described[131]. The 48 h time point was chosen as it allows assessment of the response to inflammation as well as to removal of inflammatory stimuli.

Briefly, a solution of Trizol and Chloroform (Sigma-Aldrich) in a ratio of 5 to 1 was used. Total RNA was recovered by the addition of isopropyl alcohol (Sigma-Aldrich) and glycerol (Thermo Scientific). The mixture was incubated on ice and centrifuged for 45 min at 13,000 rpm. The total RNA pellet was washed with 75% ethanol and solubilized in RNase-free water. Genomic DNA was removed by a DNA removal kit (Life Technologies, Carlsbad, California, USA). Two nanograms of RNA from each sample was used for the qPCR reaction (qPCR One-Step Eva Green kit, Bio&Sell, Feucht, Germany).

We measured gene expression of tendon markers (TNC, TNDM, SCX), collagens (COL1, COL3, COL5), matrix-metalloproteinases (MMP1, MMP3, MMP13), inflammatory factors (IL6, COX2, NFkB, p53), a marker for aberrant tenocyte differentiation (ALP) and for focal adhesion and migration (FAK) in the four model species and humans under healthy, transiently and constantly inflamed conditions. All primers were designed using the Primer3 software. Primer sequences are shown in supplementary table 2. The transcript level for the 15 genes of interest was normalized to the transcript level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented as ratio to GAPDH[58]. The ratio between COL 1 and COL 3 was also evaluated for further matrix remodelling characterization, with a higher COL1:COL3 ratio indicating a stronger tenogenic phenotype[132,133]. For the parametric analysis, we used the $\log_2$ transformed ratios of the target gene to GAPDH as target variable.

Statistical analysis. For statistical analyses, the R statistical programming language[134] and GraphPad (version 8.4.2) were used. Target and regression variables (where appropriate) are given in the respective subsections. Data are presented descriptively as mean and standard deviation. Generally, linear models (analyses of variance and covariances, ANCOVA) were used, e.g., for wound healing the untransformed area was the target variable, *time* a regression variable, and *species* and *condition* factors; as interactions of two explanatory variables

stimulation; no significant differences were observed in MMP1 expression in any species in any condition compared to humans. This corresponds well with other studies showing upregulation of MMP1 in ruptured tendons suggesting a high level of collagen degradation by this enzyme[120]. In total, for the functional group of ECM remodelling genes, horses again provided the best and rats the worst match to humans as shown in the Mahalanobis distance analysis.

For the expression of inflammatory mediators, the Mahalanobis distances of all species were larger than for the other functional gene groups. Although the immunophysiology of larger animal species has traditionally been presumed to be closer to humans than rodents[47,121], rat tenocytes most closely approximated human tendon cells in this category. Additionally, in healthy condition, mice presented the lowest distance from all animals, rising again the question if larger animals truly are more similar to human. Remarkably, healthy and transiently inflamed tenocytes of all four model species, as well as constantly inflamed ovine and equine tenocytes, showed significant differences to human in their expression of COX2. Following inflammatory stimulation, COX2 was only significantly upregulated in humans, mice and rats. Upregulation of COX2 plays an important, multifaceted role in the inflammatory cascade in injured tendons through the synthesis of prostaglandins[125]. COX2 is essential in the early injury response as evidenced by impaired tendon repair following administration of selective COX2 inhibitors in the early repair phase[122]. The lacking upregulation of sheep and horses therefore invites further investigation into the early tendon healing response in the different species in vivo.

Correspondingly, IL6, a cytokine with strong association with inflammation in tendon disease[38,123,124], displayed significantly different expression in transiently inflamed tenocytes of all species except rats. Statistically significant differences in IL6 expression compared to human were also evident under constant inflammatory conditions for mice and horses. IL6 plays an essential role in tendon healing as repair processes in IL6 knockout mice are impaired[38]. It tenocytes in two ways: i) IL6 stimulates tenocyte proliferation and survival and ii) it inhibits their tenogenic differentiation via the Janus tyrosine kinases/Stat3 signaling pathway[44,125].

Cell properties may be influenced not only by species and interdonor differences but also by cell isolation and processing methods[126,127]. In the present study, two isolation methods, enzymatic digestion and cell migration out of tendon explants, have been used depending on the available sample size. Enzymatic digestion was used for smaller sample sizes as higher cell yields are achieved with this method, while the explant technique is less invasive and requires less manipulation and labour. As both methods were used for all species and alterations in experimental conditions have been shown to be of minor importance to cell behaviour compared to cell source and interdonor variability[126], the isolation method is unlikely to have significantly influenced the species-specific gene expression profiles observed in this study.

In summary, the results of our study show that all four model species approximate some aspects of the behaviour of human tenocytes well and others poorly. No animal model sufficiently emulates human tenocytes' cellular and molecular features and response to inflammation to be considered the gold-standard for tendon research. Translational medicine will need to continue to rely on a fit-for-purpose selection of animal models to approximate the human condition, based on the essential characteristics that must be mimicked for a particular research question[19]. Peculiarities, strengths, and weaknesses of the model species need to be accounted for in the study design, analysis and interpretation[19,128,129]. Data from multiple animal models should be combined to optimize translational predictive validity.

Materials and methods

Tenocytes of four mammalian species (mouse, rat, sheep, horse) were compared with human tenocytes (n = 3 donors, i.e., biological replicates, per species). All methods and experimental protocols in this study were carried out in accordance and compliance with relevant institutional and national guidelines and regulations.

Tenocyte isolation from animals. All animals were euthanized for reasons unrelated to this study. Based on the "Good Scientific Practice. Ethics in Science und Research" regulation implemented at the University of Veterinary Medicine Vienna, the Institutional Ethics Committee ("Ethics and Animal Welfare Committee") of the University of Veterinary Medicine Vienna does not require approval of in vitro cell culture studies, if the cells were isolated from tissue, which was obtained either solely for diagnostic or therapeutic purposes or in the course of institutionally and nationally approved experiments.

Species-specific, energy-storing, weight-bearing tendons were harvested from skeletally mature animals immediately following euthanasia: Achilles tendons from sheep (Merino-cross breed, female, aged 2–5 years), rats (Fischer344 breed, female, aged 3–4 months) and mice (c57bl/6 breed, female, aged 8–12 weeks); superficial digital flexor tendons from the front limb of horses (7–15 years, geldings). Under sterile conditions, the paratenon was removed and the tendons were sectioned into small pieces (< 0.5 × 0.5 × 0.5 cm). Isolation of cells was performed either by enzymatic digestion using 3 mg/ml collagenase type II (Gibco Life technologies, Vienna, Austria) for 6–8 h or migration from explants (explants were removed after 7–10 days) or a combination of both. Cells were expanded until 80—90% confluency before passaging.

Human tenocytes. Human tenocytes obtained with ethical approval and informed consent from the Achilles tendon of three male human donors (aged 60–90 years) in accordance with relevant guidelines and regulations (Declaration of Helsinki) were purchased in cryopreserved condition in passage two from two different providers (Pelo Biotech GmbH, Germany and Zen-Bio, North Carolina, USA with review of the protocols and consent forms by an independent review board (Institutional Review Board, Pearl Pathways, LLC) which is accredited by the Association for the Accreditation of Human Research Protection Program Inc.).

*time*species*, *time*condition*, *condition*species*, and *biological replicate* nested within *species* were included; furthermore, the three-way interaction *time*species*condition* was also included. Note that all terms with *time* are to be interpreted as slopes or differences in slopes. The Tukey's HSD (honestly significant difference) test was used to account for multiple testing, where appropriate. Confidence intervals of parameter estimates were calculated.

Note that many different target variables are available, i.e., data are multidimensional. With qPCR alone, 15 genes of interest were measured under three conditions. For each gene separately, an ANOVA with species and condition was calculated. Furthermore, we condensed information by calculating the multivariate (Mahalanobis) distance of the $\log_2$-transformed mRNA concentrations, for the three conditions of each gene, and report the distance of each of the four mammalian species from the human values. For a single condition, all 15 genes of interest could be used for calculating the multivariate distance. Additionally, we grouped genes into classes, e.g., all collagens or all matrix-metalloproteinases and calculated multivariate distances for the classes separately, this time jointly for the different conditions. We also calculated a principal component analysis (PCA) of the $\log_2$-transformed qPCR data for all gene, treatment, and species combinations together. The proportions of the variance explained by the different PC's are reported and the rotated data for the different treatment and species combinations are shown in graphs for the most important components.

Received: 1 September 2020; Accepted: 24 May 2021
Published online: 14 June 2021

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Acknowledgements
The authors acknowledge Sinan Gültekin for his technical and John Breteler for his graphical support. This research was supported by the Austrian Research Promotion agency (grant number 856858) and the University of Veterinary Medicine Vienna tandem PhD programme.

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G.O.: study conception and design, data acquisition and interpretation, manuscript preparation.M.F.: study conception and design, data acquisition and interpretation, manuscript preparation.C.V.: data analysis and interpretation, manuscript preparation.I. R.: data acquisition, figure preparation.F. J.: study conception and design, data analysis and interpretation, manuscript preparationall authors reviewed the manuscript.

Funding
Open access funding provided by University of Veterinary Medicine Vienna.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-91914-9.

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Discussion

In the past decades major achievements in molecular biology and biochemistry have led to a broader understanding of cell biology and to considerable progress in modern medicine, prompted by the unravelling of cellular components, molecular functions and biological processes. However, translation of the groundbreaking lab results into clinical applications is still a major challenge. Only an unsatisfying rate of 10 % new treatment ideas made the way into clinics (Denayer, Stöhr, and Roy 2014; Prabhakar 2012). Reasons for that are manifold and can be found in the setup of *in vitro* as well as *in vivo* models (Denayer, Stöhr, and Roy 2014; Prabhakar 2012). However, only if we can identify and address the reasons we will succeed in a more efficient development of new and effective therapeutics. For both, *in vitro* as well as *in vivo* experiments, reproducibility and representability are essential. One crucial point is the choice of animal models and cell sources used to address particular research questions (Kilkenny et al. 2010; Prabhakar 2012). Although it might appear obvious, it is essential to recognize that there are often significant disparities between various animal models and even cells derived from different anatomical structures within the same species (Denayer, Stöhr, and Roy 2014; Zhang et al. 2022). These differences not only pertain to the inherent properties of the tissues or organs but also extend to the underlying pathomechanisms. Due to these differences, currently available animal models can not be presumed to fully represent the properties or pathophysiological mechanisms of interest. Consequently, this lack of congruence may not only result in overly optimistic conclusions but also hinder the translatability of research findings (Denayer, Stöhr, and Roy 2014; Prabhakar 2012.

Tendinopathies, complex musculoskeletal disorders characterized by chronic inflammation, microdamage, altered matrix composition, and abnormal cell behavior , pose a growing concern in the field of musculoskeletal disorders (Cook et al. 2016). In the context of tendinopathy research, our studies address some of the key challenges as the development of a standardized method to study cell migration in a wound healing environment and the comparison of cellular features of different species of typically used as animal model in tendinopathy reseacrh Specifically, we focus on elucidating the dynamics of cell migration within a wound healing environment, which is an essential factor in understanding the body's natural repair mechanisms during tendon injuries (Docheva et al. 2015; Sabine A. Eming, Krieg, and Davidson 2007). Furthermore, these processes are fundamental in other tissue repair processes and the progression of various degenerative diseases such as osteoarthritis (Gonzalez et al. 2016). However, existing techniques, like the conventional scratch

assay, often come with limitations, particularly in controlling gap size and ensuring replicable results (Jonkman et al. 2014).

Our primary objective was to develop a user-friendly, high-throughput wound healing assay with enhanced standardization for studying cell migration. We achieved this by using commercially available materials, offering the potential for broader adoption in laboratories, thereby promoting greater standardization of this relevant assay. Compared to manual approaches and existing techniques, our magnetic-based method consistently and precisely creates cell-free gaps, thus addressing the issue of experiment-to-experiment variability. The greater reproducibility can improve the reliability and comparability of experimental results obtain by different operators and even in different laboratories and consequentially, in conjunction with the 3Rs policy, to a reduction of animals used in experiments. Furthermore, applicability of our magnetic scratch assay can transcend tendinopathy research (Jonkman et al. 2014). For instance, in the study of osteoarthritis, a condition characterized by joint inflammation and cartilage damage, our assay could be instrumental in investigating cell migration and inflammation within the joint microenvironment (Johnson, Argyle, and Clements 2016).

We compared the gaps created with our method with a commercially available device which creates cell free gaps in confluent monolayers of cells by physically impeding cell adhesion and growth in a specific area. While the dimensional gap analyses (geometrical features of the cell free gap) were demonstrated to be comparable between the commercially available device and our novel method, life/dead staining at consecutive time points demonstrated a consistent presence of dead cells at the margins of the gaps created with the magnetic scratch in contrast to the commercially available system. The presence of dead and apoptotic cells at the margins of the cell free gaps was clearly linked to the mechanical stress inflicted by the movement of the magnets through the cell layer. Indeed, the resulting shear stress can lead to both cell death and apoptosis We could further verify the presence of apoptotic cell along the gap periphery through the use of caspase 3/7 fluorescent staining. Caspase 3/7 is the primary caspase effector involved in apoptosis triggered by intrinsic factors, such as cellular stress, or extrinsic factors, such as ligand-receptor interactions like TNFα binding to its receptor (Hengartner 2000; Julien and Wells 2017). Apoptotic cells can secrete pro-inflammatory molecules i.e. reactive oxygen species (ROS), cytokines and chemokines similar to a wound environment *in vivo*, hence offering a model that closely mimics the clinical situation (Santabárbara-Ruiz et al. 2019). Manual techniques for migration assays have failed in standardization/replicability of cell apoptosis induction, likely due to difficulties in standardization of the forces exerted to inflict the scratch (S.A. Eming, Krieg, and Davidson 2007). On the other hand, techniques relying on physically exclusion of cell adherence in a defined area do not result in mechanical cell injury or cell death. As previously

demonstrated, the presence of a cell free area will trigger cells to migrate towards this area (Anon et al. 2012). However, in response to cell injury, tenocytes showed higher cell migration speed (gap closure rates) as compared to techniques using a silicon insert to create a simple cell free area. This confirmed the importance of inflicting cell injury into an *in vitro* wound healing model to truly study cell migration in a wound setting. Altogether, these analyses revealed that the method we developed provided a highly replicable standardized, scalable assay which closely mimics the *in vivo* wound healing environment.

To further improve the applicability of the scratch to mimic tendon injuries, we included inflammatory stimulation. Inflammatory responses are another critical aspect of tendinopathy research (Dirks and Warden 2011; Docheva et al. 2015). Tendon injuries typically trigger inflammatory reaction, and the successful resolution of inflammation is a prerequisite for proper healing and tissue regeneration. However, in tendinopathies, this resolution often fails to occur. (Cook et al. 2016; Docheva et al. 2015; Gomez-Florit et al. 2022). Understanding the intricate interplay between cell migration and inflammation is crucial for developing effective therapies (Sabine A. Eming, Krieg, and Davidson 2007). By adding inflammatory cytokines TNF-α and IL1-β to the growth media, our magnetic scratch assay offers the possibility to evaluate cell migration during acute inflammation, providing a valuable tool for studying these complex processes (Joos et al. 2013). We investigated the impact of inflammation on cell migration in different species using the same magnetic scratch assay(Oreff et al. 2021). Our findings revealed that inflammation significantly reduced tenocytes migration and this effect is partially recovered in after removal of inflammatory stimuli.

However, a clear limitation of our scratching system is its constraint to a 2D culture environment, which does not fully mimic the native 3D context of the human/animals´ body (Blache et al. 2022; Muntz et al. 2022). Recent research has highlighted the critical importance of the 3D microenvironment on cell behaviours such as migration (Petrie and Yamada 2016; Yamada and Sixt 2019) . To address this limitation, future studies could extend the use of the magnet scratching system to cell culture plates coated with various adhesive proteins like collagen and fibrin to create a 2.5D environment to offer a closer approximation of the *in vivo* conditions (Pebworth, Cismas, and Asuri 2014). Additionally, our current model only includes musculoskeletal tissue derived cells, overlooking the role of other cells such as immune cells, in a wound environment, which play significant roles in wound healing (Julier et al. 2017; Schulze-Tanzil et al. 2022). To create a more comprehensive model, a possible avenue would be to employ a trans-well system that allows for co-culturing different cell types and thereby enabling a more holistic exploration of cell migration dynamics in wound healing. These enhancements would contribute to a more accurate modelling of *in vivo* wound healing processes.

We set out to establish an in vitro model replicating the acute inflammatory phase of tendinopathies and the response to transient inflammation. Through the temporary addition of inflammatory factors, we successfully achieved this objective. This platform holds the potential to contribute significantly to a more comprehensive understanding of the intricate pathophysiology of tendinopathies.

Replicability and fidelity of experiments and models play a pivotal role in translational medicine, as they ensure the robustness and reliability of scientific findings, ultimately paving the way for effective clinical applications (European Medicines Agency n.d.). However, another equally crucial aspect is the choice of the appropriate cell source for *in vitro* models. Indeed, the conservation of biological pathways and cellular and molecular features across different animal species is limited, a fact that has gained surprisingly little attention in the past and present scientific discourse (Denayer, Stöhr, and Roy 2014; Prabhakar 2012). While imperative to bridge this knowledge gap, as high-lighted by recent guidelines, only a few studies have addressed this issue (Burden et al. 2015; European Medicines Agency n.d.; Kilkenny et al. 2010). Currently available literature primarily focused on the comparison of species-specific responses of immune cells to external agents such as viruses, bacteria, and fungi (Enkhbaatar et al. 2015; Frazier et al. 2013; Wong et al. 2020). However, the species specific differences in inflammatory response of other cells such as tenocytes have not yet been investigated. For instance, in the context of tendon healing, we have limited knowledge about how tenocytes respond to chronic release of inflammatory factors, which may potentially delay the healing process or result in suboptimal outcomes (Arvind and Huang 2021). Equally important is our lack of understanding regarding the modulation of pro-inflammatory signaling molecules by tenocytes during different phases of tendon healing. This modulation is a key feature in the transition from the inflammatory phase to the proliferative and remodeling phases of the physiologic healing process in tendons (Docheva et al. 2015; Riley 2008).

Therefore, the second objective of this work was to address this knowledge gap by comparing the inflammatory response and relevant cell properties of tenocytes obtained from different animal species.

The study we conducted was the first to compare the inflammatory response of tendon cells from multiple species. As a result, we provide an indication guidance on selecting the most representative model for human pathophysiology in the context of tendon injury and tendinopathies. We assessed the differential behavior of tenocytes during the recovery phase (transient inflammation) and continuous exposure to inflammatory stimuli such as TNF-alpha and IL1-beta. During our investigation, we found differential expression of key inflammatory mediators, including COX-2

(Cyclooxygenase-2) and IL-6 (Interleukin-6), in response to cytokine exposure. COX-2 is known for its role in prostaglandin synthesis, contributing to inflammation, while IL-6 is a pro-inflammatory cytokine (Rainsford, 2007; Scheller et al., 2011). Furthermore, our results indicated a consistent effect of inflammatory cytokines on the migration of tendon cells from all species, resulting in lower gap closure rates during continuous inflammation and a partial recovery during transient inflammation. Gene expression analyses indicated an upregulation of FAKs (Focal Adhesion Kinases) both upon transient and constant exposure to inflammatory stimuli. FAKs are localized within focal adhesions, which are macromolecular structure connecting the cytoskeleton to transmembrane adhesion molecules and are involved in force transmission between the cell cytoskeleton and the substrate (Mitra, Hanson, and Schlaepfer 2005). FAKs play a key role in cell motility by regulating several processes including cytoskeletal re-arrangement, expression of adhesion molecules and protrusion edges (Katoh 2020; Mitra, Hanson, and Schlaepfer 2005). Our results match previous research findings, which show that ligament derived fibroblasts responded to inflammatory stimulation with a decrease in migration activity (Takemura et al. 2006). Furthermore, in the same study an increase in FAKs in response to TNF-α stimulation was linked to higher actin stress fiber formation with consequent inhibition/reduction of cell migration was reported (Takemura et al. 2006). Our results strengthen these findings demonstrating that tenocytes from all species respond similarly and suggest that this mechanism of action is conserved among different species.

We further explored the intricate web of molecular factors contributing to tendinopathy. Tendinopathies are characterized by a complex interplay of inflammatory responses, matrix remodeling, and altered cell behaviors (Docheva et al. 2015). One of the key aspects we explored was the regulation of extracellular matrix proteins such as collagens and matrix degrading enzymes as MMPs, particularly collagenases, in response to inflammatory cytokines. Collagens and MMPs play a pivotal role in tendon extracellular matrix (ECM) turnover, and their dysregulation has been implicated in tendon pathologies (Sorsa et al., 2016). Our study revealed that both human and animal models derived tenocytes responded to inflammatory stimuli by regulating collagens and MMPs gene expression, mirroring the dysregulation observed in tendinopathy in vivo. However, the magnitude of this regulation varied greatly among the species, including humans (Buono et al. 2013). In our study, we used the Mahalanobis Distance to assess how certain properties from tenocytes from different species correspond or differ from human cells. This method goes beyond traditional statistical approaches and considers the entire covariance matrix, providing a more robust normalization(El Bendadi, Lakhdar, and Sbai 2018; Chilingaryan et al. 2002; Suzuki et al. 2008). This innovative approach has the potential to reshape how we evaluate existing databases, establish control groups, and design experiments in research fields like biology or medicine (Anders and Huber 2010). Our findings revealed

that no single species closely mirrors human cells across all compared aspects, underlining the importance to consider cellular and molecular characteristics for the selection of an appropriate animal model.

While our study contributes valuable insights into species-specific responses of healthy tenocytes to acute inflammation, several limitations should be noted. Firstly, our research primarily addresses the acute inflammatory phase, offering only a partial view of the pathophysiology of tendinopathies (Cook et al. 2016; Stauber, Blache, and Snedeker 2020). Tendinopathies are chronic conditions which go along with long-term inflammation and tissue remodelling, which makes addressing the entire spectrum of the disease crucial for creating an accurate *in vitro* model (Cook et al. 2016; Docheva et al. 2015; Theodossiou and Schiele 2019). Our studies serve as an important steppingstone, laying the foundation for future investigations that should encompass the chronic phase of tendinopathies. To achieve a more comprehensive understanding, it is imperative to incorporate a broader array of inflammatory mediators, better mimicking the complexity of the *in vivo* environment (Cook et al. 2016; Theodossiou and Schiele 2019). Another limitation of our study is the absence of the immune system in our *in vitro* model. *In vivo* tendinopathies involve intricate interactions between tendon cells and immune cells, contributing to the chronic inflammatory state characteristic of these (Cook et al. 2016; Docheva et al. 2015; Steinmann et al. 2020). Our model, focused on tendon cells' response to inflammatory cytokines, does not encompass the complexity of immune cell involvement. Therefore, while we have gained insights into tendon cell behavior, we cannot replicate the entire spectrum of inflammatory responses present in tendinopathy (Cook et al. 2016; Xu and Murrell 2008). To address this limitation and gain a more holistic understanding, future studies may consider co-culture systems that incorporate immune cells and other relevant cell types. Moreover, our studies are limited to 2D cell culture, which, while beneficial for controlled experiments, doesn't mimic the native 3D complexity of tendon tissues (Blache et al. 2022; Frank and Hart 1990). Tendons are intricate, hierarchical structures composed of multiple cell types embedded in a highly organized extracellular matrix. Transitioning to advanced 3D models not only allows for a more realistic representation of the cellular microenvironment but will also facilitate to incorporate the critical element of mechanical loading (Muntz et al. 2022; Romani et al. 2021) . Mechanical forces are fundamental in both the normal function of tendons and the pathology of tendinopathies (Cook and Purdam 2009; Steinmann et al. 2020; Wang, Iosifidis, and Fu 2006). Tendons are subjected to continuous mechanical stresses *in vivo*, and understanding the mechano-response and mechano-transduction pathways within these tissues is of paramount importance (Stauber, Blache, and Snedeker 2020). These processes govern how tenocytes perceive and respond to their surrounding mechanical environment, orchestrating cellular behaviors, matrix remodeling, and secretion of pro

inflammatory mediators (Schulze-Tanzil et al. 2022; Snedeker and Foolen 2017). Investigating these mechanosensitive aspects is crucial, as disrupted biomechanics and the accumulation of microdamage in tendons are significant contributors to the development of tendinopathies (Cook et al. 2016; Stauber, Blache, and Snedeker 2020). Microdamage, often a consequence of repetitive use or excessive mechanical loading, is a hallmark of tendinopathy development, and its effects on cellular responses, including cell damage and death, are critical to comprehend (Stauber, Blache, and Snedeker 2020). Transitioning to advanced 3D models that consider these mechanistic factors will enable a more holistic understanding of tendinopathies (Blache et al. 2022).

Our aim was to analyse and compare the cellular features of tenocytes from different species. While we did not identify a single species that closely mirrors human cells across all aspects of the inflammatory response, our analyses revealed that some species are better mimicking human tenocytes in specific aspects than others. Our study serves to raise awareness in the field about the necessity of carefully selecting an appropriate animal model tailored for specific research purposes, as a one-size-fits-all approach may not be suitable. This consideration is crucial in advancing our understanding of the complex interplay between cellular and molecular characteristics in translational research.

The comparative analysis of cellular features among human and various animal-derived tenocytes has significantly enhanced our understanding of translatability and interpretability within the field of tendon research. Our own study, which investigated species-specific disparities in tenocytes´ response to inflammation, served as a foundational reference point for subsequent research efforts. For example, a study conducted by Palomino Lago et al. referenced our work to emphasize the critical importance of discerning species-specific differences in tenocyte responses (Palomino Lago et al. 2023). Their research highlighted that while human tenocytes may respond to a particular inflammatory agent in a specific manner, murine tenocytes might exhibit significantly different responses. Such disparities underscore the necessity of carefully considering the choice of animal models when designing studies aimed at developing treatments for tendinopathies. Similarly, the study performed by Peñin-Franch et al. built upon our findings, recognizing that murine tenocytes may exhibit certain similarities to human tenocytes in their responses to galvanic current treatment (Peñin-Franch et al. 2022). However, they also acknowledged the potential differences in the inflammatory response, as indicated by our results. This insight calls for a critical assessment of the suitability of various animal models, considering the potential disparities in their tenocyte behaviors. Furthermore, our study has been cited in the work of Furukawa et al., although their study did not directly investigate species-specific differences (Furukawa et al. 2023). Instead, they utilized our research to emphasize the importance of using human tenocytes in human tendinopathy research and

to discuss potentially controversial studies in different animal species. This broader recognition underscores the understanding that no single animal model can precisely replicate human tenocyte behavior. It highlights the complexity of species-specific responses, particularly regarding inflammatory processes and cellular reactions to stimuli, thus emphasizing the significance of our research in unravelling these nuanced distinctions and their potential implications for translational research.

Conclusion

In conclusion, our research contributed to the broader mission of enhancing translational potential, improving reproducibility, and ethical rigor in biomedical investigations. At the core of our message is the urgent need for more sophisticated *in vitro* models that replicate the *in vivo* environment. While animal models have been invaluable in scientific research, they have limitations in mimicking human diseases and responses. At best, they can replicate only small and narrowly defined aspects of the human condition, highlighting the imperative need for the validation of models tailored to specific research questions. Yet, the emphasis should shift towards developing humanized *in vitro* models that better represent the intricacies of *in vivo* situations. Our research highlights the importance of using *in vitro* models as a powerful tool to bridge the gap between basic laboratory research, *in vivo* models and clinical applications. These models should not be generic but tailored specifically to address the research questions. As we strive to develop more effective treatments and therapies for conditions like tendinopathies, we must recognize that the future lies in the refinement and expansion of *in vitro* models that faithfully capture the complexity of the human body. However, it is essential to acknowledge that our journey has only just begun, and there is much more work to be done to maximize the impact of our findings. One of the critical areas where we can further increase the relevance of our work is by integrating additional elements into our wound healing and tendinopathy models. This includes the incorporation of immune cells, a crucial component in the aetiopathogenesis of these diseases. By developing co-culture systems which include the interactions between tenocytes and immune cells, we can better mimic the *in vivo* environment and gain a more comprehensive understanding of the pathophysiology of tendinopathies. Furthermore, we should explore the adoption of 2.5D and 3D matrices in our models to better mimic the native extracellular matrix (ECM) in tendons. The inclusion of mechanical forces, which play a fundamental role in tendon pathophysiology, should also be a priority. This approach aligns with the 3Rs principle, aiming to reduce and in the future potentially replace animal testing in research.

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