



**UNIVERSITY OF
BIRMINGHAM**

**MAGNETIC NANOPARTICLE-MEDIATED
ACTIVATION OF THE
ACTIVIN IIA RECEPTOR FOR
TENOGENIC DIFFERENTIATION**

by

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A Thesis submitted to the University of Birmingham for the degree of
DOCTOR OF PHILOSOPHY

School of Chemical Engineering
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October 2022

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Abstract

Tendon injuries are among the most frequent orthopaedic conditions affecting a large part of the population worldwide and represent a huge burden for the healthcare system. In fact, tendon tears and lacerations can lead to chronic or degenerative tendinopathies as a common complication caused by tendon limited intrinsic healing properties. Despite the high frequency of tendon injuries and their major socioeconomic impact, an effective and widely-accepted treatment is missing. Current strategies include non-surgical and surgical treatments which are often not fully successful due to recurrent injury of the same tendon. Hence, there is an immediate need to find an alternative treatment strategy which could offer an effective and durable resolution of tendon injuries.

Tissue engineering offers a potential alternative approach to treat tendon injuries, wherein the use of stem cells has been shown to improve the healing response and minimise scar formation. In this Thesis, stem cells were studied in conjunction with remotely-controlled magnetic nanoparticles (MNPs) to target and stimulate the mechano-receptor Activin RIIA towards the initiation of the tenogenic differentiation pathway. Several relevant cell types were investigated along with different antibodies targeting different regions of the receptor, in order to better stimulate the mechano-responsive domain. The remote mechanical activation of the Smad2/3 signalling cascade and the expression of some tenogenic proteins was verified in human tenocytes, human bone marrow stem cells (hBMSCs) and human adipose stem cells (hASCs); raising the possibility to remotely control cell behaviour towards the tenogenic commitment. Furthermore, two 3D scaffolds were developed and characterised as potential cell delivery methods for *in vivo* applications or *in vitro* tendon 3D modelling. The first 3D scaffold consisted of an oriented fibres collagen gel which allowed for the parallel alignment of hASCs *in vitro*. The second developed scaffold was an injectable fibrin gel which was shown to be

cytocompatible and stable *in vitro* for 21 days. Finally, the tenogenic regenerative potential of remotely-activated MNPs-labelled human adipose stem cells was studied *in vivo*, as an essential step prior to translation into clinic. This study revealed that remotely-activated MNPs-labelled hASCs might have a positive impact in the deposition of an organised ECM during tendon healing.

The obtained results suggest that the application of a mechanical stimulus can enhance stem cell tenogenic potential and can be considered as a valid integrated approach for tissue engineering strategies in tendon injuries treatment.

Acknowledgments

I would like to recognize all the people who contributed to the accomplishment of this Thesis, supporting my journey on the scientific or personal level.

Thanks to my supervisor Prof. Alicia El Haj for giving me the opportunity to join her Research Group and for helping me in developing my potential. I would also like to thank my co-supervisor Prof. Martyn Snow for the clinic insights and the collaboration with the ROH.

I wish to thank Prof. Manuela Gomes for receiving me to the 3B's Research Group at the University of Minho, in Portugal. I have learned a lot from the experience in your lab and the experiments carried out at your facilities have been of highest importance for my Thesis.

Thank you to my lab mates, in particular to Hari, Iria, Nicky and Ana for the invaluable support in the lab and for also being my friends outside the lab. Thank you to all the beautiful people I met during these 3 years. Thank you to my friends in Italy, especially to Jessica, for the comfort of a deep and true friendship.

A special thanks to Laura, for being there during one of the hardest times of my life. It would not have been the same without you, I am so grateful I have met you. You know.

Thank you to my mum and dad, for always believing in me. Thank you for always being there for me. For keeping a light on when it was dark, so I could come home. Thank you to my grandma for all the letters, and to my grandad for adding the four-leaf clovers.

To Fabio, you made everything worth it. Thank you for your unconditional love. Thank you for making me believe in myself. I cannot wait to build memories with you.

Finally, I acknowledge the financial support from ERC Dynaceutics for funding the work described in this Thesis.

*Prendete la vita con leggerezza,
che leggerezza non è superficialità,
ma planare sulle cose dall'alto,
non avere macigni sul cuore.*

Italo Calvino, Lezioni Americane

*Take life lightly,
'cause lightness is not superficiality,
but gliding over things from above,
not having boulders on your heart.*

Italo Calvino, Lezioni Americane

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General abbreviations

2D – Two-dimensional

3D – Three-dimensional

A

A/A – Antibiotic/Antimycotic

aa – Amino Acids

AA – Ascorbic Acid

Ab – Antibody

ACL – Anterior cruciate ligament

Activin RIIA – Activin Receptor Type IIA

ALK – Activin-like kinase

Anti-Activin RIIA MNPs – MNPs labelled
with anti-Activin Receptor Type IIA
antibody

APC – Allophycocyanin

APTES – (3-Aminopropyl)triethoxysilane

B

BMPs – Bone morphogenetic proteins

bFGF – Basic fibroblast growth factor

BSA – Bovine Serum Albumine

BV421 – Brilliant Violet 421

BV510 – Brilliant Violet 510

BV650 – Brilliant Violet 650

C

CaCl₂ – Calcium Chloride

CO₂ – Carbon dioxide

Col I – Collagen type I

Col III – Collagen type III

CTGF – Connective Tissue Growth Factor

Cy5 – Cyanine 5

Cy7 – Cyanine 7

D

DAPI – 4',6-diamidino-2-phenylindole

dH₂O – Distilled water

DMEM – Dulbecco's Modified Eagle

Medium

DMSO – Dimethyl sulfoxide

DPBS++ – Dulbecco's Phosphate Buffered

Saline with MgCl₂ and CaCl₂

E

ECM – Extracellular matrix

EDAC – N-(3 -Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

EDS – Energy dispersive spectrometer

EDTA – Ethylenediaminetetraacetic Acid

ELISA – Enzyme-linked immunosorbent assay

ERG – Early growth response gene 1

ERK – Extracellular signal-regulated kinase

ESWT – Extracorporeal shockwave therapy

EthD-1 – Ethidium homodimer-1 in the presence of DNA

F

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

Fe – Iron

Fe₃O₄ – Iron oxide

FEMM – Finite Element Method

Magnetics Software

FGF – Fibroblast growth factor

FISH – Fluorescent *in situ* hybridisation

FITC – Fluorescein isothiocyanate

G

g – g force

GDF – Growth and differentiation factor

GFs – Growth factors

H

h – Hour(s)

H&E – Hematoxylin and Eosin

H₂O₂ – Hydrogen peroxide

hASCs – Human adipose stem cells

hBMSC – Human Bone Marrow Stem Cells

HCl – Hydrochloric acid

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hz – Hertz

I

ICC –Immunocytochemistry

ICP – Inductively coupled plasma

IGF-1 – Insulin-like growth factor 1

IHC – Immunohistochemistry

IONPs – Iron oxide nanoparticles

IP – Intraperitoneal

L

L-Glut – L-Glutamine

LLLT – Low-level laser therapy

LN – Liquid nitrogen

M

MAPK – Mitogen-activated protein kinase

MES buffer – 2-(N-morpholino)ethanesulfonic Acid

μCT – Micro-computed tomography

MICA – Magnetic Ion Channel Activation

Min – Minute(s)

MKX – Mohawk homeobox

MNPs – Magnetic nanoparticles

MRI – Magnetic resonance imaging

MSC – Mesenchymal stem cell

NNa₂CO₃ – Sodium Carbonate Anhydrous

NaF – Sodium Fluoride

NaOH – Sodium hydroxide

NaCl – Sodium chloride

NdFeB – Neodymium

NHS – N-Hydroxysuccinimide

Ni/N42 – Nickel coated, N42 grade

P

PBS – Phosphate-buffered saline

PCL – Polycaprolactone

PDGF – Platelet-Derived Growth Factor

PE – R-Phycoerythrin

PEG – Polyethylene glycol

PEMF – Pulsed electromagnetic field therapy

Pen-Strep – Penicillin/Streptomycin

PET – Positron emission tomography

PHBHHx – Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

SPIONs – Superparamagnetic iron oxide nanoparticles

PLGA – Poly (L-lactic-co-glycolic acid

T

PolR2a – RNA Polymerase II Subunit A

TBS – Tris Buffered Saline

PPIB – Peptidyl-prolyl cis-trans isomerase

TBST – Tris Buffered Saline with 0.1%

B

Tween 20

PS – Picosirius Red

TEM – Transmission electron microscopy

pSmad2/3 – Phosphorylated Smad2/3

TGF- β 3 – Transforming Growth Factor β 3

R

TNMD – Tenomodulin

RGD – Arg-Gly-Asp

TSPCs – Tendon Stem Progenitor cells

RPM – Rotations per minute

U

RT – Room temperature

UCB – Ubiquitin C

S

UV – Ultraviolet

SCXA – Scleraxis

V

SD – Standard deviation

VEGF – Vascular endothelial growth

SEM – Scanning electron microscopy

factor

SFM – Serum Free Media

W

WB – Western Blot

Dissemination of research findings

The results obtained from this Thesis have been disseminated as follows:

Publications

Some of the work described in Chapter IV provided the protocols for the following publication:

Wright, A.L., Righelli, L., Broomhall, T.J., Lamont, H.C. & El Haj, A.J. Magnetic Nanoparticle-Mediated Orientation of Collagen Hydrogels for Engineering of Tendon-Mimetic Constructs. *Front. Bioeng. Biotechnol.* 10, 797437 (2022).

Oral presentations (speaker underlined)

- Achilles Conference, Braga (Portugal), 2019.

Righelli, L., Markides, H., El Haj, A.J. Magnetic nanoparticle mediated activation of the Activin A receptor for tenogenic differentiation.

- ICORS (International combined orthopaedic research societies), Edinburgh (UK), 2022.

Righelli, L., Gonçalves, A.I., Rodrigues, M.T., Gomes, M.E., El Haj, A.J. Prospects for Tendon Regeneration: Magnetic nanoparticle mediated activation of Activin A in a rat tendon repair model.

Poster presentations

- TERMIS (Tissue Engineering and Regenerative Medicine International Society), Maastricht (The Netherlands), 2021.

Righelli, L., Markides, H., Gonçalves, A.I., Gomes, M.E., El Haj, A.J. Magnetic nanoparticle mediated activation of the Activin A receptor for tenogenic differentiation.

CHAPTER I

INTRODUCTION

1.1 Clinical demand for tendon tissue engineering strategies

Tendon injuries and related pathologic syndromes are among the most frequent orthopaedic conditions affecting a large part of the global population every year. For instance, in 2013 the 19.2% of all the medical diagnoses in the United States were represented by musculoskeletal disorders, leading to more than 9 million procedures involving joints or tendons.¹ Orthopaedic conditions such as major tendon tears can be extremely impairing and can reduce patient motility for a long period of time limiting therefore their productivity. In 2017 for example, almost half million full time employees were affected by tears or traumatic injuries of tendons and muscles.¹ Expensive physiotherapy regimens are often required as tendon healing is slow and can encounter several complications, which results in about 50 lost work days per injury, culminating in a major socioeconomic impact.² Moreover, tears and lacerations are only two of the many injury mechanisms that could lead to tendinopathy or tendon ruptures. In fact, tendinitis can happen in tendons exposed to overuse or intrinsic degeneration, for example age-related.³ In particular, Achilles tendon, rotator cuff and flexor tendon of the hand are the tendons more commonly injured and requiring a surgical intervention.¹

Aside from the high frequency of the injuries, the situation is even more complicated by tendon limited healing properties^{4,5} often leading to further complications such as fibrotic scar formation. Disorganized and excessive extracellular matrix (ECM) is deposited in the injury site, instead of a proper regeneration of the native tendon structure. This ECM is able to provide some stability to the tissue but without restoring the mechanical properties and elasticity of the original tissue and, as a consequence, increasing the risk of reinjury.⁶ Furthermore, fibrotic healing is often considered the cause of subsequent degenerative and chronic tendinopathies.¹

Despite the important socioeconomic impact and the high frequency of tendon injuries and tendinopathies, at present, an effective and widely-accepted treatment is missing.^{7,8} Current strategies including physiotherapy,⁹ shock-wave therapy,¹⁰ injections¹¹ and surgery,¹² are often inconclusive as patients often get re-injured on the same tendon. New treatment strategies are therefore required to solve this major clinical concern. Tissue engineering is a relatively new research field of regenerative medicine that aims to create biological constructs and substitutes mimicking native tissues and organs for diagnostic, research and treatment purposes. Tissues particularly prone to injury, degeneration or disease such as skin, cartilage and bone, are the main targets of tissue engineering.¹³ Most tissue engineering technologies use living cells, from multiple sources or phenotype, often in conjunction with a 3D scaffold and/or growth factors, with the purpose to recreate the native tissue from a biological, biochemical and biomechanical point of view. Regenerative medicine and tissue engineering approaches are showing promising results in several applications including tendon repair,¹⁴⁻¹⁶ offering a possible solution for this unmet clinical need.

1.2 Tendon anatomy

Tendons can be considered as mechanical bridges between muscles and bones. Their role is to maintain the body posture and to transmit muscles contractions to bones, allowing the final movement.¹⁷ Healthy tendons are stiffer than muscles and have a white translucent aspect with a fibroelastic texture. Their morphology can be different depending on the muscle of insertion and its function; for example, they can be rounded, flat ribbon-like or strap-like.¹⁸ Two of the main peculiarities of tendons are their great tensile strength and elasticity, which allow them to bear great loads but with a minimal deformation. These characteristics are fundamental for transmitting the forces from the muscle to the bone, without losing energy to elongate the tendon.¹⁷ The Achilles tendon, the Patellar tendon and the Rotator cuff are the biggest tendons

in the human body, in fact, they do not simply represent the connection between muscles and bones but, being located in the main body joints, they also have a key role in modulating the movements speed of the distal limbs where they are inserted.¹⁸

1.2.1 Cells and ECM

The two main types of cells co-existing in the tendon tissue are tenocytes and tenoblasts, which are specialised fibroblasts and represent the 90-95% of the cellular component, while the remaining 5-10% is formed by chondrocytes in the bones insertion sites, vascular cells and synovial cells of the tendon sheaths (Table 1.1).¹⁷ Tenocytes and tenoblasts differ in shape and function, in particular, tenoblasts are immature cells with an ovoidal shape which are involved in the tissue repairing process and in the deposition of collagen matrix. In the final repair step, tenoblasts differentiate into tenocytes, the mature and final cell phenotype with a characteristic elongated shape. The main functions of tenocytes are to respond to the mechanical load (Chapter 1.4.3.1) and to produce ECM, which is mainly composed of Collagen (86%), Proteoglycans (1-5%), Elastin (2-4%) and other glycoproteins (<2%). Collagen and elastin are embedded in a matrix made of proteoglycans and water, where collagen represents 65-80% of the dry mass of the tendon and elastin approximately 1-2%.

Table 1.1 Tendon main cellular and ECM components.

	Components		Location
Cells (18-35%)	Tenocytes and tenoblasts (90-95%) ¹⁸		Tendon’s body
	Other cells (5-10%) ¹⁸	Chondrocytes	Bone attachment and insertion sites
		Synovial cells	Tendon sheath
		Vascular cells	Blood vessels
		TPSCs ¹⁹	FM and IFM
ECM (65-82%)	Collagen (86%) ¹⁸	Type I (97-98%)	Collagen fibres, endotenon, epitenon, paratenon, MTJ
		Type II (0.2-0.8%)	Cartilaginous area of OTJ
		Type III (1-1.5%)	Endotenon, paratenon, vessel walls, MTJ
		Type IV (<0.2%)	Basal membrane of vessels, MTJ
		Type V (<0.2%)	Vessel walls, MTJ
	Proteoglycans (1-5%) ^{17,18}	Decorin	IFM
		Aggrecan	IFM
	Elastin (2-4%) ^{17,18}		IFM
	Glycoproteins (<2%) ^{17,18}	Fibronectin	
		Thrombospondin	
		Tenascin C ²⁰	Tendon’s body, OTJ and MTJ
TSPCs=Tendon stem/progenitor cells, FM=fascicular matrix, IFM=interfascicular matrix, MTJ=myotendinous junction, OTJ=osteotendinous junction.			

Collagen type I (Col I) is the main component of the collagen portion (97-98%), followed by a smaller amount of Collagen III (Col III) (1-1.5%), Collagen II, IV and V.¹⁸ On the other hand, proteoglycans provide the visco-elastic properties typical of tendons and with their hydrophilic nature, they enable the diffusion of soluble molecules and cell migration.²⁰ Other adhesive glycoproteins are involved in the repair and regeneration of tendons, such as fibronectin and

thrombospondin. Tenascin-C is another important component of tendon ECM and it is particularly abundant in the tendon-to-bone insertions and tendon-to-muscle junctions. Tenascin-C can also act as an elastic protein, in fact, it contains repeating units of fibronectin type III domains that can be unfolded as a consequence of stress stimuli.^{20,21} Interestingly, Tenascin-C expression is regulated by mechanical forces and it has been found to be upregulated in tendinopathies.²² Collagen, fibronectin and elastin can be found between the proteoglycan complexes, highly organised in a way that provides mechanical resistance to the tendon.

Collagen molecules are formed by chains of polypeptides bounded together to form a tropocollagen helix which, combined in groups of five, to create a microfibril. Multiple microfibrils compose a fibril, which are then grouped into fibres (primary bundles), organised in fascicles (secondary bundles)¹⁸ which form the fascicular matrix (FM).¹⁹ A thin layer of connective tissue called “endotenon”, also known as interfascicular matrix (IFM), is interposed between fibres, easing the sliding of adjacent fibres and also creating a channel for blood vessels.²³ More fibres are kept together by the “epitenon”, a connective tissue sheet which surrounds externally the whole tendon, containing the vascular, nerve and lymphatic supplies as well. In some cases, tendons are surrounded by an additional structure called “paratenon” which consists of a looser connective tissue mainly formed by collagen I and III, elastic fibrils and synovial cells (Figure 1.1).¹⁸

IFM is a crucial region in tendon anatomy as it represents the energy storing of tendons, thanks to its fatigue-resistance and great extensibility properties.^{24,25} Furthermore, another important cell population resides within the IFM and FM areas: tendon stem/progenitor cells (TSPCs).¹⁹ TSPCs include an heterogeneous cell population with various degree of differentiation potential, primarily involved in tendon homeostasis, healing and development.¹⁸ Even though the exact role and activation mechanism of these cells remain unclear *in vivo*, they

are predicted to participate in tendon repair intrinsic and extrinsic mechanisms²⁶ (Paragraph 1.4) by differentiating into the tenogenic lineage after a tendon injury.²⁷ Nevertheless, recent research revealed the existence of a delicate equilibrium in the so-called “stem niches”, involving both biochemical properties and structural patterns of the native tendon matrix, which is essential to maintain the stemness of TSPCs.²⁸ Bi et al.²⁹ showed how biglycan and fibromodulin, two proteins of the ECM, are essential in regulating the differentiation fate of TSPCs, with biglycan also playing a role in enhancing their proliferation and tenogenic differentiation.

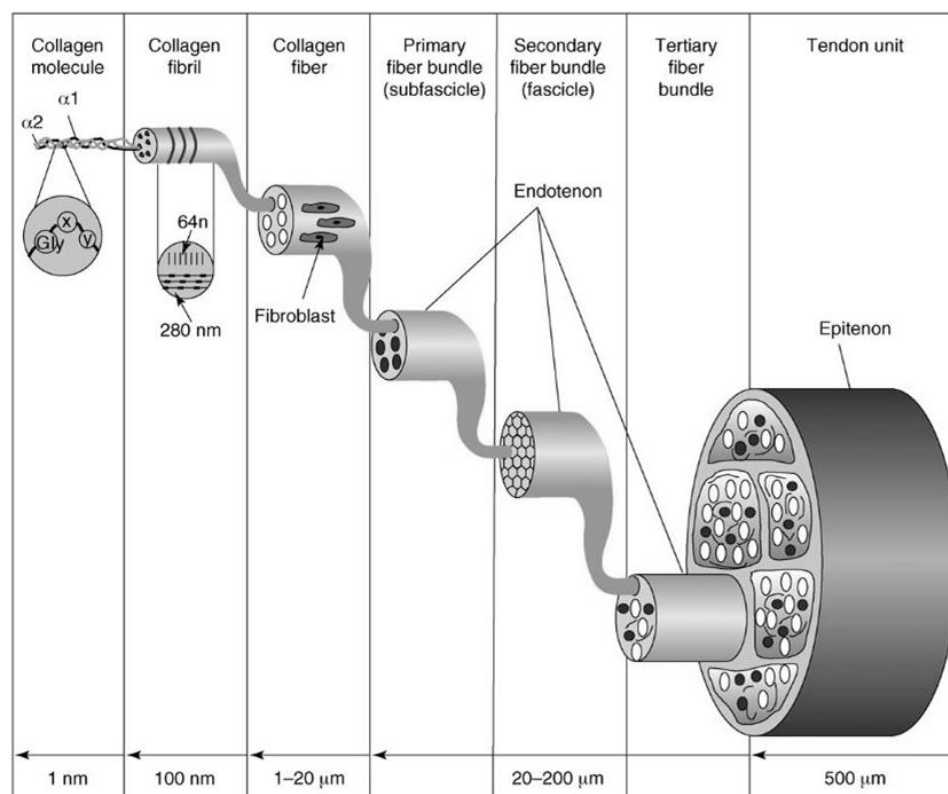


Figure 1.1 Schematic representation of tendon anatomy. Adapted from Liu et al., 2008.³⁰

1.2.2 Blood supply

Tendon vascularisation is an important limiting factor for tissue healing and regeneration. In fact, tendons are characterised by a lower blood supply compared to other tissues, like muscles.⁴ Tendons receive the blood supply through three different channels; two intrinsic systems, one

at the myotendinous junction and the other at the osteotendinous insertion, and one extrinsic system running between the synovial sheath and the paratenon. Moreover, vascularization decreases with age and mechanical loading, in fact, in the sites of torsion, compression and friction the vascularisation is compromised. In these areas, the angiogenesis is decreased by the production of inhibitor molecules like endostatin and by a reduction of some stimulatory peptides like Vascular endothelial growth factor (VEGF).¹⁸

1.3 Mechanical properties

Elasticity and resistance to mechanical stress are the main characteristics of tendons. Nevertheless, not all tendons react the same way to a mechanical stress in fact, the shape and thickness of a tendon are strictly related to its biomechanical behaviour. For instance, as muscles of the hand are smaller than quadriceps or triceps, the same way their tendons are longer and thinner compared to shorter and more robust tendons typical of bigger muscles. Furthermore, shorter tendons have a greater tensile strength compared to longer tendons with the same diameter, therefore shorter tendons are less likely to break compared to longer ones.¹⁷ Tendon mechanical properties are strongly related to the arrangement and diameter of the collagen fibrils, in particular fibrils of greater diameter are less flexible than smaller fibrils and they can be found in tendons subjected to great mechanical stress.¹⁷ During the rest phase, collagen fibres and fibrils have a crimped configuration³¹ which allows them to elongate during straining and then to return to their crimped shape when the stimulus ends. Nevertheless, there is a limited percentage of straining that tendons are able to bear without breaking. In particular, if the strain is up to 2% the crimps flatten and collagen triple helices slide on each other³² allowing the stretching of the tendon. If the strain remains below 4%, tendons can return to their original shape once the mechanical stress ends; on the contrary, strains bigger than 4% generate

microscopic failures which become macroscopic if the strain increases to 8-10% (Figure 1.2).^{20,33}

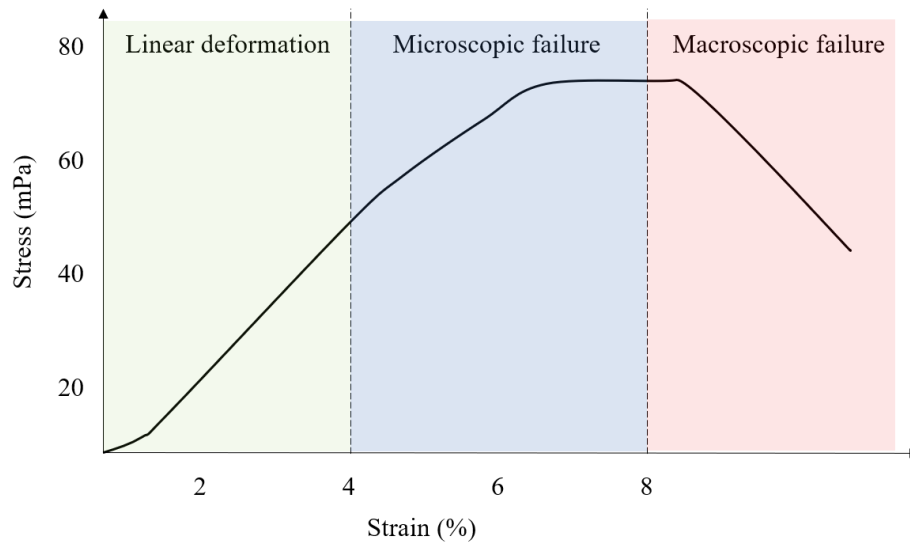


Figure 1.2 Stress-strain curve in normal tendons.

1.4 Natural tendon repair

The healing process is crucial for the mechanisms of tissue regeneration and can typically be divided in three main stages involving distinctive cells and molecular cascades: inflammatory, proliferative and remodelling phase (Figure 1.3). The inflammatory stage lasts for a few days and it is characterized by an acute inflammatory response that initiates after injury with an influx of inflammatory cells, such as neutrophils, monocytes and macrophages, along with red cells and platelets. The latter also serve as important chemo-attractants for growth factors (GF) like Transforming Growth Factor β 3 (TGF β), Insulin-like growth factor 1 (IGF-1) and Platelet-Derived Growth Factor (PDGF). The role of these GF is to encourage the recruitment of fibroblast and intrinsic tenocytes, as well as the release of other bioactive factors like Basic fibroblast growth factor (bFGF) and VEGF to promote angiogenesis.³⁴ At the same time, temporary stiffness is provided by the formation of a fibrin clot, while the necrotic tissue is digested by macrophages.³⁵ The second stage, called proliferative or repair stage, begins after

2-4 days from the injury and is mainly a reparative process in which a high synthesis of ECM components is observed. In particular, the main protagonists of this phase are tenocytes, which deposit a temporary and roughly-arranged ECM mainly composed of Col III, and macrophages which after an initial phagocytic role, start the release of growth factors to induce cell recruitment.³⁶ The final remodelling stage is characterized by a decrease in cellularity, reduction of vascular vessels and reorganization of the ECM. In this phase, which begins 1-2 months after the initial injury and can last more than a year, a reorganisation of the ECM begins with a gradual change in collagen architecture where Col III is replaced by Col I. Nevertheless, the resulting scar tissue will never recover the elastic and biomechanical properties of the tendon prior to injury³⁵ (in the native tendon, the tensile strength equals to $500\text{-}1000\text{ kg}^3/\text{cm}^2$).¹⁸

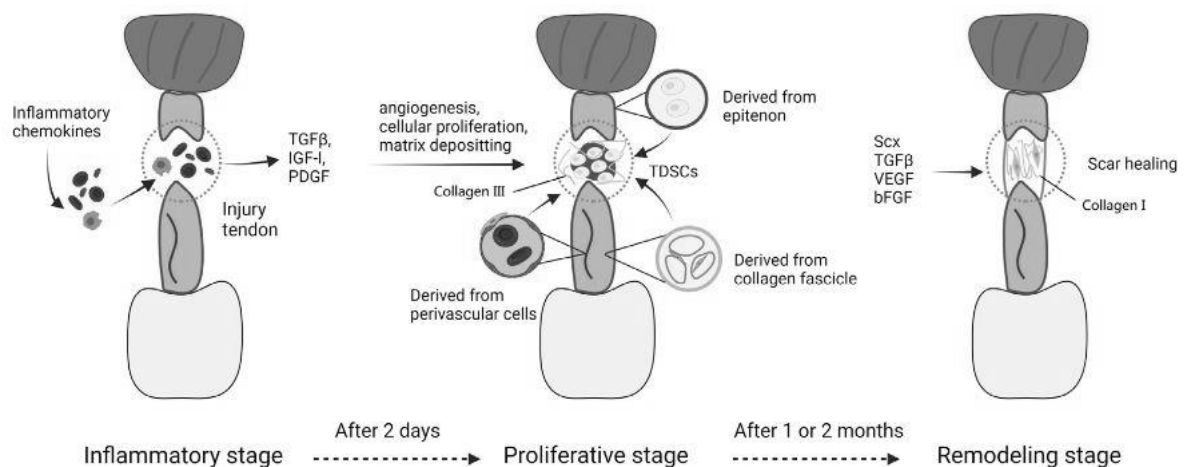


Figure 1.3 Schematic representation of natural tendon healing phases. Adapted from He et al., 2022.³⁴

The healing mechanism described above can occur intrinsically or extrinsically, depending on the tissue of origin of the tenocytes and cells recruited for the repair process. The intrinsic healing mechanism results from the proliferation and matrix deposition of epitenon and endotenon tenocytes, while the extrinsic healing starts when fibroblasts and inflammatory cells from tendon sheaths and synovium invade and proliferate forming adhesions in the healing site.²⁰ In principle, intrinsic healing mechanisms preserve the normal sliding within the tendon

sheath leading to fewer complications and stronger biomechanical characteristics. On the other hand, extrinsic healing leads to adhesions formations between tendons and the surrounding soft tissue preventing the normal sliding within the tendon sheath. The activation mechanisms of the extrinsic healing over the intrinsic depends on trauma severity, postoperative motion and tendon location,³⁵ for example, the extrinsic healing predominates in rotator cuff ruptures.²⁰

1.4.1 Fibrotic scar formation

The fibrotic healing of tendons is the main substantial obstacle preventing injured tendons from recovering and regaining adequate mechanical properties, similar to the intact tendon tissue. Disorganized and excessive ECM is deposited in the injury site, instead of a proper regeneration of the native tendon structure. This ECM is able to provide some stability to the tissue but without restoring the original mechanical properties and elasticity and, as a consequence, not only the risk of reinjury is higher,⁶ but it is also often considered the cause of subsequent degenerative and chronic tendinopathies¹. Tendon low cellularity and vascularity can be recognised as one of the main reason for this poor healing response.^{4,5} Moreover, several factors can have a negative impact on the healing response, such as old age, feminine gender³⁷ and other comorbidities (high cholesterol levels, obesity, smoking).³⁸⁻⁴⁰ It has also been shown⁴¹ how the complex anatomy surrounding certain tendons, further complicates their healing. This is the case of the flexor tendon of the hand which is often subjected to fibrous adhesion between the tendon and the surrounding tissues, which adds another limitation to the tendon's mobility.

Despite the molecular mechanisms behind the fibrous scar formation are still under study and remain unclear, improvements have been achieved in identifying the proteins responsible for the deposition and organisation of the collagen fibres during tendons maturation and healing. Scleraxis (SCXA) and Tenomodulin (TNMD) are two well-recognised proteins involved in fibrillogenesis and therefore participate to the spatial organisation of the collagen

fibres in particular of Col I, during many processes such as homeostasis, repair and development.⁴² More recently, two more transcription factors, the zinc finger transcription factor Early growth response gene 1 (EGR1) and the homeobox Mohawk (MKX), were found to upregulate the expression of Collagen I and its deposition within the tendon matrix, even though, as for TNMD as SCXA, MKW and EGR1 are not specific for tendons as they are expressed in other tissues.⁴²

1.4.2 Calcifications in tendon healing

Another possible complication that can occur during tendon healing is represented by the calcific tendinopathy (CT) which consists of single or multiple calcium-phosphate deposits in the tendon or in the subacromial bursa,⁴³ affecting more often the rotator cuff, the Achilles tendon or the patellar tendon.⁴⁴ CT symptomatology is similar to inflammatory symptoms including swelling, pain and joint stiffness⁴⁵ and, even though in the majority of cases the disease can be treated with a conservative therapy and solves itself, some patients express pain for a protracted period of time without any noticeable reabsorption of calcium deposits.⁴³ The causes of this phenomenon are still under debate, one of the first theories from 1938 stated that local ischemia and the consequent necrosis of the tendon tissue were the trigger to the calcium deposition.⁴⁶ Later studies identified other possible causes like minor traumas,⁴⁷ an initial cartilage metaplasia,⁴⁸ or a calcium intracellular accumulation consequent to tenocytes necrosis.^{46,49} Other theories address the disease to an active cell-mediated calcium deposit⁵⁰ or, most recently, to an erroneous differentiation of tendon-derived stem cells, resulting in an increased expression of Tissue transglutaminase 2 (TG2) and osteopontin.^{44,51} As previously mentioned, calcific tendinopathy often solves itself through re-absorption of the calcium crystals.^{44,52,53} One of the most accredited theory has been suggested by Uhthoff and

colleagues,⁵⁴ who indicated the involvement of cathepsin K-positive multinucleated giant cells and macrophages during an acute inflammatory response as part of the reabsorption phase.

1.4.3 Pathways involved in tendon healing and regeneration

While the signalling pathways and genes involved in bone, muscle and cartilage lineage have been identified, the same cannot be said for the tenogenic lineage. In fact, although some transcription factors and proteins like SCXA, TNMD and MKX have proved to have a strong influence on tendon development, their role has not been completely clarified, and their tissue-specificity still has to be demonstrated. For instance, TNMD is expressed also in a multitude of other tissue including adipose tissue and dermis.⁵⁵ In a similar way, SCXA can be found also in fibroblasts, muscle, heart, etc.^{56–58} Current theories indicate SCXA as the primary signalling molecule involved in the initiation of tendon progenitor cells, while MKX and EGR1 and EGR2 are involved in differentiation and maturation of immature tendon cells. TNMD is a later marker expressed by mature tendon cells which have been shown to be critical for maintaining cells self-renewal potential, regulating collagen deposition and TSPCs senescence,⁵⁹ as well as in the expression of other ECM-related genes.³⁴ Nevertheless, research is still ongoing and much still has to be clarified. Likewise, the signalling pathways involved in tendon regeneration and healing remain unclear. While several signalling pathways have been studied, Transforming Growth Factor β (TGF β) and SCXA seem to be the more accredited factors involved in tendons repair and regeneration.³⁴

- TGF β

In 2014, Havis et al.⁶⁰ showed how TGF β signalling pathway is involved in limb tendon development in mice, as well as at later development stages. The activation of TGF β pathway promotes the differentiation of TSPCs and, through Smad2/3 complex activation, it increases the expression of tenogenic genes leading to mature tenocytes. The expression of SCXA is

strictly bounded to TGF β pathway as proved by SCXA decreasing expression in zebrafish embryos when the TGF β pathway is suppressed,⁶¹ moreover, tendon related genes, including SCXA, Col 1a1 and Col 1a2, are down-regulated in mouse models when either TGF β or Smad2/3 signalling pathways are blocked.⁶⁰ Other ligands of the TGF β superfamily has shown to play a role in tendon formation. For example, animal models deficient in production of Growth and differentiation factor (GDF) -5, -6 and -7, showed an altered collagen structure and deposition. In contrast, higher collagen deposition have been found in tendon cells with increased GDF-5 activation;⁶² a similar behaviour was also found in mesenchymal stromal cells (MSCs) where an over-activation of GDF-5 increased the expression of tenogenic genes, downregulating the chondrogenic genes expression.⁶³ In other studies, GDF-7 promoted the expression of tenogenic protein in MSCs via the TGF β signalling pathway, which were induced to differentiate in tendon progenitor cells expressing increased levels of SCXA and Col 1a1.^{64,65} Nevertheless, despite the positive role of TGF β in tenocyte differentiation and tendon development, TGF β is also involved in fibrotic scar formation in a number of tissues including tendon.⁶⁶ Several studies have been carried out to understand the controversial role of TGF β but further investigations are still needed to solve this ambiguity. In 2020, for example, Kaji et al.⁶⁷ published a study where they found that TGF β signalling pathway does not have an influence in the early proliferation of SCXA+ tenocytes but, at the same time, the proliferation of SCXA- tenocytes and the consequent cells recruiting during functional recovery were TGF β -signalling dependant.

- FGF

The effects of Fibroblast growth factor (FGF) signalling pathways on tendon development has also been studied, but the results have been controversial depending on the species investigated.⁶⁸ In chicken embryos for example, FGF activates the Extracellular signal-regulated kinase/Mitogen-activated protein kinase (ERK/MAPK) signalling pathway, which

results in up-regulated SCXA expression.⁶⁹ Nevertheless, an opposite effect has been observed in mice, where the inhibition of ERK/MAPK leads to an increased expression of SCXA, Col 1a1 and Col 1a2 in tendon progenitors.⁶⁰ The role of FGF in humans is still not completely understood, but it is more likely to be analogous to the observations in the mouse model.³⁴ On the other hand, FGF positively participates in tendon repair, as showed in various studies demonstrating FGF2 positive effect in MSCs tenogenic differentiation both with and without⁷⁰ additional cell scaffolds.^{71,72} In 2015, Tokunaga et al.⁷³ studied the effects of FGF2 on rotator cuff healing in a rat model, and showed how the presence of the growth factor increased the mechanical strength of the tendon and induced TSPCs proliferation.

- Other growth factors

Other than the above-mentioned signalling pathways, there are other growth factors playing crucial roles in the different phases of tendon repair, even though their efficacy have been studied *in vitro* but it is still not completely proven *in vivo* (Table 1.2).³⁴ During the initial inflammatory phase, there is an upregulation of some growth factors like IGF-1, which is involved in fibroblasts and inflammatory cells recruitment in the injury region, while, in the later remodelling phase, it is involved in ECM production and collagen deposition.^{34,74} Similarly, VEGF is involved in the earlier inflammatory phase stimulating angiogenesis and increasing the nutrients provision, growth factors and extrinsic cells recruitment in the site of injury.^{75,76} Lee et al.⁷⁷ showed how connective tissue growth factor (CTGF) induces the recruitment of CD146+ TSPCs, stimulating cell proliferation and tenogenic differentiation. On the other hand, PDGF upregulates the expression of other growth factors by enhancing their gene translation and transcription.³⁴ It has also been shown that PDGF delivery in animal tendon injuries is able to increase the synthesis of ECM and to promote cell proliferation.⁷⁴ bFGF is another GF involved in the later proliferative and remodelling phases of tendon healing playing a role in angiogenesis, cell migration and proliferation and it has also been localised in

fibroblasts and inflammatory cells during tendon healing.⁷⁴ Lastly, bone morphogenetic proteins (BMPs), a group of growth factors from the TGF β superfamily, are not only involved in bone formation, but also in tendon cell mitogenesis and tendon repair.⁷⁸ In fact, it was shown that by delivering BMP-12, -13 and -14 it was possible to stimulate the formation of tendon-like connective tissue in ectopic sites.⁷⁴ BMP-12 has also been shown to have a positive role in collagen deposition and matrix organisation,⁷⁹ as well as in the expression of SCXA and TNMD in adipose-derived stromal cells, inducing tenogenic differentiation.⁸⁰ Nevertheless, even though it is evident that BMPs play a role in tendon healing, the mechanism remains unclear and requires further study.⁷⁸

Table 1.2 Growth factors involved in tendon healing and their main roles.

GF	Main roles in tendon healing
TGF- β	• Smad2/3 pathway activation, tenogenic genes transcription (SCXA, TNMD), • Fibrotic scar formation.
GDF	• Collagen deposition, • Expression of tenogenic genes (SCXA, Col 1a1).
FGF	• Tendon repair and healing, • MSCs tenogenic differentiation.
IGF-1	• Fibroblasts and inflammatory cells recruitment in the injury region, • ECM production and collagen deposition.
VEGF	• Angiogenesis stimulation.
CTGF	• CD146+ TSPCs recruitment, • Cell proliferation and tenogenic differentiation.
PDGF	• Upregulation of the expression of other growth factors, • ECM synthesis and cell proliferation.
bFGF	• Angiogenesis, • Cell migration and proliferation.
BMPs	• Collagen deposition and ECM organisation • TNMD and SCXA expression

1.4.3.1 Mechano-transduction mechanisms

Tendons are characterised by high elasticity and resistance to mechanical stress; these properties can be attributed to highly organised and aligned collagen fibres. It is not surprising that mechanical stimulation also plays an important role in tendon development, healing and homeostasis; even though the mechanisms by which the mechanical stimuli are sensed and processed by the cells are not yet completely clear.⁸¹ Nevertheless, some studies have shown how tendons can adapt to mechanical stress and transmit the stimuli to resident cells.⁸² In particular, *in vitro* and *in vivo* studies showed an upregulation of some tenogenic genes such as SCXA, TNMD, Col I, Col III and Tenascin C,^{83–86} as well as an increase in collagen fibril size,⁸⁷ when tendons were exposed to certain mechanical stimuli. Currently, TGF- β /Smad2/3 and FGF/ERK/MAPK are the signalling pathways considered to be involved in the mechano-transduction, referring to the activation of intracellular pathways in response to mechanical stimuli. In fact, it was demonstrated that the TGF- β /SMAD2/3 pathway can be activated by mechanical stimulation, leading to an upregulation of SCXA expression *in vitro*.⁸⁸ EGR1 also seems to be involved in tendon mechano-transduction as it has been shown that, in engineered tendon models, EGR1 was activated following a mechanical stimulus affecting the expression of tenogenic genes.^{89,90} Moreover, the regulation of EGR1 has been shown to be directly bounded to TGF β 2 gene expression.⁴² Therefore, one possible explanation could be that EGR1 can activate TGF β 2 in response to a mechanical stimulus, finally leading to the expression of tenogenic genes.⁴²

In particular, it was found that tendon-derived stem cells are induced to differentiate in different lineages when stimulated by either uniaxial or biaxial strain. In 2018, Wang et al.⁹¹ showed how uniaxial loading induces the phosphorylation of protein kinase B leading to the differentiation of tendon-derived stem cells in the tenogenic or osteogenic lineage, while biaxial

strain activated ERK phosphorylation, stimulating the differentiation towards the adipogenic or osteogenic lineage. They also showed how tendon markers like SCXA, MKX, Col I and TNMD were upregulated by uniaxial strain and inhibited by biaxial loading. Nevertheless, the optimum parameters setting such as amplitude, frequency, duration and percentage of strain still have to be determined. Several studies compared the impact that different loading regimens have on cell survival and tenogenic markers expression. For example, Wang et al.⁹² showed how a 9% strain, falling therefore outside the linear elastic region of tendons, causes more cell damage when compared to 6% or 3% strains. Likewise, tendon-derived stem cells were induced to differentiate towards tenogenic lineage when subjected to low strain stimulation, while higher strains were associated to osteogenic, adipogenic or chondrogenic differentiation.⁹³ Several studies^{94,95} also investigated the effects of different loading frequencies on tenogenic differentiation, coming to the conclusion that continuous cyclic strains negatively affect cell proliferation and increase collagen production, while intermittent cyclic strains lead to better results, increasing cell proliferation and cell alignment.

1.4.3.2 Activin A Receptor

The Activin family is a group of proteins of the TGF- β superfamily, acting through parallel signalling pathways to transduce an extracellular signal to the nucleus.⁹⁶ Activin is involved in several mechanisms such as hormonal homeostasis, embryonic development and organogenesis, follicle development, growth and differentiation of musculoskeletal tissues.^{97–99} Its receptor is a mechano-receptor which can be found in a variety of tissue and can be activated biochemically by its ligand, but also mechanically inducing the activation of the TGF- β /Smad2/3 pathway, leading to the transcription of tenogenic genes.^{100,101}

Activin is formed by heterodimers or homodimers of Inhibin subunits (β a, β b, β c, β e), held together by a disulphide bond. The different combination of the subunits leads to the

formation of the diverse Activin protein types, whose Activin A (Inhibin β _a heterodimers), B (Inhibin β _b heterodimers) and AB (Inhibin β _a and β _b heterodimers) are the most well-known and studied. Activin receptor is a heteromeric transmembrane protein formed by two types of receptors, type I and II. Activin A binds to one of the two type II subunit ACVRIIA (Activin A Receptor Type IIA) or ACVRIIB (Activin A Receptor Type IIB), which then recruits and activates a subunit type I, such as ACVRIB (Activin A Receptor Type IB, also known as activin-like kinase 4, ALK4). Three different type I receptors have been identified so far: ACVRI (Activin A Receptor Type I, also known as ALK2) which is mainly involved in the BMP signalling pathway;¹⁰² ACVR1B also known as ALK4, mainly takes part in Activin signalling pathway; and ACVRIC (Activin A Receptor Type IC, also known as ALK7) which predominantly participate in the Nodal signalling pathway.¹⁰³ Activated type II receptors recruit type I receptors and start the phosphorylation of the GS-box on the cytoplasmatic domain of the type I receptor, which leads to the activation of the Activin type I receptor kinase domain. The kinase domain then phosphorylate receptor-regulated Smad proteins (R-Smad).⁹⁸ The pathway activation of different TGF- β superfamily led to the recruitment of distinct R-Smad proteins. TGF- β and Activin signalling pathways phosphorylate Smad2 and Smad3, while Smad1, Smad5, and Smad8 are involved in BMP signalling.⁹⁸ In the case of Activin, Smad2 and Smad3 form a complex (the Smad2/3 complex) which, after recruiting and binding Smad4, moves in the nucleus. Here, Smad2 and Smad4 recognise and bind a Smad-binding element (5'-CAGAC-3') in the DNA sequence regulating gene transcriptions.⁹⁹ As previously mentioned, the TGF β /Smad2/3 is one of the most important pathways involved in tenogenic differentiation,^{104,105} whose activation leads to the transcription of tenogenic genes like SCXA and TNMD, towards the tenogenic phenotype (Figure 1.4).¹⁰⁶

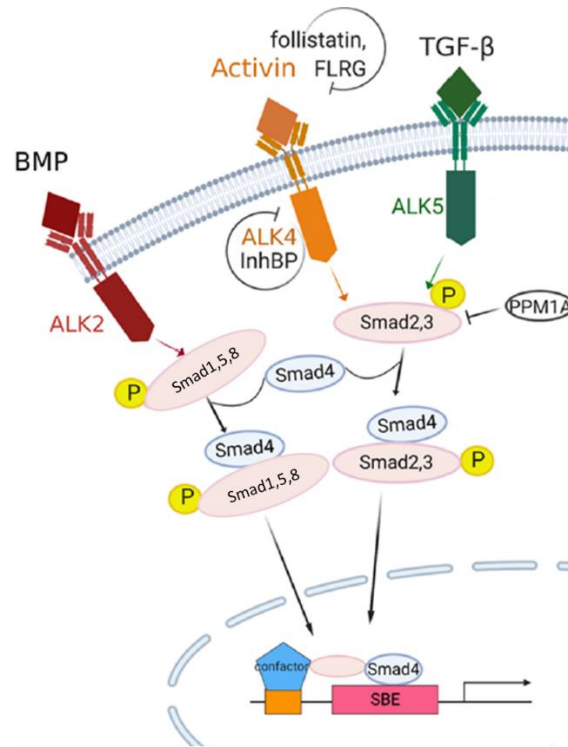


Figure 1.4 Schematic representation of the Activin A Receptor signalling pathway. Adapted from Qiu et al. 2021.⁹⁸

Protein Phosphatase PPM1A is one of the key regulators of Activin signalling pathway, in fact it is able to dephosphorylate the phospho-Smad2/3 complex leading to the relocation of Smad2 to the cytoplasm and preventing the genes transcription.¹⁰⁷ Other proteins can negatively regulate the Activin pathway, such as the extracellular antagonists inhibin, follistatin and follistatin-related gene (FLRG),⁹⁸ but also other membrane co-receptors, like Cripto,⁹⁹ which is necessary for the activation of the Nodal pathway but inhibits Activin signalling. Inhibin competes with Activin for the binding to type II receptors and its inhibitory activity can be enhanced by other co-receptors, like a membrane betaglycan which form a complex with inhibin and type II receptor, blocking the recruitment of type I subunit. Inhibin-binding protein (InhBP) is another inhibin co-receptor which, together with inhibin, associates to ACVRIB/ALK4 disrupting the binding to type II receptors. Similarly, FLRG can bind to receptor type II, antagonising Activin binding. On the other hand, follistatin whose two main

isoforms are Follistatin-288 and Follistatin-315,⁹⁶ inhibits Activin by directly binding the protein in the extracellular matrix or at the cellular membrane, disrupting the binding to its receptor. Alternatively, follistatin-288 can bind to activin inducing its endocytosis and lysosomal degradation.⁹⁸

In summary, depending on the mechanisms Activin signalling can be down-regulated at different stages of the pathway by direct binding the ligand, the receptor, or the downstream mediators.

1.5 Current clinical approaches to therapy

Despite the frequency of tendon injuries and their associated morbidity, the optimal treatment for tendons injuries and ruptures remains controversial.^{7,8} In general, it is possible to divide the current approaches to tendon repair into surgical or non-surgical treatments. The general trend in the recent years saw a decline of the incidence of surgical repairs compared to non-surgical treatments, considering surgical repair as last resort.^{7,108}

1.5.1 Non-surgical treatments

Non-surgical treatments include a wide range of approaches, such as low-level laser therapy (LLLT), extracorporeal shockwave therapy (ESWT) and injections (corticosteroid or platelet-rich plasma).

- Low-level laser therapy (LLLT)

LLLT was initially used in soft-tissues injuries and inflammation, but more recently its utilisation has also been applied to musculoskeletal injuries, such as tendinopathies. The exact mechanism involved in the improvement of tendons regeneration is still not clear, but the main theories sustain that LLLT is able to reduce the inflammation and to stimulate collagen

deposition.^{109,110} Other studies suggest LLLT's main role is to increase Adenosine 5'-triphosphate (ATP) production, protein synthesis and vascularisation through the release of specific cytokines like FGF2, VEGF.^{111,112} Still, the optimal treatment parameters, such as the timing of the treatment, the power intensity and the extension of the exposure region, are currently under debate.¹¹³

- Extracorporeal shockwave therapy (ESWT)

Shockwaves are generally biphasic and include a peak pressure that is a thousand times stronger than ultrasound waves. Shock waves are defined by their waveform, the number and frequency of the impulses, and energy flux density. As per LLLT, the mechanisms by which ESWT acts on tendon repair remain unknown, but the main suggested hypothesis implies an increased tenocytes proliferation followed by an increased collagen deposition and turnover, along with an improved vascularisation of the injured tendon.^{10,114} Moreover, it has been shown that the use of ESWT leads to an increased production of Lubricin, a glycoprotein naturally present in tendons ensuring the appropriate sliding of tendons within the surrounding tissues.¹¹⁵ In the musculoskeletal system, ESWT has shown excellent clinical outcomes, especially for the treatment of calcifying tendonitis of the shoulder, proximal plantar fasciitis of the heel, lateral epicondylitis of the elbow, non-union of long-bone fracture, patellar and Achilles tendinopathy.¹¹⁶

- Corticosteroids

Injections of corticosteroids are one of the most widely used therapeutic techniques for tendinopathy treatment, even though the therapeutic mechanism is poorly understood. In the past years, corticosteroids were employed in the treatment of tendinopathies as they are able to modulate anti-inflammatory cytokines production.¹¹⁷ Nevertheless, more recent studies found an increase of inflammatory cytokines levels after the injection, presumably in response to a

glucocorticoid-induced damage.¹¹⁸ The actual efficacy of the treatment is being questioned also considering the controversial outcomes in tendon mechanical properties restoration in patients undergoing steroids injections. Moreover, Smidt et al.¹¹ showed how corticosteroids injections reduce pain and bring a general improvement in the short term (<6 weeks), while on the long term (between 6 weeks and 6 months) no relevant positive outcomes were registered in comparison to placebo.

- Platelet-rich plasma (PRP)

By centrifuging large volumes of a patient's own plasma, it is possible to obtain the so-called "platelet-rich plasma" which consists in human plasma with a high platelet concentration.¹¹⁹ The PRP is currently used for a wide range of musculoskeletal indications including tendon injuries and tendinopathies. Platelets naturally contain a wide spectrum of growth factors, such as TGF- β 1, EGF, VEGF, IGF-1, PDGF and bFGF, which are concentrated after centrifugation of the patient's blood. Autologous PRP can be injected directly in the injury site or in conjunction with a surgical procedure to improve the tissue healing process, as well as promoting neovascularisation.^{120,121} PRP solutions can be either leukocyte-rich PRP (LR-PRP) or leukocyte-poor PRP (LP-PRP) depending on the neutrophil concentration. Nevertheless, there is not a generally-accepted strategy in terms of blood components concentrations or blood collection technique and processing. Furthermore, these variations often depend on the commercial provider rather than a more scientifically-accredited strategy compared to the others.^{120,122,123} Moreover, individual variabilities, like concomitant medications, increase the number of variables in PRP preparations, making particularly challenging the identification of the best protocol, as well as the interpretation of the clinical validity of this approach.¹²³ PRP showed positive outcomes in some tendinopathies treatment such as lateral epicondylitis, plantar fasciitis and patellar tendinopathy. While fewer or

inconclusive results have been reported for the treatment of Achilles tendinopathy, anterior cruciate ligament (ACL) reconstruction, rotator cuff surgical augmentation and Achilles tendon repair. Overall, PRP safety has been widely proved, but further studies are needed to find a generally-accredited protocol and medical indications for PRP use in musculoskeletal pathologies.¹¹⁹

1.5.2 Surgical treatments

Surgical treatments are considered the last resort for tendon repair and are applied in case of complete rupture or extensive tendon tears. The reason for this reluctance in relying on surgical procedures is the high rate of re-ruptures of the same tendon, which goes up to 94% for large rotator cuff tears.¹²⁴ Moreover, the outcome of surgical repairs has been associated with several risk factors such as patient age, smoking, other concomitant diseases, muscle atrophy or fatty infiltrations.¹²⁵ Surgical options include open or minimally invasive repair techniques (arthroscopy); these last, have decreased the risk of complications, like wound infections, but without showing a significant difference in the rate of post-surgery re-rupture compared to the normal surgical procedures.^{7,8} Surgeons may operate using different suturing techniques depending on the tendon to be fixed; interestingly, it has been shown that the kind of suture has an impact on the strength of the repair and on the surgery outcome. For example, braided sutures should be preferred to monofilaments for arthroscopic rotator cuff repairs.¹²⁶ When primary repair is not possible, as in case of tendon loss or retraction, tendon autografts can be used to help tendons reconstruction. For example, semitendinosus autografts have been used for distal biceps tendon rupture¹²⁷ or chronic patellar tendon rupture,¹²⁸ while hamstring autograft has been employed for pectoralis major tendon tear.¹²⁹ On the other hand, autografts are harvested from other donor sites which can lead to donor site morbidity.³⁵

Surgical procedures are usually followed by rehabilitation regimens, which can be painful for the patient and extremely time-consuming. Nevertheless, controlled mechanical loading and mobilization has shown to be useful in promoting tenoblasts activity, increasing tendon tensile strength and fibres diameter.³⁵ In the clinic, physical exercise post-surgery proved to increase the production of new collagen and proteoglycans in the long term, as well as the release of pro-inflammatory cytokines (IL-1 β) and growth factors. It was also shown that tensile forces enhance the production of Decorin, the proteoglycan that creates crosslinks between collagen fibres.¹⁸ In some cases, like the supraspinatus tendon, a reduced load can be beneficial for tendon's healing, as showed by the improved ECM organisation and tendon's stiffness in a rat model in comparison to other treatments.¹³⁰ At the same time, complete immobilisation proved to be detrimental for supraspinatus injuries treatment, decreasing the overall mechanical properties, stiffness and cross-sectional tendon area.^{131,132} Similarly, in a rat Achilles tendon injury model, a prolonged immobilisation showed to be counterproductive, as it caused a decrease in tendon healing-related genes such as bFGF and IGF-1.¹³³ Taken together, while reduced load can be beneficial in some cases, early rehabilitations regimens should be preferred to tendons immobilisation. The intensity of the physiotherapy sessions is driven by a delicate balance between the risk of re-rupture associated with excessive loading, and the risk of stiffness and adhesions formation in response to immobilisation; therefore, rehabilitation regimens need to be carefully planned depending on the tendon affected and the severity of the lesion.¹¹³

1.6 Tissue engineering in tendon repair

Tissue engineering is a relatively new research field of regenerative medicine emerging with potential alternative treatment strategies for tendon injuries. Resorting to cells, bioactive factors, nanotechnology tools and bioengineered constructs, it aims to create biological

substitutes mimicking native tissues and organs for diagnostic, research and treatment purposes. With the purpose to recreate the native tissue from a biological, biochemical and biomechanical point of view, tissue engineering targets organs and tissues particularly susceptible to injury or degeneration with limited healing properties.¹³ Regenerative medicine and tissue engineering approaches are showing promising results in several applications including tendon repair,^{14–16} aiming to replace the injured tendon or to repair the damaged site offering a possible solution for this unmet clinical need.

1.6.1 Growth factors and stem cells

In the past few years, stem cell transplantation and growth factors have been proposed as an alternative strategy to surgery for tendon repair, showing the possibility to improve the healing response and decrease the scar formation.^{134,135} In terms of growth factors, despite several recent studies investigated the effects of the supplementation of different cocktails of growth factors, a well-defined media formulation for tenogenic differentiation is still missing.^{80,136} The limiting factor is the poor knowledge of tendon biology and tendon markers, compared to other more defined tissues like bone or cartilage.^{55–58} Perucca-Orfei et al.¹³⁷ highlighted the controversial effects of TGF- β 3 in adipose stem cells (ASCs), bone marrow stem cells (BMSCs) and tenocytes as it promoted the expression of SCXA, but also inhibited the maturation of collagen fibres at later stage. This suggested the possible involvement of TGF- β 3 in the early phases of tenogenic differentiation rather than in later ECM deposition stages.¹³⁸ In the same study, it was suggested the involvement of other factors like CTGF and IGF-1 in matrix production rather than in the initiation of the tenogenic differentiation.¹³⁸ Other works focused on the role of ascorbic acid (AA) which showed to induce collagen production and SCXA synthesis,^{139–141} as its absence in culture media led to the complete loss of tendon ECM markers.¹³⁷ BMP-12 is another growth factor that showed to be critical in tenogenic

differentiation, as it promoted the synthesis of SCXA and TNMD in ASCs.^{64,79,80} Interestingly, some studies^{139,142} highlighted the importance of serum reduction in GF-enriched media preparation, as a decisive factor for tenogenic differentiation enhancement. In particular, Stanco et al.¹⁴² found that the supplementation of AA, TGF- β 3, BMP-12 and CTGF was able to stimulate ASCs towards tendon markers expression, when used in correlation with a 5% Human Platelet Lysate-enriched media.

At the same time, several studies have been carried out with different sources of stem cells, delivery systems and cells densities, pointing out not only the possibility to use stem cells as a treatment strategy, but also showing the advantages of the use of these cells compared to mature and differentiated cells (such as tenocytes and fibroblasts). In fact, MSCs are characterised by a high proliferation and differentiation rate, being also able to provide the signals to induce tendon regeneration, compared to differentiated cells.¹³⁵ Moreover, MSCs can secrete a wide range of immunomodulatory cytokines and growth factors such as TGF β 1 and prostaglandin E2, increasing these cell immunomodulatory and paracrine capacity.¹⁴³

The most used sources of MSCs in tendons regenerative medicine are BMSCs and ASCs. Several studies and reviews have been published exploring the regenerative potential of these two stem cells sources. One of the main aspects that would push the research towards the use of ASCs rather than BMSCs, is the higher cells number that is possible to isolate from the same amount of tissue.¹⁴³ In fact, it is reported that for every mL of bone marrow aspirate it is possible to isolate about 6×10^6 cells, but only 0.01% being stem cells. On the other hand, it is possible to isolate 2×10^6 cells from every gram of adipose tissue, with about 10% being stem cells.^{144,145} ASCs also show a higher proliferation rate, compared to BMSCs, without losing their multipotency.¹⁴⁶ It has been shown that injections of adipose derived perivascular cells can enhance Achilles tendon stiffness and mechanical properties during healing.¹⁴⁷ Norelli et al.¹⁴⁸ showed that the use of tenogenically-induced ASCs on a collagen/alginate hydrogel can

significantly improve the tissue organisation and gene expression in a mouse tendon model. Nevertheless, bone marrow still represents a valid source for stem cells in regenerative medicine. For example, Ge et al.¹⁴⁹ reported that BMSCs have a higher collagen excretion *in vitro* and a longer survival rate compared to fibroblasts. It has also been shown that BMSC immunogenicity is low^{150,151} and it can be controlled by modulating the cells culture conditions.⁴³ In another study,¹⁵² ASCs, BMSCs and PRP were compared in the treatment of tendon lesions induced in a horse model. They recorded a better outcome for the treated tendons compared to the control group and, despite the differences being quite small, they found an increased gene expression of tenogenic genes such as Decorin, collagen I and Tenascin in the group treated with BMSCs.

1.6.2 3D scaffolds and delivery methods in tissue engineering

In tissue engineering, scaffolds play a vital role in enhancing cell attachment, proliferation and tissue regeneration. Several constructs have been investigated in order to find the most suitable material that recapitulates the shape, function and mechanical properties of native tendons. The ideal tissue engineered construct should meet several requirements: i) being biocompatible, ii) not inducing a host immune response, iii) holding and supporting cells, iv) being biodegradable, v) having an adequate structure that mimics the native tissue and allows for nutrients and cells infiltration, vi) being cost effective.^{153,154} Moreover, effective tendon engineered constructs need to have excellent mechanical and elastic properties, considering the high loads and mechanical stresses tendons are usually subjected to. It is also important that the scaffold does not degrade faster than the ECM deposition speed, otherwise the reconstructed tissue would be too weak and likely to rupture (Figure 1.5).¹⁵³

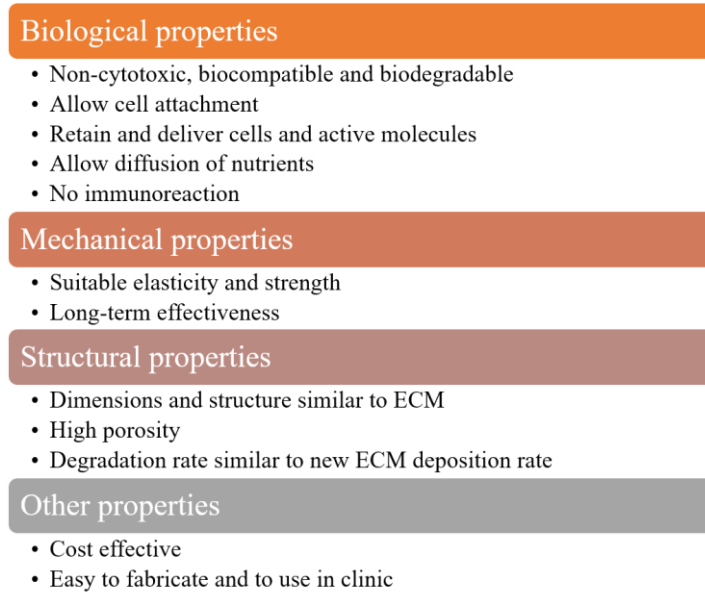


Figure 1.5 Summary of the ideal scaffold main characteristics for tissue engineering purposes.

Scaffolds can be divided in three categories: biologic, synthetic or composite. Biologic scaffolds include natural polymers and decellularized ECM of human or animal origin.¹⁵⁵ For example, they can be decellularized non-tendinous tissues like dermis or small intestine, or autologous decellularized tendinous tissues.^{153,155} The main advantages of biologic scaffolds are the biocompatibility, biodegradability and capacity to mimic the native tissue. On the other hand, their availability can be limited, especially if they require harvesting from another biological source, which also implies destructive harvesting techniques that might damage the harvested tissue and the organ of origin.¹⁵³ In contrast, synthetic scaffolds have unlimited availability and their properties can be adjusted by changing the starting material, composition, architecture, size, etc. The main disadvantages are, between the others, the possible unwanted host immunoreactions, undesired degradation compounds or poor cell attachment.^{153,155} Lastly, composite scaffolds consist of a combination of natural and synthetic materials. Several materials combinations have been investigated, such as poly (L-lactic-co-glycolic acid (PLGA) and fibrin,^{79,156,157} polycaprolactone (PCL) and collagen,¹⁵⁸ silk/fibroin with PLGA and collagen¹⁵⁹ or methacrylated gelatin with PCL.¹⁶⁰

1.6.2.1 Collagen

Among biologic polymers, collagen is one of the most studied materials for tendon tissue engineering. In fact, collagen is biocompatible, biodegradable and, importantly, it is the main component of the ECM in native tendon tissue. In the native tendons, collagen is highly organised in fibrillar parallel structures that provides the elastic properties and mechanical strength typical of tendons. For years, researchers have tried to recreate this collagen alignment *in vitro*, sometimes combining it with other materials to increase the mechanical strength. Chen et al.¹⁶¹ prepared a composite scaffold made of collagen and woven silk fibres that showed to have better mechanical properties, more similar to the natural tendon, than silk on its own. Moreover, they showed that the cells were better aligned and they preserved a good proliferation rate within the scaffold matrix. In another study, Webb et al.¹⁶² found that a composite scaffold of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), collagen and tenocytes was useful in accelerating the return to function post-surgery in an Achilles tendon repair rat model. Additionally, tenocytes were able to migrate to the scaffold core also becoming more organised on the fibre surface within the scaffold body. Other studies investigated the use of cell-seeded collagen hydrogels for tendon repair strategies. Awad et al.¹⁶³ used MSCs-seeded collagen gels in the repair of a patellar tendon defect in a rabbit model and they observed how the scaffold increased tendon's mechanical load bearing capacity. Nevertheless, the tensile strength of the tendon post-recovery was not the same as the native tissue, indicating that a full recovery was not achieved. A similar study¹⁶⁴ obtained better results in terms of mechanical strength by seeding tenocytes instead of MSCs in a collagen scaffold to repair a tendon-bone junction defect of the infraspinatus tendon in a sheep model. After 12 weeks, the tensile strength of the treated tendon was only 10% lower than the native tendon tissue. Moreover, they also observed an increase of proteoglycans and Col III production compared to controls.

Importantly, several techniques have also been employed to prepare oriented-fibres collagen scaffolds rather than scaffolds with randomly-aligned collagen fibres. In fact, by recapitulating the native tendon tissue structure, the alignment of collagen fibres proved to have an importance as strong as the material itself in the regeneration of damaged tendons. Extrusion,¹⁶⁵ electrochemical^{166,167} and microfluidic¹⁶⁸ are just a few examples of the range of strategies investigated to create aligned collagen scaffolds. For instance, Butler et al.¹⁶ used counter-rotating extrusion to manufacture an aligned collagen membrane that was then used as scaffold for BMSCs seeding. The membrane was tested *in vitro* and *in vivo* in Achilles tendon defect repair in a rat model, where it promoted a better regeneration of the damaged tendon compared to non-aligned scaffold, including tendon protein expression, fibres thickness and cell density. Nevertheless, despite these good results, the mechanical properties of the recovered tendon were still quite inferior compared to the native tendon tissue.

1.6.2.2 Fibrin

Fibrin is another well-known biopolymer used as biologic scaffold in tendon tissue regeneration. By combining the right ratio of thrombin and fibrinogen, fibrin gels can recreate the last step of the coagulation cascade resulting in a clump of fibrin.^{169,170} One of the interesting characteristics of fibrin gels, is that they can adhere to the surface or tissue where they are delivered on. This not only implies that fibrin gels can be used in surgeries as a bioadhesive for haemostasis and wound closure,^{171–173} but they can also provide a binding site for cells implantation promoting tissue regeneration.^{174,175} Fibrin has nonlinear elastic properties and it is therefore able to stretch when small strains are applied, or to stiffen with bigger strains.¹⁷⁶ Interestingly, fibrinogen and thrombin concentrations can be adjusted to achieve final fibrin gels with different mechanical properties, for example, it has been shown that fibrin gels at high fibrinogen and thrombin concentrations were stiffer and had higher failure loads compared to

gels prepared with lower concentration of both components.^{153,177} Fibrin has unquestionable biocompatibility as testified by the use at clinical grade of many fibrin sealants, like Tisseel from Baxter¹⁷⁸ or Beriplast from CSL Behring,¹⁷⁹ which are currently used in a variety of surgical procedures including haemostasis, tissue sealing and wound closure.^{171,173,177,180} The above mentioned fibrin sealants are characterised by a very high concentrations of fibrinogen (80-120 mg/mL) and thrombin (300/600 NH U/mL) compared to physiological values (circulating fibrinogen ~2/4 mg/mL), in order to ensure quick polymerization and setting time during surgeries.¹⁸¹ Nevertheless, high concentrations lead to a very dense fibrin network which inhibits cell migration, suggesting that intermediate concentrations should be preferred for cell delivery purposes.^{177,181}

Fibrin gels have recently been investigated as cell delivery methods for a wide number of tissue engineering applications, including bone, cartilage, tendon and skin,¹⁷⁰ especially thanks to several characteristics making them advantageous for cell delivery purposes. First of all, fibrinogen has a porous architecture that facilitates cells infiltration, as well as a number of binding motifs for cells (like fibroblasts, platelets, epithelial cells) and growth factors (including TGF- β , PDGF, VEGF, FGF) promoting tissue healing, cell regeneration and angiogenesis.¹⁸¹ Fibrin is also biodegradable and can be enriched with growth factors while still in the liquid phase, in order for them to be gradually released during the scaffold degradation *in situ*.¹⁸¹ Another important advantage of fibrin is that it can be used as injectable therapy for cell delivery purposes, in particular liquid-phase fibrin can be injected *in situ* and allowed to polymerise filling the wound space reducing significantly the gap between the native tissue and the scaffold.^{170,175} This approach has already been adopted in a few tendon tissue engineering applications even though it is a quite unexplored field compared to other tissues. For example, Hankemeier et al.¹⁸² suspended BMSCs in a liquid fibrin gel which was then injected into a patellar tendon defect in a rat model. The study revealed how the cells and scaffold combination

led to a more homogeneous ECM deposition and a more even cell distribution. A similar experiment¹⁸³ was performed on a rabbit Achilles tendon defect model, and it was shown how the injection of BMSCs and fibrin improved the biomechanical properties of tendons in the early phases of healing. The last advantage is that fibrinogen and thrombin could be isolated from the patient's own blood and, in addition to autologous embedded cells, this would minimise the risk of rejection or adverse immune-reactions.¹⁸⁴

1.7 Animal models of tendon injuries for tendon tissue engineering

As previously discussed, a wide number of biological constructs, materials and tools have recently been developed in tendon tissue engineering research. However, even though *in vitro* tests are necessary for protocol optimisations and initial testing, they can only provide preliminary information on the mechanical characteristics and biocompatibility of a new material or construct. *In vivo* testing in an appropriate animal model is a fundamental step in the development of new tissue engineering techniques before translation into clinic. In order to conduct a critical and realistic evaluation of a new technology *in vivo*, it is paramount to choose the most appropriate animal model for each investigation. Furthermore, it is essential to choose a model allowing the reproducibility of the condition across animals (injury creation, either with or without repair), as well as an adequate tendon manipulation in consideration of the intervention to be performed. Several parameters need to be considered for an appropriate choice, such as the anatomical structure of the tendon (which can be intrasynovial or extrasynovial) and the type of injury to be modelled.¹⁸⁵

- Anatomical structure of the tendon

Intrasynovial tendons differ from extrasynovial tendons by the presence of an external synovial sheath, containing the synovial fluid lubricating the tendon, which in some cases is also covered by an additional fibrous sheath.^{17,20} Because of this anatomical arrangement, the blood supply

in this type of tendon is more limited and, as a consequence, they recover differently from injuries compared to extrasynovial tendons.¹⁸⁵ The flexor tendons of the hand are examples of intrasynovial tendons and have historically been considered a valid model for adhesion formations. Several animal models have been employed for *in vivo* modelling of flexor tendons healing and adhesion resolution, including chicken,^{186,187} rabbits^{188,189} and dogs.^{190,191} On the other hand, extrasynovial tendons include the patellar tendon, the Achilles tendon and the supraspinatus. Intuitively, the Achilles tendon is among the most chosen models for tendon injuries both because of the high frequency of Achilles tendon injuries^{192,193} but especially because of its size. This tendon is one of the largest extrasynovial tendons and it is therefore easy to handle even in small animal models. *In vivo* models of Achilles tendon injuries, predominantly rat and rabbit, are often employed in the development of prostheses¹⁹⁴ or in tendon-to-bone injury repairs.^{194,195} The patellar tendon, because of its size and easy accessibility due to its superficial location, has been used in several animal models including mice,¹⁹⁶ rabbits⁶ and rats,¹⁹⁷ often in the modelling of a partial tendon injury.¹⁹⁴ While different animals are considered as acceptable in Achilles and patellar tendon injury models, rats represent the most valid choice for supraspinatus injury models.^{194,198,199} This is due to a high similarity with human rotator cuff anatomy, which make the rat as the preferred choice in supraspinatus injury modelling.

- Type of injury

The wide number of tendon injury types can be classified in three main categories: i) partial tendon injury/removal, ii) tendon rupture without gap formation and iii) complete tendon rupture with formation of a gap between the resected ends. In the first two cases, no significant gap is created within the tendon and healing usually occurs spontaneously over time even without treatment. These models are therefore preferred when investigating the acceleration or the improvement of the repair in a partial tendon injury.¹⁸⁵ Beason et al.¹⁹⁶ evaluated the

feasibility and reproducibility of partial injuries in different tendon models in mice. They found that, in comparison to the Achilles tendon and the flexor tendon of the hand, the patellar tendon gave the best results in terms of reproducibility of the injury, general handling and overall differences compared to the native tissue. On the other hand, the rotator cuff and the ACL are the two main tissues employed in the modelling of a complete rupture creation with gap formation.¹⁸⁵ Contrarily to partial tendon ruptures, this kind of injury does not heal spontaneously and requires a reconstructive surgical intervention adding a prosthesis between the torn ends.²⁰⁰ Such prosthesis can be engineered constructs, like collagenous scaffolds, but also autografts, or decellularized allografts and xenografts.²⁰¹ The last two have recently been investigated in different animal models, including rabbits,^{202,203} dogs,²⁰⁴ and mice,²⁰⁵ for the repair of complete tendon ruptures as they possess limited immunogenicity and can be repopulated with different cells types (tenocytes, hASCs or hBMSCs).²⁰¹

To summarise, there is a wide range of tendon injuries and potential animal models available and, as there are no widely-accepted indications about a most suitable animal/defect model for tendon injuries repair, each final choice is case-specific.

1.8 Magnetic strategies for tendon tissue engineering

1.8.1 Magnetic nanoparticles and magnetic scaffolds

The use of nanotechnologies is being widely investigated as a way to improve tissue regeneration strategies. Nanoparticles have gained a particular interest due to their great versatility as their shape, composition and size can be tuned depending on the required application.²⁰⁶

Since 1970s, when magnetic resonance imaging (MRI) moved from the laboratory to the clinic, the possibility to use magnetic nanoparticles (MNPs) for medical applications

became more tangible and, from the 1990s, research publications in this field increased dramatically.²⁰⁷ Generally speaking, four main applications of MNPs can be identified in biomedical applications: imaging, such as MRI contrast agents and positron emission tomography (PET); cell sorting; immunoassays, like Enzyme-linked immunosorbent assay (ELISA); and therapy, where MNPs can be used for targeted drug delivery, stem cell therapy or tissue engineering (Figure 1.6).^{206,207}

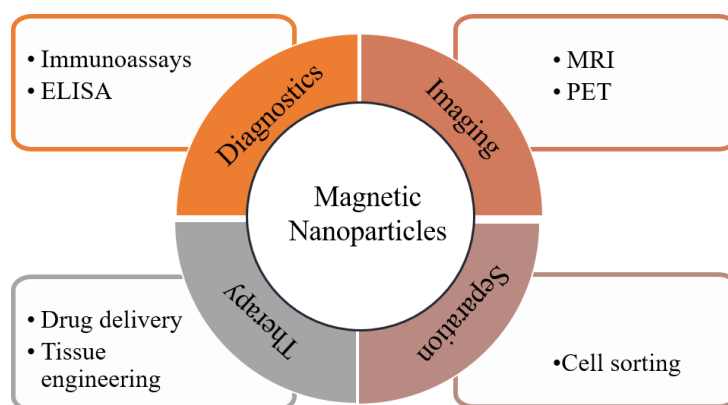


Figure 1.6 Summary of Magnetic Nanoparticles main applications.

Iron oxide nanoparticles (IONPs) are one of the most promising and investigated nano magnetic materials for medical purposes due to their magnetic properties, wide size range and the possibility to be functionalised with different bioactive substances.^{206,208} In fact, IONPs versatility can be mostly addressed to the possibility to coat their surface with organic materials which increase the nanoparticles stability and biocompatibility. There is a wide range of materials suitable for MNPs coating, with polymers (like polyethylene glycol (PEG), dextran,²⁰⁹ polyvinylalcohol or chitosan)^{210–213} and single small molecules or proteins (such as albumins)^{210,214,215} being the most popular. Moreover, reactive groups available on the primary coating (such as -COOH, -NH₂, -OH or -SH) can be functional to bind additional bioactive molecules like antibodies, peptides, enzymes or drugs to the MNPs, widening their field application range.^{211,216–219} Another factor playing an important role in determining MNPs bioactive function is the nanoparticles size, since it plays a key role in regulating MNPs

biodistribution and their capacity to overcome biological barriers.²¹⁹ It has been found that smaller particles with a diameter below 10 nm are eliminated by the kidneys, while bigger MNPs are picked up by the reticuloendothelial system in liver and spleen, and by macrophages. Bigger particles have therefore been employed in spleen and liver imaging or in the identification of areas of inflammation.²¹⁰ Even larger MNPs (20 nm-150 nm) have a longer circulating time in blood vessels and they can also accumulate in other organs like the stomach, which makes them a good instrument for drug delivery or cancer imaging.²⁰⁶

Magnetic nanoparticles have been widely investigated in a multitude of tissues for regenerative purposes, such as bone,²²⁰⁻²²² cartilage,^{223,224} heart²²⁵ and neuronal cells.²²⁶⁻²²⁹ Only in more recent years, MNPs have also been applied to tendon tissue as a cell-tracking method or as tools to induce stem cells differentiation. Scharf et al.²³⁰ were able to detect by MRI MSCs labelled with IONPs up to 7 days from the implantation in a sheep model of tendinitis. A similar study carried out by Kremen et al.²³¹ demonstrated how IONPs-labelled murine mesenchymal progenitor cells were detectable by MRI after implantation in surgically-created Achilles tendon defects in nude rats. Both studies showed how IONPs allowed the monitoring of the implanted cells by MRI, evaluating their viability, distribution and turnover over the time. In another study,²³² superparamagnetic iron oxide (SPIONs)-labelled mesenchymal stromal cells were used to investigate the cytocompatibility of tendon scaffolds obtained by decellularization of horse tendons specimens. By MRI-imaging, they verified the effective decellularization and biocompatibility of the grafts, achieved by subsequent freeze-thaw cycles in combination with Triton X-100.

MNPs have also been employed in the preparation of aligned scaffolds to recreate native tendon ECM architecture. Hiraki et al.²³³ generated aligned scaffolds using synthetic electrospun fibres with embedded SPIONs. They used an external magnetic field to adjust the alignment of the fibres which were then functionalised with cell adhesive peptides promoting

the uniaxial alignment of seeded tendon fibroblasts. An oriented collagen hydrogel was achieved by Xu et al.²³⁴ by remotely-aligning IONPs embedded in a Col I matrix. They showed how the alignment of the fibres induced the orientation of seeded human TSPCs and the expression of tenogenic genes.

Furthermore, the combined use of MNPs and a magnetic field have been recently explored to drive stem cells tenogenic differentiation. Gonçalves et al.,^{235,236} built a magnetic starch and polycaprolactone scaffold with oriented fibres showing how a magnetic stimulation together with the fibres alignment were useful to promote tenogenic differentiation in hASCs. In another study, a tendon mimetic fibrous scaffold decorated with MNPs were used to seed and magnetically stimulate hASCs, which revealed an improved anisotropic organisation of the cytoskeleton and an increased expression of tenogenic markers after magnetic stimulation.²³⁷ In a study published in 2016,²³⁸ MNPs were employed to develop a magnetic responsive tendon-to-bone interface construct. In particular, hASCs and tendon derived stem cells were seeded on MNPs-embedded hydrogel constructs enriched with platelet lysate and stimulated with an external magnetic field. They showed how the magnetic field application had an impact on cell morphology and matrix deposition, as well as on the degradation and release of platelet-derived growth factors. Interestingly, a magnetic scaffold-free construct has also been developed by Gonçalves et al.;^{239,240} briefly, MNPs were internalized in hASCs cultured at high density in order to obtain a magnetic cell sheet which can take advantage of a self-deposited ECM with the mechanical reinforcement provided by the MNPs. Moreover, the cell sheet could also be magnetically stimulated to modulate the tenogenic differentiation.

Interestingly, Wu et al.²⁴¹ investigated *in vivo* the effects of exosomes produced by MNPs-actuated BMSCs on tendon-to-bone healing. Briefly, an ACL reconstruction was performed by using a tendon autograft which was then injected with exosomes produced by

MNPs-actuated BMSCs (IONP-Exos). They showed how the use of IONP-Exos, in comparison to normal exosomes, significantly improved the repair, preventing bone loss, increasing fibrocartilage formation and enhancing the mechanical strength of the grafted tendon.

To summarise, the application of magnetic nanoparticles and magnetic materials in tendon tissue engineering is quite recent and more unexplored compared to other tissues or other medical applications. Nevertheless, the outcomes of the research studies performed so far are extremely promising and there is good potential for further improvements in magnetic nanoparticle applications for tendon healing and regenerative treatments.

1.8.2 Bioreactors in tissue engineering

The importance of recreating *in vitro* the natural microenvironment has become evident for tissue engineering applications. Nevertheless, its realisation is one of the biggest challenges of this research field, as the native microenvironment is characterised by a complex equilibrium of cells, signalling molecules, structural components and physiological stimuli. In fact, as previously discussed in Paragraph 1.4.3.1, biochemical signals are not the only factors influencing the biological processes such as tissue development, organisation and healing, but biomechanical stimuli are able to activate intracellular pathways impacting cell fate and behaviour. For these reasons, regenerative medicine has been focusing on the development of bioreactors able to provide biophysical stimuli to the cells/scaffolds in culture replicating tissue-specific requirements.^{242,243} Depending on the bioreactor type, it is possible to provide static and/or dynamic culturing conditions to scaffold-seeded cells. In static conditions, the bioreactor only provides a culture medium motion in order to equally distribute nutrients and shear stress stimuli, while in dynamic conditions, the bioreactor also provides a time-dependent stimuli, whose characteristics depend on the specific tissue under investigation.²⁴²

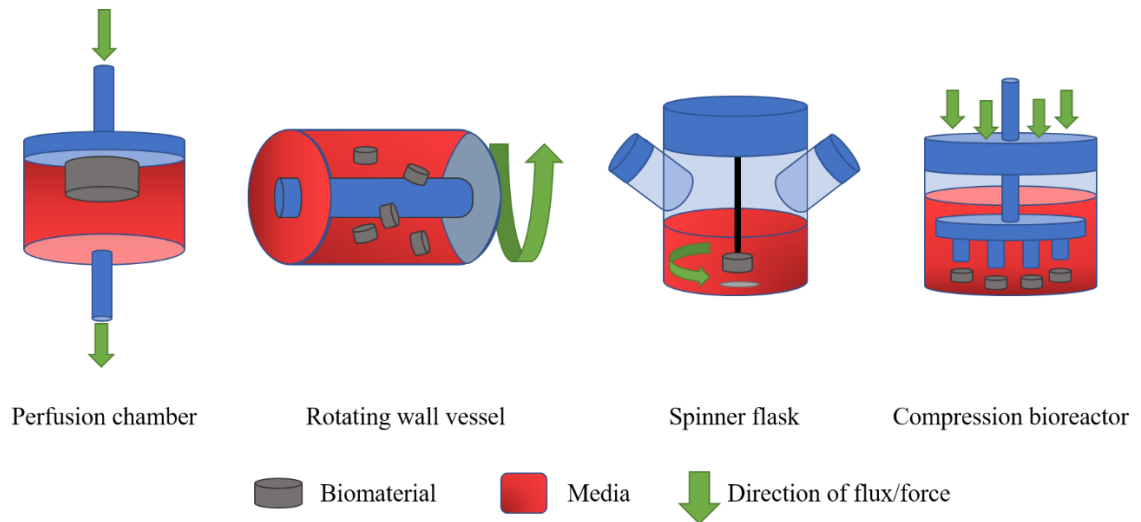


Figure 1.7 Schematic representation of the main bioreactors categories for tissue engineering applications.

Bioreactors can generally be classified in four main categories (Figure 1.7), depending on the system structure and the type of stimulus provided to the cells and constructs:^{242,244}

- Perfusion bioreactors; in these bioreactors the cells constructs are placed in a main chamber which is connected to a pump making the medium flow either from the bottom to the top of the chamber or vice-versa.²⁴⁴ They can be designed in several configurations, for example, the medium can recirculate in “closed loops” or in “single pass”, where fresh medium is provided continuously.²⁴²
- Rotating wall vessels; these systems are formed by a static inner cylinder and an outer rotating cylinder containing the medium and the biomaterial. The main key of this bioreactor consists in an equilibrium between the rotation rate, the centrifugal force and drag force. This way, a sort of microgravity is created within the bioreactor allowing the biomaterial to grow in a dynamic culture environment, but with a low shear stress exposure.²⁴⁵
- Spinner flasks; also known as stirred bioreactors, they consist in a closed tank, usually a cylinder, containing a stirring system such a magnetic stirrer. The cell construct is

immersed in the stirring medium by means of a metallic structure hanging from the lid of the tank. While stirring, the medium improves the oxygen and nutrients delivery to the cells and provides shear stress that proved to be useful for some tissue engineering applications. Nevertheless, the appropriate stirring speed must be accurately evaluated, in order to avoid an excessive destructive turbulence.²⁴⁴

- Mechanical loading bioreactors; considering the broad variety of stimuli that human body is exposed to in daily life, a wide range of mechanical bioreactors have been designed depending on the kind of stimulus to recreate.²⁴² For example, compression bioreactors have been used for bone²⁴⁶ and cartilage²⁴⁷ tissue engineering, while stretching bioreactors have been employed in muscles²⁴⁸ and tendons.²⁴⁹ Even electrical stimuli bioreactors were designed and they could be used either alone²⁵⁰ or in conjunction with another mechanical stimulus.²⁵¹

1. Bone

In bone tissue engineering several bioreactors designs have been used. Perfusion bioreactors for example, have shown to be useful in improving cells differentiation in the osteogenic lineage when used in conjunction with a 3D scaffold. For instance, bone marrow stromal osteoblasts seeded on titanium fibres mesh scaffolds increased their mineralised matrix production when cultured in presence of fluid-induced shear stress, compared to static conditions.²⁵² In fact, the continuous media pumping through the chamber mimics the fluids movement in bone trabeculae, stimulating the cells to differentiate, as testified by the increased expression of osteopontin and alkaline phosphatase activity.²²¹ The use of spinner flask bioreactors have been studied as well and it was demonstrated how, when using these systems, bone engineered constructs showed an improved cells distribution and viability when compared to static conditions.²⁴⁶ Similar interesting results were achieved also with rotating wall vessels, which demonstrated to improve the expression of osteogenic markers and nodule mineralisation;

moreover, cell proliferation was higher compared to spinner flasks and static conditions.²⁵³ A wide range of mechanical bioreactors have been used to study the impact of mechanical stimuli on engineered bone tissue constructs. Perfusion compression bioreactors have been studied in human bone mesenchymal stromal cell 3D constructs, revealing a positive influence in procollagen I synthesis and equilibrium modulus enhancement.²⁵⁴ The same bioreactors also had positive effects on an osteosarcoma cell line seeded on poly-L-lactic acid scaffolds, as it helped to increase significantly the expression of osteogenic markers when cells were exposed to a loading regime of 0.1% strain at 1 Hz frequency, 1 hour a day for one week in combination with a media perfusion rate of 0.1 mL/min.²⁵⁵ Compression and tensile forces have also been applied to bone tissue engineering constructs, showing promising results in RNA synthesis²⁵⁶ and alkaline phosphatase production,²⁵⁷ respectively.

2. Cartilage

Different types of bioreactors were used to investigate the effects of biophysical stimuli on engineered cartilage constructs. Shear loads bioreactors, such as spinner flasks, perfusion systems and rotating wall vessel bioreactors, have been important in understanding the impact that shear forces have on cartilage homeostasis. In particular, it has been shown that high shear forces can have a negative impact on chondrocytes, causing cell apoptosis¹⁵ or osteoarthritis earmarks expression.^{258,259} Therefore, rotating wall vessel bioreactors by providing a low shear flow, should be preferred in cartilage engineering as they have an impact in cartilage growth and differentiation, in comparison to static conditions or other shear loads bioreactors.²⁶⁰ Nevertheless, considering the shock-absorber function of natural cartilage, the majority of studies have been focusing on the investigation of hydrostatic pressure and compressional forces on cartilage constructs. For example, cartilage constructs cultured under cyclic hydrostatic pressure loading regimens showed a more effective ECM deposition and a higher chondrogenic gene expression when compared to constructs cultured at atmospheric

pressure.²⁶¹ Similarly, dynamic compression cycles demonstrated to have a statistically significant impact in increasing chondrogenic gene expression in mesenchymal stem cells in comparison to static compression. These results appear to be in line with cartilage every-day usage which undergoes dynamic compression during natural movements.

3. Tendon

As previously discussed, tendon tissue is constantly undergoing mechanical stress *in situ* and research showed how mechanical stimuli have an important impact on tendon development and repair. Mechanical bioreactors have been widely used to deliver a mechanical load to tendon tissue and tendon-engineered constructs, to investigate the role of different types of stimuli on cell fate. Considering tendon elastic properties and natural function, tensile loading bioreactors are the most widely used devices to study tendon-mimetic constructs, with a prevalence of uniaxial tensile bioreactors over biaxial loading systems.²⁶²

There are currently several commercially available uniaxial bioreactors models, such as from CellScale,²⁶³ which have been used to study phenotypic and genotypic expression of tenogenic markers on a variety of cell types, including MSCs, tenocytes and dermal cells.²⁶² Another Company, Flexcell,²⁶⁴ has made available on the market another bioreactor able to provide biaxial strain to a silicone membrane with high cell attachment affinity. This model was used by Matheson et al.²⁶⁵ to investigate the alignment of actin filament and tendon cell-cell interaction when undergoing biaxial strain. The study revealed that cells subjected to the stretching stimulus showed an improved cell-cell attachment and actin filaments organised in the direction of the strain.²⁶⁶ The same device was employed by Riboh et al.⁹⁴ to investigate the collagen deposition rate in different cell sources when subjected to a stretching stimulus, revealing a higher collagen production in BMSCs compared to ASCs or sheath fibroblast. FlexCell bioreactor as well as other commercially available bioreactors, such as TA

instruments,²⁶⁷ could be used on 3D constructs, including cell-seeded scaffolds, but also on explanted tendon tissue. In fact, several studies revealed the great impact that the 3D environment has on tendon genes/protein expression and on Young's Modulus values, compared to 2D constructs. For example, stimulated 3D constructs have shown higher Col 1a1²⁶⁸ and SCXA²⁶⁹ expression compared to stimulated 2D constructs, probably because of a better mechano-activation of the TGF β 2 signalling pathway.

Overall, these studies confirmed the importance of mechanical stimulation in tendon regeneration, making highly appropriate the use of mechanical stimulating devices in tendon tissue engineering.

1.8.2.1 Magnetic Bioreactors

Recent studies showed how magnetic forces have an influence on biological processes, and magnetic therapy is currently under evaluation for the treatment of tendon injuries. Evidence suggests that magnetic fields are able to improve tendon healing, improving pain relief, blood circulation and cell proliferation.^{270–272} Some evidence²⁷³ suggests that the field might have an influence on the production of anti-inflammatory cytokines and angiogenic factors (IL-6, IL-1B, TGF- β and IL-8), as well as promoting cells. Nevertheless, even though several devices are commercially available for Pulsed electromagnetic field therapy (PEMF) in clinical use, such as Physio-Stim®²⁷⁴ from Orthofix or SomaPulse®²⁷⁵ developed by Dr Pawluk, the impact of magnetic forces over biological processes remains unclear, as well as the role of the magnetic field in tendon healing and regeneration. PEMF showed to be useful in pain relief in lateral epicondylitis²⁷⁶ and rotator cuff post-operative pain²⁷⁷ while poor outcomes were registered in terms of repair improvement. In addition, a clear indication on exposure, frequency and intensity of the magnetic field is still missing.²⁷⁸

Interestingly, the use of a magnetic field has been investigated as an alternative strategy to deliver mechanical stimuli in tissue engineering constructs. In particular, the magnetic field generated by a magnetic bioreactor can be used in conjunction with magnetic tools such as MNPs, magnetic scaffolds or magnetic bioinks, in order to provide a remote stimulus to a culture system, being minimally invasive and maintaining sterility at the same time. In fact, by tagging the cells with MNPs, the magnetic field acts on the magnetic particles/scaffolds, delivering a mechanical stimulation to the cells without being in direct contact with the magnets.²⁴² This approach can be applied to *in vitro* experiments, where the magnetic field is generated outside the tissue culture plate, but also *in vivo*, as the field can be applied outside a living organism. The magnetic field can be initiated by a permanent magnet or by an electromagnet. In the first case, the magnet can be used either statically, to create a uniform magnetic field, or in motion, to create a variable magnetic field, for example, by changing the position of the magnet in relation to the cells either longitudinally or perpendicularly. In the case of electromagnets, it is not necessary to change the position of the magnets to have a variable magnetic field, as the current amplitude and frequency can be changed and adapted depending on the desired effect.²⁴² Magnetic bioreactors have been used on several tissues for regenerative and engineering purposes. For example, magnetic-responsive 3D scaffolds have been used in conjunction with a magnetic bioreactor to promote the proliferation of pre-osteoblasts in a bone-mimetic environment.²⁷⁹ Other research groups have focused on cartilage regeneration, showing how the synergistic effect of magnetic scaffolds and a magnetic field is able to improve chondrogenesis enhancing, for instance, the production of Col XI,²⁸⁰ Col II and aggrecan.²²⁴

1.8.2.2 MICA (Magnetic Ion Channel Activation) technology

In the field of magnetic bioreactors, a new technology able to combine the cells mechanical stimulation and the possibility to use MNPs for regenerative purposes, has recently been developed.^{221,281} The MICA (Magnetic Ion Channel Activation)²⁸² technology (Figure 1.8) consists in a magnetic force bioreactor with an oscillating magnetic array which, providing an external magnetic field, is able to remotely deliver a mechanical stimuli to single cells *in vitro* or *in vivo*. As a first step, stem cells are functionalised with labelled MNPs targeting specific mechano-receptor on the surface of the cells; then, cells are stimulated with an external magnetic field with MICA bioreactor, inducing the movement of the MNPs attached to the cells and therefore mechanically stimulating the relative receptors. The stimulation of the receptors causes the activation of some intracellular pathways (like the activation of calcium or potassium ions flux or the phosphorylation of intracellular proteins) leading to a well determined cell differentiation.¹⁴ The perks of this technology is to be minimally invasive, in fact no physical contact is required between the bioreactor and any component of the cell culture, also minimising the risk of infections. Moreover, it is possible to adjust the frequency and the displacement of the magnetic array, allowing a high precision control of the physical stress transmitted to the cells.²⁸¹

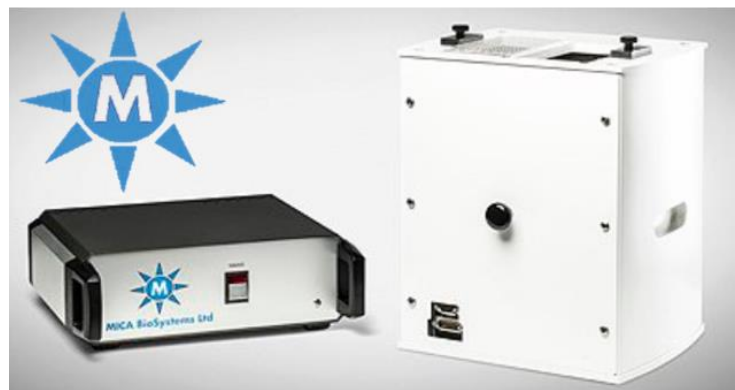


Figure 1.8 MICA (Magnetic Ion Channel Activation) bioreactor.

MICA technology has already been applied to different fields of tissue engineering, stimulating specific receptors and ion channels by means of antibody-labelled MNPs:

- TREK-1; a potassium channel involved in the mechano-transduction signalling pathway in bone formation.¹⁴ *In vitro* studies showed how the remote activation of the channel with MICA bioreactor leads to an increased mineralisation and matrix density in human mesenchymal stem cells.²⁸³ Furthermore, MICA technology was also translated to a pre-clinical ovine model, highlighting the possibility to enhance bone growth with remote magnetic stimulation of TREK-1 channel.¹⁴
- Frizzled; a family of ten G-protein-like 7-transmembrane spanning receptors whose ligand is Wnt, a protein involved in many pathways including tissue formation and regulation of stem cells. Wnt pathway mechanical activation with MICA bioreactor was studied *in vitro* and it showed to promote osteogenesis in human mesenchymal stem cells.²⁸⁴
- Integrins; the Arg-Gly-Asp (RGD) binding sites of integrins, that connects the extracellular matrix with the cytoskeleton, can be mechanically stimulated towards the activation of osteogenesis. *In vitro* studies showed that osteoblasts²²⁰ and human mesenchymal stem cells²⁸³ tagged with RGD-coated MNPs and stimulated with MICA, showed increased mineralisation and osteoblastic activity.
- Activin Receptor IIA; as previously described in Paragraph 1.4.3.1, Activin RIIA is a mechanoreceptor which, once stimulated, leads to the activation of the tenogenic pathway. Gonçalves et al.²⁸⁵ demonstrated *in vitro* the activation of the Smad2/3 pathway in hASCs labelled with anti-Activin RIIA MNPs and stimulated with MICA (Figure 1.9). In particular, the remote activation of the receptor via MICA stimulation led to increased levels of phosphorylated Smad2/3 complex, testifying the activation of

the tenogenic pathway. Furthermore, they also registered an increased expression of some tenogenic proteins like TNMD and fibromodulin when compared to controls.

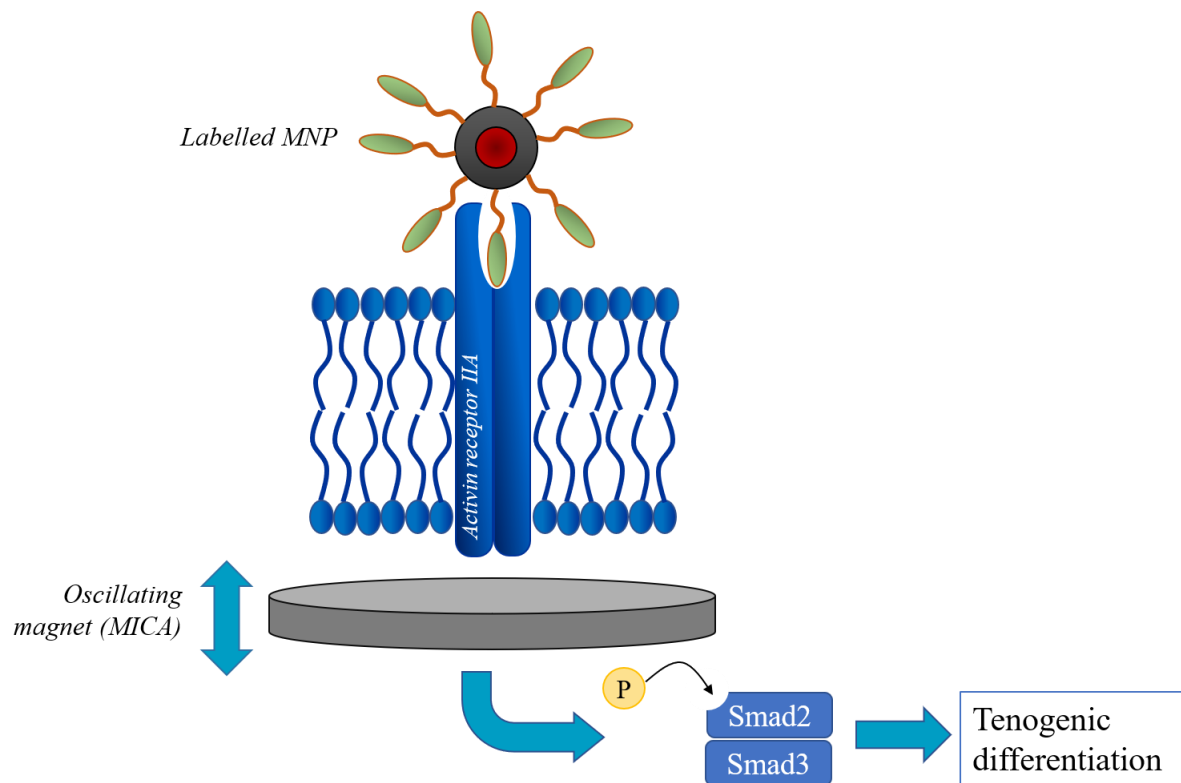


Figure 1.9 Schematic representation of antibody-labelled MNPs-driven activation of Activin RIIA.

1.9 Project aim

The work presented in this Thesis aims to develop a stem cell-based therapy for the treatment of tendon injuries. MICA technology will be used to deliver an external magnetic field to stem cells labelled with anti-Activin RIIA MNPs, inducing the differentiation towards the tenogenic commitment. The hypothesis is that the remote mechanical activation of the Activin RIIA via MICA stimulation will be able to promote tendon healing by:

1. Driving the tenogenic differentiation of MSCs,
2. Promoting proliferation and maturation of MSCs,
3. Promoting the deposition and remodelling of extracellular matrix.

Furthermore, two 3D scaffold will be developed to further explore tendons healing mechanisms and to investigate an effective MNPs/cells therapy delivery method. Finally, an *in vivo* rat model will be used to investigate the tenogenic regenerative potential of remotely-activated MNPs-labelled hASCs, as an essential step prior translation into clinic.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Consumables used for *in vitro* studies:

Table 2.1 List of consumables, product codes and manufacturers for *in vitro* experiments.

Consumable	Product code	Manufacturer
(3-Aminopropyl)triethoxysilane	A3648	Sigma Aldrich
0.5 mL Microtubes	72.699	Starsted
0.5% Trypsin-EDTA 10x	15400-054	Gibco
10X Bolt™ Sample Reducing Agent	B0009	Thermo Fisher
12 well plate	3513	Corning
24 well plate	3526	Corning
4X Bolt™ LDS Sample Buffer	B0007	Thermo Fisher
50 ml centrifuge tubes	430921	Starsted
6 well plate	3516	Corning
8 well chamber slide	354118	Corning
96 well plate	3596	Corning
Acetic acid glacial >99%	A/0360/PB17	Fisher Scientific
Acetone >99.8%	A/0600/17	Fisher Scientific
Anti-Dextran Antibody	60026FI	Stemcell Tech
Aprotinin from ovine lung	A3428-10MG	Sigma Aldrich
Ascorbic Acid	A4544	Sigma Aldrich
Atto 488 NHS ester dye	41698	Sigma Aldrich
Blotting-Grade Blocker	1706404	Bio-rad

Bolt™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gels	NW04120BOX	Thermo Fisher
BOND Dewax Solution	AR9222	Leica
Bond TM Epitope Retrieval 2	AR9640	Leica
Boric acid powder, 99.5%	B0394	Sigma Aldrich
Bovine serum albumin	9998S	Cell Signaling
Calcium Chloride	793639	Sigma Aldrich
Calcium Chloride	C/1500/53	Thermo Fisher
Cell Counting Slide for TC10™/TC20™ Automated Cell Counter	145-0011	Bio-rad
Cell Culture Cryogenic Tubes	377267	Thermo Fisher
Citric Acid monohydrate	C1909	Sigma Aldrich
Collagen I, High Concentration, Rat Tail	354249	Corning
Corning® 100 mm TC-treated Culture Dish	430167	Corning
Cover slips, round	631-0148P	VWR
Coverslips, rectangular	1237-3128	Fisher Scientific
CTGF	120-19	PeptoTech
DAPI	D9542	Sigma Aldrich
Dimethyl Sulfoxide	D2650	Sigma Aldrich
DMEM, high glucose, pyruvate, no glutamine	21969035	Gibco
DPBS++	D8662	Sigma-Aldrich
DPX Mountant for histology	06522	Sigma Aldrich
DynaMag™-2 Magnet	12321D	Thermo Fisher
EDAC	E2247	Life technologies

EDTA	AM9260G	Thermo Fisher
Embed-812 resin	14120	Electron Microscopy sciences
Embedding cassettes	M480-10	Simport
Eosin Y solution	HT110216	Sigma Aldrich
Fetal bovine serum	FBS-70831- EPS117650	Labtech
Fibrinogen	F3879-250MG	Sigma Aldrich
Fluoromount aqueous mounting media	F4689	Sigma Aldrich
Formaldehyde	15713	Electron Microscopy sciences
Formalin 10%, Q Path, buffered	11699404	VWR
GDF-7	120-37-10uG	PeproTech
Glacial acetic acid	64-19-7	Sigma Aldrich
Glutaraldehyde	16316	Electron Microscopy sciences
Glycine	G8898	Sigma Aldrich
HALT Protease and Phosphatase Inhibitor cocktail	78440	Thermo Fisher
Hematoxylin Solution, Gill No. 2	GHS216	Sigma Aldrich
HEPES	H0887	Sigma Aldrich
Histoclear II	HS-202	National diagnostics
Histofluid mounting medium	6900002	Biosystems
Histogel™	HG-4000-012	Thermo Fisher

Histoplast	6774060	Thermo Fisher
Human Adipose Stem Cells, Batch 18TL212639	PT-5006	Lonza
Human Bone Marrow, Unprocessed	1M-125	Lonza
Hydrochloric acid	258148	Honeywell
iBlot™ 2 Transfer Stacks	IB23001	Thermo Fisher
Industrial Methylated Spirit 99% (v/v)	11482874	Fisher Scientific
Lead citrate	11300	Electron Microscopy sciences
L-Glutamine	25030-081	Gibco
LIVE/DEAD Viability/Cytotoxicity Kit	L3224	Thermo Fisher
Magnet-Cuboid 40x40x15 mm, NdFeB/Ni-N42	-	HKCM Engineering
Magnesium sulphate	M7506	Honeywell
MES	m3671	Sigma Aldrich
MES SDS Running buffer	B0002	Thermo Fisher
Microtubes 1.5ml	3810X	Eppendorf
Mr. Frosty Freezing Container	5100-0001	Thermo Fisher
Neodymium Magnets, N42, 40x20 mm	EP352	Bunting Magnetics
Nanomag-D 250nm COOH 10 mg/mL	09-02-0252	Micromod
Neubauer counting chamber	0650010	Marienfeld
N-Hydroxysuccinimide	130672	Sigma Aldrich
Opal Polaris 7-Color Automation IHC Detection Kit	NEL871001KT	Akoya Biosciences
Osmium tetroxide	19190	Electron Microscopy sciences

PathScan Phospho-Smad2 (Ser465/467) Sandwich ELISA Kit	7348	Cell Signaling
Penicillin-Streptomycin	15070-063	Gibco
Phalloidin, CF dye conjugate	00045-T	Biotium
Phosphate Buffer Saline	14190-094	Gibco
Pierce BCA Protein assay	23228	Thermo Fisher
Potassium hexacyanoferrate(II) trihydrate	P3289	Honeywell
Presto Blue Cell Viability Reagent	A13261	Thermo Fisher
ProFreeze CDM (2x)	BEBP12-769E	Lonza
ProLong Diamond Antifade Mountant	15205739	Fisher Scientific
Propan-2-ol, >99.5%	P/7507/17	Fisher Scientific
Recombinant Human/Mouse/Rat Activin A Protein	338-AC-010	R&D System
Restore™ Western Blot Stripping-Buffer	21059	Thermo Fisher
Ripa Buffer	89900	Thermo Fisher
RNAscope® 2.5 LS Multiplex Positive Control Probe- Hs	321808	ACD
RNAscope® LS Multiplex Reagent Kit	322800	ACD
Saturated aqueous solution of Picric Acid (1.3% in water)	88-89-1	Sigma Aldrich
SeeBlue Plus2 Prestained Standard	LC5925	Thermo Fisher
SiMAG-Carboxyl	1402-1	Chemicell
Sirius Red	2610-10-8	Sigma Aldrich
Slide-A-Lyzer Dialysis cassette G2	87735	Thermo Fisher

Sodium Carbonate Anhydrous	S/2920/53	Thermo Fisher
Sodium Chloride	S7653	Sigma Aldrich
Sodium hydrogen carbonate	A17005	Alfa Aesar
Sodium hydroxide	S8045	Honeywell
SuperFrost™ Microscope Slides	12392098	Thermo Fisher
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	34577	Thermo Fisher
TGF- β 3	100-36E	PeptoTech
Thrombin from human plasma	T4393-100UN	Sigma Aldrich
Tissue Culture Flask 175cm ²	431080	Corning
Tissue Culture Flask 25cm ²	430639	Corning
Tissue Culture Flask 75cm ²	430641U	Corning
TRIS-buffered saline 10x pH 7.6	J62662	Alfa Aesar
Triton X-100	437002A	VWR
Tween 20	663684B	VWR
Uranyl acetate substitute	11000	Electron Microscopy sciences
Xylene	X/0250/17	Fisher Scientific
α MEM with Glutamax, no nucleosides	32561037	Gibco

Consumables used for *in vivo* study, if different from *in vitro* studies:

Table 2.2 List of consumables, product codes and manufacturers for *in vivo* experiments

Consumable	Product code	Manufacturer
Adhesion microscope slides	J1800AMNZ	Epredia

Antibiotic/Antimycotic	15240062	Gibco
Antisedan	7418046	NOVAVET
Biopsy punch, Ø2mm	BP-20F	Kai medical
Buprenorphine	1170046	Richter Pharma AG
Eosin	05- M10003	Bio-Optica
Fetal bovine serum	10270	Gibco
Formalin buckets	Q49071	Sicura Countainers
Hematoxylin	H3136	Sigma Aldrich
Histofluid	6900002	Paul Marienfeld
Iron, plasma standard solution, Specpure®, Fe 1000µg/ml	13830.AD	Alfa Aesar
Ketamine/ Imalgene 1000	7410928	Merial S.A.S.
Male RNU RAT 49-55* Days	316RNU	Charles River
Medetomidine/ Domitor	7418335	Orion Corporation
Nitric Acid 69%	1.01799.2500	Fisher Scientific
Perls Van Gieson	04-181807	Bio-Optica
Phentobarbital solution, Eutasil/ Euthanimal	7412877	Alfasan Nederland BV
Sirus Red Picrate	04-121873	Bio-Optica
Sodium bicarbonate	S5761	Sigma Aldrich
Sodium citrate tribasic dihydrate	71406	Sigma-Aldrich
Th Complete 14-Plex Rat ProcartaPlex Panel	EPX140-30120-901	Thermo Fisher
αMEM powder, no nucleosides	12000063	Gibco

2.2 General cell culture techniques

All the described procedures were performed in sterile conditions. In this section, “cells” can refer either to hBMSCs, hASCs or human tenocytes unless specified otherwise, as they were all cultured, handled and experimentally treated following the same protocols here described. Nevertheless, different media were used depending on the cell type:

Table 2.3 Media composition depending on cell type.

hBMSc	Basal media	DMEM supplemented with 10% FBS, 1% Pen-Strep and 1% L-Glut.
	Tenogenic media	DMEM supplemented with 5% FBS, 1% Pen-Strep, 1% L-Glut, AA (50 mg/mL), CTGF (20 µg/mL), GDF-7 (10 µg/mL) and TGF-β3 (10 µg/mL).
	Serum free media	DMEM supplemented with 1% Pen-Strep and 1% L-Glut.
hASCs	Basal media	αMEM with Glutamax supplemented with 10% FBS, 1% Pen-Strep.
	Tenogenic media	αMEM with Glutamax supplemented with 5% FBS, 1% Pen-Strep, AA (50 mg/mL), CTGF (20 µg/mL), GDF-7 (10 µg/mL) and TGF-β3 (10 µg/mL).
	Serum free media	αMEM with Glutamax supplemented with 1% Pen-Strep.
Human tenocytes	Basal media	DMEM supplemented with 10% FBS, 1% Pen-Strep and 1% L-Glut.
	Serum free media	DMEM supplemented with 1% Pen-Strep and 1% L-Glut.

To maintain sterility, the cell culture procedures described below were performed in a class II Microbiological Safety Cabinet (51029645, Thermo Fisher) and disposable sterile plastic consumables were used to avoid contaminations. Cells and 3D constructs were cultured in humidified, tri-gas controlled incubators with 21% oxygen, 5% carbon dioxide and 93% nitrogen (MCO-170AICUVH-PE, Panasonic).

2.2.1 Resuscitating cells

Upon removal from liquid nitrogen (LN) storage, cryovials were gently thawed in a water-bath at 37°C until about 90% of the frozen content had defrosted. The cells suspension was then transferred to a 50 mL tube containing 10 mL of 37°C-warm media. Afterwards, cells were centrifuged at 350g for 5 min (Megafuge 8R, Thermo Fisher). After discarding the supernatant, the cell pellet was carefully resuspended in the required amount of appropriate media and seeded at the desired concentration in T175 cell culture flasks.

2.2.2 Cells culturing and passaging

Cells thawed from LN (Paragraph 2.2.1) and seeded in cell culture flasks were incubated at 37°C and 5% CO₂ until 80% confluent, the media was changed three times a week with fresh pre-warmed basal media. When confluent, the cells were washed with 20 mL of phosphate-buffered saline (PBS) and detached by incubation for 5 min at 37°C with 12 mL of 1X Trypsin-EDTA (5 mL of 0.5% Trypsin-EDTA 10x were diluted in 45 mL of PBS). After checking the actual cells detachment under an optic microscope (EVOS XL Core, Thermo Fisher), 12 mL of basal media were added in the cell culture flask and the cells dispersion was transferred to a 50 mL centrifuge tube and centrifuged for 5 minutes at 350g. The supernatant was carefully discarded and the cell pellet was resuspended in 3 mL of basal media. Cells were then counted and seeded at the desired density.

2.2.3 Cells counting

After detaching seeded cells with 1X Trypsin-EDTA, centrifuging and resuspending the cells in 3 mL of fresh media (Paragraph 2.2.2), 10 µL of the cell dispersion was withdrawn and placed in a Hemocytometer chamber for cell counting (Figure 2.1).

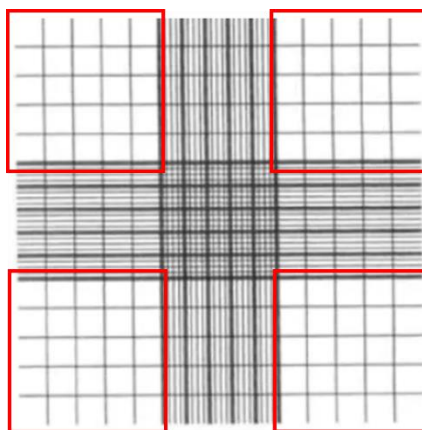


Figure 2.1 Hemocytometer chamber scheme. The cells present within the four external squares (underlined in red) were used for the cell count.

Under an optical microscope (EVOS XL Core, Thermo Fisher), the cells in the four external squares of the chamber were counted. The total number of cells counted in the four squares was divided by 4, in order to have the average number of cells per square. The number was then multiplied by the correction factor (10^4), and by the dilution factor (3, in case the cells pellet was previously resuspended in 3 mL of fresh media) to have the total number of cells in the cells dispersion volume as final result.

2.2.4 Cells counting with Trypan Blue exclusion procedure

The cells were first detached with 1X Trypsin-EDTA, centrifuged and resuspended in 3 mL of fresh media (Paragraph 2.2.2). Afterwards, 10 μ L of the cell suspension was mixed with 10 μ L of Trypan Blue (dilution 1:1). 10 μ L of the final suspension were withdrawn and loaded into a Cell Counting Slide and counted at an Automated Cell Counter (TC20, Bio-Rad).

2.2.5 Cells cryopreservation

For long-term preservation, cells in culture were detached and counted, as described in Paragraphs 2.2.2 and 2.2.3. Cells were then centrifuged for 5 minutes at 350g and an appropriate amount of freezing media was added (50% basal media, 35% ProFreeze, 15% DMSO),

considering 1×10^6 cells for every mL of freezing media. 1 mL of the final cell suspension was then transferred to a cryovial and placed in a Mr Frostie freezing container, which was immediately stored at -80°C . 24 h later, cells were transferred to a LN storage tank.

2.3 Cells isolation from human specimens

2.3.1 Tenocytes isolation from human tendons

Human tendons explants were obtained from the Royal Orthopaedic Hospital-ROH Research Tissue Bank (East Midlands-Derby Research Ethics Committee, Ref:17/EM/0030. Application Ref: ROH19002) prior patient's informed consent. The tendon specimen was placed in a sterile 50 mL tube filled with PBS supplemented with 3% antibiotic/antimycotic solution (A/A), transferred on ice to the lab and processed within 4 hours from the extraction. Alternatively, the specimen was stored in DMEM serum free media (SFM) overnight at 4°C . Upon arrival to the lab, the sample was weighted and subsequently processed in sterile conditions. Firstly, any extra tissue was removed from the tendon and the specimen was washed with a solution of 3% wide spectrum antibiotic in PBS. Using a scalpel, the tendon was then minced into approximately 1-2 mm³ pieces, while constantly keeping the tissue hydrated with the 3% antibiotic in PBS solution. Subsequently, the minced tendon pieces were put in dry Petri Dishes, considering ~6 pieces every dish. The dishes were then moved to an incubator at 37°C for 30 min, allowing the tendon pieces to loosely adhere to the plastic surface. After the incubation time, one drop of basal media was added on top of each small tendon piece, and the dish was then incubated at 37°C for 10 min. Afterwards, about 20 mL of basal media was carefully added into the dish until the tendon pieces were completely covered with media, with care being taken not to detach the specimens. The samples were kept in an incubator at 37°C , with half media change being performed every week. After around a week, the cells started to migrate out of the explants. When the cells reached the confluence, the tendon explants were carefully moved to a new dry

Petri Dish and the process was repeated following the same protocol as previously described. The confluent cells were detached from the first dish as described in Paragraph 2.2.2 and counted with Trypan Blue to check cells viability (Paragraph 2.2.4). The cells were seeded in T75 or T175 cell culture flasks with basal media at a minimum seeding density of 5×10^3 cells/cm². This process was repeated for the second dish the tendon pieces adhered into, up to a maximum of 3 times.

2.3.2 Stem cells isolation from human patellar fat

Human patellar fat samples were obtained from the Royal Orthopaedic Hospital-ROH Research Tissue Bank (East Midlands-Derby Research Ethics Committee, Ref:17/EM/0030. Application Ref: ROH19002) prior patient's informed consent. After being harvested, specimens were placed in a sterile 50 mL Falcon tube filled with DMEM SFM, transferred on ice to the lab and processed within 4 hours from the extraction. Alternatively, the sample was stored overnight in SFM at 4°C. Upon arrival to the lab, the sample was weighted and subsequently processed in sterile conditions. Firstly, the fat specimen was cleaned from any extra tissue using a sterile scalpel and any visible blood was removed by washing the sample several times with a solution of 3% A/A in PBS. The fat sample was then minced into ~1-2 mm³ pieces using a sterile scalpel, while constantly being kept hydrated with the 3% antibiotic in PBS solution. For every gram of tissue, a digestion solution was prepared by mixing 4 mL of 750 U/mL of Chlostridium Collagenase in SFM. The digestion solution was then sterile-filtered with Ø0.22 µm syringe filters and added to the minced fat tissue in a spinner flask. The tissue digestion was initiated by incubation at 37°C under stirring for about 3 hours, or until approximately 95% of the sample was digested. After the digestion, 2 volumes of 20% DMEM (DMEM supplemented with 20% FBS, 1% Pen-Strep and 1% L-Glut) were added to the suspension and mixed well. The suspension was sieved using a 140 µm nylon mesh strainer into new 50 mL tubes, in order to

remove any matrix debris and undigested material. After centrifuging the filtrate at 650g for 5 min, the supernatant was discarded and the pellet was resuspended in 20 mL of 20% DMEM. The suspension was sieved again using a 40 μ m nylon mesh strainer into new Falcon tubes and centrifuged at 650g for 5 min. After discarding the supernatant, the pellet was resuspended in 3 mL of α MEM and a cell count was performed using Trypan blue to check the cells viability (Paragraph 2.2.4). The cells were plated in T75 or T175 cell culture flasks, considering a minimum seeding density of 5×10^3 cells/cm². After adding the appropriate volume of α MEM, the cells were incubated at 37°C. Media changes were performed every other day and the cells were split when 80% confluent.

2.4 Tenogenic media

2.4.1 Reagent preparation

Ascorbic acid (AA) was reconstituted in sterile dH₂O to give a stock concentration of 50 mg/mL. 50 μ L of the solution was aliquoted in 500 μ L tubes and stored at -20°C up to six months. Transforming Growth Factor β 3 (TGF- β 3) was initially reconstituted in 10 mM Citric Acid to reach an initial stock concentration of 1 mg/mL, which was then diluted with 0.1% BSA to a final stock concentration of 10 μ g/mL. 50 μ L aliquots were stored in 500 μ L tubes at -20°C up to six months. Connective Tissue Growth Factor (CTGF) and Growth and differentiation factor 7 (GDF-7) were initially reconstituted in sterile dH₂O to an initial stock concentration of 1 mg/mL, which was further diluted with 0.1% BSA to a final stock concentration of, respectively, 20 and 10 μ g/mL. 250 μ L aliquots of both the solutions were transferred in 500 μ L tubes and stored at -20°C up to six months.

2.4.2 Preparation of the media

Tenogenic media¹⁴² was prepared by adding 50 μ L of AA (50 mg/mL), 250 μ L of CTGF (20 μ g/mL), 250 μ L of GDF-7 (10 μ g/mL) and 50 μ L of TGF- β 3 (10 μ g/mL) to 50 mL of DMEM or α MEM supplemented with 5% FBS, 1% L-Glut and 1% Pen-Strep. Final working concentrations of AA, CTGF, GDF-7 and TGF- β 3 were, respectively, 50 μ g/mL, 100 ng/mL, 50 ng/mL and 10 ng/mL. Media changes were performed three times a week.

2.5 MNPs antibody functionalization

1 mL of 1 mg/mL MNPs stock dispersion was prepared by dissolving 12 mg of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) and 24 mg of N-Hydroxysuccinimide (NHS) in 2 mL of 0.5 M 2-(N-morpholino)ethanesulfonic Acid (MES buffer) in water (adjusted to pH=6.3 with 2M Na₂CO₃ in water). After sterile filtering the solution, 20 μ L were withdrawn and mixed with 0.1 mL of MNPs (10 mg/mL) in a 1.5 mL Eppendorf tube to activate the particles (MNPs were either dextran-coated COOH-functionalised Nanomag-D 250 nm MNPs, or silica-coated COOH-functionalised SiMAG-Carboxyl 1 μ m MNPs). The solution was mixed continuously for one hour at room temperature (RT) using a rotating mixer (Rotator SB3, Stuart). Afterwards, the MNPs were washed with 0.2 mL of 0.1 M MES buffer in water (adjusted to pH=6.3 with 2M Na₂CO₃ in water) at a permanent magnet (DynaMag-2 Magnet, Thermo Fisher). Briefly, the tube was left on the magnetic rack for about 2 minutes until the solution got clear, the solution was then discarded paying attention not to remove the MNPs from the wall of the tube. The tube was dismounted from the array and the MNPs were washed with 0.2 mL of 0.1 M MES buffer. This washing step was repeated two more times with 0.1 mL of 0.1 M MES buffer. The MNPs were resuspended in 0.1 mL of 0.1 M MES buffer and 20 μ L of the appropriate secondary antibody

were added (Table 2.4). The MNPs were incubated at 4°C overnight or at RT for 3 h under continuous mixing.

Table 2.4 Secondary antibodies used for MNPs labelling.

Secondary antibody	Product code	Stock conc.	Host
Anti-Rabbit	Ab97196	1 mg/mL	Goat
Anti-Rabbit	Ab97048	1 mg/mL	Goat
Anti-Mouse	Ab97261	1 mg/mL	Goat

After the incubation time, the MNPs were washed and resuspended with 0.1 mL of 0.1 M MES buffer. The MNPs were incubated under continuous mixing at 4°C overnight or at RT for 3 h with primary antibody, considering 10 µg of primary antibody for 1 mg of MNPs (Table 2.5). After the incubation time, 10 µL of 25 mM of glycine in PBS were added to the MNPs and mixed continuously for 30 minutes at RT. The MNPs were washed with 0.1 mL of 0.1% BSA in PBS and then resuspended in 1 mL of 0.1% BSA.

Table 2.5 Primary antibodies used for MNPs labelling.

Primary antibody	Product code	Stock conc.	Host	Ab amount
Anti-Activin RIIA	Ab135634	1.5 mg/mL	Rabbit	6.7 µL
Anti-Activin RIIA	MAB340	0.5 mg/mL	Mouse	20 µL
Anti-Activin RIIA	MBS606695	0.5 mg/mL	Mouse	20 µL
Anti-Collagen I	Ab260043	0.5 mg/mL	Rabbit	20 µL

The primary/secondary antibody combinations employed for MNPs labelling are summarised here below:

Table 2.6 Primary and secondary antibodies combinations for MNPs labelling.

Secondary antibody		Associated primary antibody	
Target	Code	Target	Code
Anti-Rabbit	Ab97196	Anti-Activin RIIA	Ab135634
Anti-Mouse	Ab97261	Anti-Activin RIIA	MAB340
Anti-Mouse	Ab97261	Anti-Activin RIIA	MBS606695
Anti-Rabbit	Ab97048	Anti-Collagen I	Ab260043

Unspecific MNPs were prepared following the same protocol, skipping the incubation with the primary and secondary antibody. In particular, the MNPs were activated by the incubation with the EDAC and NHS buffer, washed with 0.1 M MES buffer, resuspended in the same buffer and incubated with 10 μ L of 25 mM of glycine for 30 minutes at RT under continuous mixing. The MNPs were finally washed with 0.1% BSA and resuspended in 1 mL of 0.1% BSA.

2.6 Zetasizer and Zeta potential measurements

Ø250 nm iron oxide MNPs and Ø1 μ m Simag MNPs, either non-labelled or antibody-labelled (Paragraph 2.5), were characterized for their size and charge using a Zetasizer Nano ZS (Malvern Instruments, United Kingdom) in water dispersant (dilution 1:10).

2.7 Cells on monolayer/in suspension

2.7.1 MNPs cells labelling on monolayer

Sterile round glass coverslips were added at the bottom of 24-well plates and the desired cells amount was seeded following the procedure describe in Paragraph 2.2.2. The cells were

incubated at 37°C with 0.5 mL of the appropriate media, until 80% confluent. After discarding the media, the cells were washed once with 0.5 mL PBS and then incubated at 37°C for 90 min with a dispersion of the desired concentration of MNPs in SFM. The cells were washed twice with PBS and incubated at 37°C with 0.5 mL of fresh appropriate media until further use.

2.7.2 Fixing cells on monolayer

After seeding the cells in tissue culture well plates on monolayer (Paragraph 2.7.1), the media was discarded and the cells were washed with PBS and fixed with 0.5 mL formalin for 30 minutes at RT. Afterwards, the formalin was discarded and the cells were washed twice with PBS and stored at 4°C in 1 mL PBS until further use.

2.7.3 MNPs cells labelling in suspension

After being trypsinized with 1X Trypsin-EDTA solution in PBS (Paragraph 2.2.2), the required number of cells was withdrawn, moved to a 1.5 mL tube, centrifuged at 350g for 4 minutes (accuSpin micro 17R, Fisher Scientific), washed with 2 mL of PBS and resuspended in a dispersion of labelled MNPs in serum free media (considering 1 mL of SFM and 125 µg of MNPs for 1 million of cells). After 3 hours incubation at 37°C, the cells were centrifuged at 350g for 4 minutes, washed with 2 mL of PBS and resuspended in the required media or solution.

2.7.4 Immunocytochemistry on cells on monolayer

Cells seeded on glass coverslips in well plates (Paragraph 2.7.1) were fixed with formalin and washed with PBS (Paragraph 2.7.2). The cells were incubated for 10 minutes at 37°C with 0.5 mL of antigen retrieval solution (0.1% Trypsin-EDTA in 1% CaCl in dH₂O) and washed with PBS. If needed, cells were permeabilized with 0.5 mL of 0.5% Triton-X100 in PBS for 10

minutes at RT and washed three times with PBS for 5 min. Cells were then blocked for 1 h at RT with 0.5 mL of 3% BSA in 0.01% Tween in PBS and washed with PBS. Afterwards, samples were incubated overnight at 4°C with 0.25 mL of a primary antibody solution diluted in 1% BSA, using the following concentrations:

Table 2.7 Primary antibodies used for immunocytochemistry.

Primary antibody	Product code	Stock conc.	Dilution	Host
Anti-Activin RIIA	Ab135634	1.5 mg/mL	1:50	Rabbit
Anti-Activin RIIA	MAB340	0.5 mg/mL	3:50	Mouse
Anti-Activin RIIA	MBS606695	0.5 mg/mL	3:50	Mouse
TNMD	Ab203676	1 mg/mL	1:100	Rabbit
SCXA	Ab58655	0.5 mg/mL	1:200	Rabbit
Col I	Ab34710	1.1 mg/mL	1:500	Rabbit
Col III	Ab7778	1 mg/mL	1:500	Rabbit
Anti-Dextran-FITC	60026FI	-	1:500	Mouse

The cells were then washed three times with PBS for 5 minutes and incubated in the dark for 2 hours at RT with 0.25 mL of a secondary antibody solution diluted in 1% BSA (Table 2.8).

Table 2.8 Secondary antibodies used for immunocytochemistry.

Secondary antibody	Product code	Stock conc.	Dilution	Reactivity
AlexaFluor 488	A11059	2 mg/mL	1:1000	Anti-Mouse
AlexaFluor 594	A11012	2 mg/mL	1:1000	Anti-Rabbit
AlexaFluor 647	A21245	2 mg/mL	1:1000	Anti-Rabbit
AlexaFluor 647	A21236	2 mg/mL	1:1000	Anti-Mouse

After washing the cells three times with PBS for 2 minutes each, the nuclei were stained with 0.25 mL 4',6-diamidino-2-phenylindole (DAPI) (0.5 µg/mL in PBS) for 15 minutes at RT in

the dark. Finally, the cells were washed three times with PBS. The glass coverslips were then carefully removed from the wells and mounted on glass slides using Fluoromount as mountant media. Stained samples were imaged with a Cytation5 microscope (BioTek). ImageJ Software (ImageJ 1.52n, Image processing and analysis in Java, National Institutes of Health) was used to quantify the fluorescent signal from the acquired pictures, the values were normalised for the nuclei number.

2.7.5 Phalloidin staining

After being fixed with formalin (Paragraph 2.7.2), cells seeded on glass coverslips were incubated for 10 minutes at 37°C with 0.5 mL of antigen retrieval solution (0.1% Trypsin in 1% CaCl₂ in dH₂O) and washed with PBS. Cells permeabilization was performed by incubation with 0.5 mL of 0.5% Triton-X100 in PBS for 10 minutes at RT. Cells were washed three times with PBS for 5 min and blocked for 1 hour at RT with 0.5 mL of a solution 3% BSA in 0.01% Tween in PBS. After the incubation, the cells were rinsed three times with PBS and stained with Phalloidin (#00041-T, Biotium) for 20 minutes at RT in the dark following manufacturer's instructions. The cells were washed three times with PBS. The glass coverslips were then carefully removed from the wells and mounted on glass slides using Fluoromount as mountant media. Stained samples were imaged with a Cytation5 microscope (BioTek).

2.7.6 Samples preparation for FACS analysis

After culturing and expanding the cells, they were detached with 1X Trypsin-EDTA solution in PBS (Paragraph 2.2.2), transferred to a 50 mL tube and the old media was discarded and replaced with fresh media. After counting the cells (considering that the cells concentration of each FACS sample has to be between 5×10^4 and 1×10^6 cells in total), the desired cells suspension was moved to a 500 μ L tube and centrifuged at 500g for 4 minutes. The media was

discarded and the cells were fixed in suspension with 0.5 mL of formalin for 30 minutes. After washing with 0.5 mL of PBS, the cells were incubated with 0.5 mL of 2% BSA at RT for 30 minutes. After centrifuging the cells at 500g for 2 minutes, the solution was discarded and replaced with 100 μ L of a solution of primary antibody (Table 2.9) diluted in FACS buffer (0.5% BSA and 2 mM EDTA in PBS).

Table 2.9 Primary antibodies used for FACS samples preparation.

Primary antibody	Product code	Stock conc.	Dilution	Host
CD105-PE	560839	-	1:20	Mouse
CD14-Cy7	557742	-	1:20	Mouse
CD19-Cy5	561295	-	1:20	Mouse
CD31-PE	563652	-	1:20	Mouse
CD34-BV241	562577	-	1:20	Mouse
CD45-APC	566041	-	1:20	Mouse
CD73-FITC	561254	-	1:20	Mouse
CD90-APC	559869	-	1:20	Mouse
HLA-DR-BV510	563083	-	1:20	Mouse
IgG2a-BV421	562602	-	1:20	Rat
IgG2b-BV650	563233	-	1:20	Rat
Anti-Activin RIIA	Ab135634	1.5 mg/mL	1:150	Rabbit
Anti-Activin RIIA	MAB340	0.5 mg/mL	1:50	Mouse
Anti-Activin RIIA	MBS606695	0.5 mg/mL	1:50	Mouse

After 30 minutes of incubation at RT with the primary antibody, 400 μ L of FACS Buffer were added to the antibody solution and the cells were centrifuged for 2 minutes at 500g. The solution was discarded and, for samples labelled with one of the Anti-Activin RIIA antibodies, the cells were incubated for 30 min in the dark at RT with 100 μ L of secondary antibody diluted in FACS buffer (Table 2.10).

Table 2.10 Secondary antibodies used for FACS samples preparation.

Secondary antibody	Product code	Stock conc.	Dilution	Reactivity
AlexaFluor 647	A32733	2 mg/mL	1:1000	Anti-Rabbit
AlexaFluor 488	A11059	2 mg/mL	1:200	Anti-Mouse

After the incubation time, 400 μ L of FACS buffer were added to the cells and the tube was centrifuged for 2 min at 500g. The cells were washed twice with 500 μ L of FACS Buffer and once more with 1 mL of FACS Buffer. Finally, the cells were resuspended in 300 μ L of FACS buffer and analysed at CytoFLEX instrument (Beckman Coulter).

2.7.7 Tenogenic differentiation with MICA stimulation

1×10^4 cells (hBMSCs or hASCs) were seeded on glass coverslips in 24-well plates (Paragraph 2.7.1) and incubated with the appropriate tenogenic media overnight at 37°C. The old media was discarded and the cells were washed once with PBS. The cells were labelled with a dispersion of Ø250 nm Anti Activin-MNPs in SFM, considering 50 μ g of MNPs every 5×10^4 seeded cells (for hBMSCs), or 25 μ g of MNPs every 2×10^5 seeded cells (for hASCs) (Paragraph 2.7.1). After the incubation time and the PBS washings, 1 mL of the appropriate tenogenic media was provided and the plates were stored in an incubator at 37°C. The following seeding conditions were considered: i) unlabelled cells (hereafter referred as “cells only”) in tenogenic media stimulated with external magnetic field, ii) cells labelled with MNPs in tenogenic media stimulated with external magnetic field (hereafter referred as “magnetically stimulated group”). For the magnetic stimulation, the well plates were placed on a commercially available vertical oscillating magnetic bioreactor (MICA Biosystems Ltd) placed inside an incubator at 37°C. The bioreactor provided a magnetic field ≤ 540 mT from an array of permanent magnets (NdFeB) situated beneath the culture plates at a frequency of 1 Hz. The stimulation was provided every

day for 1 h, 5 days/week, up to 21 days. Non-stimulated groups were cultured in identical conditions but kept static inside the incubator at 37°C.

2.7.8 Phosphorylation assay

The Phosphorylation assay was performed testing different combinations of cells seeding densities (hBMSCs, hASCs or human tenocytes), Ø250 nm Anti-Activin RIIA-MNPs concentrations and incubation periods. All the cells were seeded in tissue-culture 6-well plates and incubated with tenogenic media, subsequently the following combinations were tested:

- Human tenocytes and hBMSCs:
 - 5×10^4 seeded cells incubated until confluent (4 days), further labelled with 50 µg MNPs,
- hASCs:
 - 5×10^4 seeded cells incubated until confluent (4 days), further labelled with 50 µg MNPs,
 - 2×10^5 seeded cells incubated overnight, further labelled with 25 µg MNPs,
 - 1×10^6 seeded cells incubated overnight, further labelled with 25 µg MNPs.

After the required incubation period, the old media was discarded and the cells were washed with PBS. After 90 minutes incubation at 37°C with SFM, the desired amount of Ø250 nm Anti Activin-MNPs were added to each well and incubated at 37°C for 30 min. As positive control of Activin RIIA activation, 20 ng/mL recombinant human/mouse/rat Activin A protein were added to the wells and incubated for 30 min. After the labelling period, the media and unattached MNPs were discarded and replaced with fresh tenogenic media. The cells were stimulated for 2, 5, 10 or 30 min with a vertical oscillating magnetic bioreactor (MICA Biosystems) placed inside an incubator at 37°C. The bioreactor provided a magnetic field ≤ 540 mT from an array of permanent magnets (NdFeB) situated beneath the culture plates at a

frequency of 1 Hz. The non-stimulated cells were kept as static control in identical conditions in the incubator. After each stimulation period, the media was discarded and the cells washed with ice-cold PBS. The cells were lysed by incubating twice with 0.5 mL of Lysis Buffer (HALT Protease and Phosphatase Inhibitor cocktail in Ripa Buffer, 1:100) for 5 minutes on ice. The cells were scraped from the bottom of the well with a pipette and transferred to a 1.5 mL Eppendorf tube. The lysates were sonicated in ice-cold water and then centrifuged for 10 minutes at 4°C at $14 \times 10^3 g$. The supernatant was transferred into a new Eppendorf tube and the endogenous levels of phospho-Smad3 (Ser423/425) protein were analysed with an ELISA kit (#7348), following the manufacturer's instructions. The Smad2/3 complex levels were quantified by spectrophotometry (Spark, Tecan), reading the absorbance at 450 nm.

2.7.9 Picrosirius Red staining on cells on monolayer

Formalin-fixed cells in 24-well plates (Paragraph 2.7.2) were incubated for 1 hour at RT with 0.5 mL of Picrosirius Red staining (0.5 g Sirius Red in 500 mL of saturated aqueous solution of picric acid). The samples were washed twice with 1 mL of acidic water (5 mL of acetic acid in 1 L of water) and then imaged in PBS. Stained samples were imaged with an EVOS Core XL microscope.

2.7.9.1 Picrosirius Red quantification at the plate reader

Picrosirius Red-stained cells in 24-well plates (Paragraph 2.7.9) were washed once with PBS and an equal volume of a solution of NaOH 1M was added to all wells (400 μ L for a 24 well plate) followed by 30 min incubation at RT. After the incubation time, 100 μ L of the NaOH solution were withdrawn and moved to a new 96 well plate. The absorbance was read at 540 nm at the Plate Reader (Spark, Tecan).

2.7.10 SEM and EDS analysis

2×10^4 hASCs were seeded on glass coverslips in 24-well plates and incubated at 37°C until confluent. The cells were labelled with Ø250 nm anti-Activin RIIA-MNPs, considering 25 µg MNPs/ 2×10^5 seeded cells (Paragraph 2.7.1) and fixed with formalin (Paragraph 2.7.2). Afterwards, the cells were dehydrated by incubation with ethanol-in-water solutions (50% IMS, 70% IMS, 90% IMS and 100% IMS, for 15 min each) and air-dried overnight at RT. Subsequently, the samples were sputter-coated with gold (30 s at 20 mA, Cressington, C5219, Model 108A, USA) and then imaged at the SEM (Auriga Compact, ZEISS, Germany). The elemental characterization was performed at the same time, using an energy dispersive spectrometer (EDS, Pegasus X4M).

2.7.11 Western Blot

12×10^4 hASCs were seeded into 6 well plates (7×10^5 hASC for Day 0 timepoint), cultured with tenogenic media and incubated overnight at 37°C. The following day, the cells were labelled with Ø250 nm anti Activin-MNPs (Paragraph 2.7.1) considering 25 µg of MNPs/ 2×10^5 seeded cells, and incubated with tenogenic media at 37°C up to 14 days. The following experimental groups were investigated:

- i) Unlabelled cells stimulated with MICA (hereafter referred as “cells only”),
- ii) Cells labelled with MNPs stimulated with MICA.

Identical static groups were kept unstimulated at 37°C.

2.7.11.1 Lysate preparation

At day 0, 7 and 14, cells lysates were prepared by firstly discarding the media and washing the cells with PBS. Subsequently, 200 µL of lysis buffer was added to each well and incubated for

15 min at 4°C. The lysis buffer was prepared freshly every time, following the protocol described in Table 2.11. The single inhibitors were reconstituted following the manufacturer instructions.

Table 2.11 Cell lysis buffer composition for Western Blot.

Inhibitor	Function	Stock conc.	Working conc.	Prod code
Protease inhibitor cocktail mix	Serine, cysteine, and metallo-proteases inhibitor	10x	1x	Sigma, P2714
PMSF	Serine protease inhibitor	100 mM	1 mM	Sigma, P7626
Pepstatin A	Aspartic proteases inhibitor	1.5 mM	1.5 µM	Sigma, P5318
NaF	Phosphatase inhibitor	0.8 M	40 mM	Sigma, S1504
Sodium Orthovanadate	Phosphatase inhibitor	200 mM	1 mM	Sigma, 450243
RIPA Buffer	Enables cells lysis	1x	1x	Sigma, R0278

After the incubation time, a sterile cell scraper was used to fully detach the cells. The sample was homogenised by pipetting the cells suspension several times, afterwards the lysate was collected in a 1.5 mL Eppendorf tubes and store at -80°C until further processing.

2.7.11.2 Total protein quantification with BCA analysis

The lysates were thawed on ice and centrifuged at $15.3 \times 10^3 g$ for 10 min to pellet insoluble debris. Following the manufacturer instructions, a BCA total protein assay was performed on

the supernatant to determine the sample total protein concentration (the sample was diluted with milliQ water, dilution 1:1). The samples were then stored at -80°C until further processing.

2.7.11.3 Gel electrophoresis

After defrosting the samples on ice, 10 µg of total protein content were withdrawn and added to a new Eppendorf tube. 10 µL of 4x LDS Sample Buffer and 4 µL of 10x Bolt Reducing Agent were added to the samples and an appropriate volume of milliQ water was added to make up the total volume to 40 µL. To initiate the protein denaturation, the final suspension was heated at 70°C for 10 min on a heating block (HC100-Pro, DLab) and then centrifuged at $15.3 \times 10^3 g$ for 30 sec. An electrophoresis tank (Mini Gel Tank, A25977, Invitrogen) was then filled with 1x MES SDS Running Buffer and 40 µL of the samples were loaded onto a polyacrylamide gel for electrophoresis together with 10 µL of Prestained Standard, following the manufacturer instructions. The electrophoresis run was performed at 200 V and 250 mA for 25 min. When the run was complete, the gel was incubated in a solution of 20% EtOH for 10 min, to help the fixation of the protein bands.

2.7.11.4 Blotting

The pre-run gel was carefully moved to a nitrocellulose membrane placed between an iBlot™ 2 Transfer Stack, following the manufacturer instructions. The iBlot™ 2 Gel Transfer Device (IB21001, Invitrogen) was used to transfer the protein bands to the membrane, as instructed by the manufacturer. The blotted membrane was stored dry at 4°C until further processing.

2.7.11.5 Membrane antibody-staining

Before proceeding with the antibody staining, the blotted membrane was rehydrated in Tris Buffered Saline (TBS) for 5 min at RT. The rehydrated membrane was incubated with a

blocking solution of 5% milk in TBST (0.1% Tween in TBS) for 1 h at RT under gentle agitation. The blocking buffer was used to dilute the primary antibodies, following the dilutions described in Table 2.12. The membrane was incubated with the primary antibody overnight at 4°C under gentle agitation.

Table 2.12 Primary antibodies used for Western Blot.

Antibody	Product code	Molecular weight	Stock conc.	Dilution	Diluent	Host
TNMD	ab203676	37 kDa	1 mg/mL	1:100	Milk buffer	Rabbit
SCXA	ab58655	22 kDa	0.5 mg/mL	1:500		Rabbit
β -Actin	ab8224	42 kDa	1 mg/m	1:1000		Mouse

After incubation with primary antibody, the membrane was washed with TBST 3 times for 5 min each, under gentle agitation at RT. The blot was then incubated with the appropriate HRP-linked secondary antibody diluted in the blocking buffer (Table 2.13).

Table 2.13 HRP-linked Secondary antibodies used for Western Blot.

Antibody	Product code	Diluent	Dilution
Goat anti Rabbit-HRP	#1706515	Milk buffer	1:3000
Goat anti Mouse-HRP	#1706516		1:3000

When the incubation was completed, the membrane was washed 5 times in TBST for 5 min each at RT.

2.7.11.6 Detection

Enough chemiluminescent substrate solution was prepared to evenly cover the membrane (HRP Chemiluminescent substrate Reagent A and B, dilution 1:1). After 3 min incubation with the chemiluminescent substrate solution, the membrane was imaged in a Digital Imager (iBright 1500, Invitrogen) within 30 min from staining, as the light emission decreases with time. After imaging, the membrane was rinsed with dH₂O and stored dry at 4°C. Blots were quantified with ImageJ. β -Actin protein levels were used for data normalisation.

2.7.11.7 Stripping and re-staining

An already stained membrane could be counter-stained for β -Actin control by firstly stripping off the previous antibody staining. To do so, the dry membrane was rehydrated for 5 min in TBS and then incubated for 15 min at RT with the Restore™ Western Blot Stripping-Buffer. The buffer was then discarded, the membrane washed with TBS and then processed again for blocking and antibody staining as previously described (Paragraph 2.7.11.5).

2.7.12 TEM analysis

To check antibody-labelled MNPs cells internalization, 13 T25 flasks were seeded with 1.5×10^6 hASCs and the cells were allowed to attach for 2 h at 37°C. The cells were then labelled with Ø250 nm anti-Activin RIIA-MNPs (Paragraph 2.7.1) considering 25 μ g MNPs/ 2×10^5 seeded cells. The following experimental groups were considered: i) cells only, ii) 30 min, iii) 1 h, iv) 3 h, v) 6 h, vi) 24 h and vii) 48 hours. An identical experiment was run in parallel but incubating the flask on permanent magnets (NdFeB, N42) for the whole length of the experiment. After 90 min incubation with MNPs (or after 30 min and 1 h for the earliest timepoints), the cells were washed twice with PBS, fresh basal media was provided and the flasks were incubated at 37°C. After every cited timepoint, cells were washed with PBS, trypsinized (Paragraph 2.2.2)

and resuspended in 1 mL of a solution 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH=7.4). Afterwards, the cells were centrifuged at 400g for 10 min to create a pellet, which was stored in the same glutaraldehyde/formaldehyde solution at 4°C until processing. The pellet was then resuspended in HistogelTM and stained with aqueous 1% uranyl acetate solution overnight, dehydrated and embedded in Embed-812 resin. 50 nm-thick sections were cut on a RMC Ultramicrotome (PowerTome, USA) using Diatome diamond knives. The sections were mounted on mesh copper grids (Electron Microscopy Sciences) and stained with uranyl acetate substitute and lead citrate for 5 min each. Samples were imaged at JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan) and images were digitally recorded using a CCD digital camera Orius 1100W (Tokyo, Japan). The TEM analysis was carried out at i3S Scientific Platform HEMS facilities, member of the national infrastructure PPBI - Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122).

2.7.13 Presto Blue on monolayer

1.3×10^3 cells were seeded in 96 well plates and incubated at 37°C with the appropriate media. At the desired timepoint, the media was discarded and 100 μ L of a solution of Presto Blue in media were added (dilution 1:10) following the manufacturer's instructions. The cells were incubated for about 2 h (or until the colour of the solution changed to light purple) in the dark at 37°C. After incubation, the solution was withdrawn and put in a new 96 well plate. The fluorescence was read at 560/590 nm at a Plate Reader (Spark, Tecan). Cells were then washed twice with PBS and incubated at 37°C with fresh media until further use.

2.8 Collagen gels

2.8.1 Non-oriented collagen gels

hBMSCs were cultured with basal media in culturing flasks until 80% confluent. After being trypsinized (Paragraph 2.2.2), for every 500 μL gel prepared, the desired cells amount (1×10^4 cells/mL, 2.5×10^4 cells/mL, 5×10^4 cells/mL or 10×10^4 cells/mL) was centrifuged at 350g for 5 min, washed with PBS and resuspended in a solution of 20% HEPES in basal media, according to the final desired collagen concentration. A certain volume of Rat Tail Collagen I was added to the cells dispersion in order to have a final collagen concentration of 1.5 mg/mL, 2 mg/mL or 3 mg/mL. The final cells/collagen suspension was pipetted into a 1 mL cropped syringe working as a mold for the gel³⁰⁴ (Figure 2.2). The syringe was incubated in a water-bath at 37°C for 1 hour until complete setting. Afterwards, the gels were squeezed out of the syringes in a 6 well plate, covered with basal media and incubated at 37°C.



Figure 2.2 Cropped syringe used as mold for 500 μL collagen gels.

2.8.2 Oriented fibres collagen gels

Similar to the preparation of non-oriented collagen gels, hBMSCs were cultured in basal media until confluent and trypsinized (Paragraph 2.2.2). For every 500 μL gel, 5×10^4 cells/mL were washed with PBS and resuspended in a dispersion of Ø250 nm or Ø1 μm anti Activin-MNPs (Antibody2) in SFM (considering 1 mL of SFM for 1 million of cells and 250 μg of MNPs every 500 μL gel total volume)²²⁶. After labelling in suspension for 3 h at 37°C (Paragraph 2.7.3), the cells were centrifuged at 350g for 5 min, washed with PBS and resuspended in the

appropriate amount of 20% HEPES in basal media. To reach a final collagen concentration of 3 mg/mL, the appropriate volume of Rat Tail Collagen I (according to the collagen stock concentration) was added to the cell suspension and the final mixture then was pipetted into a 1 mL cropped syringe. The gel/syringe was placed in between two permanent magnets (Neodymium Magnets, N42, 40x20 mm), in parallel length-to-length 7.2 cm apart (Figure 2.3). The north and south poles of each magnet were interfacing each other and a magnetic field established. The magnetic device was then put in a water-bath at 37°C for 1 hour until the complete setting of the gel. Afterwards, the gels were squeezed out of the syringes in a 6 well plate, covered with basal media and incubated at 37°C elevated from metal shelf present inside incubator.

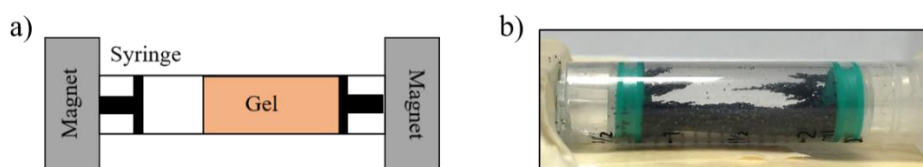


Figure 2.3 Magnetic setup used for collagen gels alignment. a) Schematic representation of the syringe/magnets model used for preparing oriented collagen gels; b) Empty cropped syringe filled with iron filings and placed between two permanent magnets, as a proof of concept.

2.8.2.1 FEMM modeling

A 2D prediction of the magnetic field within the two parallel magnets was performed via FEMM Software (Finite Element Method Magnetics Software),^{286–288} setting “N42 Neodymium” for permanent magnets and “air” as filling space.

2.8.3 Collagen gels fluorescent labelling

A 5 mL 3 mg/mL Rat Tail Collagen I solution was prepared in a Ø10 cm Petri Dish, by diluting the collagen stock solution with the required amount of 20% HEPES in DMEM. 40 µL of sterile

NaOH 1M were carefully added, until the collagen solution turned bright pink. Keeping the solution sterile, the collagen was allowed to polymerize at RT for about 1 h. The completely gelled collagen was then covered with PBS and stored at 4°C overnight. The PBS was discarded and the gel was incubated for 15 min at RT with 10 mL of sterile 50 mM borate buffer (1.55 g boric acid in 400 ml dH₂O, corrected to pH=9). The volume of dye (VD) required to label the amount of collagen protein was calculated using the following equation:

$$VD = \frac{(\text{mg collagen protein/MWC}) \times \text{molar excess of dye} \times \text{MWD}}{CD}$$

Where MWC, MWD and CD were, respectively, the molecular weight of collagen, the molecular weight of the dye and the concentration of the dye stock solution. 45.28 µL of Atto-488 NHS ester dye (5 mg/mL, reconstituted in DMSO) were added to 50 mM borate buffer to give a final volume of 5 mL. The collagen was labelled with the dye solution for 1 h at RT in the dark (or overnight at 4°C) while rocking slowly. After the incubation, the dye solution was discarded and the labelling reaction was quenched by incubating the collagen with 10 ml of sterile TBS 50 mM for 10 min at RT while rocking slowly. The excess of dye was then removed by rinsing the collagen six times for 20 min with sterile Dulbecco's Phosphate Buffered Saline with MgCl₂ and CaCl₂ (DPBS++). All the DPBS++ was carefully discarded and the gel was incubated with 750 µL of sterile acetic acid 500 mM for 1 h at 4°C in the dark under slow rocking. When the collagen gel was completely turned into a liquid and with no visible clumps, the labelled collagen was transferred to a glass vial and stirred for 1 h at 4°C. The solution was stored overnight at 4°C. The collagen solution was pipetted into a wet Slide-A-Lyzer cassette and the solution was dialyzed by immersing the cassette into 2-4 L of 20 mM acetic acid. The dialyzing solution was equilibrated to 4°C and stirred in a cold room in the dark. After 4 h, the acetic acid solution was replaced with one freshly prepared and the dialysis was carried out overnight in the dark. Finally, the dialyzed collagen solution was removed from the cassette

and centrifuged for 1 h at $2 \times 10^4 g$ at $4^\circ C$ to remove any collagen clumps. The supernatant was collected and the dye-labelled collagen was stored in the dark at $4^\circ C$ up to 1 year.

2.8.4 Non-oriented and oriented fluorescent collagen gels

The protocol used for the preparation of non-oriented and oriented fluorescent collagen gels was similar to the one used in Paragraph 2.8.1 and 2.8.2, but starting from a fluorescent collagen stock solution obtained diluting 1:10 the fluorescent collagen stock solution with a non-fluorescent collagen stock solution.

2.8.5 Live/dead assay on collagen gels

7 days after the preparation of the collagen gels, the media was discarded and the gels were washed with PBS. The cells viability was checked using a Live/Dead assay following the manufacturer instructions. In particular, a solution of 5 μL of Calcein and 20 μL of EthD-1 in 10 mL of PBS was added to the gels until completely covered. The gels were incubated in the dark at RT for 30 minutes. After the incubation time, the gels were washed twice with PBS and imaged in PBS at a fluorescent microscope (EVOS M5000, Thermo Fisher).

2.8.6 Collagen gels contraction study

Non-oriented collagen gels were prepared freshly as previously described (Paragraph 2.8.1) seeding different concentrations of hBMSCs (1×10^4 cells/mL, 2.5×10^4 cells/mL, 5×10^4 cells/mL or 10×10^4 cells/mL). The gels were incubated at $37^\circ C$ for 21 days. At day 0, 7, 14 and 21, the length and diameter were measured with a sterile metallic ruler.

2.8.7 Oriented collagen gels preparation in chamber slides

To establish magnetic collagen type I hydrogels, the appropriate amount of Ø250 nm anti-Col I MNPs, were separated from aqueous suspension in a magnetic field generated by a permanent magnet in order to have a final MNPs concentration of 1 µg/µL. MNPs were then resuspended in PBS to give a final solution of 20% MNPs in PBS. The pH of the collagen/MNP suspension was adjusted to pH=7.4 by adding 1 M NaOH (approximately 5% NaOH final concentration). The MNP suspension was then homogenized with a volume of fluorescent collagen solution (fluorescently labelled rat-tail collagen I and non-fluorescent rat-tail collagen I solution at a ratio of 1:10) to give a final collagen I concentration of 3 mg/mL. 20% HEPES-full DMEM was added to make up the final 100 µL gel volume. Further to this, hASC (PT-5006, Lonza) were resuspended in the collagen/MNP solution at a density of 2.5×10^4 cells/ml prior to being aliquoted into the each well of an 8 well chamber slide. The chamber was then placed between two N42 40x20 mm neodymium magnets (Bunting Magnetics, EP352), in parallel length to length 7.2 cm apart. The north and south poles of each magnet were interfacing each other and a magnetic field established. The chamber-well slides were parallel to the length of the magnets. Simultaneous to application of magnetic fibrillogenesis was initiated by incubating at 37°C for 30-minutes. After, the magnetic field was then removed and gels immersed in basal media and further incubated at 37°C, elevated from metal shelf present inside incubator.

2.8.8 Immunohistochemistry of collagen hydrogels in chamber slides

3 mg/mL 3D fluorescent collagen gels were prepared as previously described (Paragraph 2.8.7), with a cell density of 2.5×10^4 cells. After culturing in basal media for 7 days, gels were fixed in formalin for 20 min, washed three times in PBS and incubated in 0.1 M glycine in PBS for 5 min. Cells were then permeabilized in 0.1% Triton X-100 in PBS for 3 min. Cellular actin filaments were stained with Rhodamine Phalloidin (R415, Thermo Fisher) (1:2 phalloidin:PBS)

for 50 min at RT. The gels were washed three times with PBS and then immersed in DAPI for 10 min at RT. The samples were washed three times with PBS, immersed in PBS, and imaged by confocal microscopy. If required, gels were stored at 4°C in PBS.

2.8.9 Collagen fibres alignment quantification

To quantify the orientation of the fibre-like collagen structures, light and confocal microscopy images were analyzed with the Directionality plugin for ImageJ Software (ImageJ 1.52n, Image processing and analysis in Java, National Institutes of Health). Fourier spectrum analysis provides a Gaussian distribution of the orientation of fibre-like structures. The orientation is relative to deviation from a transect across the width of the image, considered 0°. Measurement of the amount of structures was taken every 2° from 0-180°. Orientation values were plotted as an average of the amount measured for three different gels, for each experimental group. The same analysis was adopted for determination of hASC f-Actin orientation, following conversion of confocal images into binary format.

2.9 Fibrin gels

2.9.1 Reagent preparation

A solution 0.9% NaCl in dH₂O was used to reconstitute fibrinogen, thrombin and aprotinin to the get stock concentrations as follows:

Table 2.14 Reagent stock concentrations for fibrin gels preparation.

Reagent	Stock conc.
Fibrinogen	83.3 mg/mL
Thrombin	100 IU/mL
Aprotinin	1 mg/mL

500 μ L aliquots of each reagent were stored at -20°C in 1.5 mL centrifuge tubes for up to six months. To obtain working concentrations, these reagents were thawed at RT and further diluted with 0.9% NaCl.

2.9.2 Fibrin gels preparation

hASCs were labelled in suspension with $\text{Ø}250$ nm anti Activin-MNPs as previously described in Paragraph 2.7.3. To prepare a 50 μ L gel, 15 μ L of cell suspension in basal media at the desired cells density (20×10^4 cells/gel or 3×10^4 cells/gel), was mixed with 20 μ L fibrinogen at different working concentrations (20 mg/mL, 30 mg/mL, 40 mg/mL or 50 mg/mL). Separately, 2 μ L aprotinin (1 mg/mL) was combined with 10 μ L calcium chloride (100 mM) and 5 μ L thrombin at different working concentrations. 17 μ L of the aprotinin/ CaCl_2 /thrombin solution was mixed to 35 μ L of the cells/fibrinogen dispersion. The final mixture was resuspended once and then squeezed in a 96 well plate. The fibrin gel begins to set after about 30 seconds. After incubating the gels at 37°C for 30 minutes, 150 μ L of basal medium supplemented with aprotinin (20 $\mu\text{g/mL}$) were added to each gel. For acellular gels, the cell suspension was substituted with 15 μ L of appropriate medium. 150 μ L of fresh media supplemented with aprotinin was provided every day.

2.9.3 Presto Blue on cellular fibrin gels

Similarly to the procedure used for Presto Blue assay on cells on monolayer (Paragraph 2.7.13), after preparing the gels in a 96 well plate, the media was discarded and 100 μ L of a solution 1:10 of Presto Blue in media supplemented with 20 $\mu\text{g/mL}$ aprotinin were added, following the manufacturer's instructions. The gels were incubated for about 2 hours (or until the colour of the solution changed to light purple) in the dark at 37°C . After incubation, the solution was withdrawn and put in a new 96 well plate. The fluorescence was read at 560/590 nm at a Plate

Reader (Spark, Tecan). If the gels were still needed after the assay, they were washed twice with PBS, fresh media was provided and the plate was incubated at 37°C.

2.9.4 Gelation time study

A plate reader (Spark, Tecan) was used to test the fibrin gels gelation time at different thrombin concentrations. In particular, acellular 100 μ L fibrin gels were prepared in 96 well plates as described in Paragraph 2.9.2 considering 30 mg/mL fibrinogen and 20 IU/mL, 50 IU/mL, 60 IU/mL, 70 IU/mL, 80 IU/mL, 90 IU/mL or 100 IU/mL thrombin final working concentrations. Firstly, once the gels were completely set, the complete absorbance spectra were acquired between 200 nm and 1000 nm, at a controlled temperature of 25°C. This was to identify an appropriate wavelength at which it was possible to appreciate a distinguishable difference between the spectra of the different fibrin gels tested. The selected wavelength was 405 nm, which was also adequately distant from the background absorption of the plastic well plate ($\sim$ 200/300 nm).^{289,290} The fibrin gels at each thrombin concentrations were prepared again freshly one at a time and placed immediately in the plate reader, at a controlled temperature of 25°C. The absorbance spectra were acquired at 405 nm over the time, for up to 5 min.

2.9.5 Degradation study

3×10^4 hASCs, either or not labelled with Ø250 nm Anti-Activin RIIA-MNPs (considering 25 μ g MNPs/ 2×10^5 cells), were used to prepare 50 μ L fibrin gels as described in Paragraph 2.9.2, with a final fibrinogen and thrombin working concentration of respectively 30 mg/mL and 90 IU/mL. Once the fibrin gels were completely set, they were carefully moved to a 24 well plate and incubated with 500 μ L of basal media without aprotinin at 37°C. After 0, 7, 14 and 21 days the gels were weighted and the media was collected and stored at -80°C. 300 μ L of the collected media were mixed to 200 μ L of nitric acid and heated at 60°C in a water-bath for 1 hour.

Afterwards, the tubes were allowed to cool down at RT and then the solution was diluted by adding 4.5 mL of milliQ water, to reach a final minimum volume of 5 mL at 5% nitric acid. The solution was first filtered with Ø0.45 µm syringe filters to remove any insoluble debris and then an ICP analysis was performed (JY2000-2, Horiba Jobin, Yvon). Iron Plasma Standard (1,000 ppm) in milliQ water solutions were used as standards. Basal media without aprotinin was used as blank.

2.10 Histology

2.10.1 Samples preparation for paraffin sectioning

Once the collagen or the fibrin gels were prepared (Paragraph 2.8.2 and 2.9.2), the media was discarded and the gels were washed with 3 mL of PBS. The gels were fixed in formalin overnight at 4°C, washed once with 3 mL of PBS and dehydrated by incubation at RT with increasing concentration ethanol-in-water solutions (70% IMS, 80% IMS, 90% IMS and 100% IMS), for 2 h each. Afterwards, the gels were incubated with xylene for 20 min at RT and then they were immersed in 60°C melted paraffin wax for 1 h (Histo Star, Thermo Scientific). The samples were then loaded in histology cassettes and aligned in the direction of future sectioning. The wax was solidified on a cold plate for about 20 min (Histo Star, Thermo Scientific).

2.10.2 Slides coating for sectioned samples

In order to improve sections adhesion, glass slides were coated with (3-Aminopropyl)triethoxysilane (APTES). In particular, the slides were subsequently dipped in: 100% acetone, a solution of 2% APTES in acetone, and DI water for 2 minutes each at RT. The slides were then dried overnight in the oven at 60°C.

2.10.3 Samples sectioning

A microtome (HistoCore BioCutR, Leica) was used to create 7 µm-thick sections of paraffin embedded samples. Sections were placed on APTES-coated glass slides (Paragraph 2.10.2) and incubated overnight in the oven at 60°C to dry out water before deparaffinization.

2.10.4 Paraffin sections rehydration and dehydration

After overnight incubation at 60°C, the sectioned samples were deparaffinized by immersion in xylene twice for 3 min each. Slides were then rehydrated in a reverse ethanol-in-water sequence (100% IMS twice for 3 min each, 90% IMS for 3 min and 100% dH₂O for 3 min). This procedure was performed prior to every histology staining. After any histology staining, the sections were washed with dH₂O and dehydrated by immersion in an ethanol-in-water sequence (90% IMS for 3 min, 100% IMS twice for 3 min each, 100% xylene twice for 3 min each). Coverslips were finally mounted with DPX Mountant for histology and the slides were dried overnight at RT.

2.10.5 Picrosirius Red staining on paraffin sections

After rehydrating the samples (Paragraph 2.10.4), the slides were stained with Picrosirius Red staining for 1 h at RT. The slides were washed twice with acidified water (5 mL of glacial acetic acid in 1 L of dH₂O) to remove any stain excess. The samples were then dehydrated (Paragraph 2.10.4) and glass coverslips were mounted with DPX. Stained samples were imaged with an EVOS Core XL microscope or at a Polarised Microscope (Kern Optics).

2.10.6 Prussian Blue staining on paraffin sections

Paraffin sectioned samples were rehydrated (Paragraph 2.10.4) and then incubated for 20 min at RT with Prussian Blue staining, obtained from mixing 1:1 a solution 20% hydrogen chloride

in water and a solution 1% potassium ferrocyanide in water. The samples were washed with dH₂O, dehydrated and the coverslips were mounted on the slides following the protocol described in Paragraph 2.10.4. Stained samples were imaged with an EVOS Core XL microscope.

2.11 *In vivo* study

The *in vivo* study was conducted under the supervision of Professor Manuela Gomes at the facilities of 3B's Research Group, I3Bs - Research Institute on Biomaterials, Biodegradables and Biomimetics (University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Avepark - Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal) and ICVS/3B's – (PT Government Associate Laboratory, Braga/Guimarães, Portugal). Animal protocols were conducted in accordance with Portuguese legislation (Portaria no1005/92) and international standards on animal welfare as defined by the EC Directive 2010/63/EU. All animal manipulations were performed only by qualified personnel to minimize distress and discomfort and the principle of the 3Rs were rigorously applied. The host institution is authorized by the DGVA (Direção Geral de Alimentação e Veterinária) to perform animal experimentation.

2.11.1 hASCs culturing

2.11.1.1 Media preparation

hASCs culture media was prepared by mixing 5 g of α MEM powder and 1.1 g NaHCO₃ in 500 mL dH₂O. The solution was corrected to pH = 7.4 and sterile filtered. 50 mL of FBS and 5 mL of A/A solution were then added in order to have a media solution at, respectively, 10% and 1% v/v (for the *in vivo* study, this media will be hereafter called “basal media”). Serum free media was prepared in identical way, but without adding FBS.

2.11.1.2 hASCs general handling

For the *in vivo* study, unless otherwise specified, cells general handling was performed following the same protocols followed for the *in vitro* studies and described in Paragraph 2.2.

2.11.2 hASCs MNPs labelling

The anti-Activin RIIA antibody MAB340 was used to functionalise Ø250 nm MNPs following the protocol described in Paragraph 2.5. hASCs were then labelled in suspension with previously tagged MNPs, as described in 2.7.3, considering 25 µg MNPs every 2×10^5 cells.

2.11.3 Fibrin gels preparation

Fibrin gels were prepared following the protocol described in Paragraph 2.9.2. In particular, 25 µL gels were prepared with a final fibrinogen concentration of 30 mg/mL and a final thrombin concentration of 90 IU/mL. 5×10^5 hASCs tagged with anti-Activin RIIA-labelled MNPs were included during the preparation of each gel. 10 µL of the final gel were injected in the tendon defect (2×10^5 cells labelled with 25 µg MNPs were implanted in each rat).

2.11.4 Animal surgeries

An *in vivo* patellar tendon defect was used to investigate the host response to the developed remotely-activated anti-Activin RIIA MNPs-labelled hASCs in the context of tendon regeneration. A total of 22 male nude rats (49-55 days old RNU male rats with an average weight of 250 g) were used to conduct this experiment. Animals were given an initial anaesthesia by inhalation of isoflurane (1-3% in O₂), followed by intraperitoneal (IP) injection of an anaesthetic solution of ketamine (75 mg/kg, IP) and medetomidine (0.5 mg/kg, IP). The right rear limb knee was incised to expose the patellar tendon, where a window defect was then created with a Ø2 mm biopsy punch. The defect was filled with 10 µL of the cellular fibrin

construct previously prepared (Paragraph 2.11.3) (Figure 2.4). The gel was allowed to set for 2 min and the incision was carefully closed using surgical stitches. As control, a non-treated group was also included in the study (hereafter called “Sham group”), which received the patellar defect but no treatment was administered. After surgery, anaesthesia was reversed with Antisedan (1 mg/Kg, IP). Animals were fed ad libitum and allowed unrestricted cage activity. During follow-up, the animals were monitored every day looking for signs of infection or discomfort according to 3Rs regulation. One of the animals died during the experimental timeline and it was replaced.

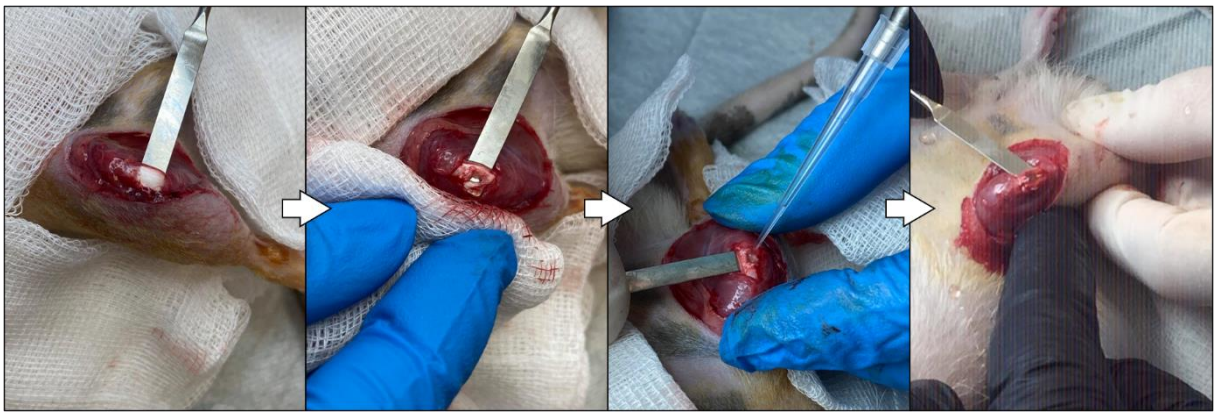


Figure 2.4 In vivo surgical procedure. From left to right: exposure of the patellar tendon; creation of a window defect with Ø2mm biopsy punch; injection of the cellular fibrin construct; setting of the construct before closing the wound.

2.11.5 Euthanasia and organs collection

Inhalation of isoflurane (1-3% in O₂) was provided to the animals as initial anaesthesia, followed by IP injection of an anaesthetic solution of ketamine (75 mg/kg, IP) and medetomidine (0.5 mg/kg, IP). For future analysis, the blood was collected via intracardiac puncture. Afterwards, an intracardiac overdose of pentobarbital (200 mg/kg) was delivered to the animals, followed by cervical dislocation as an additional method to confirm euthanasia. Subsequently, the experimental patellar tendon and the contralateral patellar tendon (left back

limb) were collected, together with liver, spleen and lungs. Organs and tendons were extensively washed in PBS and fixed in formalin at RT for 2 weeks.

2.11.6 Magnetic Box

To recreate *in vivo* the remote magnetic activation of MNPs-labelled hASCs initiated *in vitro* by MICA, a box with fitted magnets was designed and assembled (the Physics Workshop at the University of Birmingham provided a Solidworks CAD model of assembly, showed in Figure 2.5, and the box manufacturing). The aim was to create a contained space where the rats could move being subjected to an external magnetic field. For this project, 64 permanent magnets (Magnet-Cuboid L40xW40xH15, NdFeB/Ni-N42, HKCM Engineering e.K.) were carefully arranged in order to create a variable magnetic field in the box (NHF Consulting, Cheshire, UK, carried out the magnets assembly within the box). This way, the anti-Activin RIIA-MNPs tagged to hASCs implanted in the patellar tendon were subjected to a changing magnetic field as the rat moved inside the box. Considering the rat housing guidelines, the box was designed considering a minimum rat walking area of 800 cm² and a minimum 20 cm cage height (in accordance to the Portuguese Decree-Law n° 113/2013 of 7th August, and to the European Directives 86/609/EEC of 24 November 1986 and 2010/63/UE of 22 September 2010). Therefore, the box was designed to be 22 cm tall and consisted of two sides (42 cm) containing an array of 8x3 permanent magnets each side, two short sides (29.5 cm) containing no magnets and a central wall (20 cm) containing a 4x4 array of magnets. The box dimensions would have allowed to include a 8x4 magnet array on the long sides, but 3D magnetic field computational modelling (COMSOL 5.1 Software, Paragraph 2.11.6.2) showed that the first row of magnets from the top, was not bringing any additional contribution to the field. Considering this and the average rat knee distance of about 5 cm from the floor, it was decided to not include the highest row of magnets when assembling the box. The central wall was

included in the project with the purpose to increase the magnetic field in the walking area on the box, which otherwise would have been too low as being the long magnetic walls almost 30 cm apart. To reduce the dispersion of the magnetic field outside the box, while re-directing it towards the inside, two 1 cm-thick iron plates were applied on the outside of the long magnetic sides. Other two identical boxes were built, but without including the permanent magnets.

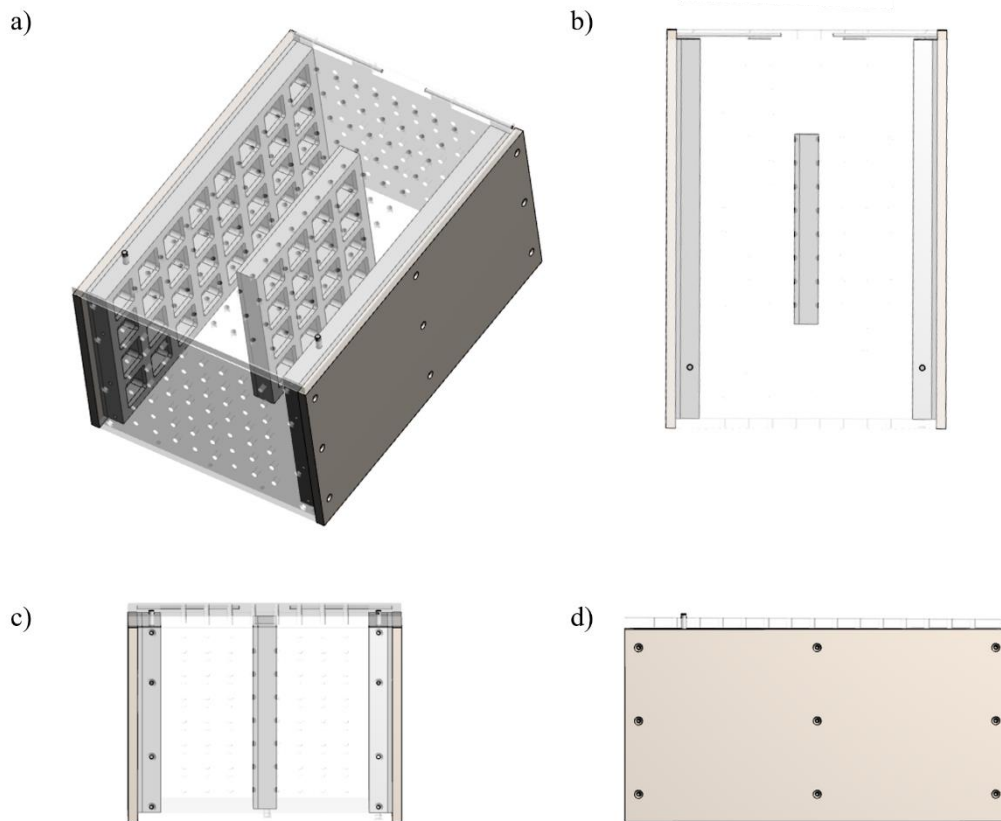


Figure 2.5 Solidworks CAD model of assembly of the box. a) general view, b) top view, c) front view and d) lateral view.

2.11.6.1 2D magnetic field mapping

A 2D magnetic field prediction within the box was performed via FEMM Software (Finite Element Method Magnetics Software),^{286–288} setting “N42 Neodymium” for permanent magnets, “Pure iron” for the iron shielding plates and “air” as filling space.

2.11.6.2 3D computational modelling

A 3D magnetic field computational model was generated with COMSOL 5.1 Software.^{291–294} All nonmagnetic parts were removed to reduce the computational demand. Magnetic Fields physics module was used, solving Amperes Law in 3D. Each permanent magnet was initialised with static value of remnant Magnetic Flux at 1.28 T (+1.28 T in Z-axis designated “North” facing, -1.28 T in Z-axis designated “South” facing). The shielding plates on the outer walls were initialised with standard BH curve for Iron, with a relative permeability of 4000 to ensure no flux leaked outside the box. Course meshing was employed for permanent magnets (due to no change in flux) with a much finer mesh (maximum mesh size = 0.0082 m, convergence = 1×10^{-5}) employed for shield plates and air to accurately map flux densities within system.

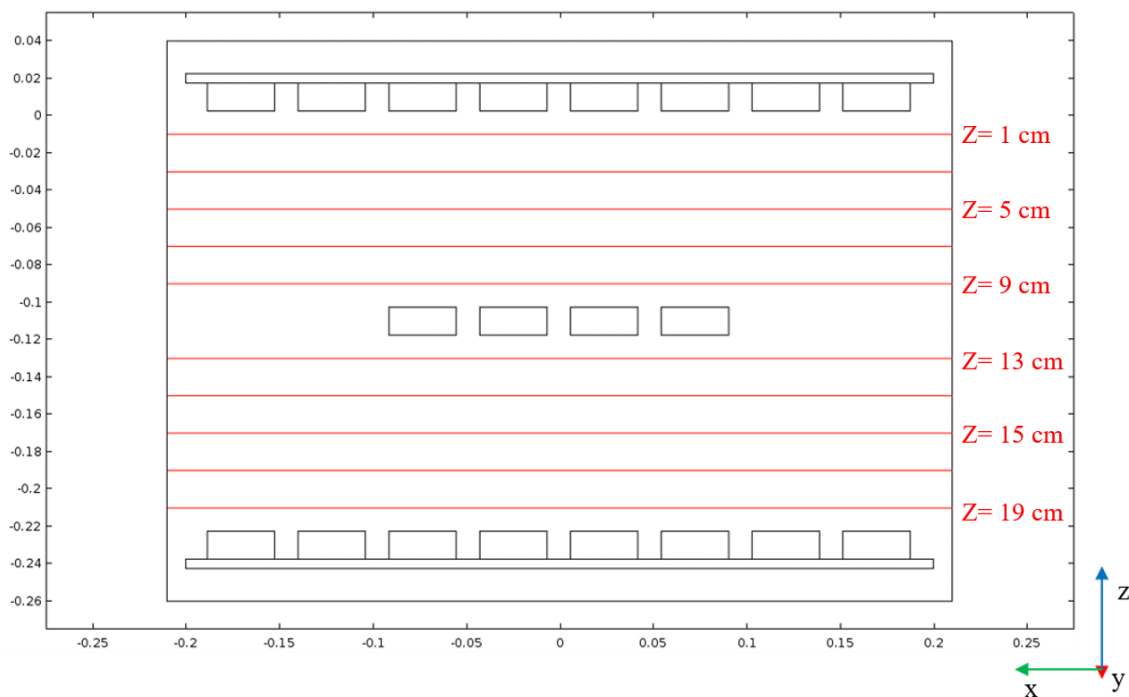


Figure 2.6 Map of linear paths along which the magnetic field was investigated.

Considering that the fibrin scaffold would be implanted in rat rear limbs patellar tendons, approximately 5 cm from the floor, the magnetic field profile and the forces applied to a Ø250 nm MNP ($\chi = 8.26$) were calculated from slices of the 3D model taken at 1 cm and 5 cm from

the external magnetic wall (Z-axis) at a fixed height of $y = 5$ cm (Figure 2.6). $Z=1$ cm represented the closest Z-slice to the external wall, while $Z=5$ cm was the middle of the corridor between the external and the middle magnetic walls. In particular, the magnetic flux in the X-, Y- and Z- planes were calculated, as well as normalized total magnetic flux ($|B|$) at each co-ordinate position along the slice, considering the following equation:

$$|B| = \sqrt{Bx^2 + By^2 + Bz^2}$$

With B_x , B_y and B_z being the magnetic flux in x-, y- and z- planes respectively.

Subsequently, the force applied to a $\varnothing 250$ nm MNP moving along a linear path in the X- plane at a fixed height of $y=5$ cm was calculated by the following equation:

$$F = \frac{V_p}{\mu_0} (\chi_p - \chi_f) B \cdot \nabla B$$

Where V_p was the particle volume, χ_p and χ_f were the magnetic susceptibilities of the particle and fluid respectively. B and ∇B were the applied magnetic field and resulting field gradient, respectively. The 3D magnetic field simulations described above were performed by Dr Thomas J. Broomhall (Healthcare Technology Institute, University of Birmingham).

2.11.6.3 Magnetic field empirical measurement

The magnetic field within the box was measured manually using a Gaussmeter GM07 with transverse probe (Hirst, GM07). 40 measurements were acquired along the magnetic wall at 1 cm from it and at 5 cm from the floor.

2.11.7 Study design and animal stimulation

The study was performed twice at two different timepoints. In particular, a first experiment was carried out for 4 weeks and a second study for 8 weeks, to test short-term and long-term effects of the treatment, respectively. The following experimental groups were considered:

Table 2.15 Experimental groups with animals involved and type of stimulation performed.

	Group	Implant	Stimulation	Euthanasia
i)	Sham	Empty defect	No stimulation	8 weeks
ii)	Cells only -mag	Fibrin/hASCs	Box w/o magnets	4 weeks
iii)	Cells +MNPs -mag	Fibrin/MNPs/ hASCs	Box w/o magnets	
iv)	Cells +MNPs +mag	Fibrin/MNPs/ hASCs	Box w/ magnets	
v)	Cells only -mag	Fibrin/hASCs	Box w/o magnets	8 weeks
vi)	Cells +MNPs -mag	Fibrin/MNPs/ hASCs	Box w/o magnets	
vii)	Cells +MNPs +mag	Fibrin/MNPs/ hASCs	Box w/ magnets	
	Fibrin/hASCs: fibrin gels with embedded hASCs			
	Fibrin/MNPs/hASCs: fibrin gels with embedded MNPs-labelled hASCs			

3 animals were included in each group. While group i) was left unstimulated in the cage as control, groups iv) and vii) were stimulated in the box with permanent magnets. Groups ii), iii), v) and vi) were stimulated in the boxes without magnets in order to allow them to exercise and freely move in a space with the same area and shape as the animals stimulated in the box with magnets. Animals were stimulated in the respective boxes 1 h/day, 3 days a week, with first stimulation happening 48 h after surgery. To minimise the number of animals, in accordance to

3Rs principles, only one Sham group was included in the study and sacrificed after 8 weeks from surgery.

2.11.8 Organs and tendons paraffin embedding and sectioning

After 2 weeks fixation in formalin, organs and tendons were placed in an automatic Tissue Processor (Spin Tissue Processor, STP120-2, Microm). Here, samples were incubated 2 h in formalin and then dehydrated by incubation in increasing concentration ethanol-in-water solutions (70%, 80%, 90% for one h each and 100% ethanol for 3 h), followed by 2 h incubation in xylene. Dehydrated organs and tendons were embedded in paraffin for 2.5 h and 4 µm thick sections were obtained using a microtome (HM355S, Microm, Thermo scientific). Sections were incubated in the oven at 60°C overnight to dry out water before deparaffinization.

2.11.9 FISH

A Fluorescent *in situ* hybridisation (FISH) analysis was carried out on tendon sections to identify and locate any human content, which would testify the presence of implanted hASCs. The analysis was performed automatically creating a 4-plex *in situ* hybridization software protocol on the Leica BOND RX System, based on ACD LS Multiplex protocol. Briefly, the slides were firstly de-paraffinized by incubation with BOND Dewax Solution, followed by a pre-treatment for epitope/enzyme retrieval. In particular, a heat-induced epitope retrieval was performed by incubating the slides for 15 minutes with Bond TM Epitope Retrieval 2 at 95°C, followed by a 15 min incubation with RNAscope Protease III for enzyme retrieval. Afterwards, sections were incubated for 2 h at 42°C with RNA probes anti Ubiquitin C (UCB), anti-Peptidyl-prolyl cis-trans isomerase B (PPIB) and anti RNA Polymerase II Subunit A (PolR2a) (Table 2.16) and then extensively washed with BOND Wash Solution from the reagent excess. The hybridisation was then pre-amplified by incubation with pre-amplifier solutions for 30 min at

42°C, followed by extensive washes with BOND Wash Solution and incubation for 15 min at 42°C with HRP-linked antibody solutions, one for each probe. The slides were then incubated with three fluorophores diluted in Tyramide Signal Amplification buffer (TSA), which also provided a further signal amplification and a minimisation of the background noise. The slides were then extensively washed with BOND Wash Solution.

Table 2.16 Probes and fluorophores used for FISH analysis.

Probe	Fluorophore	Dilution	Diluent
POLR2A	Opal 520	1:150	TSA buffer
PPIB	Opal 570	1:150	TSA buffer
UBC	Opal 620	1:150	TSA buffer

The proteins were then blocked for 15 min at 42°C and then extensively washed with BOND Wash Solution. The slides were counterstained for DAPI for 10 min at RT and then washed three times with dH₂O. Glass coverslips were finally mounted with ProLong Diamond Antifade Mountant and the slides were imaged using Vectra Polaris, Automated Quantitative Pathology Imaging System. The FISH analysis and imaging were performed at the Institute of Inflammation and Ageing facilities (University of Birmingham), with the support of the Rheumatology Research Group.

2.11.10 Histology

Before any histological staining, tissue paraffin sections were deparaffinised and rehydrated using an automatic staining equipment (HMS740, Microm, Thermo scientific). Slides were incubated twice in xylene for 5 min and 3 min, followed by rehydration in ethanol-in-water solutions (100%, 95%, 70%) for 2 min each. Slides were rinsed in water for 2 min and stained appropriately. Stained slides were dehydrated in reverse ethanol-in-water sequence (70%, 95%,

100%) for 2 min each and incubated twice in xylene for 5 min and 3 min. Subsequently, glass coverslips were mounted with Histofluid and dried overnight at RT.

2.11.10.1 H&E on animal tissues

Rehydrated tissue sections were stained with H&E using an automatic staining equipment (HMS740, Microm, Thermo scientific), following a standard H&E protocol. Stained samples were imaged with a Cytation5 microscope (BioTek).

2.11.10.2 Perls van Gieson

Iron deposition was investigated in organs, using Perls van Gieson staining kit, following the manufacturer instructions. Reactive ferric iron was coloured in blue, collagen in purple/red, while cytoplasm, muscle, corny layer of the skin, neuroglia and erythrocytes were stained in yellow. Stained samples were imaged at an optic microscope (DM750, Leica).

2.11.10.3 Sirius Red Picrate

Collagen deposition and matrix arrangement in tendon samples, were analysed using a Sirius Red Picrate staining kit following manufacturer instructions. Collagen fibres were stained in red, bilirubin was stained in green, nuclei in blue and erythrocytes in green. Stained samples were imaged at a Fluorescence Inverted Microscope with Incubation, under polarized light (Axio Observer 3851000135, Zeiss). Collagen fibres orientation was analysed with Directionality plug-in of ImageJ Software (ImageJ 1.52n, Image processing and analysis in Java, National Institutes of Health) in Fourier spectrum analysis. Collagen fibres orientation distribution was expressed as ratio Col I/Col III. ImageJ Software was also used to quantify the signal intensity from the acquired pictures. The refractive signal intensity was expressed as ratio Col I/Col III.

2.11.11 Immunostaining on animal tendons

Tendons sections, once rehydrated (Paragraph 2.11.10), were incubated for 30 min with a boiling solution of antigen retrieval (10 mM sodium citrate in 0.05% Tween in dH₂O, corrected to pH=6). After washing with PBS, the sections were blocked with a solution of 3% BSA in PBS for 30 min at RT. The slides were then incubated overnight at 4°C with the primary antibody:

Table 2.17 Primary antibodies used for immunohistochemistry.

Primary antibody	Product code	Stock conc.	Dilution	Host
TNMD	Ab203676	1 mg/mL	1:200	Rabbit
CD45-FITC	A163B0135	-	1:50	-
CD163	MCA342GA	-	1:50	Mouse

Afterwards, sections were washed with PBS for 5 min and then incubated at RT for 2 h with the secondary antibody diluted in 1% BSA (Table 2.18).

Table 2.18 Secondary antibodies used for immunohistochemistry.

Secondary antibody	Product code	Stock conc.	Dilution	Reactivity
AlexaFluor 488	A11059	2 mg/mL	1/200	Anti-Mouse
AlexaFluor 594	A11012	2 mg/mL	1/200	Anti-rabbit

The slides were washed twice with PBS for 5 min and a glass coverslip was mounted with Fluoromount. Stained samples were imaged with a Cytation5 microscope (BioTek). ImageJ Software (ImageJ 1.52n, Image processing and analysis in Java, National Institutes of Health) was used to quantify the signal intensity from the acquired pictures.

2.11.12 μ CT analysis

Prior fixation in formalin, tendon regeneration and architecture was analysed using a high-resolution X-Ray Microtomography System Skyscan 1072 scanner (Skyscan, Kontich, Belgium) and a Microtomography System 1272 scanner (Skyscan, Belgium). Data sets from the acquisitions were reconstructed using NRecon Software (version 1.6.6.0, SkyScan). DataViewer Software (version 1.4.4, Skyscan) and CT Vox Software (version 2.3.0, Skyscan) were used to build tendons 3D virtual models of the from acquired data sets.

2.11.13 Plasma analysis

Immediately after collection, the blood was centrifuged at $1 \times 10^3 g$ for 10 min at $4^\circ C$. The pellet, containing erythrocytes, coagulation factors and white cells, was discarded, while the supernatant, containing the plasma, was stored at $-80^\circ C$ in 1.5 mL tubes until further processing. Plasma levels of 14 cytokines (G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, TNF- α) were analysed with Multiplex Assay (ProcartaPlex, Thermo Fisher), following the manufacturer instructions. Plasma was used diluted 1:3 with the Universal assay buffer (1x) provided in the kit. The fluorescence was measured using a Luminex instrument (Luminex, MAGPIX, MAGPX20114721, Luminex BV) following the manufacturer instructions. Each cytokine level of Cells+MNPs-mag and Cells+MNPs+mag was expressed as ratio between the value of the cytokine in each animal and the average of the cytokine levels in all the three animals of Cell only-mag group.

2.12 Statistical analysis

Statistical analysis was performed using the Column analysis tool in GraphPad Prism® Software version 7.00 (San Diego California, USA). The significance of difference between

two groups was determined using unpaired, two-tailed Student t-test, while 2-way ANOVA with Post-hoc Sidak's Multiple Comparison analysis were used to analyse differences between three or more groups. For experiments repeated three times, the means of technical replicates of each experiment were used to calculate an overall mean for every analysed condition. These values were then compared using an ordinary 2-way ANOVA with Post-hoc Sidak's Multiple Comparison analysis. Significance was set to * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and results were presented as mean $\pm$ standard deviation (SD).

CHAPTER III

ACTIVIN RIIA ACTIVATION:

COMPARISON OF ANTIBODY BINDING AND CELL TYPES

3.1 Introduction

Tendons are known to be mechano-sensitive tissues¹⁷ and it has been shown how the application of a certain mechanical stimuli has a positive impact in collagen deposition¹⁸ and in the expression of tenogenic markers like SCXA, TNMD and Tenascin C.^{83–86} Therefore, the magnetic mechano-activation of mechanosensitive receptors on stem cells surface might be an effective strategy for stem cells differentiation towards the tenogenic commitment. Nanotechnologies have often been used in conjunction with stem cells for tissue regeneration strategies²⁰⁶ and, in particular, MNPs have been widely used in the regeneration of a multitude of tissues, including bone^{220–222} cartilage^{223,224} and neuronal cells.^{226–229} MNPs are in fact highly biocompatible and they can be functionalized with a broad range of polymers, proteins or antibodies (Ab), widening their application range.^{206,208} Used in conjunction with a magnetic bioreactor, they can deliver a remote mechanical stimulus to the cells they are tagged to, with the advantage of maintaining sterility and being minimally invasive.²⁴²

In a previous work from Gonçalves et al.,²⁸⁵ Anti-Activin RIIA MNPs have been employed in conjunction with an oscillating magnetic bioreactor (MICA Biosystems Ltd) to promote the activation of the receptor towards the initiation of the tenogenic signalling cascade. With this approach, the activation of the tenogenic Smad2/3 pathway in hASCs was successfully achieved leading to the expression of some tenogenic proteins, like TNMD and SCXA. In this Chapter, the remote activation of Activin RIIA via MICA bioreactor was studied in three different cell types (tenocytes, ASCs and hBMSCs) aiming to investigate different cell susceptibility to an external mechanical stimulus and to identify the most suitable cell source for tenogenic differentiation. Furthermore, MNPs were labelled with three different Anti-Activin RIIA antibodies targeting different

regions of the receptor, with the purpose to identify and stimulate the mechano-responsive region for an optimal activation of the Smad2/3 tenogenic pathway.

3.2 Aims

The primary aims of this Chapter were:

- To assess Activin RIIA mechanical activation towards stem cells tenogenic differentiation,
- To identify the antibody that best targets the mechano-responsive region of the Activin RIIA,
- To compare Activin RIIA mechanical activation within different cell types.

3.3 Methods

3.3.1 MNPs labelling

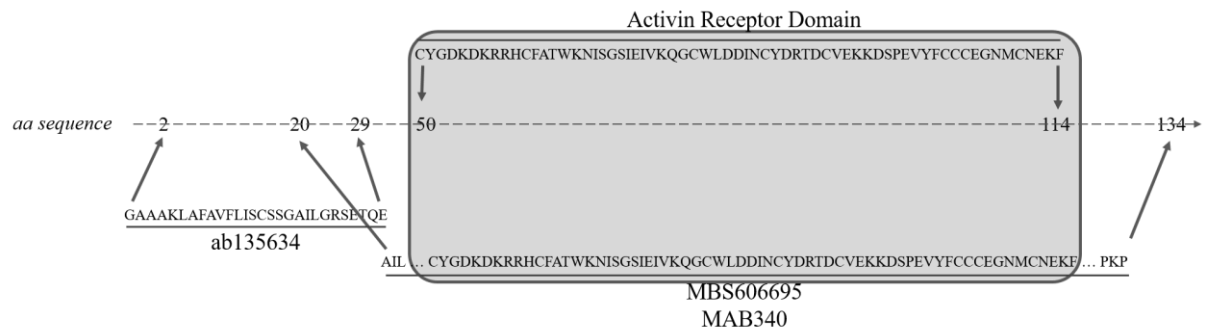


Figure 3.1. Schematic representation of the binding sites of the three different anti-Activin RIIA antibodies tested. “Ab135634” will be referred as “Antibody 1”, “MAB340” as “Antibody 2” and “MBS606695” as “Antibody 3”.

As mentioned in Chapter 2.5, three anti-Activin RIIA antibodies were tested targeting different regions of the receptor. In particular, the binding region of the Abcam-provided antibody (ab135634) is between the amino acid (aa) 2 and 29, outside the receptor domain (50-114aa),²⁹⁵

while the NovusBiologicals (MAB340) and MyBioSource (MBS606695)-provided antibodies have the same binding site (20-134aa) which include the Activin Receptor domain (Figure 3.1).

Hereafter, Abcam antibody will be called “Antibody 1” and the relatively labelled MNPs will be referred as MNPs1. Similarly, NovusBiologicals and MyBioSource antibodies will be called “Antibody 2” and “Antibody 3”, respectively. The relatively labelled MNPs will be referred as MNPs2 and MNPs3 (Table 3.1).

Table 3.1 Summary of the tested Anti-Activin RIIA antibodies, with their relative binding site and future in-text references.

Manufacturer and product code	Binding site	Antibody, in-text reference	Ab-labelled MNPs, in-text reference
Abcam (ab135634)	2-29aa	Antibody 1	MNPs 1
NovusBiologicals (MAB340)	20-134aa (Active site)	Antibody 2	MNPs 2
MyoBioSource (MBS606695)	20-134aa (Active site)	Antibody 3	MNPs 3

3.3.2 Tenogenic protein detection

3.3.2.1 Phosphorylation assay

All the cell types were labelled with Anti-Activin RIIA MNPs on monolayer as specified in Chapter 2.7.1. Subsequently, the magnetic stimulation efficacy in the initiation of the phosphorylation of the Smad2/3 complex was tested by ELISA as described in Chapter 2.7.8. Briefly, different combination of cell seeding densities and MNPs concentrations were tested:

- Tenocytes or hBMSCs: 5×10^4 cells were seeded in 6 well plates and incubated with tenogenic media (Chapter 2.4) for 4 days. After labelling the cells with anti-Activin

RIIA MNPs (Antibody 1, 2 and 3) considering 50 μg MNPs/ 5×10^4 seeded cells, they were stimulated with MICA Bioreactor for 2, 5 and 10 minutes.

- hASCs:
 - 6-well plates seeded with 5×10^4 cells incubated at 37°C with tenogenic media until confluent, subsequently labelled with 50 μg MNPs/ 5×10^4 seeded cells. Stimulation with MICA Bioreactor for 2 minutes.
 - 6-well plates seeded with 2×10^5 cells incubated overnight at 37°C with tenogenic media, subsequently labelled with 25 μg MNPs/ 2×10^5 seeded cells. Stimulation with MICA Bioreactor for 2, 10 or 30 minutes.
 - 6-well plates seeded with 1×10^6 cells incubated overnight at 37°C with tenogenic media, subsequently labelled with 25 μg MNPs/ 1×10^6 seeded cells. Stimulation with MICA Bioreactor for 2 minutes.

For all cell types, as positive control cells were treated with Activin A protein (20 ng/mL in tenogenic SFM) and as negative control the cells were cultured with unlabelled MNPs. Another group was kept unstimulated in identical conditions at 37°C as the static control. After each incubation period, the cells were lysed and analysed by ELISA that recognizes the endogenous levels of pSmad2/3 complex.

3.3.2.2 Tenogenic differentiation assay

The expression of tenogenic proteins following remote mechanical stimulation was tested by ICC (Chapter 2.7.7). Briefly, 1×10^4 cells (hBMSCs or hASCs) were seeded on glass coverslips in 24-well plates and incubated with tenogenic media overnight at 37°C . Afterwards, cells were labelled on monolayer with Anti Activin-MNPs, considering 50 μg of MNPs every 5×10^4 seeded cells (for hBMSCs), or 25 μg of MNPs every 2×10^5 seeded cells (for hASCs). Cells were stimulated with MICA Bioreactor for 21 days, considering the experimental groups and

following the protocol described in Chapter 2.7.7. After 21 days, the protein expression of TNMD, SCXA, Col I and Col III were detected by ICC (Chapter 2.7.4) and ImageJ Software was used to quantify the fluorescent signal from the acquired pictures. Total collagen deposition was analysed by Picrosirius Red staining (Chapter 2.7.9) and quantified at the plate reader (Chapter 2.7.9.1).

3.3.2.3 Western Blot

The amount of TNMD protein expressed by hASCs after mechanical stimulation was quantified by WB after 7 days and 14 days MICA stimulation. As extensively described in Chapter 2.7.11, 12×10^4 hASCs were seeded into 6 well plates, cultured with tenogenic media and incubated overnight at 37°C. The cells were then labelled with anti-Activin RIIA-MNPs, considering 25 µg of MNPs/ 2×10^5 seeded cells, and incubated with tenogenic media at 37°C up to 14 days. At day 0, 7 and 14 cells were lysed and a WB analysis was performed on the lysates, investigating TNMD protein levels. β-Actin protein levels were used for data normalisation.

3.3.3 MNPs cellular internalisation

The cellular internalisation timing of Anti-Activin RIIA-MNPs was studied by labelling hASCs on monolayer with MNPs (25 µg MNPs/ 2×10^5 seeded cells), followed by TEM analysis at different timepoints (30 min, 1 h, 3 h, 6 h, 24 h and 48 hours). Any potential influence of an external magnetic field was tested as well, by incubating MNPs-labelled cells on permanent magnets (NdFeB, N42) until processing for TEM analysis. As described in Chapter 2.7.12, at each timepoint MNPs-labelled cells were trypsinized, pelleted and fixed in a glutaraldehyde solution. The cell pellet was then sectioned at a microtome and visualised at a TEM (JEOL JEM 1400, Tokyo, Japan).

3.3.4 Statistical analysis

Statistical analysis was performed as described in Chapter 2.12.

3.4 Results

3.4.1 Labelled MNPs size changes

MNPs functionalisation with anti-Activin RIIA antibody occurred by carbodiimide activation, as represented schematically in Figure 3.2. MNPs characterisation at the Zetasizer (Chapter 2.6) revealed a significant size increase after antibody functionalisation in comparison to plain MNPs (243.6 ± 0.6 nm) confirming the successful Ab labelling. In particular, the diameter of MNPs functionalised with Antibody 1, 2 or 3 was respectively 599.3 ± 22.8 nm, 371.03 ± 19.5 nm and 324.97 ± 4.3 nm.

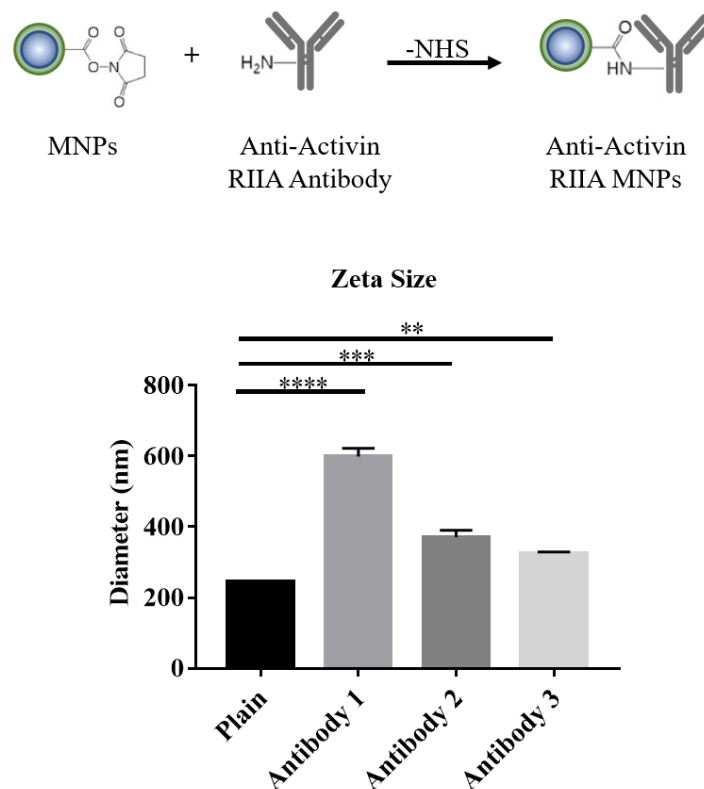


Figure 3.2 Schematic representation of MNPs functionalisation with anti-Activin RIIA antibodies. The antibody labelling occurred via carbodiimide activation (top). The MNPs diameter was analyzed via Zetasizer analysis, comparing plain and anti-Activin RIIA labelled MNPs with either Antibody 1, 2 or 3 (bottom). Data are expressed as the mean $\pm$ standard deviation, $n=3$, $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

3.4.2 Isolated human tenocytes express Activin RIIA

Human tenocytes were isolated from human tendons specimens by explants method (Chapter 2.3.1). Cells started to migrate from the tendon specimen after one week in culture with basal media. By day 21, as shown in Figure 3.3a, cells reached 80% confluency.

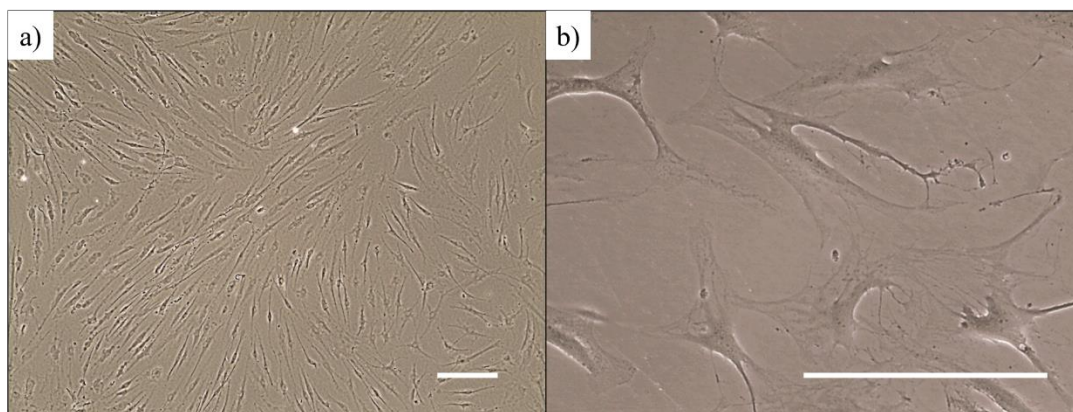


Figure 3.3 Human tenocytes migrating from a tendon explant. Picture acquired on day 21 in culture with basal media. Scale bar=200 μm . $n=3$. Cells were imaged at x10 (a) and x40 (b), using EVOS Optic Microscope.

ICC analysis was performed to verify the presence of Activin RIIA on tenocytes surface. The analysis showed a positive expression of the receptor, exhibiting a high fluorescence signal for the all the three Abs tested (Figure 3.4).

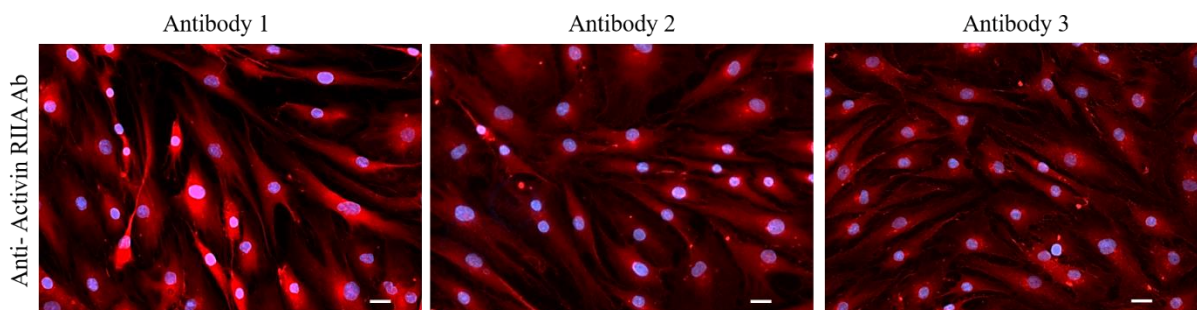


Figure 3.4 Binding of the three Abs was observed in tenocytes in culture with basal media. Red: anti-Activin RIIA Ab; Blue: nuclei. Scale bar=25 μm . $n=3$. Cells were imaged using Cytation5 Microscope at x10 magnification.

After confirming the presence of Activin RIIA on the surface of differentiated tenocytes, the possibility to activate the Smad2/3 pathway by MNPs-driven mechanical stimulation was assessed. The graphs in Figure 3.5 show a general increase of the pSmad2/3 complex levels in MICA-stimulated groups compared to the static control, especially after 2 min stimulation. MNPs 1 and 3 induced a significantly higher production of the complex after 2 min. While,

MNPs 2-labelled cells show significantly increased Smad2/3 complex levels after 5 min stimulation. Higher pSmad2/3 levels were detected in MICA-stimulated groups also in non-labelled cells (“cells only”) and cells labelled with unspecific MNPs after 2 min and 10 min stimulation, respectively.

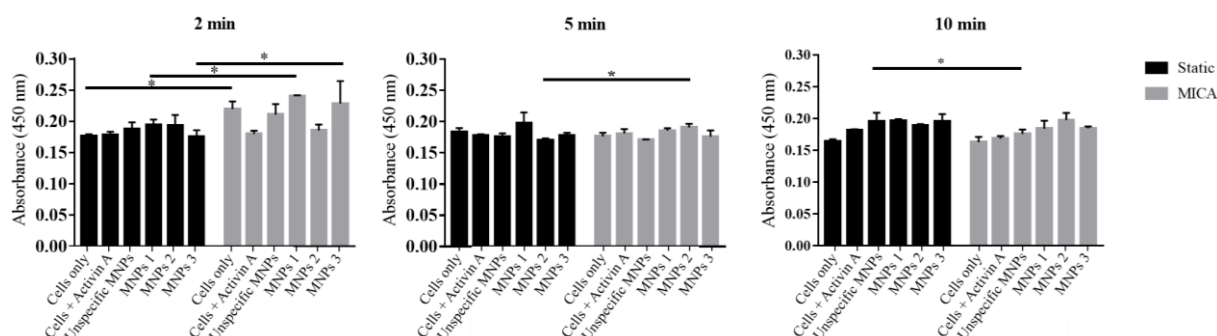


Figure 3.5 Phosphorylation assay of Smad2/3 complex in human tenocytes. The cells were stimulated with MICA bioreactor for (from left to right) 2, 5 and 10 mins, while a static group was kept as control. Significant elevated expression patterns were shown in cells treated with MNPs 1, MNPs 3 at 2 min, MNPs 2 at 5 min, non-labelled cells and cells labelled with unspecific MNPs at, respectively, 2 min and 10 min. Black: static group, Grey: MICA-stimulated group. Data are expressed as the mean $\pm$ standard deviation, $n=3$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

3.4.3 hBMSCs

3.4.3.1 Anti-Activin RIIA MNPs positive binding

The ICC analysis displayed a positive expression of Activin RIIA on the surface of hBMSC cultured in basal media (Chapter 2.2.2). As shown in Figure 3.6, all the three primary antibodies tested successfully bound to the receptor. This data was further corroborated by FACS analysis (Chapter 2.7.6), which showed a percentage of Activin RIIA-positive cells of 99.16%, 94.29% and 97.63% for Antibody 1, Antibody 2 and Antibody 3, respectively. Moreover, the co-localisation of functionalised MNPs and hBMSCs was validated via ICC. The dextran shell of

the MNPs was labelled with a green fluorescent antibody, while cell Actin filaments were stained in red with Phalloidin. As shown in Figure 3.6 it is possible to notice how some of the anti-Activin RIIA MNPs are associated with the cells.

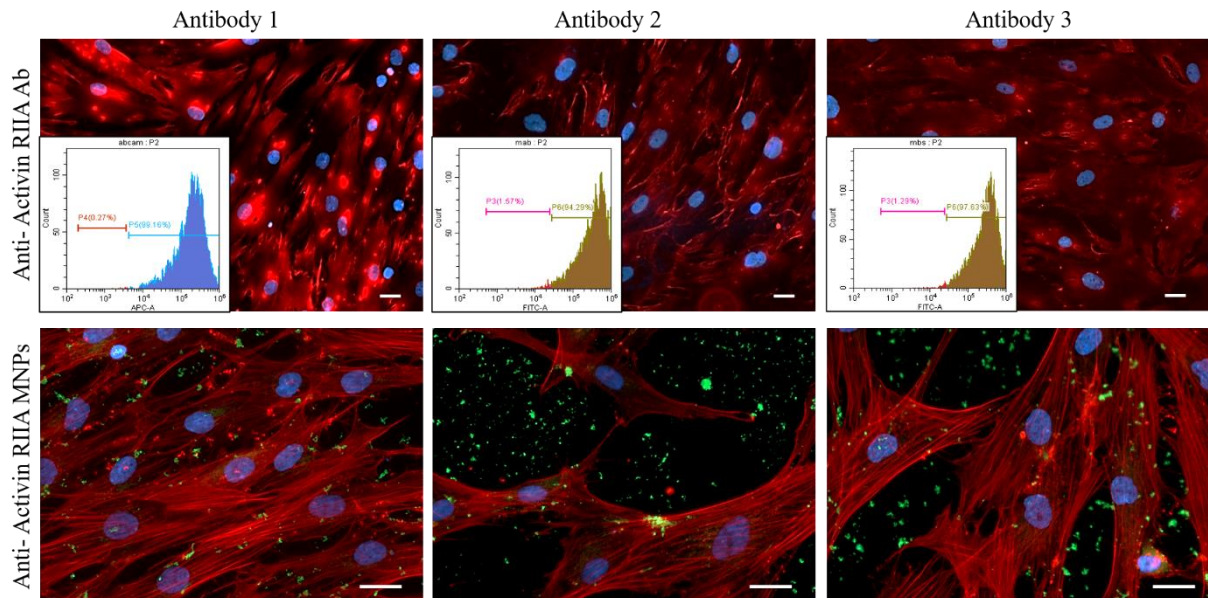


Figure 3.6 Binding of the three anti-Activin RIIA Abs and relative MNPs was verified in hBMSCs. The cells were labelled with (top) anti-Activin RIIA antibodies and (bottom) MNPs anti-Activin Activin RIIA. FACS analysis confirmed anti-Activin RIIA antibodies positive binding to hBMSCs. Red: anti-Activin RIIA Ab (top) or L-Actin filaments (bottom); Blue: nuclei; Green: MNPs. Scale bar=25 μ m. n=3. Cells were imaged using Cytation5 Microscope at x10 (top) and x20 (bottom) magnification.

3.4.3.2 MICA stimulation enhances the activation of the Smad2/3 pathway in hBMSCs

After checking the presence of Activin RIIA and the positive labelling of anti-Activin RIIA MNPs on hBMSCs, the possibility to mechanically activate the tenogenic Smad2/3 pathway was assessed. Anti-Activin RIIA MNPs-labelled hBMSCs were stimulated with MICA for 2 min, 5 min and 10 min, followed by ELISA assay detecting pSmad2/3 complex levels. The graphs in Figure 3.7 show an overall increase of the phosphorylated complex levels in the

MICA-stimulated groups compared to the static controls, which become more evident after 10 minutes stimulation. MNPs 2-labelled cells showed significantly higher complex levels after 2 min and 10 min stimulation, while MNPs 1 and 3 induce a higher production of the complex after, respectively, 10 and 2 minutes. An increase of pSmad2/3 complex was also registered in cells incubated with Activin A protein and stimulated with MICA for 5 min. The data presented are the results of three independent experiments, the statistical analysis was performed as described in Chapter 2.12.

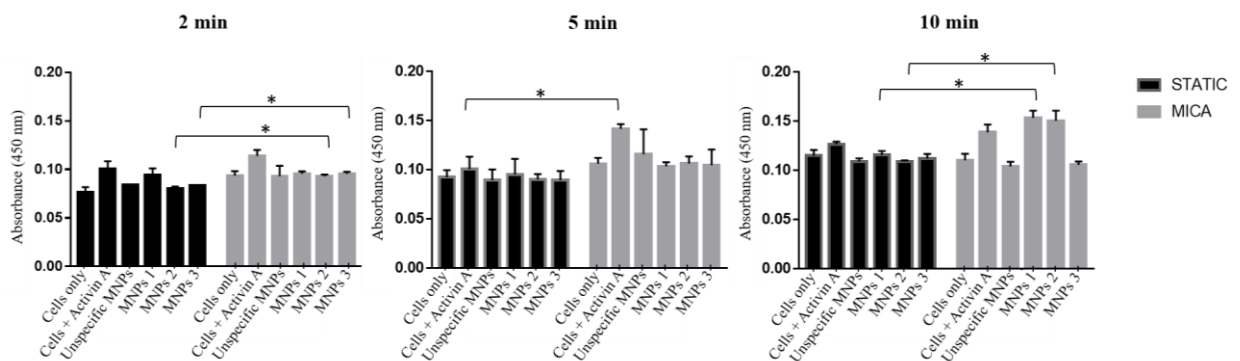


Figure 3.7 Phosphorylation assay of Smad2/3 complex in hBMSCs. The cells were stimulated with MICA bioreactor for (from left to right) 2, 5 and 10 mins, while a static group was kept as control. Results show significant elevated expression patterns in cells treated with MNPs 2 and MNPs 3 at 2 min, Activin A at 5 min, MNPs 1 and MNPs 2 at 10 min. Black: static group, Grey: MICA-stimulated group. Data are from three independent experiments ($n=3$) and expressed as the mean $\pm$ standard deviation, $n=3$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

3.4.3.3 Tenogenic markers expression is enhanced in MICA-stimulated cells

hBMSCs were seeded in 24 -well plates, incubated with tenogenic media, labelled with MNPs 1, 2 or 3 and stimulated with MICA 1h/day for up to 21 days. Stem cells tenogenic transition was analysed by assessing the total collagen deposition with Picrosirius Red (PS) staining and

by investigating the tenogenic protein expression by ICC. PS staining on cells stimulated with MICA showed an increased collagen deposition by day 14 and 21 in MICA-stimulated cells labelled with MNPs 1, 2 and 3 when compared to unlabelled cells or cells labelled with unspecific MNPs (Figure 3.8). The staining quantification (Figure 3.9) confirmed the increasing of collagen deposition by day 14 and 21, in particular cells labelled with MNPs 2 showed the highest levels of total collagen at day 14 and 21.

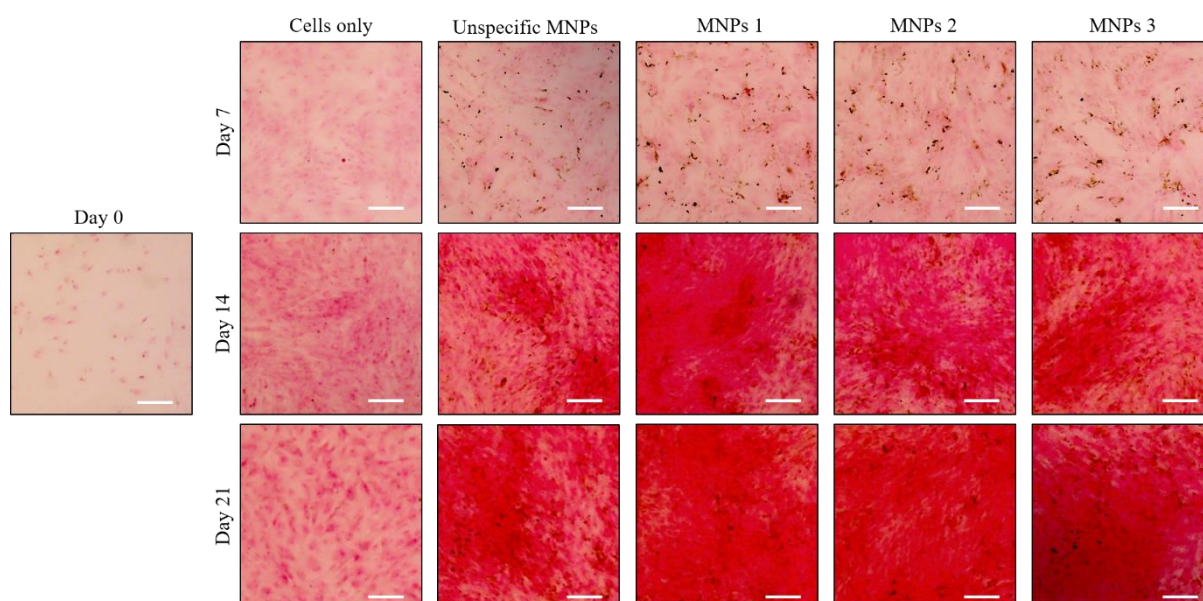


Figure 3.8 Picrosirius Red staining on hBMSCs. Cells were cultured with tenogenic media, labelled with MNPs 1, 2 and 3 and stimulated with MICA for 21 days. Pictures were acquired at day 0 (left), 7 (top), 14 (middle) and 21 (bottom). Unlabelled cells and cells labelled with unspecific MNPs were kept as controls. Scale bar=250 μ m. Cells were imaged using EVOS Optic Microscope.

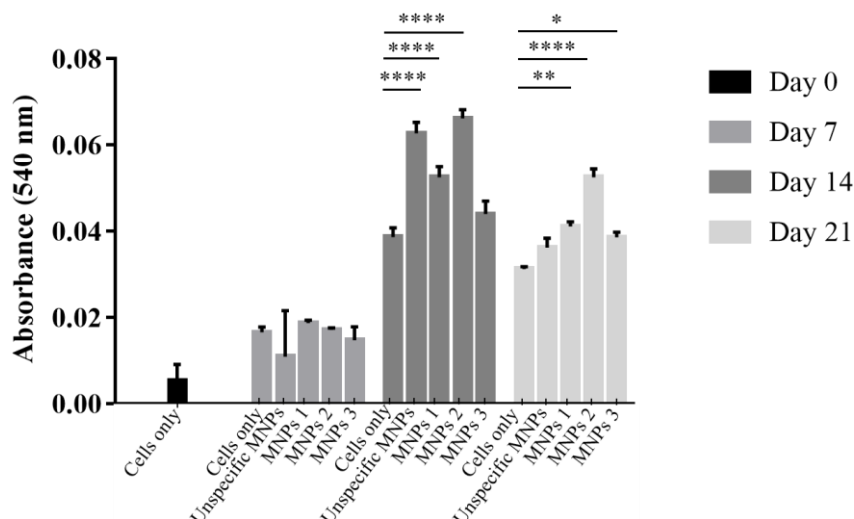


Figure 3.9 Picrosirius Red quantification. Picrosirius Red-stained cells were incubated with NaOH 1M for 30 minutes. Data show the highest levels of collagen deposition in MNPs2-labelled cells at day 14 and 21. The absorbance of the NaOH solution was read at the plate reader at 540 nm. $n=3$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

For the same differentiation experiment with MICA stimulation, ICC analysis was performed to assess the expression levels of some tenogenic proteins (TNMD, SCXA, Col I and Col III) in hBMSCs after 21 days (Figures 3.10-3.13). Pictures in Figure 3.10 show how TNMD expression was higher in cells mechanically stimulated compared to non-stimulated samples, especially for cells labelled with MNPs 2. SCXA levels (Figure 3.11) were higher in the stimulated group only in MNPs 1-labelled cells, while stimulated cells labelled with MNPs 2 and 3 did not show a higher response compared to the static control. The levels of Coll I were higher in MICA stimulated groups compared to the unstimulated samples, as showed in Figure 3.12, while Coll III expression was lower in the stimulated samples compared to the unstimulated ones (Figure 3.13).

TNMD

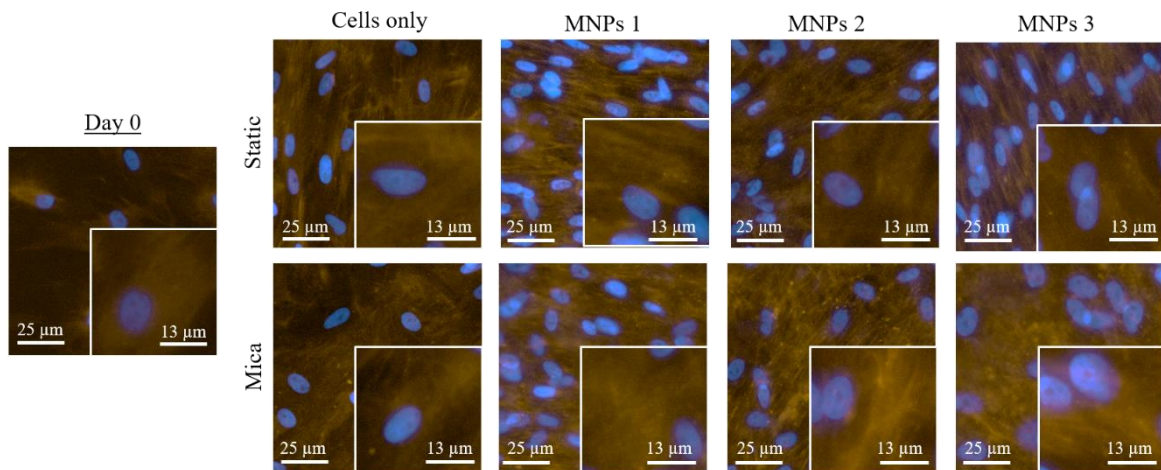


Figure 3.10 Immunocytochemistry for TNMD protein in hBMSCs. Cells were cultured with tenogenic media and stimulated with MICA bioreactor 1h/day for 21 days (bottom). A static group was kept unstimulated under the same culturing conditions (top). Orange: TNMD; Blue: nuclei. Cells were imaged using Cytation5 Microscope at x10 and x20 magnification. $n=1$.

SCXA

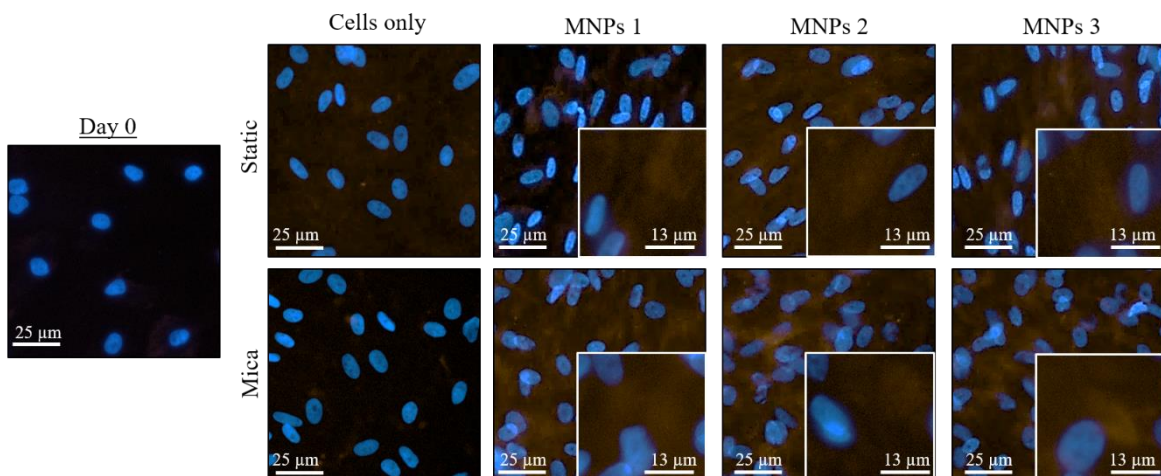


Figure 3.11 Immunocytochemistry for SCXA protein in hBMSCs. Cells were cultured with tenogenic media and stimulated with MICA bioreactor 1h/day for 21 days (bottom). A static group was kept unstimulated under the same culturing conditions (top). Orange: SCXA; Blue: nuclei. Cells were imaged using Cytation5 Microscope at x10 and x20 magnification. $n=1$.

Col I

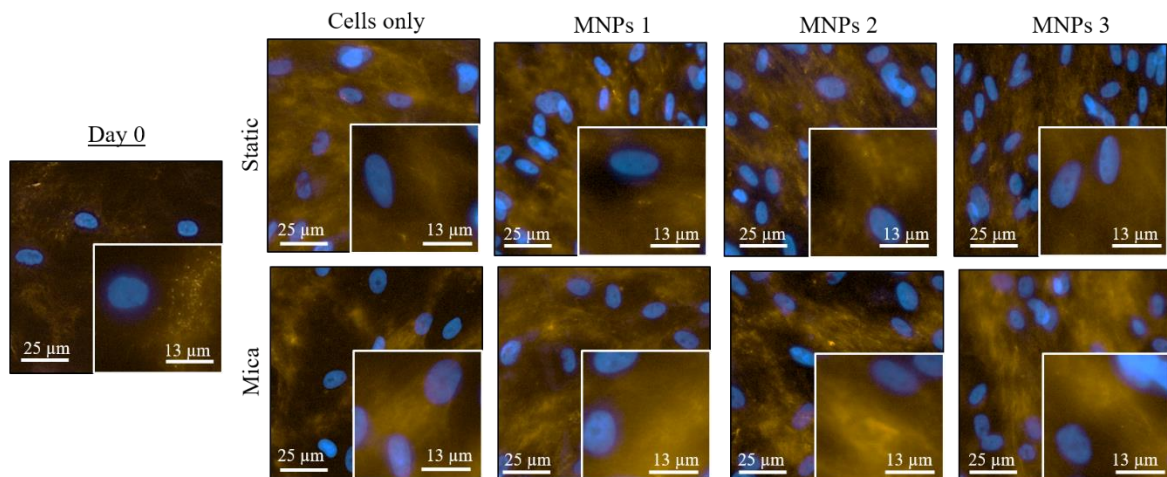


Figure 3.12 Immunocytochemistry for Col I protein in hBMSCs. Cells were cultured with tenogenic media and stimulated with MICA bioreactor 1h/day for 21 days (bottom). A static group was kept unstimulated under the same culturing conditions (top). Orange: Col I; Blue: nuclei. Cells were imaged using Cytation5 Microscope at x10 and x20 magnification. $n=1$.

Col III

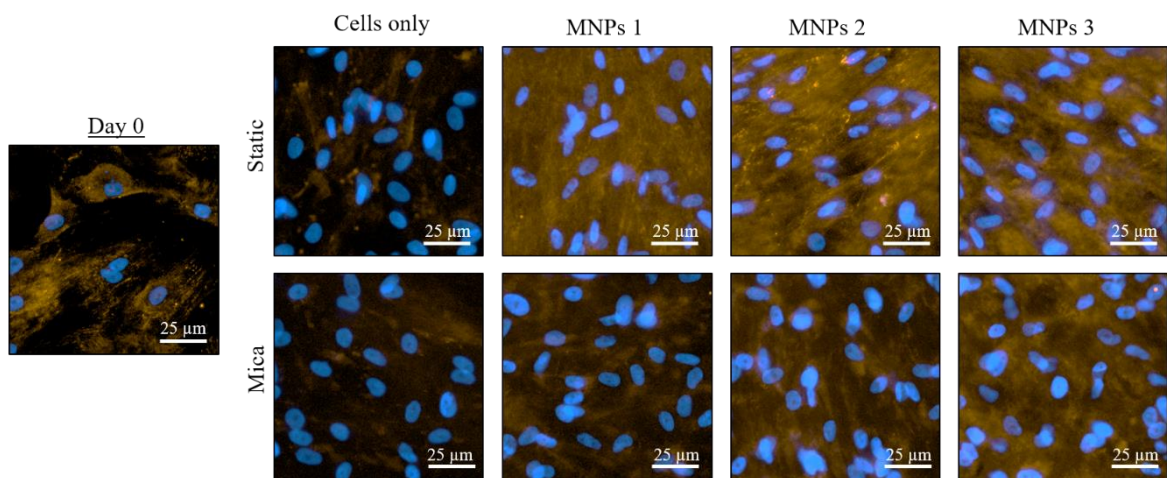


Figure 3.13 Immunocytochemistry for Col III protein in hBMSCs. Cells were cultured with tenogenic media and stimulated with MICA bioreactor 1h/day for 21 days (bottom). A static group was kept unstimulated under the same culturing conditions (top). Orange: Col III; Blue: nuclei. Cells were imaged using Cytation5 Microscope at x10 and x20 magnification. $n=1$.

3.4.4 hASCs

3.4.4.1 hASCs characterization and anti-Activin RIIA MNPs positive binding

Adipose stem cells were isolated from human fat pad samples via enzymatic digestion and cultured with basal media (Chapter 2.3.2). By day 14, as shown in Figure 3.14a, cells reached 80% confluency.

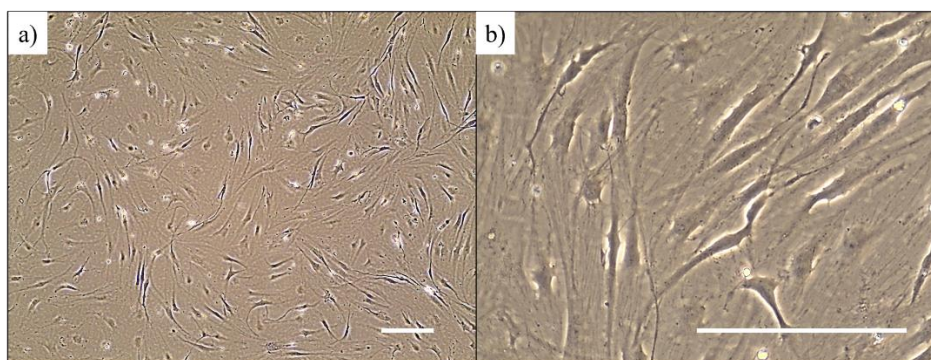


Figure 3.14 Human adipose stem cells isolated from fat pad samples. Pictures acquired at day 14 in culture with basal media. Scale bar=200 μ m. Cells were imaged at x10 (a) and x40 (b) using EVOS Optic Microscope.

Isolated hASCs were characterised for multipotency CD markers via FACS analysis as explained in Chapter 2.7.6 (Figure 3.15). Cells tested positive for CD105, CD90 and CD73 at, respectively, 96.75%, 78.4% and 97.39%. Stem cells negative CD markers were tested at the same time and, in particular, isolated cells were negative for CD31, CD45, CD19, CD14, CD34 and HLA-DR at, respectively, 75.59%, 52.92%, 55.87%, 11.43%, 24.72% and 97.63%. IgG2a and IgG2b were tested as negative controls.

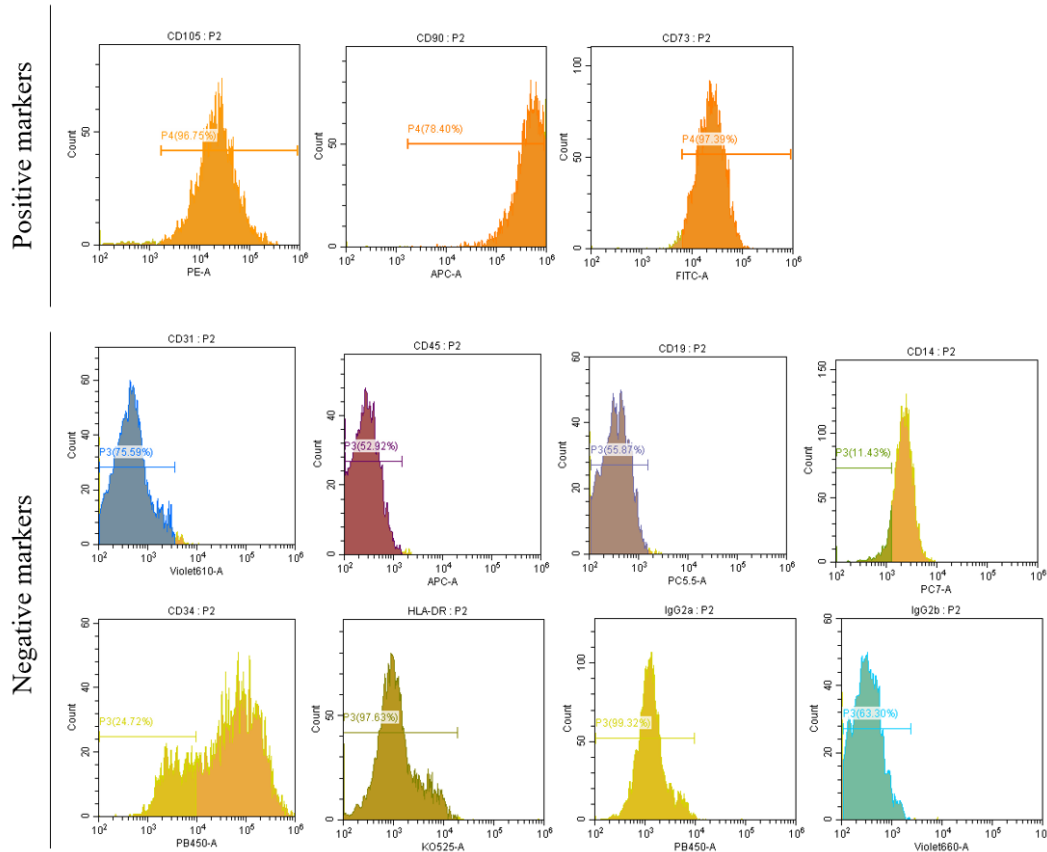


Figure 3.15 FACS characterization of cells isolated from human fat pad sample. Cells were tested for positive CD markers (CD105, CD90 and CD73) and for negative markers (CD31, CD45, CD19, CD14 and CD34). HLA-DR, IgG2a and IgG2b were tested as negative controls. Number of recorded events: 10,000, using CytoFLEX instrument (Beckman Coulter).

After characterisation for stem cells markers, isolated hASCs were tested for Activin RIIA expression via ICC analysis. As shown in Figure 3.16, hASCs tested positive for all the three primary antibodies, confirming the presence of Activin RIIA on hASCs surface. This result was corroborated by FACS analysis (Chapter 2.7.6), which showed a percentage of Activin RIIA-positive cells of 93.23%, 96.77% and 97.46% for Ab1, Ab2 and Ab3 respectively. Lastly, as performed for hBMSCs, the positive binding of anti-Activin RIIA MNPs to hASCs was tested via ICC. The analysis revealed the co-localisation of functionalised MNPs and hASCs, as showed in Figure 3.16.

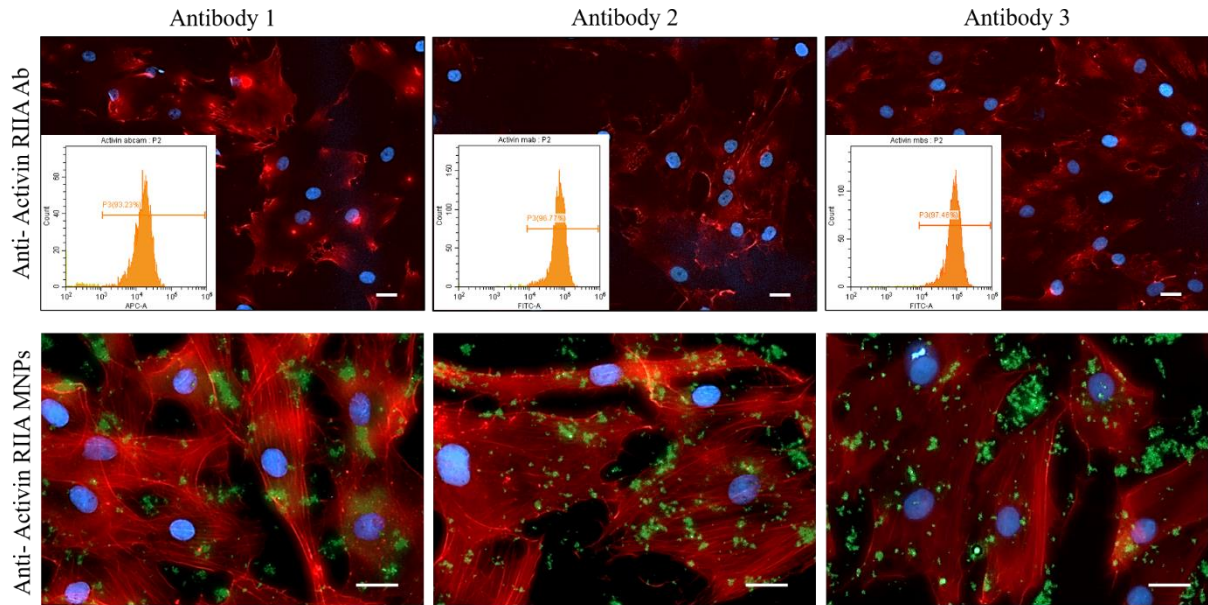


Figure 3.16 Binding of the three anti-Activin RIIA Abs and relative MNPs was verified in hASCs. The cells were labelled with (top) anti-Activin RIIA antibodies and (bottom) MNPs anti-Activin Activin RIIA. FACS analysis confirmed anti-Activin RIIA antibodies positive binding to hASCs. Red: anti-Activin RIIA Ab (top) or L-Actin filaments (bottom); Blue: nuclei; Green: MNPs. Scale bar=25 μ m. n=3. Cells were imaged using Cytation5 Microscope at x10 and x20 magnification.

Considering the results obtained from the experiments conducted on human tenocytes and hBMSCs, in particular the phosphorylation assay (Figure 3.5 and 3.7) and the differentiation assay (Figures 3.8-3.13), “Antibody 2” was identified as the one that better stimulates the cells towards the tenogenic commitment via mechanical stimulation, when compared to “Antibody 1” and “Antibody 3”. Therefore, this antibody was the one used for further experiments of stem cells tenogenic differentiation. An important limitation to the choice of the most suitable antibody was the production discontinuation of “Antibody 1”. For these reasons, for the next experiments here described, “anti-Activin RIIA antibody” will refer to “Antibody 2” and “Anti-Activin RIIA MNPs” will refer to “MNPs labelled with Antibody 2”.

Further confirmation of anti-Activin RIIA MNPs positive binding to hASCs was provided by the SEM analysis (Figure 3.17a), which showed MNPs bounded to cells surface, and by the

relative EDS analysis (Chapter 2.7.10) which displayed a peak for Iron (Fe) (Figure 3.17c) that was not detected on unlabelled hASCs, used as control (Figure 3.17b). Carbon (C) and Oxygen (O) signals indicate the presence of organic material, Platinum (Pt) peaks are imputable to the gold sputtering process during samples preparation for the analysis.

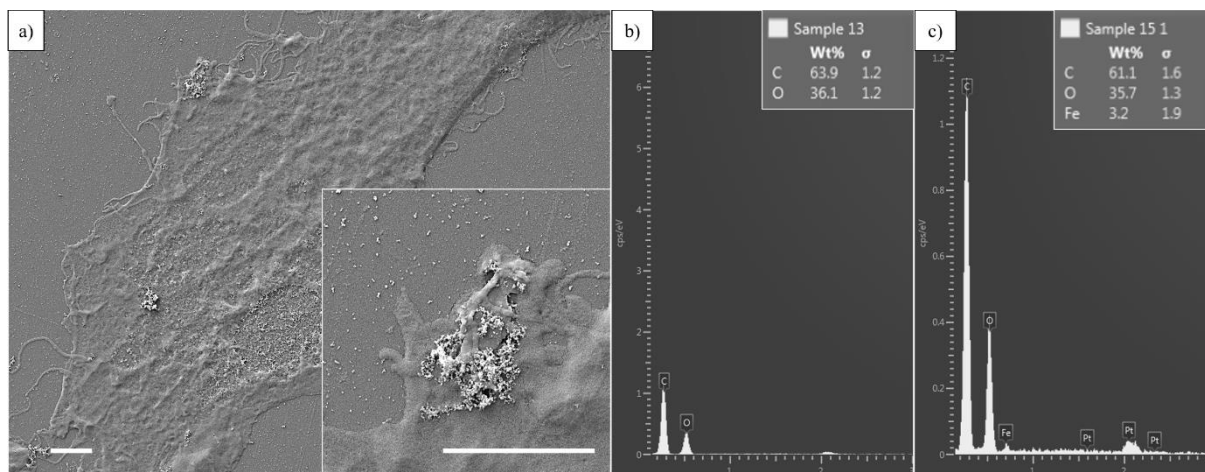


Figure 3.17 SEM and EDS analysis of anti-Activin RIIA MNPs labelled hASCs. MNPs labelled cells were imaged at the SEM (a) at x2.5k and x10k, and analysed at the EDS for iron detection (c) in comparison to unlabelled hASCs (b). Scale bar= 2.5 μm. Cells were imaged using SEM (Auriga Compact, ZEISS, Germany) and the elemental characterization was performed using an EDS (Pegasus X4M).

3.4.4.2 Phosphorylation assay at different MNPs and cells concentrations

Considering the phosphorylation assay results on human tenocytes and hBMSCs, the possibility to activate the Smad2/3 pathway was assessed in hASCs. Furthermore, the phosphorylation of the Smad2/3 complex was investigated at different MNPs concentrations and cell seeding densities (50 μg MNPs/5x10⁴ seeded cells, 25 μg MNPs/2x10⁵ seeded cells or 25 μg MNPs/1x10⁶ seeded cells) with the aim to identify the optimal MNPs/cells ratio for Smad2/3 pathway activation. The 2 min stimulation timepoint was selected over 5 min and 10 min, as

the levels of pSmad2/3 complex in human tenocytes were overall higher after 2 min rather than after 5 min or 10 min stimulation. As shown in Figure 3.18b, cells incubated overnight at 37°C and labelled with 25 µg MNPs/2x10⁵ seeded cells expressed higher levels of pSmad2/3 complex compared to cells incubated overnight and labelled with 25 µg MNPs/1x10⁶ seeded cells (Figure 3.18c) or compared to cells incubated until confluent and labelled with 50 µg MNPs/5x10⁴ seeded cells (Figure 3.18a). Following these results, cells overnight incubation and MNPs concentration of 25 µg MNPs/2x10⁵ seeded cells were considered as the best protocol and optimal MNPs/cells ratio for further experiments. For this reason, for the next experiments here described “MNPs-labelled cells” will refer to the concentration of 25 µg MNPs/2x10⁵ seeded cells, unless otherwise specified.

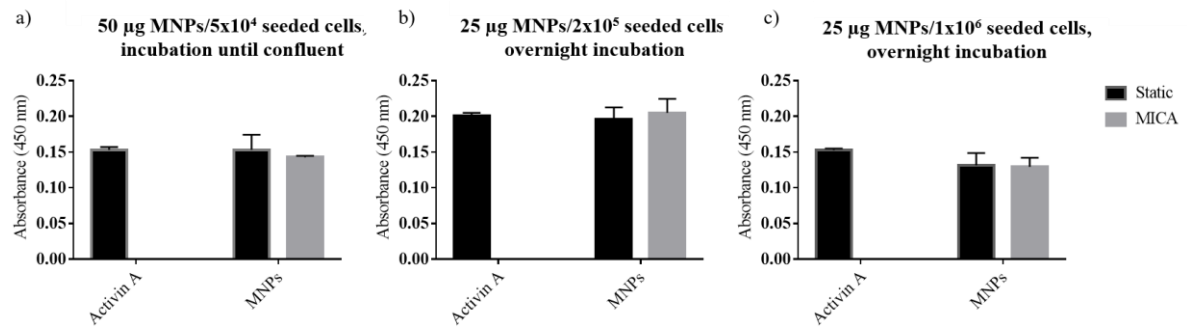


Figure 3.18 Phosphorylation assay of Smad2/3 complex in hASCs stimulated with MICA bioreactor for 2 mins. Cells were either: (a) cells incubated until confluent and labelled with 50 µg MNPs/5x10⁴ seeded cells, (b) incubated overnight at 37°C and labelled with 25 µg MNPs/2x10⁵ seeded cells, or (c) incubated overnight and labelled with 25 µg MNPs/1x10⁶ seeded cells. Cells incubated overnight at 37°C and labelled with 25 µg MNPs/2x10⁵ seeded cells (b) expressed higher levels of pSmad2/3 complex compared to other groups. Black: static group, Grey: MICA-stimulated group. Data are expressed as the mean ± standard deviation, n=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

After identifying the most suitable protocol, the assay was repeated with different stimulation periods (2 min, 10 min and 30 min) in order to test later timepoints than the maximum 10 min tested on human tenocytes and hBMSCs. The highest pSmad2/3 levels were indeed identified

after 30 min MICA stimulation, with significantly greater complex levels for cells labelled with MNPs and stimulated with MICA when compared to static control (Figure 3.19c), while non-significant results were achieved after 2 min or 10 min stimulation. The data presented are the results of two independent experiments, the statistical analysis was performed as described in Chapter 2.12.

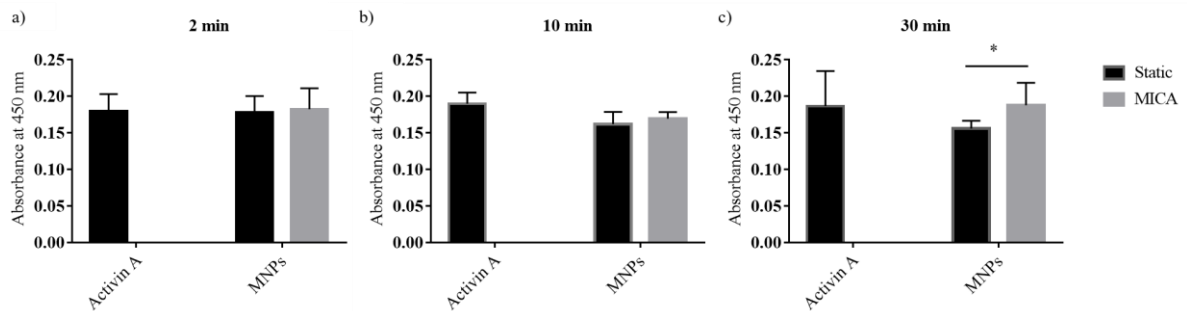


Figure 3.19 Phosphorylation assay of Smad2/3 complex in hASCs. The cells were stimulated with MICA bioreactor for a) 2 min, b) 10 min and c) 30 min, while a static group was kept as control. Results showed significantly higher levels of pSmad2/3 in cells labelled with MNPs and stimulated with MICA for 30 min. Black: static group, Grey: MICA-stimulated group. Data are from two independent experiments and expressed as the mean $\pm$ standard deviation, $n=3$, $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

A similar tenogenic differentiation experiment with MICA stimulation, as performed on hBMSCs, was also completed on hASCs. Cells were seeded in 24 -well plates, incubated with tenogenic media, labelled with MNPs and stimulated with MICA for 21 days. Picrosirius Red staining was performed to assess the total collagen deposition after 21 days. The staining showed a higher total collagen deposition in cells stimulated with MICA, in particular in cells non labelled with MNPs (“cells only” in Figure 3.20).

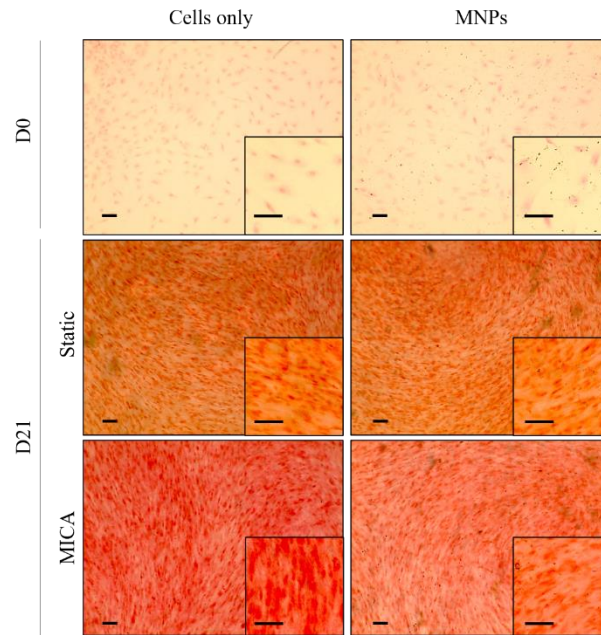


Figure 3.20 Picrosirius Red staining on hASCs. Cells were cultured with tenogenic media, labelled with MNPs and stimulated with MICA 1h/day for 21 days. Images were acquired at day 0 (top) and after 21 days (bottom). A static group was kept as control. Scale bar = 100 μ m. $n=3$. Cells were imaged using EVOS Optic Microscope.

Moreover, hASCs tenogenic differentiation was further investigated by ICC. TNMD, SCXA, Col I and Col III expression was evaluated in MNPs-labelled hASCs after 21 days incubation with tenogenic media and stimulation with MICA Bioreactor. Pictures in Figures 3.21-3.22 and the relative ICC fluorescence signal quantification in Figure 3.23 showed a general increase in tenogenic proteins expression in cells labelled with MNPs compared to unlabelled cells (Cells only) except for Col III. Similar protein levels were found between Static and MICA-stimulated groups, except for SCXA levels which were higher in static groups.

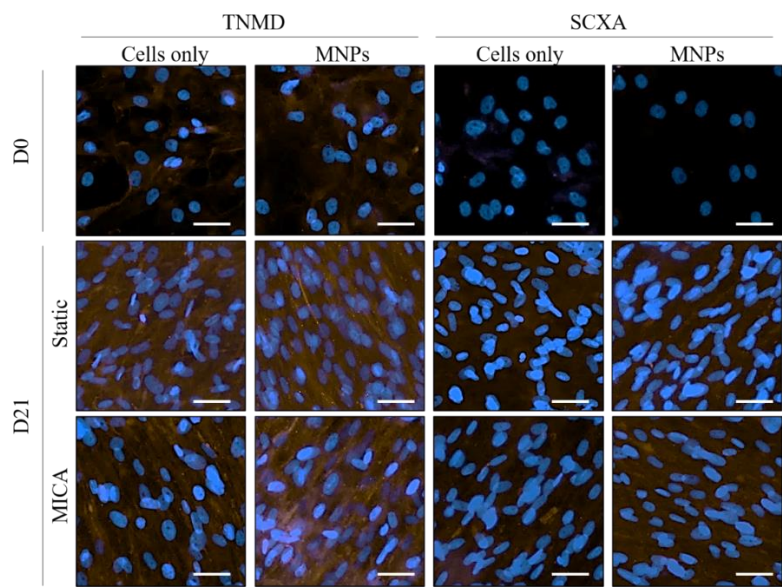


Figure 3.21 TNMD and SCXA immunocytochemistry analysis in hASCs. TNMD (left) and SCXA (right) expression was investigated in hASCs cultured with tenogenic media and stimulated with MICA bioreactor 1h/day for 21 days (bottom). A static group was kept unstimulated under the same culturing conditions (middle). Orange: tenogenic protein; Blue: nuclei. n=3. Scale bar=50 μ m Cells were imaged using Cytation5 Microscope at x10 magnification.

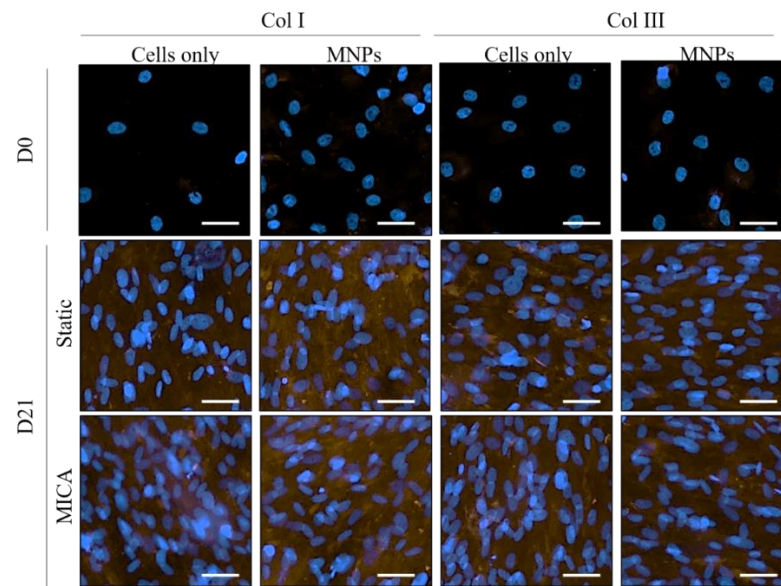


Figure 3.22 Col I and Col III immunocytochemistry analysis in hASCs. Col I (left) and Col III (right) expression was investigated in hASCs cultured with tenogenic media and stimulated with MICA bioreactor 1h/day for 21 days (bottom). A static group was kept unstimulated under the same culturing conditions (middle). Orange: tenogenic protein; Blue: nuclei. $n=3$. Scale bar=50 μm Cells were imaged using Cytation5 Microscope at $\times 10$ magnification.

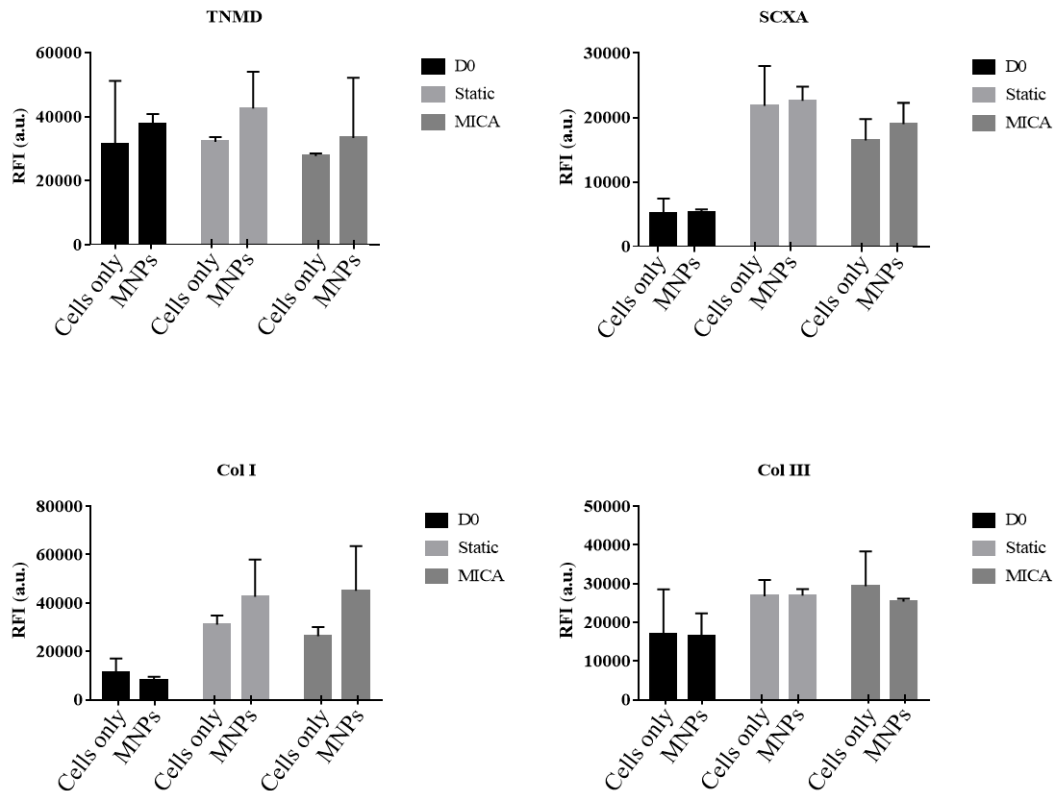


Figure 3.23 Quantification of ICC fluorescent signal for TNMD, SCXA, Col I and Col III proteins in hASCs at day 0 and after 21 of MICA stimulation. The analysis showed a general increase in tenogenic proteins expressions in cells labelled with MNPs compared to unlabelled cells (cells only). Similar levels of proteins were found between static and MICA stimulated groups. The quantification was performed with ImageJ software, normalizing the fluorescence with the cells number in each picture. Black: day 0; Light grey: Static groups; Dark grey: MICA stimulated groups. $n=3$.

A similar tenogenic differentiation experiment was carried out testing the effect of MICA stimulation on MNPs-labelled hASCs at earlier timepoints, namely after 7 and 14 days. Figure 3.24 shows how the WB analysis detected overall higher levels of TNMD after 14 days rather than after 7 days. Nevertheless, a more substantial difference in TNMD levels between groups was registered after 7 days rather than at the later timepoint. In particular, TNMD levels were higher in MNPs-labelled MICA stimulated group compared to unlabelled cells or to static controls. β -Actin protein levels were used for data normalisation.

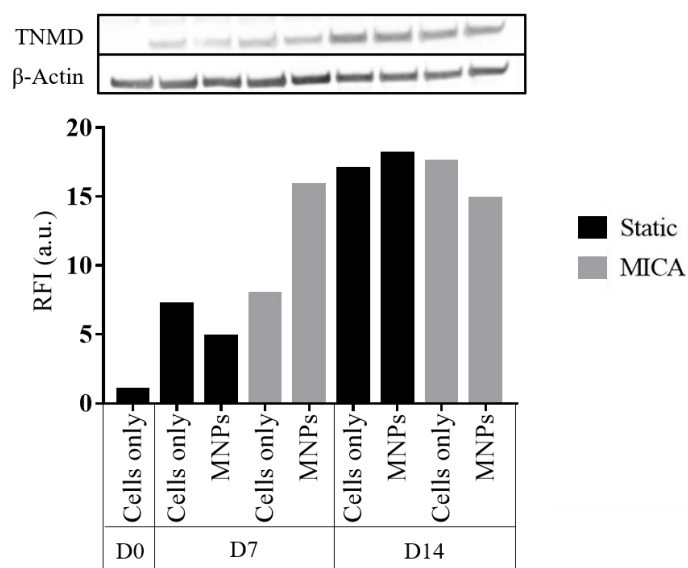


Figure 3.24 WB analysis on TNMD protein content in hASCs. Cells were cultured with tenogenic media and stimulated with MICA for 0, 7 and 14 days. Protein levels were generically higher after 14 days, but with a more substantial difference at 7 days between MNPs-labelled MICA stimulated hASCs and the other groups. Blots were quantified with ImageJ. β -Actin protein levels were used for data normalisation. Black: static; Grey: MICA. $n=1$.

Lastly, to investigate the cellular internalisation timing of anti-Activin RIIA MNPs, hASCs incubated with labelled MNPs were imaged at the TEM after 30 min, 1 h, 3 h, 6 h, 24 h or 48 h incubation. MNPs-labelled cells were either incubated on a permanent magnet (+mag) or without the influence of a magnetic field (-mag) to evaluate any potential influence of the magnetic field on MNPs internalisation. Figure 3.25 shows how after 30 min incubation, MNPs in both groups were outside the cells and reaching the membrane. After 1 h, MNPs reached the cellular membrane and, in +mag group, they started to get internalised. After 3 h most of the MNPs were internalised. After 6 h, it is possible to identify intracellular vesicles encapsulating MNPs clusters. Vesicles incapsulating MNPs clusters are appreciable also after 24 and 48 h.

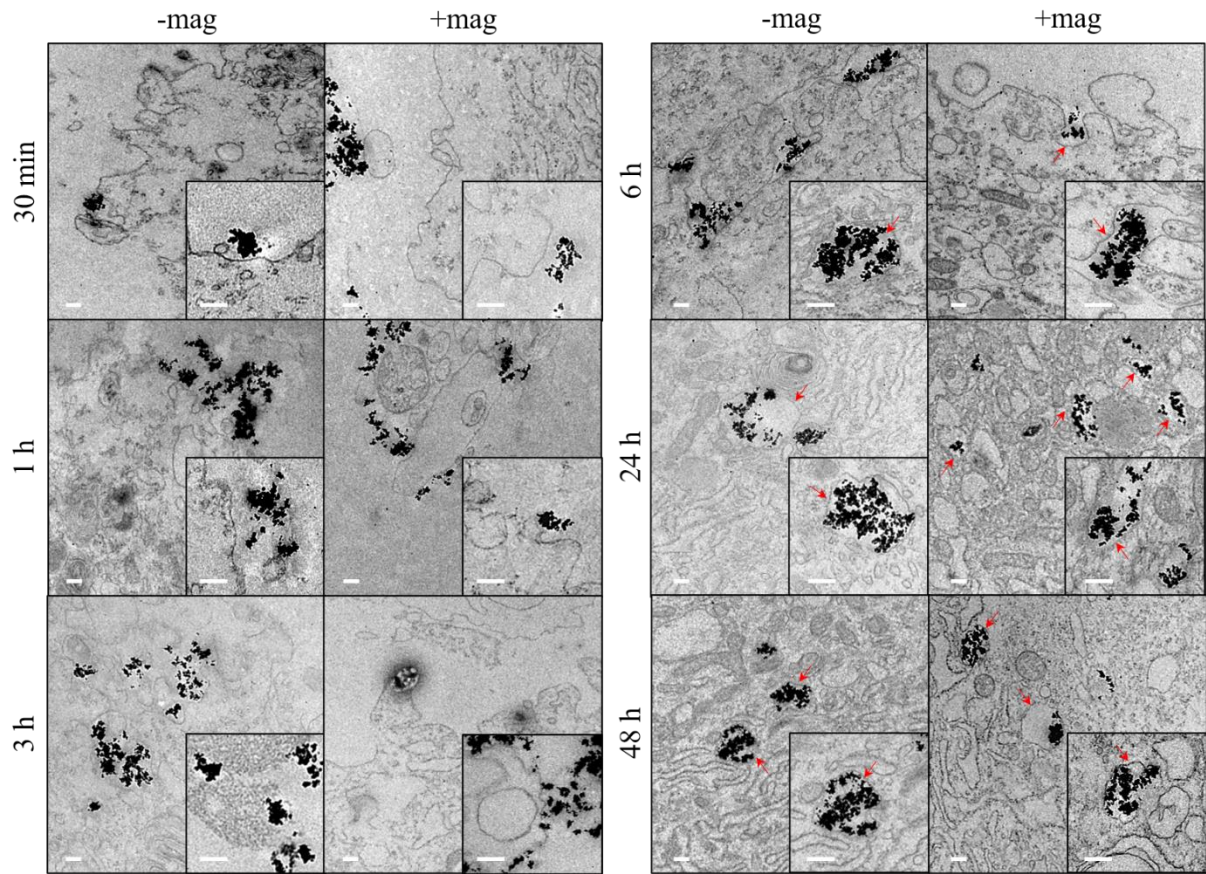


Figure 3.25 hASCs internalization timing evaluation of Anti-Activin RIIA MNPs. MNPs-labelled hASCs were analyzed at the TEM after 30 min, 1 h, 3 h, 6 h, 24 h or 48 h incubation either with (+mag) or without (-mag) constant magnetic field exposure. MNPs internalization started after 1 h in +mag group and after 3 h in -mag group. At 24 h and 48 h, intracellular vesicles encapsulating MNPs clusters were appreciable. Scale bar= 250 μ m. n=3. Red arrows show intracellular vesicles. Samples were imaged at JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan).

3.5 Discussion

In recent years, tissue engineering and regenerative medicine have offered alternative strategies for tendon injury repair showing promising results in improving tendons healing response and decreasing the scar formation.^{134,135} In the native tissue, biochemical signals are not the only factors impacting cells behaviour, tissue healing and matrix deposition. In fact, mechanical stimuli proved to have a major importance in tendons development, healing and

homeostasis.^{81,82} The mechanoreceptor Activin RIIA²⁸⁵ can be activated biochemically by Activin A binding, but also mechanically stimulating the activation of the Smad2/3 pathway leading to the synthesis of tenogenic proteins.^{100,101} In this Chapter, three anti-Activin RIIA antibodies targeting different regions of the Receptor were studied and tested on different cell types, with the aim to find the most appropriate Ab to target the mechano-responsive region of the Receptor and to evaluate the tenogenic mechano-activation across different cell types.

The presence of Activin RIIA on cells surface was assessed by ICC on the three cell types and quantified by FACS for hBMSCs and hASCs, confirming the presence of the Receptor on all cells types and antibodies tested. Functionalised MNPs were co-localised on hBMSCs and hASCs, by immunolabelling the dextran shell of the particles with anti-Dextran Ab and the cells with Phalloidin staining, suggesting a successful MNPs-cells association. The next step was to investigate the magnetic stimulation efficacy in the initiation the phosphorylation of the Smad2/3 complex which leads to the transcription of tenogenic genes,^{104,105} therefore the highest the levels of Smad2/3 complex, the highest the activation of the tenogenic pathway. For this purpose, a protocol found in the literature²⁸⁵ was slightly modified and adapted to the study. In particular, human tenocytes and hBMSCs were labelled with functionalised MNPs, but increasing the MNPs concentration in order to test if a higher MNPs/cells ratio would correspond to an increased activation of the Smad2/3 pathway. Furthermore, an additional earlier timepoint (5 min) was tested, as it was shown²⁸⁵ that the highest levels of Smad2/3 complex could be detected within 30 minutes of stimulation, probably due to the fact that longer incubation periods allow for the complex to proceed in the Smad cascade and therefore becoming undetectable by the ELISA assay. pSmad2/3 complex levels were greater in tenocytes than hBMSCs, but while in human tenocytes generally higher levels of pSmad2/3 complex were detected after 2 min, in hBMSCs the most elevated levels of complex were found after 10 min stimulation, suggesting a later activation of the Smad pathway in stem cells rather

than in differentiated tenocytes. This might be due to the fact that in stem cells the Smad cascade initiates a differentiation pathway, while in tenocytes the complex phosphorylation is triggered just to maintain the tenogenic commitment in already differentiated cells. Statistical analysis revealed that cells tagged with MNPs 2 expressed the highest levels of pSmad2/3 complex when cells were tagged with MNPs 2 after 5 min stimulation in human tenocytes and after 2 and 10 min in hBMSCs.

In line with these results, the differentiation assay on hBMSCs showed higher levels of total collagen deposition and in cells labelled with MNPs 2 and stimulated with MICA. MNPs 2-labelled hBMSCs stimulated with MICA also showed an increased expression of TNMD, an extracellular protein associated with the tenogenic phenotype.²⁹⁶ Another remarkable result was that MICA-stimulated cells produced a lower concentration of Col III compared to static groups, which can be considered a promising result as Col III is associated with fibrotic scar formation during tendon repair, which often causes chronic tendinopathies.^{1,6} As mentioned, these results have to be considered as preliminary and the experiment need to be repeated for statistic relevance, which was not possible in the first instance because of technical issues in addition to Covid-19 pandemic-related limitations (2020/2021).

Following the results achieved on human tenocytes and hBMSCs, Antibody 2 was identified as the one that better activates the Smad pathway in both the cell types when compared to the other anti-Activin RIIA antibodies tested. For this reason and for the promising results achieved in terms of tenogenic proteins expression in MNPs 2-labelled hBMSCs, Antibody 2 was selected for further tenogenic differentiation experiments on hASCs. Cells isolated from human fat pad were characterised by FACS for multipotency CD markers. Cells were either positive or negative for the markers tested as determined by literature²⁹⁷, except for CD14 and CD34 which showed negative cells percentages of 11.4% and 24.72%, respectively. CD34 is a transmembrane protein mainly known for being a surface marker for hematopoietic

stem cells and progenitor cells,^{298,299} while CD14 is expressed on monocytes and macrophages.^{300,301} The presence of these markers might be explained by a not sufficient washing of the tissue sample from blood cells during the processing of the sample, plus, the isolated cells were characterised when at culture passage 2 while further washings and cells passages might have been useful in reducing, or removing, blood cells and macrophages²⁹⁷.

Once the stemness of the isolated cells was verified, the mechano-activation of the Smad pathway was assessed by labelling hASCs with Antibody 2-tagged MNPs. To investigate the minimum MNPs concentration for Smad pathway activation, two additional MNPs labelling/cells seeding protocol were tested as obtained from literature,^{285,14} namely 50 µg MNPs/5x10⁴ seeded cells, 25 µg MNPs/2x10⁵ seeded cells and 25 µg MNPs/1x10⁶ seeded cells. The highest pSmad2/3 complex levels were detected at 25 µg MNPs/2x10⁵ seeded cells, while 25 µg MNPs/1x10⁶ seeded cells was probably a MNPs/cell ratio too low for tenogenic pathway activation. On the other hand, 50 µg MNPs/5x10⁴ seeded cells was probably over-saturating Activin RIIA availability. 25 µg MNPs/2x10⁵ seeded cells was therefore selected to be the ideal MNPs concentration for tenogenic pathway activation and it was employed for the following experiments. The phosphorylation assay on hASCs was repeated stimulating MNPs-labelled cells for 2, 10 or 30 min, to investigate the effects of MICA stimulation at earlier and later timepoints. Significantly higher levels of pSmad2/3 complex were detected after 30 min in MNPs-labelled stimulated with MICA compared to static control, while non-significant results were achieved after 2 min and 10 min stimulation, suggesting that a longer stimulation time is needed for Smad pathway activation in hASCs compared to hBMSCs.

A differentiation assay was then carried out by stimulating MNPs-labelled hASCs with MICA bioreactor one hour a day for 21 days. After 21 days, Col I and TNMD levels of MNPs-labelled cells were higher both in static and MICA stimulated groups when compared to unlabelled cells. Col III levels were slightly lower in MICA-stimulated MNPs-tagged cells

compared to other groups, while, SCXA levels were higher in the static groups rather than in the MICA-stimulated ones. SCXA levels resulted to be lower both in hBMSCs and hASCs in MICA-stimulated groups, while increased TNMD expression was registered in both cell types after 21 days. These results might be explained considering the fact that SCXA is a transcriptional activator of TNMD expression^{55,296} therefore, earlier timepoints might need to be preferred when investigating SCXA expression. This hypothesis is partly confirmed by the WB analysis of TNMD levels at earlier timepoints. The analysis revealed generally higher levels of TNMD after 14 days rather than after 7 days, but at day 7 it displayed a more substantial difference of protein content between MICA-stimulated MNPs-labelled hASCs and the other groups. This data might suggest that earlier timepoints could be more crucial for tenogenic protein expression rather than later timepoints, but a repetition of the experiment is needed to confirm this theory. In fact, a few challenges were encountered during the realisation of the experiment, which led to the successful WB results acquisition for just one replicate of each condition. In particular, already after 7 days in culture, cells deposited a big amount of ECM which was not digested by the WB cell lysis buffer, possibly leading to the loss of a considerable amount of cells which remained trapped in the undigested ECM. Therefore, the experiment needs to be repeated prior protocol optimisation and cells seeding density adjustment.

As last experiment, the cellular internalisation timing of anti-Activin RIIA MNPs was investigated at the TEM after 30 min, 1 h, 3 h, 6 h, 24 h or 48 h MNPs incubation either with or without the influence of a constant magnetic field. After 30 min incubation, MNPs in both groups were getting in contact with the membrane but still outside the cells. After 30 more minutes, MNPs reached the cellular membrane and, in the “+mag” experimental group, they started to get internalised, suggesting that the exposure to a magnetic field might speed up the internalisation of MNPs. Most of the MNPs were internalised after 3 hours in fact, at later

timepoints it is possible to identify intracellular vesicles containing MNPs clusters. The MNPs formations visualised both at the TEM and SEM, were in line with the structure of the employed MNPs which were not single spherical particles but irregular clusters with an average diameter of 250 nm, as described by the manufacturer.

3.6 Conclusion and future perspectives

Human mesenchymal stem cells offer a promising cell source for tendon tissue engineering, indeed their multipotency and high proliferation rate are key points for the potential resolution of tendon tears and injuries. In this Chapter we showed that the mechanical stimulation with MICA bioreactor of Activin RIIA is able to increase the activation of the Smad2/3 pathway leading to the expression of some tenogenic proteins, when compared to a static control. In particular, following the Phosphorylation assay and differentiation assay results, Antibody 2 was identified as the one that better activates the mechano-responsive region of the Activin RIIA, when compared to the other anti-Activin RIIA antibodies tested. Considering also the important limiting factor of the production discontinuation of Antibody 1 which prevented from performing further antibodies comparison studies, Antibody 2 was selected for future experiments of stem cells tenogenic differentiation. The stem cells sources investigated in this Chapter, bone marrow aspirate and adipose tissue, showed similar results in terms of mechanical activation of the Activin RIIA but considering works previously published,²⁸⁵ as well as the high cells number that could possibly be isolated from the same amount of tissue,^{143,144,145} hASCs were selected over hBMSCs for further experiments.

To conclude, the main results illustrated in this Chapter can be summarised as follows:

- The initiation of the phosphorylation of the Smad2/3 complex suggested the successful mechanical activation of Activin RIIA via magnetic stimulation in human tenocytes, hBMSCs and hASCs,

- Considering the results achieved in the mechanical activation of the Smad pathway and tenogenic protein expression, Antibody 2 was identified as the most suitable for the mechanic stimulation of Activin RIIA. The mentioned antibody was used for further experiments,
- Activin RIIA remote mechanical activation showed similar results in tenogenic protein expression in hASCs and hBMSCs.

CHAPTER IV

3D MODELS FOR TENDON REGENERATION

4.1 Introduction

In tendon tissue engineering it is important to develop an effective scaffold that would recreate the native tissue from a biological and mechanical point of view. In fact, mechanical resistance and elasticity are essential for tendon function,¹⁷ therefore the selected scaffold should not only act as cell delivery method and growing support, but also comply with the mechanical requirements of native tendons. The ideal tendon scaffold should also be biocompatible, biodegradable and have an adequate porosity for cells and nutrients infiltration.^{153,154}

Many different materials have been studied for tissue engineering applications, either biological, synthetic or composite.¹⁵³ Among biological materials, collagen is one of the most investigated for tendon tissue engineering, especially because of its biocompatibility and degradability. Moreover, collagen is the main component of native tendons, in fact, collagen I constitutes the almost totality of tendon ECM (97-98%)¹⁸ and its hierarchical fibres arrangement provides the elasticity and resistance typical of tendons.¹⁷ Webb et al.¹⁶² tested *in vivo* a composite scaffold of collagen, PHBHHx and tenocytes in an Achilles tendon repair model, showing an improvement in tendon function restoration after the injury. In this Chapter, a tendon-like 3D collagen gel was developed as a potential cell delivery method or *in vitro* tendon modelling for tissue engineering purposes. MNPs were used to align collagen fibres within the gel, macroscopically recreating the highly organised collagen matrix of native tendons. A similar approach was previously used by Antman-Passig et al.²²⁶ who employed MNPs to create an organised 3D collagen matrix promoting neuronal differentiation. In this study, MNPs were embedded in a collagen solution that was then set within a permanent magnetic field. In this system, the nanoparticles aggregated into strings matching the magnetic field lines, leading to the alignment of collagen fibres.²²⁶ It is also important to mention that, an

organised matrix can be beneficial for stem cell differentiation, as it has been reported in literature that stem cells cultured and stimulated to differentiate on oriented fibres are more likely to differentiate compared to stem cells cultured on a smooth surface.^{302,303}

Fibrin is another biological biopolymer that has been widely investigated in tendon tissue engineering, as it possesses several characteristics that make it ideal for *in situ* cell delivery for a range of tissues, including bone, cartilage, skin and tendons.¹⁷⁰ First of all, fibrin is a biocompatible material which, because of its capacity to adhere to tissues and surfaces, is currently used at high concentrations as wound sealant in clinical surgeries.^{171–173} Fibrin gels can also be used at lower concentrations as cell delivery method for tissue engineering purposes in fact, cells suspended in the liquid fibrin matrix can be injected *in situ* and allowed to set adapting to the wound space.^{170,175} In a study published by Hankemeier et al.,¹⁸² BMSCs were suspended in a fibrin matrix and injected into a rat patellar tendon improving ECM deposition and cells distribution. In another study,¹⁸³ fibrin-embedded BMSCs were injected in a rabbit Achilles tendon defect, improving tendon recovery during the first healing phases. In this Chapter, a fibrin hydrogel was developed and characterised as injectable cell delivery method for tendon tissue engineering *in vivo* applications.

4.2 Aims

The main aims of this Chapter were:

- To develop 3D scaffolds for cell delivery in tendon defects,
- To characterise 3D scaffold structural properties and cytocompatibility.

4.3 Methods

4.3.1 Collagen gels in syringe moulds

4.3.1.1 Collagen gels with non-oriented fibres

hBMSCs were thawed from LN following the protocol described in Chapter 2.2.1. and expanded in basal media until 80% confluent. To prepare 500 μ L non-oriented collagen gels, hBMSCs were firstly trypsinized with 1X Trypsin-EDTA as described in Chapter 2.2.2, then the desired cells amount (1×10^4 cells/mL gel, 2.5×10^4 cells/mL gel, 5×10^4 cells/mL gel or 10×10^4 cells/mL gel) was resuspended in a solution 20% HEPES in media, as described in Chapter 2.8.1. The appropriate volume of Rat Tail Collagen I was added to the cells suspension to have a final collagen concentration of 1.5 mg/mL, 2 mg/mL or 3 mg/mL, as explained in Chapter 2.8.1. The liquid collagen/cells suspension was injected into a syringe mould and allowed to solidify in a water-bath at 37°C. When the collagen was completely solidified, the gel was carefully squeezed out of the syringe into a 6-well plate and covered with basal media.

4.3.1.2 Collagen gels with oriented fibres

Collagen gels with oriented fibres were prepared similarly to non-oriented fibres collagen gels but labelling the cells with Ø250 nm or Ø1 μ m anti-Activin RIIA MNPs (250 μ g MNPs/500 μ L gel total volume). The MNPs/cells/collagen dispersion was pipetted into a 1 mL cropped syringe which was placed in a water-bath at 37°C within a magnetic field (N42 permanent magnets, B = 30 mT), until the complete setting of the gel (Chapter 2.8.2). Afterwards, the gels were squeezed out of the syringes in a 6-well plate and covered with basal media. Fluorescent oriented collagen gels were prepared similarly to non-fluorescent oriented or non-oriented

collagen gels, but starting from a fluorescent collagen stock solution, as extensively explained in Chapter 2.8.3 and 2.8.4.

4.3.2 Oriented collagen gels in chamber slides

100 μL oriented collagen gels were prepared in chamber wells as described in Chapter 2.8.7. Briefly, the appropriate amount of MNPs were homogenized with a volume of fluorescent collagen solution to have a final MNPs concentration of 1 $\mu\text{g}/\mu\text{L}$ and a final collagen concentration of 3 mg/mL . A solution of 20% HEPES in basal media was added to the collagen/MNPs suspension to make up the final 100 μL volume. 2.5×10^4 cells/ml hASC (PT-5006, Batch 18TL212639) were resuspended in the collagen/MNP suspension prior to being aliquoted into the each well of an 8 -well chamber slide. The chamber was then placed between two permanent magnets (N42) 7.2 cm apart and allowed to solidify at 37°C for 30 minutes. After the complete setting, the magnetic field was removed and the gels were covered with basal media and incubated at 37°C until further use.

4.3.3 Fibrin gels

hASCs isolated from human patellar fat samples (Chapter 2.3.2) were cultured in basal media until 80% confluent. As described in Chapter 2.9.2, to prepare 50 μL fibrin gels, 20×10^4 or 3×10^4 cells, either or not labelled with $\text{Ø}250$ nm anti Activin-MNPs, were resuspended in 15 μL basal media and mixed with 20 μL fibrinogen at different working concentrations (20 mg/mL , 30 mg/mL , 40 mg/mL or 50 mg/mL). Separately, 2 μL aprotinin (1 mg/mL) was combined with 10 μL calcium chloride (100 mM) and 5 μL thrombin at different working concentrations (20 IU/mL, 50 IU/mL, 60 UN/mL, 70 UN/mL, 80 UN/mL, 90 UN/mL or 100 UN/mL). 17 μL of the aprotinin/ CaCl_2 /thrombin solution was mixed to 35 μL of the

cells/fibrinogen dispersion, placed in 96 -well plates and incubated at 37°C for 30 min. 150 µL of fresh media supplemented with aprotinin was provided every day.

4.3.4 Statistical analysis

Statistical analysis was performed as described in Chapter 2.12.

4.4 Results

Considering the results presented in Chapter III, supported by works previously published,²⁸⁵ as well as the high cells number that could possibly be isolated from the same amount of tissue, hASCs were selected over hBMSCs. Unfortunately, during Covid-19 Pandemic (2020/2021), the Royal Orthopaedic Hospital (Birmingham, UK) stopped the provision of human patellar fat samples and human tendons specimens. 3D collagen gels were developed during this period using hBMSCs as cells source, since these cells were previously isolated from bone marrow aspirates (1M-125, Lonza) and stored in LN. When the human samples provision was restored, hASCs were used for fibrin gels development. Additionally, mechanical testing of fibrin and collagen gels were not possible in the first instance because of limited instruments accessibility during Covid-19 pandemic (2020/21) and, as further specified, these will need to be included in future investigations.

4.4.1 Collagen gels

4.4.1.1 Non-oriented collagen gels

According to a study published in 2013 by Lomas et al.³⁰⁴ non-oriented tendon-like collagen gels were prepared by injecting a liquid collagen/cells suspension into a syringe mould (Figure 4.1) which was then allowed to solidify in a water-bath at 37°C for 1 h.



Figure 4.1 1 mL cropped syringe used a mold for 3D collagen gels.

As a first step, in order to find the most suitable cells/collagen ratio, different cells densities and collagen concentrations were tested by monitoring cell viability (Chapter 2.8.5) and gel

contraction over the time (Chapter 2.8.6). After preparing 1.5 mg/mL, 2 mg/mL and 3 mg/mL collagen gels at different cells densities (5×10^4 cells/mL and 15×10^4 cells/mL), the cells viability was assessed with a Live/Dead assay after 7 days in culture (Figure 4.2).

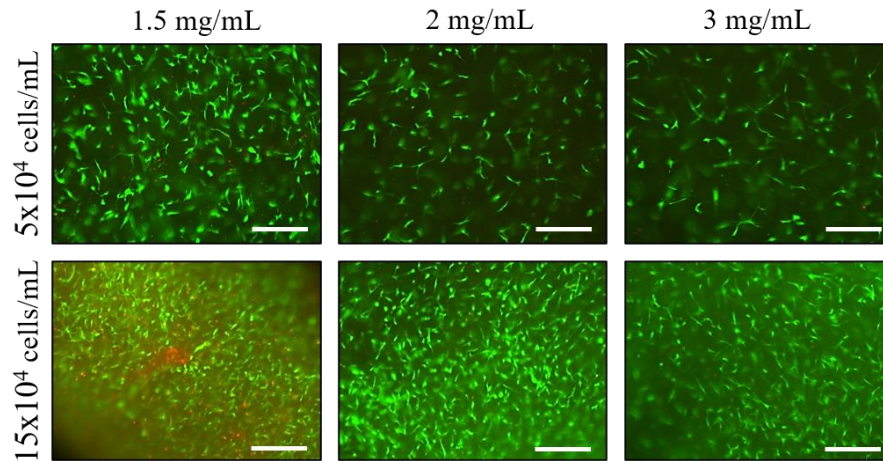


Figure 4.2 Live/dead assay of collagen gels. Different collagen concentrations (from the left; 1.5 mg/mL, 2 mg/mL and 3 mg/mL) and cell densities (top, 5×10^4 cells/mL and bottom, 15×10^4 cells/mL) were tested. Alive cells were stained in green with Calcein and dead cells were stained in red with EthD-1. Scale bar= 6.5 mm. Gels were imaged at EVOS Fluorescent Microscope at x10 magnification.

As shown in Figure 4.2, the Live/Dead assay did not reveal any substantial difference in cells viability between 2 mg/mL and 3 mg/mL collagen gels, at both the cells concentrations tested, while it showed an increased cell death in 1.5 mg/mL collagen gels at 15×10^4 cells/mL. Following these results and considering the high collagen concentration in the native tendon tissue,^{17,305} the 3 mg/mL collagen concentration was selected for further investigations.

To determine the gels contraction, 3 mg/mL collagen gels were prepared at different hBMSCs concentrations (1×10^4 cells/mL, 2.5×10^4 cells/mL, 5×10^4 cells/mL and 10×10^4 cells/mL) and measured in length and diameter after 0, 7, 14 and 21 days in culture with basal media.

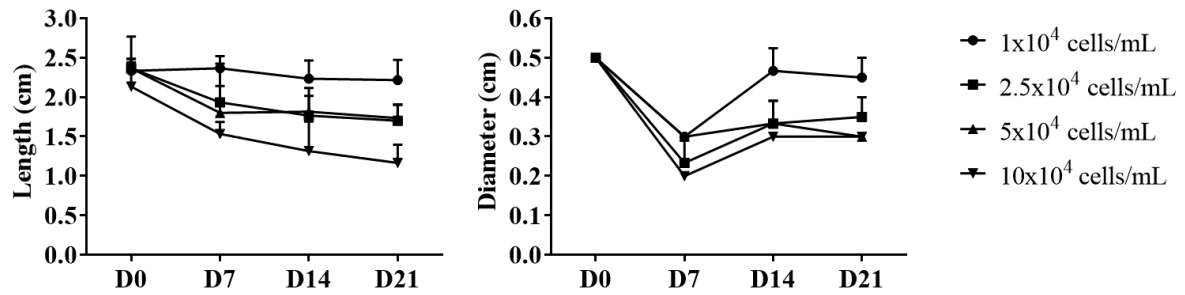


Figure 4.3 3 mg/mL collagen gels contraction study. Different cell densities were tested (1×10^4 cells/mL, 2.5×10^4 cells/mL, 5×10^4 cells/mL and 10×10^4 cells/mL). The contraction was determined by gels length (left) and diameter measurements (right) after 0, 7, 14 and 21 days in culture with basal media. $n=3$.

As shown in Figure 4.3, the contraction study showed how an increased cells density was leading to an increased collagen contraction, both in length and diameter. Following these results, considering other collagen contraction studies found in literature³⁰⁴ and taking into account the native tendon tissue cellularity,^{101,306} 5×10^4 cells/mL was considered a fair compromise between collagen contraction and cells density.

4.4.1.2 Oriented collagen gels

A more realistic tendon 3D model was prepared by labelling hBMSCs with Anti-Activin RIIA $\text{\AA}250$ nm MNPs (considering 250 μg of MNPs every 500 μL gel) and then dispersing the MNPs-labelled cells into a liquid collagen/media solution. The MNPs/cells/collagen dispersion was injected into a syringe mould and allowed to solidify between two magnets in a water-bath at 37°C (Figure 4.4). MNPs were exposed to a constant magnetic field which was measured to be of ~ 30 mT close to the magnet long sides, in line with what predicted by a 2D simulation of the magnetic flux performed via FEMM Software (Figure 4.4 c) (Chapter 2.8.2.1).

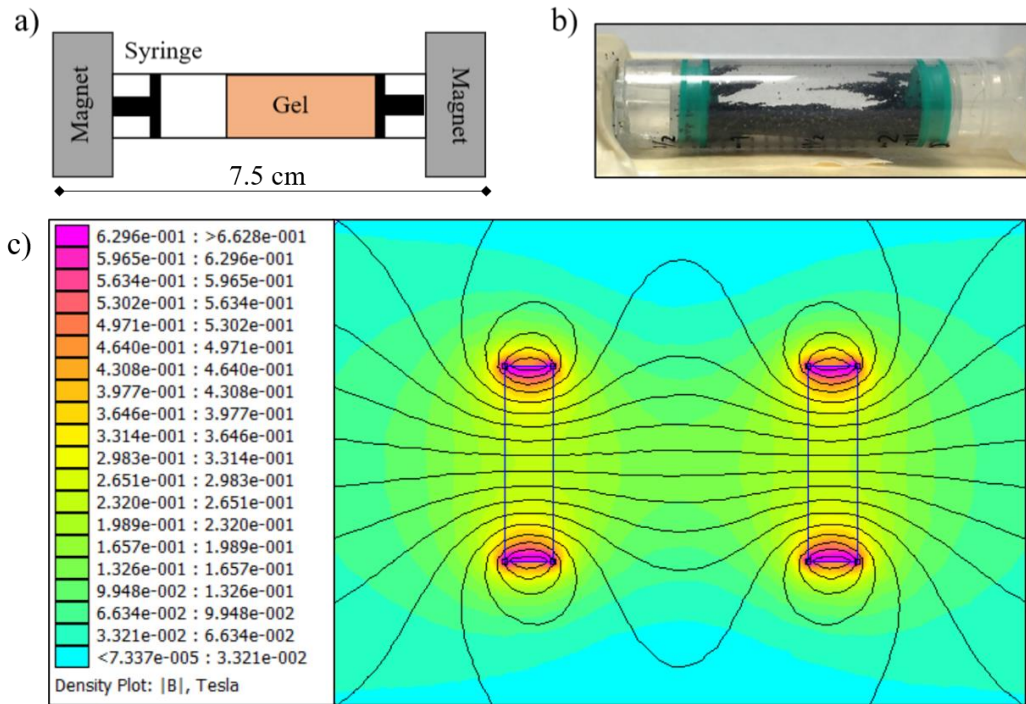


Figure 4.4 Magnetic setup for collagen gels alignment and magnetic field mapping. a) Schematic representation of the syringe/magnets setup used for preparing oriented collagen gels; b) Empty cropped syringe filled with iron filings and placed between two permanent magnets, as a proof of concept; c) FEMM Software 2D simulation of the magnetic flux in the magnets setup for collagen gels alignment. Numbers indicate the magnetic field intensity (T), lines show magnetic field gradients.

To assess the MNPs orientation, oriented fibres collagen gels were firstly embedded in paraffin and sectioned at the microtome, consequently the samples were stained with Prussian Blue staining for iron in order to visualize the MNPs (Chapter 2.10.6). As shown in Figure 4.5, the MNPs are arranged in oriented and organized strings-like structures, following the magnetic field orientation.

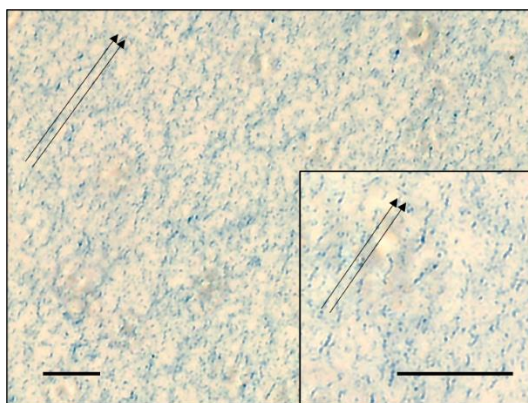


Figure 4.5 Prussian blue staining on paraffin sectioned collagen gels. The image shows MNPs oriented string-like structures following the direction of the magnetic field. Scale bar=50 μm . $n=3$. Arrows indicate the direction of the magnetic field. Cells were imaged at x20 and x40, using EVOS Optic Microscope.

To investigate any relationship between MNPs size and collagen fibres orientation and, in particular, if to an increase in MNPs size would correspond a more organised and defined collagen fibres orientation, anti-Activin RIIA $\text{\AA}1\text{ }\mu\text{m}$ MNPs were employed in the preparation of oriented collagen gels. The antibody labelling of silica-coated COOH-functionalised $\text{\AA}1\text{ }\mu\text{m}$ MNPs was performed following the same protocol used for $\text{\AA}250\text{ nm}$ MNPs (Chapter 2.5) since the functionalisation of both MNPs occurred by carbodiimide activation (Chapter 3.4.1). Anti-Activin RIIA $\text{\AA}1\text{ }\mu\text{m}$ MNPs were analysed at the Zetasizer (Chapter 2.6) and compared to functionalised $\text{\AA}250\text{ nm}$ MNPs previously employed. For both MNPs, the analysis revealed a size increase after antibodies functionalisation in comparison to plain MNPs. $\text{\AA}250\text{ nm}$ particles diameter increased from $243.6 \pm 0.6\text{ nm}$ to $371.03 \pm 19.5\text{ nm}$ after functionalisation, while $\text{\AA}1\text{ }\mu\text{m}$ MNPs increased from $1091.7 \pm 33.3\text{ nm}$ to $2378.7 \pm 133.2\text{ nm}$ (Figure 4.6). After functionalisation, the surface charge increased as well raising from $-28.9 \pm 0.3\text{ mV}$ to $-4.13 \pm 0.4\text{ mV}$ for $\text{\AA}250\text{ nm}$ particles, and from $-28 \pm 0.9\text{ mV}$ to $-6 \pm 0.1\text{ mV}$ for $\text{\AA}1\text{ }\mu\text{m}$ MNPs.

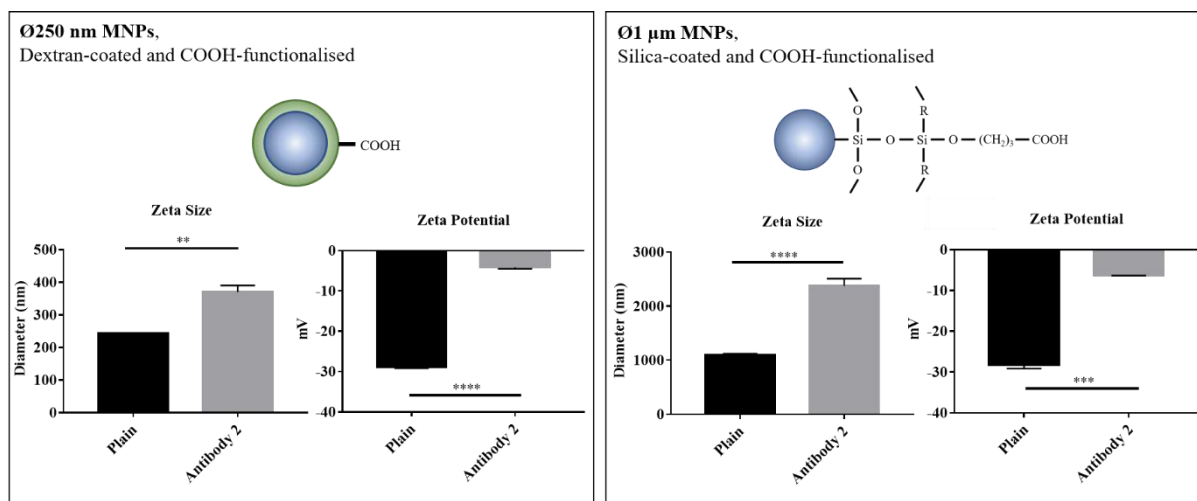


Figure 4.6 Chemical structure, Zeta Size and Zeta potential of Ø250 nm and Ø1 µm MNPs. Black: plain MNPs, Grey: anti-Activin RIIA functionalised MNPs. Size and charge measurement were performed at Zetasizer Nano ZS (Malvern Instruments, United Kingdom). Data are expressed as the mean $\pm$ standard deviation, $n=3$, $*p<0.05$, $**p<0.01$, $***p<0.001$, $****P<0.0001$.

To investigate any alteration in cell metabolism at the high MNPs dose used for the preparation of oriented collagen gels, hBMSCs were labelled on monolayer with Ø250 nm or Ø1 µm anti-Activin RIIA MNPs, considering 250 µg/2.5x10⁴ seeded cells. Presto Blue Assay (Chapter 2.7.13) was performed and the metabolism was assessed up to 21 days. As shown in Figure 4.7, a slight decrease in cells metabolism was found after 21 days for cells labelled with Ø1 µm MNPs, which was not significant if compared to non-labelled cells. No significant changes were reported for Ø250 nm tagged cells.

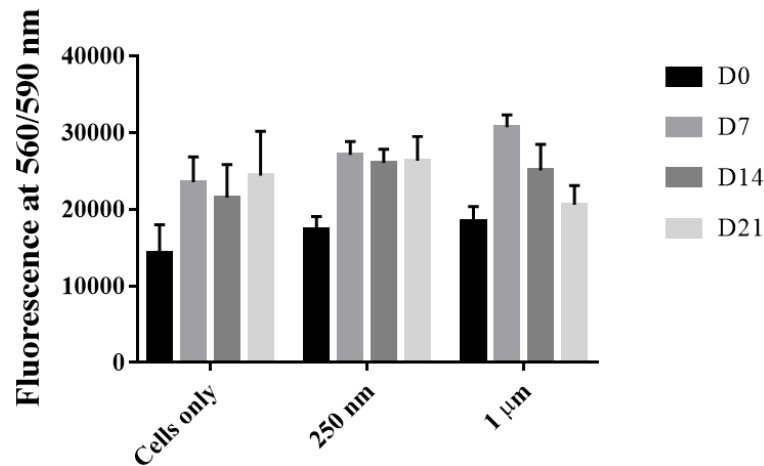


Figure 4.7 Presto Blue assay of hBMSCs labelled on monolayer with either Ø250 nm or Ø1 µm anti-Activin RIIA MNPs (250 µg/2.5x10⁴ seeded cells). No significant changes were reported between MNPs-labelled and unlabelled cells (cells only). *n*=3. Fluorescence was read at 560/590 nm at a Plate Reader (Spark, Tecan).

Subsequently, MNPs alignment and collagen fibres organisation were evaluated in gels prepared by embedding either Ø250 nm or Ø1 µm anti-Activin RIIA MNPs. The gels were imaged after 2 days using an optic microscope to visualise the MNPs organisation in the intact gels (Figure 4.8, left). The analysis confirmed the presence of oriented strings-like MNPs structures in gels exposed to a magnetic field (+mag), as expected from previous Prussian Blue staining of sectioned collagen gels (Figure 4.5), while no MNPs organisation was detectable in gels solidified without magnetic field exposure (-mag). To not only visualize MNPs strings but also to assess the alignment of the collagen fibres, oriented collagen gels were prepared, sectioned in paraffin and stained with Picrosirius Red (Chapter 2.10.5). As shown in Figure 4.8 (middle) stained collagen fibres appear in different birefringence colours under a polarized light, indicating the orientation of collagen fibres.³⁰⁷ Collagen fibres orientation was appreciable in gels prepared with both Ø250 nm and Ø1 µm MNPs.

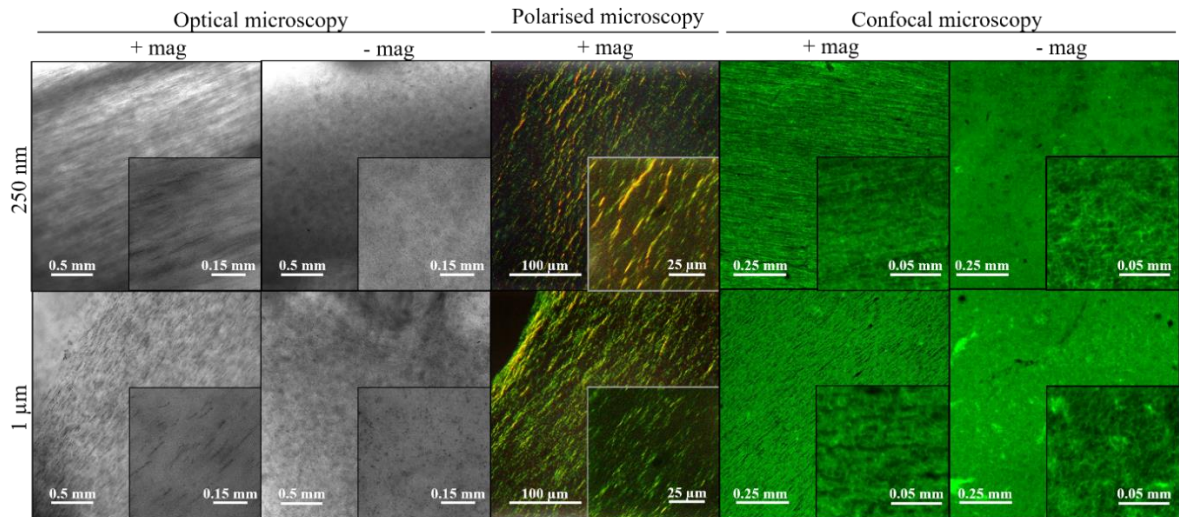


Figure 4.8 Comparison of collagen gels alignment either with or without magnetic field exposure. Pictures of collagen gels solidified under a magnetic field (+mag) and gels solidified without the magnetic field (-mag) were acquired after 2 days in culture. Pictures were acquired at (left) optic, (center) polarised and (right) confocal microscope. $n=3$.

Moreover, to better visualize the oriented collagen fibres and assess their alignment, fluorescent oriented collagen gels were prepared using a collagen stock solution labelled with Atto-488 NHS ester dye, following a procedure found in literature.³⁰⁸ The obtained fluorescent collagen gels were imaged under a confocal microscope and compared to fluorescent collagen gels set under the same temperature conditions but not being exposed to a magnetic field. As showed in Figure 4.8 (right), oriented collagen fibres are clearly visible in gels solidified under the magnetic field (+mag), while no such organisation is appreciable in gels prepared without the magnetic field exposure (-mag). This was confirmed by the quantification of collagen fibres directionality (Chapter 2.8.9) which showed a preferred fibres orientation in collagen gels prepared with Ø250 nm and Ø1 μ m MNPs and exposed to magnetic field (Figure 4.9 b and d, respectively), while no single fibre orientation was determined within collagen gels not exposed to a magnetic field (Figure 4.9 a and c). Interestingly, a larger fibre orientation variability was

observed within the Ø1 µm MNPs gels ($SD \pm 12.42$) (Figure 4.9 d) compared to Ø250 nm MNPs gels ($SD \pm 4.52$) (Figure 4.9 b).

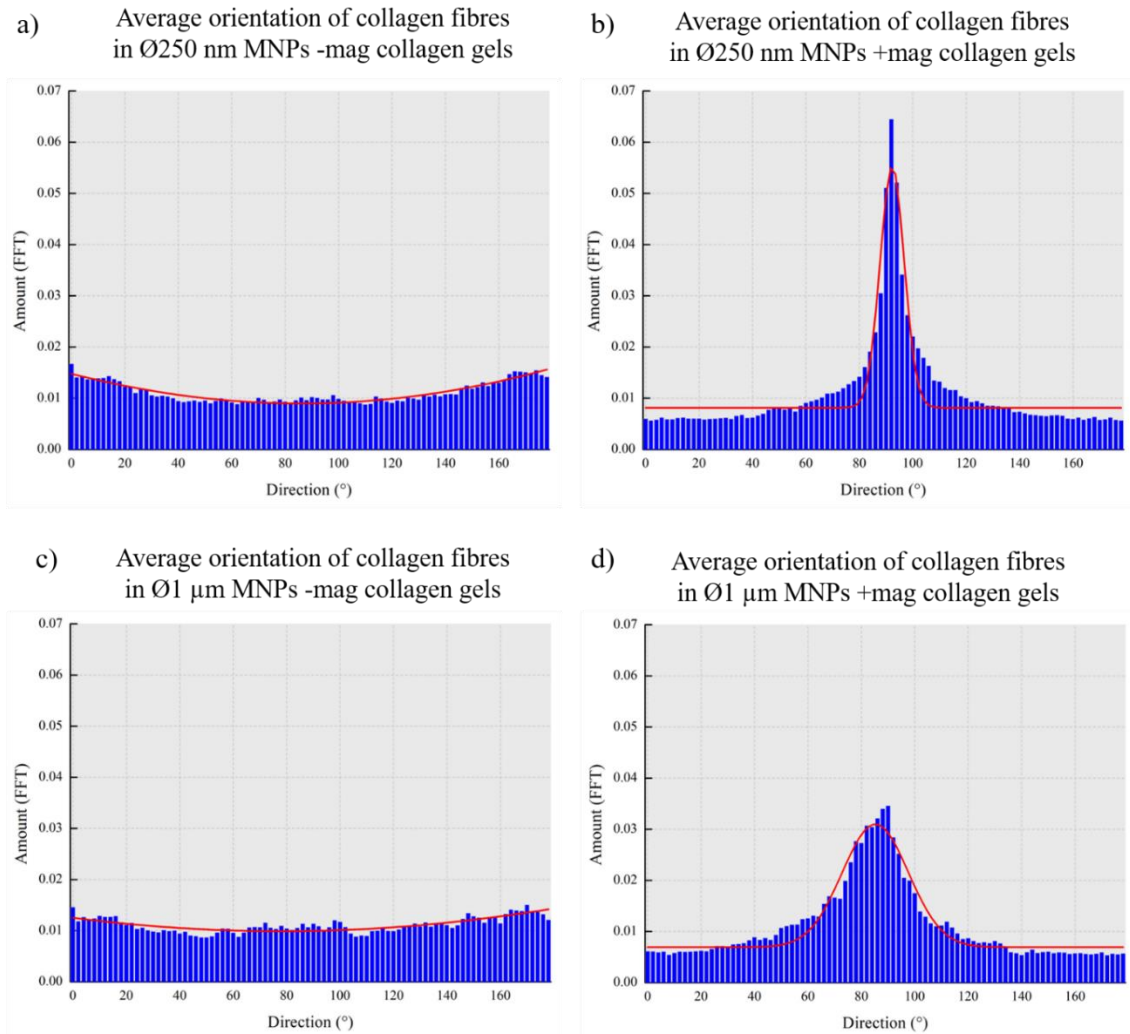


Figure 4.9 Directionality distribution histograms of collagen fibres in 3D collagen gels. Graphs were obtained by image analysis of fluorescent collagen gels visualized at confocal microscope after 2 days in culture. Gels were prepared with Ø250 nm (a, b) or Ø1 µm anti-Activin RIIA MNPs (c, d) and solidified without a magnetic field (-mag) (a, c) or under a magnetic field (+mag) (b, d). Orientation determined through ImageJ's Directionality plug-in, and averages plotted. $n = 3$, trendline = fit to the Gaussian function.

The orientation of MNPs and collagen fibres was then investigated up to 21 days from gels preparation, to determine if the deposition of extracellular matrix by hBMSCs seeded within

the gel might lead to the remodelling of the collagen fibres altering their orientation. For this reason, fluorescent oriented collagen gels were prepared with either Ø250 nm or Ø1 µm MNPs and cultured for 21 days with basal media at 37°C. After 0, 7, 14 and 21 days of culture, the fluorescent collagen gels were visualised by optic and confocal microscopy to check the orientation of MNPs strings and collagen fibres. Figure 4.10 shows how both the MNPs strings and collagen fibres preserved their orientation up to 21 days despite the extracellular matrix deposition. Nevertheless, after 21 days the collagen fibres orientation in gels prepared with Ø1 µm MNPs was more well-defined compared to Ø250 nm MNPs collagen gels, even though a MNPs and collagen fibres orientation was still appreciable after 21 days.

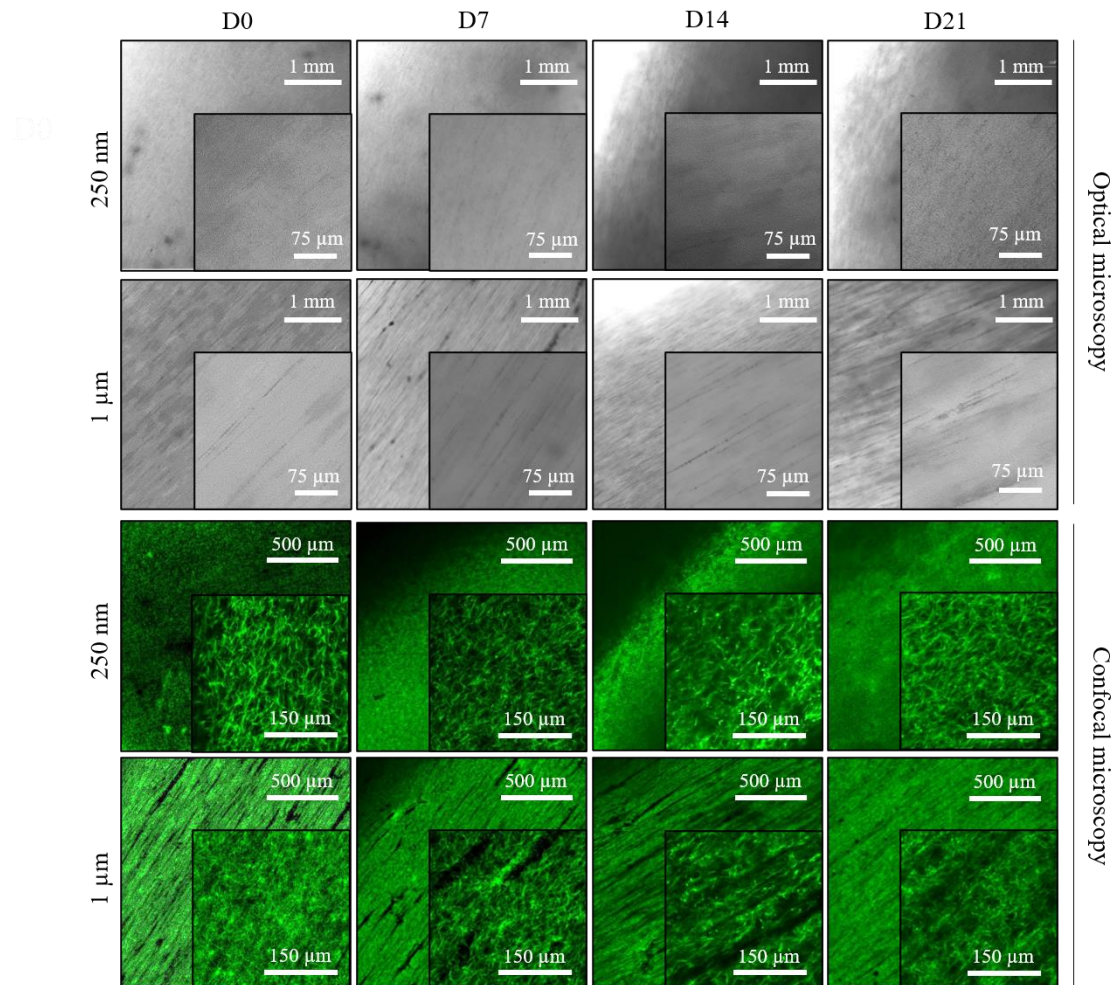


Figure 4.10 Collagen orientation assessment over 21 days. Oriented MNPs strings (top) and collagen fibres (bottom) were visualized in oriented collagen gels at day 0, 7, 14 and 21 (from left to right). The oriented collagen gels were prepared with either Ø250 nm MNPs or Ø1 μ m MNPs. Pictures were acquired at EVOS Optic Microscope (top) and Confocal Microscope (bottom). $n=3$

4.4.1.3 Oriented collagen gels in chamber slides

The work presented in this Chapter provided protocol optimisations for the following publication: “Abigail L. Wright†, Lucrezia Righelli†, T. J. Broomhall, Hannah C. Lamont and Alicia J. El Haj. Magnetic Nanoparticle-Mediated Orientation of Collagen Hydrogels for Engineering of Tendon-Mimetic Constructs, *Frontiers in Bioengineering and Biotechnology*, 2022; doi: 10.3389/fbioe.2022.797437”. In this work, aligned collagen gels were achieved by

using anti-Col I functionalized MNPs to guide collagen I fibre-like structure alignment *in situ* upon magnetic field application. For this purpose, the same magnetic device used for the alignment of collagen gels in syringe moulds was used to align 100 μ L collagen gels in chamber slides. It was shown how the exposure to the magnetic field led to the formation of a complex and aligned collagen structure which was not observed in gels prepared without MNPs and/or without the magnetic field exposure.

The effect of the matrix architecture on cell organization was investigated by seeding collagen gels with hASCs. It was hypothesized that 1) magnetically aligned collagen hydrogels would facilitate parallel, unidirectional cell organization, and 2) non-magnetic hydrogels - both with and without the application of magnetic field - would facilitate poly-directional cell organization. Following 7 days in culture, magnetically aligned and non-aligned cellular hydrogels were fixed and immuno-stained (Chapter 2.8.8) so that cell nuclei and actin filaments could be observed through confocal microscopy (Figure 4.11). In MNP-dosed hydrogels subjected to a magnetic field, cellular F-actin filaments were observed to be unidirectional in their orientation, aligning parallel to the direction of the fibre-like collagen structures and MNPs (Figure 4.11 bottom panel). In non-magnetic collagen gels, cytoskeletal structures were orientated in multiple directions, regardless of the presence of magnetic field (Figure 4.11 top and middle panels).

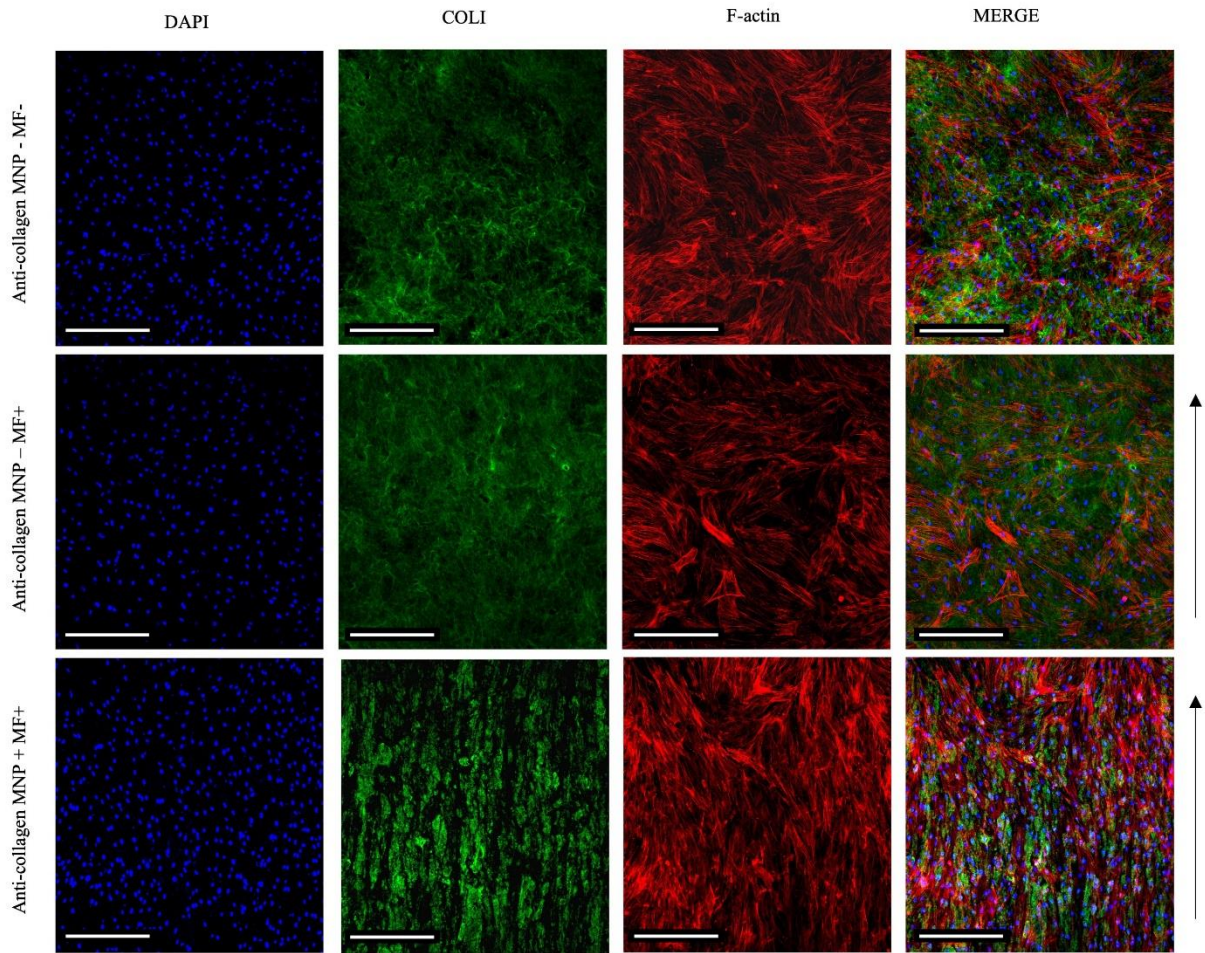


Figure 4.11 Confocal imaging of primary hASCs seeded (250 cells/ μ L) within collagen hydrogels 7 - days post-seeding. Merged images show an aligned arrangement of hASCs, in parallel to collagen fibre-like structures in anti-collagen MNP laden, magnetically aligned gels (bottom panel). Without MNP-dosing and/or application of magnetic field, f-actin orientation can be observed to be poly-directional (top and middle panel). Scale bar = 200 μ m, magnification = 10 $\times$, blue = nuclei, red = F-actin, green = collagen I, arrow = direction of magnetic field lines, $n=3$.

Further to this, cell orientation was quantified in relation to the applied magnetic field to compare the effect of MNP dosing on hASC organization (Methods 2.8.9). Plotting of the average amount of hASC aligning at 0–180° results in a single, normally distributed peak is shown in Figure 4.12 c. This demonstrates that the cells are aligning at a preferred orientation. Such preference of hASC orientation cannot be determined when laden in non-magnetic

collagen gels, regardless of the presence of the magnetic field (Figures 4.12 a, b). This is consistent with the visual assessment of the hydrogels (Figure 4.11).

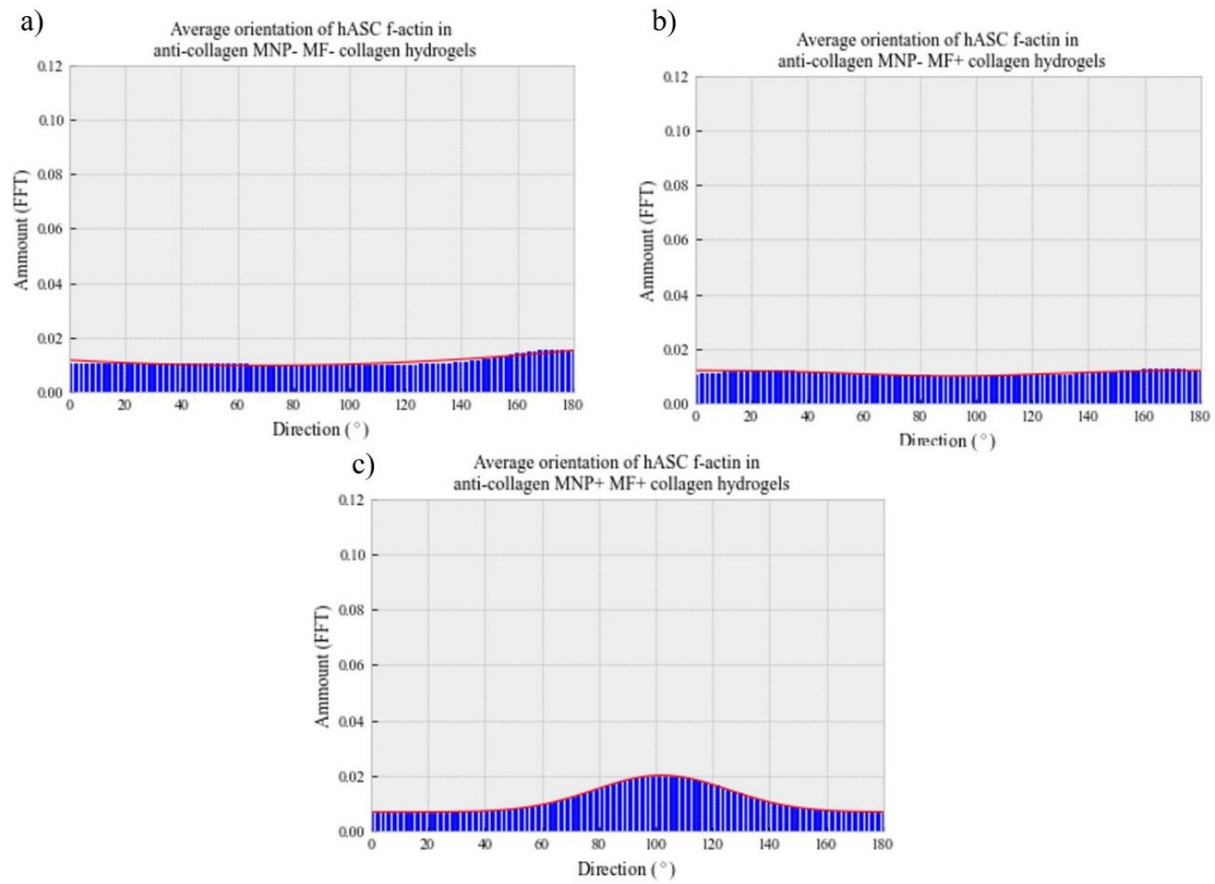


Figure 4.12 Average directionality histograms of hASC f-actin 7 days post-gelation in collagen hydrogels. hASCs were seeded (250 cells/ μ l) into depolymerized collagen suspensions, either with (c) or without (a, b) anti-collagen MNP dosing. Cell–collagen suspensions were then gelled and, if appropriate, a magnetic field applied for 30 min at 37°C. Hydrogels were then immersed in basal media and fixed following 7-day incubation. F-actin was visualized using confocal microscopy, following rhodamine staining. Orientation determined through ImageJ's Directionality plug-in, and averages plotted. $n = 3$, trendline = fit to the Gaussian function.

4.4.2 Fibrin gels

Fibrin gels were the second 3D scaffold to be investigated as potential stem cell delivery method for tendon tissue engineering applications. As first step, the optimal cells density and fibrinogen

concentration was investigated by testing different fibrinogen/cells ratios and assessing cell metabolism with Presto Blue assay (Chapter 2.9.3). For this reason, 20×10^4 hASCs were seeded within 50 μ L fibrin gels at 20 IU/mL thrombin and different fibrinogen concentrations (20 mg/mL, 30 mg/mL, 40 mg/mL or 50 mg/mL). As shown in Figure 4.13, after 3-, 7- and 14-days cells seeded in fibrin gels at 20 mg/mL, 30 mg/mL or 40 mg/mL showed a similar proliferation rate, while hASCs seeded in 50 mg/mL fibrin gels did not show any metabolic activity at the tested timepoints.

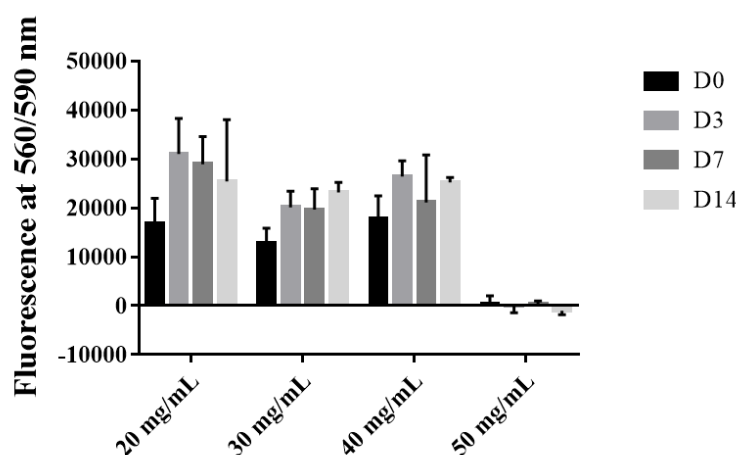


Figure 4.13 Presto Blue assay of 20×10^4 hASCs seeded within 50 μ L fibrin gels at 20 IU/mL thrombin and 20 mg/mL, 30 mg/mL, 40 mg/mL or 50 mg/mL fibrinogen concentration. No metabolic activity was registered at 50 mg/mL, while no significant changes were recorded at the other concentrations tested. $n=3$. Fluorescence was read at 560/590 nm at a Plate Reader (Spark, Tecan).

To investigate if the limiting factor of the low metabolic activity observed in fibrin gels at 50 mg/mL was the high cells density or the high fibrinogen concentration, fibrin gels were prepared freshly at 20 IU/mL thrombin and at the same fibrinogen concentrations tested previously, but seeding 3×10^4 hASCs/gel. Presto Blue assay was performed after 3, 7 or 14 days in culture. The same assay was also performed on fibrin gels with embedded hASCs labelled with anti-Activin RIIA MNPs ($\varnothing 250$ nm, considering 25 μ g MNPs/ 2×10^5 cells).

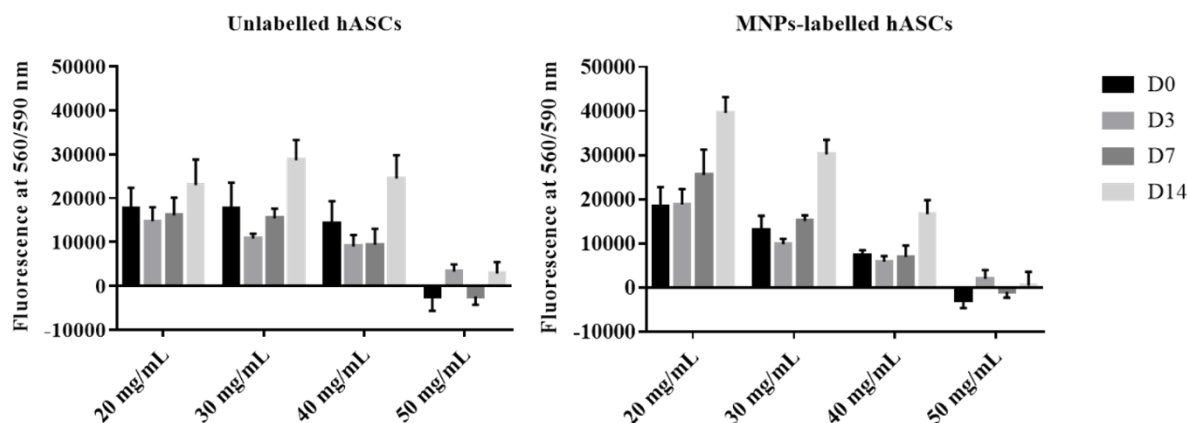


Figure 4.14 Presto Blue assay of 3×10^4 hASCs, either or not MNPs-labelled. Cells were seeded within 50 μ L fibrin gels at 20 IU/mL thrombin and 20 mg/mL, 30 mg/mL, 40 mg/mL or 50 mg/mL fibrinogen concentration. Very low cells metabolism was registered at 50 mg/mL, while no significant changes were recorder at the other concentrations tested. $n=3$. Fluorescence was read at 560/590 nm at a Plate Reader (Spark, Tecan).

Figure 4.14 shows how even at lower cells density, 50 mg/mL fibrin gels limited hASCs metabolic activity, in both unlabelled or labelled cells. It is also possible to notice how MNPs-labelled cells seeded in 40 mg/mL fibrin gels decreased their metabolism compared to lower fibrinogen concentrations. Following these results and considering that higher fibrinogen concentrations lead to more long-lasting gels,¹⁷⁷ 3×10^4 hASCs in 30 mg/mL fibrin gels was selected as the optimal cells/fibrinogen ratio for further experiments.

Once assessed the best cells/fibrinogen ratio, the ideal concentration of thrombin was investigated (Chapter 2.9.4). It is known from literature^{169,309} that it is possible to modulate gel setting time by adjusting thrombin concentration and in particular, the higher the concentration, the shorter the setting time. The gelation time was assessed by spectroscopy³⁰⁹ at the Plate Reader by firstly preparing 30 mg/mL fibrin gels at different thrombin concentrations, namely 20 IU/mL, 50 IU/mL, 60 IU/mL, 70 IU/mL, 80 IU/mL, 90 IU/mL or 100 IU/mL. The gels were allowed to set at a controlled temperature of 25°C and the complete absorbance spectra was

acquired between 200 nm and 1000 nm, in order to identify an appropriate wavelength with an appreciable difference between the spectra of the fibrin gels tested (Figure 4.15). Gels prepared at 20 IU/mL and 50 IU/mL thrombin concentrations did not set successfully at 25°C (data not shown) and therefore it was not possible to acquire the absorbance spectra. After selecting 405 nm as an appropriate wavelength, the absorbance spectra of fibrin gels undergoing gelation were acquired at 405 nm over the time, for up to 5 min (Figure 4.16).

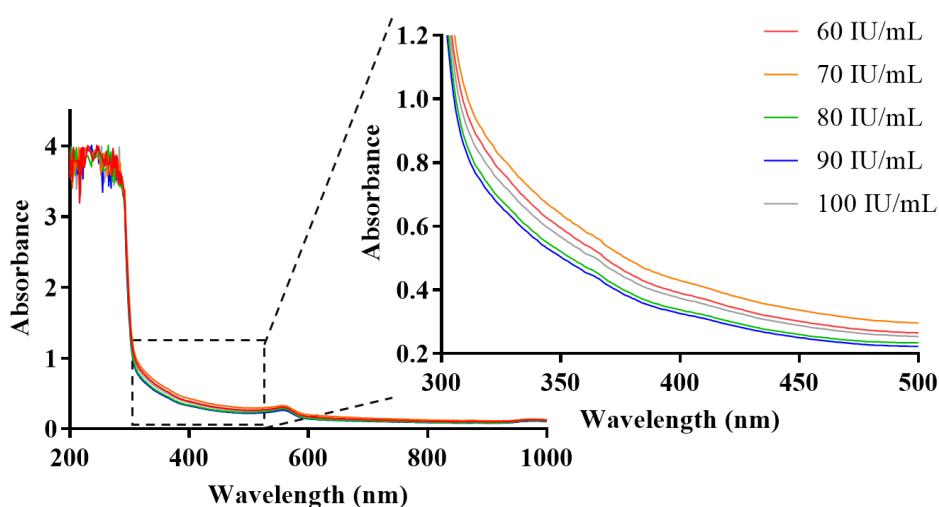


Figure 4.15 Complete absorbance scan (200-1000 nm) of completely set 30 mg/mL fibrinogen and 60 IU/mL, 70 IU/mL, 80 IU/mL, 90 IU/mL or 100 IU/mL thrombin. $n=3$. The scans were acquired at a controlled temperature of 25°C at a Plate Reader (Spark, Tecan).

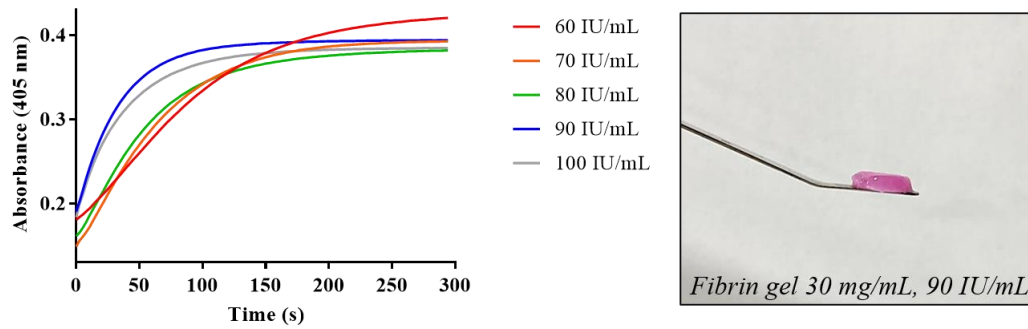


Figure 4.16 Absorbance spectra of fibrin gels undergoing gelation. The scans were acquired at 405 nm over the time for up to 5 min, at a controlled temperature of 25°C using a Plate Reader (Spark, Tecan). $n=3$.

As shown in Figure 4.16, gels absorbance spectra reached a plateau when the gels were completely set.³⁰⁹ It is possible to notice how by increasing the thrombin concentration, the time needed for the curve to reach the plateau was diminishing, and therefore the gels were setting faster. While 60 IU/mL gels were still not set after 5 min, 90 IU/mL and 100 IU/mL gels were set after about 90 sec. In particular, 90 IU/mL gels set slightly quicker than 100 IU/mL gels. Afterwards, hASCs metabolic activity was investigated in fibrin gels at the same thrombin concentrations previously considered. 3×10^4 hASCs, whether or not anti-Activin RIIA MNPs-labelled, were seeded in 30 mg/mL fibrin gels at 60 IU/mL, 70 IU/mL, 80 IU/mL, 90 IU/mL or 100 IU/mL thrombin. Presto Blue assay was performed after 3, 7, and 14 days.

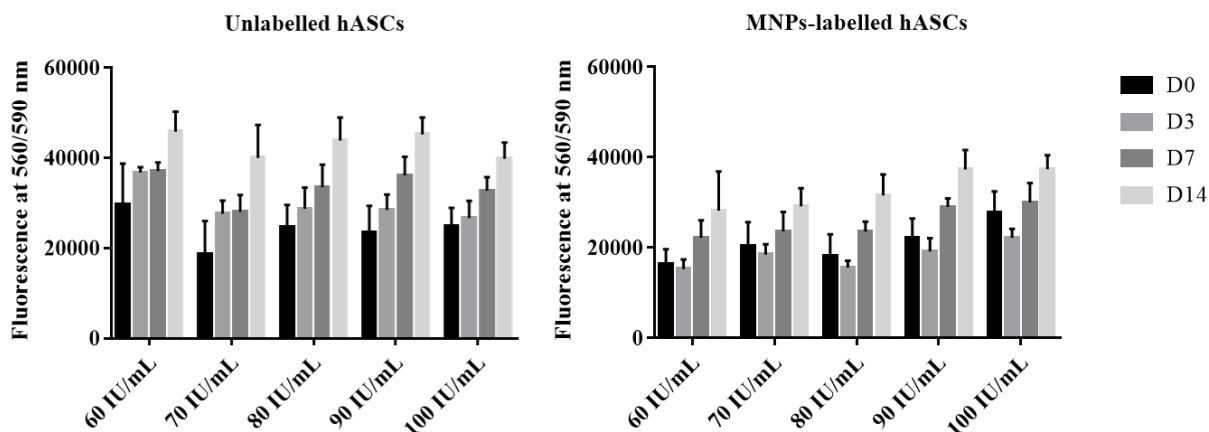


Figure 4.17 Presto Blue assay of 3×10^4 hASCs, either or not MNPs-labelled. Cells were seeded within 50 μ L fibrin gels at 30 mg/mL fibrinogen and 60 IU/mL, 70 IU/mL, 80 IU/mL, 90 IU/mL or 100 IU/mL thrombin concentration. No significant changes were recorded across the concentrations tested. $n=3$. Fluorescence was read at 560/590 nm at a Plate Reader (Spark, Tecan).

As shown in Figure 4.17, no significant difference in cell metabolism was recorded between the thrombin concentrations tested. Considering this result and the outcomes of the gelation time study, 90 IU/mL was evaluated as the most suitable thrombin concentration. To summarise, the final fibrin gel formulation, identified as optimal for this Chapter's purposes, was 30 mg/mL fibrinogen, 90 IU/mL thrombin and 3×10^4 seeded hASCs.

As last experiment, a degradation study was carried out to assess fibrin gel degradation speed when incubated with media without aprotinin supplementation (Chapter 2.9.5). After 0, 7, 14 and 21 days the gels were weighted and the media was analysed at the ICP for iron content, testifying MNPs release from gels degradation.

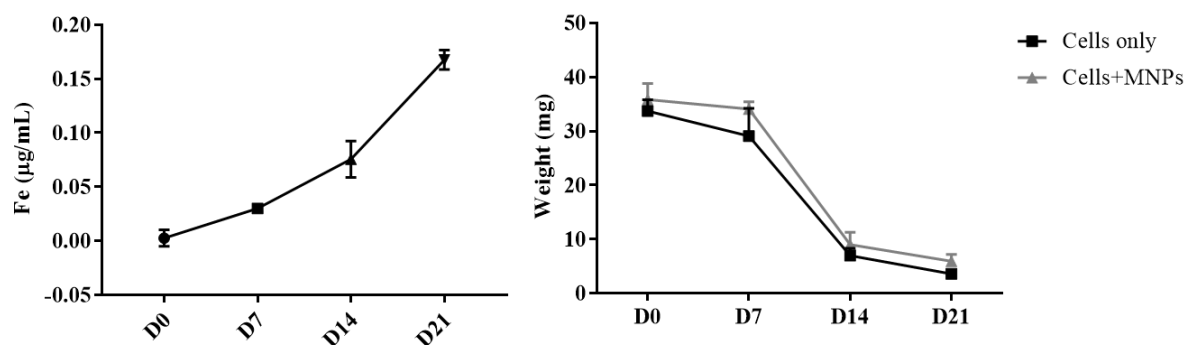


Figure 4.18 Degradation study of 30 mg/mL fibrinogen, 90 IU/mL thrombin and 3×10^4 seeded hASCs, either or not MNPs-labelled. Cellular fibrin gels were cultured in basal media without aprotinin. At day 0, 7, 14 and 21 the iron content of the fibrin gels media was analysed by ICP (left) and gel weight was registered (right). $n=3$. The ICP analysis was performed using ICP-OES JY2000-2.

Figure 4.18 shows how the iron concentration in media increased by day 21, in parallel with a decrease in fibrin gels weight which reached almost 0 mg by day 21, testifying the complete degradation of the gels. It is also possible to notice that the degradation of the gels with MNPs-labelled or unlabelled hASCs followed a similar trend.

4.5 Discussion

As previously mentioned, 3D scaffolds have been widely used in regenerative medicine to provide the cells with a more realistic growth environment compared to 2D cultures and to serve as cell delivery methods in preclinical and clinical studies^{170,182}. In this Chapter, collagen and fibrinogen have been investigated as possible materials for the preparation of 3D gels for tissue engineering purposes.

4.5.1 Collagen gels

Collagen is one of the major components of many extracellular matrices including tendons and ligaments. In particular, highly organised type I collagen constitutes more than 90% of tendons matrices, providing the structural and tensional properties required for their

function.^{304,306} For these reasons, collagen hydrogels have been widely used as 3D scaffolds in regenerative medicine, as they can simulate the natural matrix, providing good biocompatibility and biodegradability at the same time.^{226,310}

To have a realistic tendon model that could be used both for cell delivery and for *in vitro* tendon 3D modelling, a cropped 1 mL syringe was used as a mould for the preparation of 3D collagen gels.³⁰⁴ 1.5 mg/mL, 2 mg/mL and 3 mg/mL collagen gels were prepared at two different hBMSCs densities (5×10^4 cells/mL and 15×10^4 cells/mL) assessing cells viability after one week in culture with basal media. The viability test revealed a good cells survival in collagen gels at higher collagen densities and, considering the high collagen concentration in the native tendon^{17,305} and studies previously published in literature³⁰⁴, 3 mg/mL was the selected collagen concentration for further studies. A subsequent contraction study was performed in order to investigate the ideal cells concentration that would not lead to a dramatic gel contraction. In fact, it is known from literature that cellular collagen gels are subjected to contraction over the time, which can be modulated by adjusting the ratio between the collagen concentration and the amount of cells seeded within the gel.^{311,312} For this reason, several trials of collagen gels formulations were performed changing the cells seeding density. Different cells densities (1×10^4 cells/mL, 2.5×10^4 cells/mL, 5×10^4 cells/mL and 10×10^4 cells/mL) were seeded in 3 mg/mL collagen gels, which were measured in length and diameter after 7, 14 and 21 days in culture. Following the contraction study results and the low native tendon tissue cellularity,¹⁷ 5×10^4 cells/mL was considered a fair compromise between gels contraction and cells seeding density.

After the ideal collagen concentration and seeding density was established, the possibility to develop a 3D collagen model with oriented collagen fibres was investigated. In fact, human tendons are characterised by highly organised collagen fibres^{6,17,101,105,306} which confer the peculiar mechanical properties and elasticity to the native tissue. Moreover, it is

reported in literature that stem cells cultured and stimulated to differentiate on an aligned scaffold are more likely to differentiate compared to stem cells cultured on a smooth surface.^{302,303} Several methods have been investigated to create aligned-fibres collagen scaffolds, ranging from extrusion¹⁶⁵ to microfluidic.¹⁶⁸ Cheng et al.¹⁶⁷ used the application of an electric current to generate a pH gradient in collagen I solutions leading to the aggregation of collagen molecules into aligned crosslinked bundles. Antman-Passig and Shefi in 2019²²⁶ demonstrated how it was possible to organise and align collagen fibres by combining collagen to MNPs and exposing the liquid MNPs/collagen suspension to an external magnetic field. When exposed to the field, MNPs would aggregate creating strings following the magnetic field lines. Interestingly, after gels setting, the gels could be removed from the magnetic field without affecting the MNPs orientation. Following this protocol, oriented fibres collagen gels were prepared by mixing a dispersion of anti-Activin RIIA Ø250 nm MNPs-labelled hBMSCs in a liquid collagen solution (considering 250 µg of MNPs every 500 µL gel and 5×10^4 cells/mL gel as cells density). The final mixture was then injected into a cropped syringe and allowed to set under the magnetic field created by two permanent magnets placed one in front of the other. By cutting the syringe on both ends, it was possible to place the magnets closer to each other (7.2 cm apart), increasing the magnetic field exerted on the MNPs within the gel. The field was measured to have a strength of ~30 mT close to the long sides of the magnets, in line with the values predicted with FEMM Software. Firstly, the presence of the MNPs strings was verified by Prussian blue staining, which revealed MNPs alignment in the direction of the magnetic field.

Subsequently, a possible relationship between MNPs size and collagen fibres orientation was investigated by employing anti-Activin RIIA Ø1 µm MNPs in the preparation of oriented collagen gels, in particular we wanted to examine if an increase in MNPs size would lead to a more aligned and organised collagen fibres alignment. Ø1 µm MNPs were labelled

with anti-Activin RIIA antibody by carbodiimide activation (Chapter 3.4.1) and characterised for size and surface charge. Even though MNPs biocompatibility is well known from literature and has been widely investigated,^{313–317} due to the high concentration of MNPs employed in the preparation of oriented collagen gels, cell metabolic activity was verified by Presto Blue assay. hBMSCs were seeded on monolayer and labelled with Ø250 nm or Ø1 µm anti-Activin RIIA MNPs, considering 250 µg/2.5x10⁴ seeded cells. After 7, 24 or 21 days, no significant difference was reported in terms of cell metabolism, confirming MNPs cytocompatibility. Afterwards, MNPs and collagen fibres orientation were investigated by several techniques. Firstly, 3D oriented collagen gels with either Ø250 nm or Ø1 µm MNPs were imaged under an optic microscope, revealing a parallel strings pattern which was not appreciable in gels solidified without magnetic field exposure. After assessing MNPs organization, collagen fibres orientation was analysed by staining paraffin sectioned collagen gels with Picrosirius Red. Picrosirius Red is known to be a birefringent molecule that, when binds to collagen, increases the fibrils natural birefringence by disposing in parallel to the collagen.³⁰⁷ Stained collagen fibres appeared mainly in green or orange birefringence colours when imaged with a polarised microscope, indicating the orientation of collagen fibres. Collagen alignment was further investigated by using a fluorescently-labelled collagen stock solution to prepare oriented collagen gels. In fact, following a protocol found in literature,³⁰⁸ it was possible to clearly visualise collagen fibres under a confocal microscope which revealed oriented collagen fibres reflecting the MNPs aligned pattern, while no such alignment could be appreciated in gels solidified without a magnetic field exposure. The quantification of collagen fibres alignment via ImageJ evidenced a more dispersed collagen fibre orientation in Ø1 µm MNPs gels, compared to Ø250 nm MNPs gels (SD±12.42 and ±4.52, respectively) suggesting that an increase of MNP size might not correspond to a better collagen fibres alignment.

As previously mentioned, after the complete setting, collagen gels could be removed from the magnetic field without affecting the fibres orientation. Nevertheless, the deposition of extracellular matrix by the hBMSCs seeded within the gel, might have impacted on the orientation of the collagen fibres leading to a modification of their orientation. Therefore, fluorescent collagen gels were prepared with either Ø250 nm or Ø1 µm MNPs and cultured with basal media for 21 days. Collagen and MNPs orientation were preserved for 21 days despite extracellular matrix deposition. Nevertheless, collagen gels prepared with Ø1 µm MNPs preserved a better collagen and MNPs orientation compared to Ø250 nm MNPs gels, suggesting that increasing the MNPs size might have a positive impact in collagen fibre alignment preservation over the time.

Aligned collagen gels were also achieved in chamber wells, using the same alignment protocol and magnetic set up. It was observed that uniaxially aligned, fibrillar hydrogel scaffolds provided topographical cues for the parallel alignment of hASCs *ex vivo*. hASCs actin filaments orient along the collagen fibres and MNPs, into an organized complex structure that is not observed in collagen gels without MNPs and/or magnetic field exposure. Cellular characteristics, such as morphology and orientation, have been evidenced to be mediated by signalling pathways modulated in response to topographical cues.^{318,319} Therefore, controlling matrix topography would enable control of such characteristics in engineered tissues. Musculoskeletal tissues, including tendon, are reliant upon specific organization of ECM components, mediated and sensed by resident cells. Healthy native tendon is characterized by a hierarchical fibrillar cellular matrix which provides the elasticity and mechanical properties of the tissue, essential for its load transferring function.³²⁰ In tendinopathy, loss of this ECM organization and transition to isotropy is a key marker of this disease state.³²¹ Therefore, in order to engineer *ex vivo* constructs analogous to tendon tissue for disease modelling and regenerative therapies, tuning of ECM is paramount.

4.5.2 Fibrin gels

Fibrin gels were the second 3D scaffold to be investigated as potential cell delivery method for tendon defects repair. Fibrin gels were prepared from a combination of fibrinogen and thrombin, which are both naturally present in the blood stream, as key components of the coagulation cascade.^{169,170} Their biocompatibility is therefore unquestionable, as attested by the large use of fibrin sealants in surgical procedures even at clinical grade.^{172,173,322,323} Moreover, fibrin naturally contains binding motifs for cells and growth factors, as well as an architecture that promotes cells infiltration and wound repair.¹⁸¹ In this Chapter, fibrin gels were investigated as potential injectable cells therapy, as they are notably minimally invasive, cost-effective and do not require the complete replacement of the injured tendon^{170,175}. Moreover, as injectable therapy, the cells-loaded fibrin gels can be delivered *in situ* while still in the liquid phase which would then solidify adapting to the tendon defect conformation. Other studies have been published in literature^{182,183} using fibrin gels as cell delivery method for tendon tissue repair, highlighting how the combination of fibrin and stem cells improved the mechanical properties of tendons in the initial healing phases and led to a more organised ECM deposition.

A fibrin gel model was developed by firstly investigating the appropriate ratio between cells density and fibrinogen concentration. Presto Blue assay was performed on 20×10^4 hASCs seeded within 50 μ L fibrin gels at 20 IU/mL thrombin and different fibrinogen concentration, namely 20 mg/mL, 30 mg/mL, 40 mg/mL or 50 mg/mL, and cultured with basal media supplemented with 20 μ g/mL aprotinin for 14 days. Cells did not show any metabolic activity when seeded in fibrin gels at 50 mg/mL fibrinogen, in strong contrast with the other fibrinogen concentrations tested which showed a similar metabolism rate up to 14 days. A second metabolic activity experiment was performed on fibrin gels at the same fibrinogen concentrations previously tested, but reducing the hASCs seeding density to 3×10^4 cells/gel.

This was in order to investigate if the poor cell metabolism rate observed in 50 mg/mL fibrin gels was attributable to the fibrinogen concentration or to the high cell density. Presto Blue results show how reducing the cell seeding density did not lead to an improvement in cell metabolism in 50 mg/mL gels, suggesting that high fibrinogen concentrations inhibit hASCs metabolic activity, as also indicated by lower cell metabolism rates in 40 mg/mL gels. Another possible explanation would be that the fibrin matrix at 50 mg/mL fibrinogen concentration was so dense to act as a diffusion barrier preventing the assay from working. These results were in line with the literature, which suggests intermediate fibrinogen concentrations for cell delivery purposes,^{177,181} since high fibrinogen concentrations lead to stiffer gels with a very dense fibrin network which inhibits cells migration and might also act as a strong diffusion barrier. On the other hand, stiffer gels have higher cells retention and longer degradation times,¹⁷⁷ which are both desirable characteristics for a cells scaffold. Following these considerations, 30 mg/mL fibrinogen concentration was selected over 20 mg/mL for further experiments.

Once fibrinogen concentration was assessed, the appropriate thrombin concentration was investigated. In fact, by adjusting thrombin concentration it is possible to modulate the gels setting time¹⁶⁹ and, considering a preclinical or clinical application, a quick setting time was desirable in order to reduce the length of the surgical procedure and therefore the risk of infections. The gelation time was investigated by spectroscopy^{169,309} at a controlled temperature of 25°C, which was considered a fair compromise between the average room temperature (20°C) and body temperature (37°C), so that the setting time would have been relevant for arthrotomy surgical procedures. Firstly, gels at 30 mg/mL fibrinogen and 20 IU/mL, 50 IU/mL, 60 IU/mL, 70 IU/mL, 80 IU/mL, 90 IU/mL or 100 IU/mL thrombin were prepared and allowed to set at 25°C. Afterwards, the complete absorbance spectra (200 nm-1000 nm) of completely set gels were acquired in order to identify a wavelength at which an appreciable difference between the spectra of the gels at different thrombin concentration could be identified. 405 nm

was considered an appropriate wavelength which was also fairly distant from the background absorption of the plastic well plate (~200-300 nm).^{289,290} The absorbance spectra of gels undergoing gelation were acquired at 405 nm at a controlled temperature of 25°C for up to 5 min. While 20 IU/mL and 50 IU/mL gels did not set at 25°C within a sensible timeframe (data not shown), 90 IU/mL and 100 IU/mL were the quickest gels to set at the tested temperature by reaching a complete set after about 90 sec, as testified by the relative spectra reaching a plateau. 90 IU/mL gels had a slightly faster setting time compared to 100 IU/mL, suggesting that 90 IU/mL thrombin might be the saturation dose for the fibrin gels tested.

Subsequently, the same fibrin gels at different thrombin concentrations were tested for hASCs metabolic activity with Presto Blue assay. 3×10^4 hASCs, either or not anti-Activin RIIA MNPs labelled, seeded within the just-mentioned fibrin gels showed a good metabolism rate at all thrombin concentrations tested up to 14 days. Following this result and the gelation time study outcomes, 90 IU/mL was selected as optimal thrombin concentration for further studies.

Lastly, a degradation study of the ultimate fibrin gels formulation (3×10^4 hASCs in fibrin gels 30 mg/mL fibrinogen and 90 IU/mL thrombin) was performed. As previously mentioned, fibrin gels and sealants are notably subjected to degradation induced by the secretion of plasminogen and other matrix metalloproteinases from the cells seeded within the gel.^{175,322} *In vitro*, daily provisions of fresh media supplemented with aprotinin, a plasmin inhibitor, were therefore necessary to ensure and prolong gels stability.¹⁷⁰ The degradation study performed in this Chapter was designed in order to mimic an *in vivo* context, in which the preservation of the fibrin gels was not assured by daily provisions of aprotinin but from an equilibrium between fibrin degradation and new extracellular matrix deposition. For these reasons, MNPs-labelled hASCs-seeded fibrin gels were incubated with basal media not supplemented with aprotinin for 21 days at 37°C. After 7, 14 and 21 days the gels were weighed and the iron content released in the media was analysed by ICP. As indicated by the weight loss and the parallel increase in iron

release, the gels maintained their stability until day 21. Furthermore, no significant difference in degradation speed was observed between MNPs-labelled and non-labelled hASCs-seeded gels, testifying that the presence of MNPs did not impact the gels degradation rate. In native tendon tissue, the repair process after an injury include a reparative stage beginning 2-4 days after the damage in which tenocytes deposit a high amount of ECM with the purpose of providing an initial stability to the tissue.³⁶ The fibrin gels developed in this Chapter could be useful in improving the stability of the newly formed tissue and in providing support to the injected cells until the remodelling phase of tendon repair, which starts 1-2 months from the injury.³⁵

4.6 Conclusion and future perspectives

Finding the most appropriate cell delivery method is a step as challenging as fundamental to proceed from *in vitro* to *in vivo* studies. Several factors need to be considered when developing a cells scaffold, such as the material biocompatibility and the scaffold *in situ* delivery. In this Chapter, two hydrogels were developed and investigated as cell delivery methods and 3D tendon modelling. In particular, oriented collagen gels were developed by dispersing MNPs in a collagen solution allowed to set between two permanent magnets and it was demonstrated how the organisation of collagen fibres and MNPs are preserved over the time and are not affected by gel removal from the magnetic field. It was also shown how the aligned collagen gel architecture facilitated the alignment of hASCs creating a more complex and realistic structure which recapitulated in gross organisation the fibrillary structure of native tendons. possible to remotely control and direct hASC alignment through MNP-mediated modulation of the collagen hydrogel architecture after seeding. This 3D oriented collagen gel model can be considered as promising for mimicking the structure of tendons and might serve as *in vitro* tendon 3D model to explore healing mechanisms and repair. Further studies are

required to better characterise the material and mechanical properties of the collagen model in comparison with the native tendon tissue. An important investigation would also be to study any potential effect of the oriented collagen fibres in the expression of tenogenic proteins and ECM deposition of differentiating stem cells, in comparison to non-aligned collagen gels.

Fibrin gels were the second cells scaffold to be developed in this Chapter. In particular, fibrinogen and cells seeding densities were modulated in order to obtain a stiff gel at high fibrinogen concentration which would last longer *in vivo*, but without compromising cells metabolic activity. The most appropriate thrombin concentration was also investigated, with the aim to develop a fast-setting fibrin gel which would reduce the length of *in vivo* surgical procedures. The developed fibrin scaffold can be considered as a promising injectable cell delivery method for tendon tears and injuries, thanks to its high biocompatibility, quick setting time and long *in vitro* degradation times. Nevertheless, further studies are required to characterise the mechanical properties of the gels, in order to have a better understanding of the material properties.

In conclusion, the main results elucidated in this Chapter can be summarised as follows:

- A 3D oriented fibre collagen gel and an injectable fibrin gel were developed as potential cell delivery method or *in vitro* tendon modelling,
- MNPs and collagen fibre alignment was verified in 3D collagen gels with several techniques. The alignment of hASCs seeded within the oriented scaffold was also achieved, mimicking the highly organised structure of native tendon,
- The developed fibrin gel has proven to be highly cytocompatible, to set within 90 sec and to be stable *in vitro* for 21 days.

CHAPTER V

MNPs-MEDIATED ACTIVATION OF ACTIVIN RIIA IN A RAT TENDON REPAIR MODEL

5.1 Introduction

In this Chapter, the mechano-magnetic stimulation of MNPs-tagged hASCs has been translated *in vivo*, investigating the effects of an external magnetic field on the healing process of a patellar tendon defect in a rat model.

Because of the wide range of tendon injuries, there is not a specific animal model for testing tendon tissue engineering techniques *in vivo*; therefore, the clinical condition to be investigated needs to be matched appropriately to a useful animal model in order to guarantee a reproducible tissue manipulation and defect creation.^{194,324} Beason et al.¹⁹⁶ evaluated multiple tendon injuries and identified the patellar tendon as the best one for defect reproduction consistency, difference in the injured tendon compared to the native tissue and in the overall tendon manipulation. Moreover, the patellar tendon has been used in several tendon injury models, especially to investigate the healing of a partial tendon defect.¹⁹⁴ In this Chapter, a partial window defect was created in the patellar tendon in a rat model and subsequently filled with MNPs-labelled hASCs embedded in an injectable fibrin matrix. The effect of an external magnetic stimulus in driving MNPs-labelled hASCs towards the tenogenic commitment was then investigated, as well as the safety and biocompatibility of the implant. Fibrin gels have already been used in a few studies for tendon engineering applications, for example, in the repair of a patellar tendon defect in a rat model¹⁸² or in a rabbit Achilles tendon defect.¹⁸³ In both cases, fibrin was used as stem cell delivery method and its application showed to be useful in improving tendon healing

5.2 Aims

The main purposes of this Chapter were:

- To investigate the tenogenic regenerative potential of remotely-activated MNPs-labelled hASCs in an *in vivo* rat model after 4 and 8 weeks of treatment,
- To assess the safety and biocompatibility of the implant.

5.3 Methods

5.3.1 hASCs MNPs-labelling and fibrin gels preparation

hASCs were isolated from human fat sample as described in Chapter 2.3.2 and expanded in basal α MEM as in section 2.11.1.1 and 2.11.1.2. When confluent, cells were trypsinized and labelled in suspension with anti-Activin RIIA MNPs ($\varnothing$ 250 nm) as explained in 2.11.2, considering 25 μ g MNPs every 2×10^5 cells.¹⁸² Following the procedure described in Chapter 2.11.3, for each tendon defect 25 μ L fibrin gels were prepared (30 mg/mL fibrinogen and 90 IU/mL thrombin) embedding 5×10^5 hASCs, either tagged with anti-Activin-labelled MNPs or unlabelled and 10 μ L of the final gel were injected in the tendon defect (2×10^5 cells labelled with 25 μ g MNPs effectively implanted).

5.3.2 Surgical procedure

23 skeletally mature male RNU nude rats (of an average weight of 250 g) were purchased from Charles River Laboratories (#316RNU). Animals were treated as described in Chapter 2.11 and kept in authorised facilities at 3Bs Research Group (University of Minho, Portugal). Surgical procedures and animal manipulations were performed only by qualified personnel and carried out as described in Chapter 2.11.4. Briefly, after anaesthesia (ketamine 75 mg/kg, IP and medetomidine 0.5 mg/kg, IP) a window defect was created with a biopsy punch ($\varnothing$ 2 mm) on

the patellar tendon of the right rear limb of the rats. The defect was filled with 10 μ L of the cellular fibrin gels previously prepared (Chapter 2.11.3) which was allowed to set for 2 min before closing the created wound. During follow-up, the animals were monitored every day looking for signs of infection or discomfort according to 3Rs regulation (Chapter 2.11). At the end of the experiment (i.e.: after 4 or 8 weeks), the animals were euthanised and organs, blood and tendons were harvested as described in Chapter 2.11.5. A brief overview and description of the study design and experimental groups can be found in Table 5.1. Contralateral tendons were also harvested and used for histological controls.

Table 5.1 Experimental groups with animals involved and type of stimulation performed. 3 animals were included in each group.

	Group	Implant	Stimulation	Euthanasia
i)	Sham	Empty defect	No stimulation	8 weeks
ii)	Cells only -mag	Fibrin/hASCs	Box w/o magnets	4 weeks
iii)	Cells +MNPs -mag	Fibrin/MNPs/ hASCs	Box w/o magnets	
iv)	Cells +MNPs +mag	Fibrin/MNPs/ hASCs	Box w/ magnets	
v)	Cells only -mag	Fibrin/hASCs	Box w/o magnets	8 weeks
vi)	Cells +MNPs -mag	Fibrin/MNPs/ hASCs	Box w/o magnets	
vii)	Cells +MNPs +mag	Fibrin/MNPs/ hASCs	Box w/ magnets	
	Fibrin/hASCs: fibrin gels with embedded hASCs			
	Fibrin/MNPs/hASCs: fibrin gels with embedded MNPs-labelled hASCs			

“Sham group” animals received the tendon injury but no treatments, the defect was allowed to heal naturally. “Cells only -mag” received the fibrin gel with embedded hASCs without MNPs and they were stimulated with the box without magnets. “Cells +MNPs -mag” and “Cells

+MNPs +mag” group animals received the fibrin gel with embedded MNPs-labelled hASCs and they were stimulated with the box without magnets and with the box with magnets, respectively.

5.3.3 Rat magnetic stimulation

To deliver an external magnetic stimulus to MNPs-labelled hASCs, a magnetic box was designed and assembled as described in chapter 2.11.6. Briefly, 64 permanent magnets (1.42 T each) were fitted on the long sides and on the inner wall of the box, in particular they were arranged to create a variable magnetic field which would be sensed by the hASCs tagged with anti-Activin RIIA MNPs. As the animals move in the box area, the variable magnetic field would mechanically activate the Activin RIIA towards tenogenic differentiation. Rats in the experimental groups iv) and vii) as indicated in Table 5.1, were allowed to move freely in the magnetic box one hour a day, three days a week, for the whole duration of the experiment. To provide the same exercise intensity, groups ii), iii), v) and vi) were allowed to move in other identical boxes built without magnets, following the same exercise regimen adopted for group iv) and vii). Group i) was left unstimulated as control.

5.3.4 Implant localisation

After 4- and 8-weeks post-surgery, the localisation and presence of the fibrin implant in the patellar tendon explants were investigated by FISH analysis (Vectra Polaris, Automated Quantitative Pathology Imaging System) following the protocol described in Chapter 2.11.9. Tendons sections were checked for human content by FISH analysis, targeting human POLR2A, PPIB and UCB RNA. The slides were imaged using Vectra Polaris, Automated Quantitative Pathology Imaging System.

5.3.5 Tendons repair evaluation

A structural and morphological evaluation of tendon repair was performed by H&E and Picrosirius Red histology staining as described in section 2.11.10.1 and 2.11.10.3. H&E and Picrosirius Red stained sections were imaged respectively with a Cytation5 microscope (BioTek) and Fluorescence Inverted Microscope under polarized light (Axio Observer 3851000135, Zeiss). Tenogenic proteins expression was evaluated by immunohistochemistry as described in Chapter 2.11.11, in particular TNMD, CD45 and CD163 expressions were investigated. Slides were imaged at an Inverted Confocal microscope (TCS SP8, Leica). Moreover, the regeneration of the injured site and tendon overall architecture was evaluated by μ CT analysis as explained in Chapter 2.11.12.

5.3.6 Iron accumulation

Any potential iron accumulation was investigated in some of the main detoxification organs (liver, spleen and lungs). Iron deposition was monitored by Perls van Gieson histological staining (Chapter 2.11.10.2). Organs were harvested, fixed in formalin and dehydrated in ethanol as in paragraph 2.11.10. 4 μ m-thick sections were stained with Perls van Gieson and imaged at an optic microscope (DM750, Leica).

5.3.7 Immune response analysis

The immune response was analysed by measuring the blood levels of 14 inflammatory cytokines (G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, TNF- α). Blood samples were collected via intracardiac puncture and plasma was isolated via centrifugation as described in Chapter 2.11.13. Plasma was analysed by a Multiplex assay following the manufacturer instructions, using a Luminex (MAGPIX) equipment.

5.3.8 Statistical analysis

Statistical analysis was performed as described in Chapter 2.12.

5.4 Results

5.4.1 Magnetic box computational modelling

An initial prediction of the magnetic field generated within the box was performed in 2D via FEMM Software (Finite Element Method Magnetics Software) (Chapter 2.11.6.1). It was calculated that the magnetic field at 1 cm from the external magnetic wall was varying between ~ 0.15 T and ~ 0.45 T. At 5 cm from the wall, in the centre of the region between side and central wall (“corridor”), the field was varying between ~ 0 T and ~ 0.15 T (Figure 5.1).

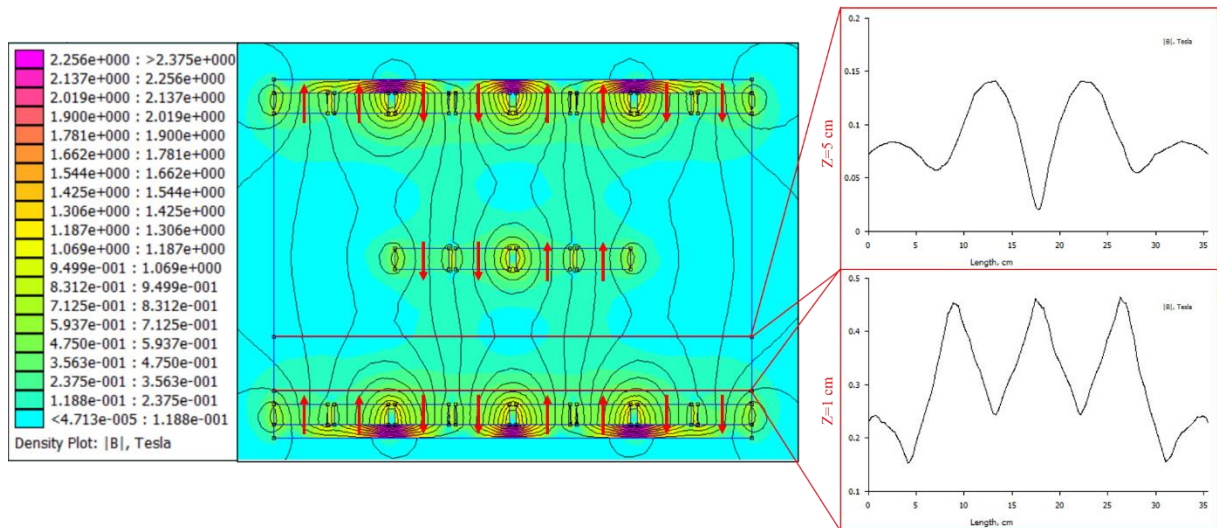


Figure 5.1 2D simulation of Magnetic Flux was performed via FEMM Software. Top view of the magnetic box with predicted magnetic field mapping (left) and graphs of the magnetic flux variation predicted at $Z=5$ cm and $Z=1$ cm (right).

2D FEMM modelling was used to initially plan the arrangement of the magnets, predicting the consequent magnetic field. However, due to the coarseness of the mesh, several field details were missing. For this reason, COMSOL 5.1 Software was used to generate a more accurate 3D computational model of the magnetic field within the box (Chapter 2.11.6.2). This software was also useful in finding the most suitable arrangement of the magnets on the X-Y plane on external and inner walls, which was not possible with FEMM Software as it only allowed for

the calculation of more basic profiles. The magnetic field predictions were calculated at 1 cm and 5 cm distance from the magnetic walls, as representative of the peak and lowest magnetic field areas, respectively. In the final 3D arrangement, evaluated as the best in providing the highest magnetic field strength and variable gradient, the magnets were arranged in groups of 2x2 arrays with the same orientation, with the inner wall magnets having an opposite direction of side wall magnets (Figure 5.2 a). The magnetic field at 1 cm distance from the external wall was predicted to have a peak strength of ~ 0.2 T at ~ 12 cm from the short side of the box (X plane), in correspondence to the beginning of the “corridor” between external and inner wall (Figure 5.2). As described in Chapter 2.11.6.3, the magnetic field along the box was also empirically measured with a Gaussmeter which detected field values comparable to the ones simulated with COMSOL Software (Figure 5.2).

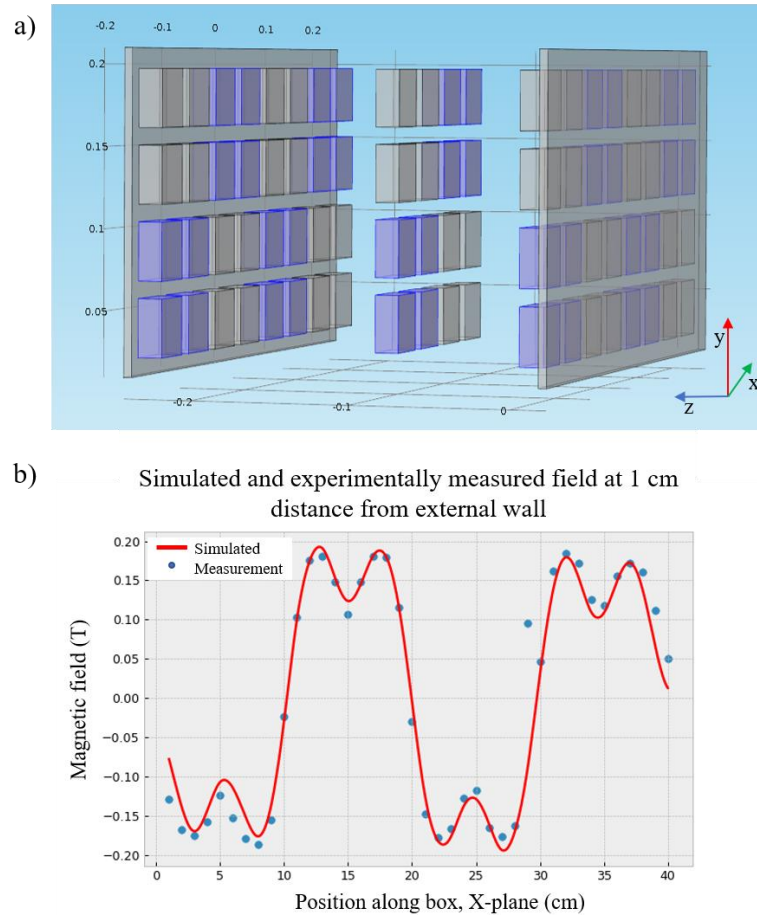


Figure 5.2 3D computational model of the magnetic field within the magnetic box. a) Blue-highlighted magnets are “North” poles with $B = 1.28 \text{ T}$ in $+Z$ direction, while grey-highlighted magnets are “South” poles with $B = 1.28 \text{ T}$ in $-Z$ direction, b) simulated (red line) and measured (blue dots) magnetic field at 1 cm distance from the external wall at 5 cm height. The computational modelling was performed with COMSOL 5.1 Software.

The forces acting on a magnetic nanoparticle followed a similar trend. The greatest forces were applied closest to the external magnetic wall (Figure 5.3, blue) with peaks at approximately 1.5 pN, which was expected due to the stronger applied fields (Figure 5.2). The magnetic force on MNP decayed to $\sim 0.15 \text{ pN}$ at 5 cm from the wall (Figure 5.3, red).

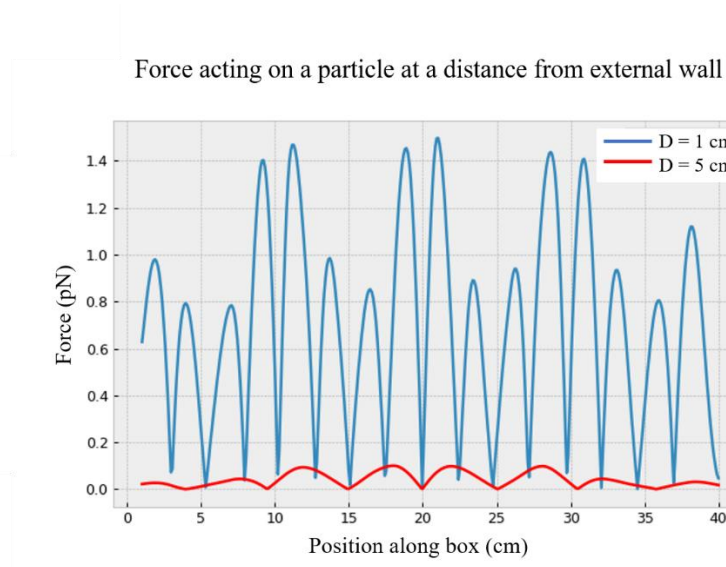


Figure 5.3 3D computational model of magnetic forces acting on a $\varnothing 250$ nm MNP moving along a linear path in the X- plane at a fixed height of $y=5$ cm. The forces were calculated at 1 cm (blue) and 5 cm (red) from the external magnetic wall. The computational modelling was performed with COMSOL 5.1 Software.

5.4.2 Implant localisation

To investigate the presence of human content in tendon sections, attributable to hASCs implanted in tendon defect, FISH analysis was carried out at 4 weeks and 8 weeks after surgery, exploring the presence of human UCB, PPIB and PolR2a RNA. UCB was subsequently excluded from the image analysis as the anti-UCB RNA probe cross-reacted with rat tendon tissue, leading to unspecific binding (data not shown). A human tendon section was used as positive control, while a rat contralateral tendon section and a Sham group sample were employed as negative control. As shown in Figure 5.4, human tendon showed the presence of green and yellow fluorescent signal, attributable respectively to human PolR2a and PPIB, while no signal was detected in the rat contralateral tendon or in the Sham sample. On the contrary, expression of PolR2a and PPIB were detected both at 4 weeks and 8 weeks, suggesting the presence of hASCs even at later timepoints. In particular, in 4 weeks samples PolR2a and PPIB

expression were appreciable especially in groups without magnetic stimulation [ii) and iii)], while a weaker PolR2a signal can be observed in Cells+MNPs+mag group [iv)]. Interestingly, after 8 weeks from surgery, human cells could still be detected in tendon samples and in particular, Cells+MNPs+mag [vii)] showed a strong presence of PolR2a in comparison to groups without magnetic stimulation [v) and vi)] which showed a weaker presence of the human genes (Figure 5.4).

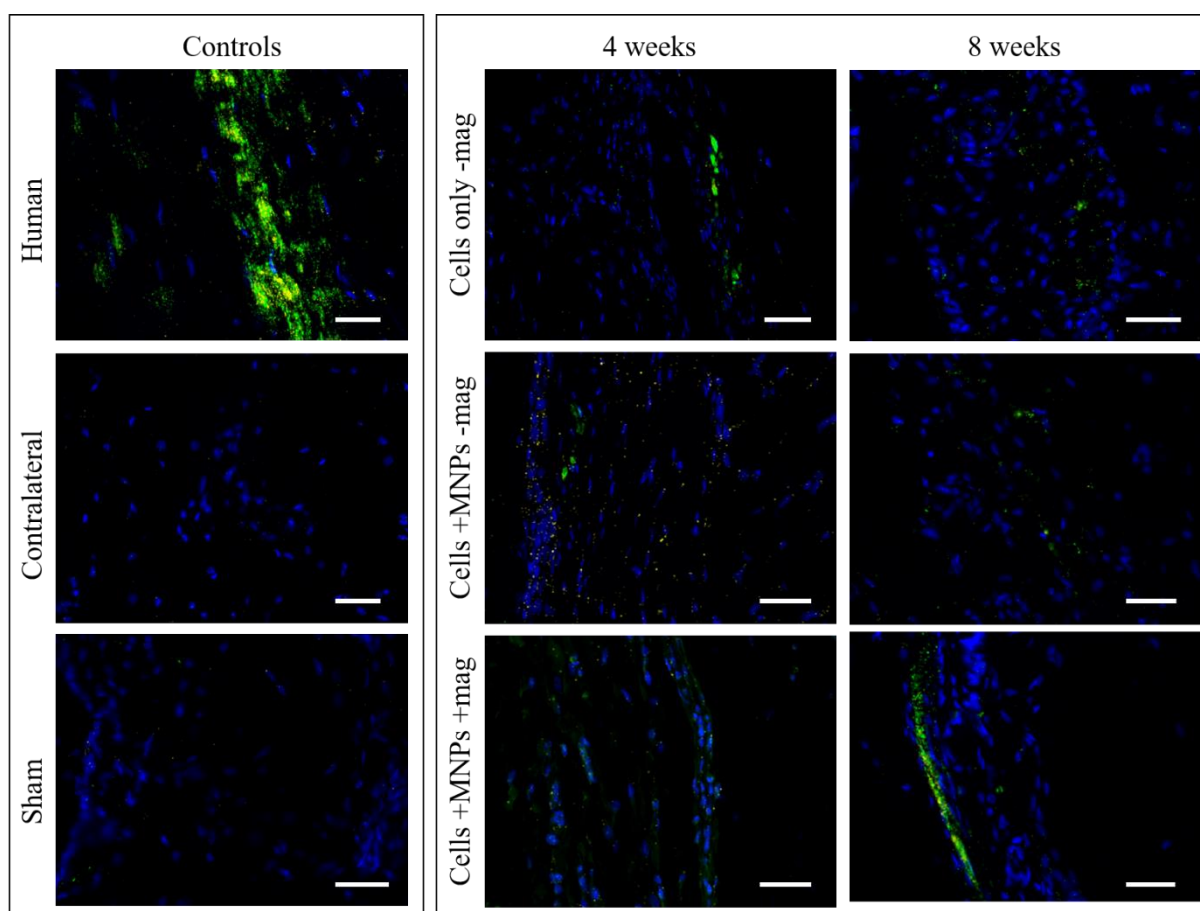


Figure 5.4 FISH analysis on tendon sections. Left: Human tendon section was used as positive control (top), while rat contralateral and Sham group tendons were employed as negative control (middle and bottom). Right: 4 weeks (middle) and 8 weeks tendon sections of, from the top, Cells only-mag, Cells+MNPs-mag and Cells+MNPs+mag. Green: PolR2a RNA, yellow: PPIB RNA, blue: nuclei. Scale bar= 40 μ m. Slides were imaged using Vectra Polaris, Automated Quantitative Pathology Imaging System.

5.4.3 Tendon repair assessment

5.4.3.1 Early repair assessment

H&E staining was performed on tendons sections to investigate ECM organisation and remodelling after tendon injury. As expected, remodelling areas can be found in the central region of the sections and they can be associated to the locations where the window defect was created. These areas are characterised by an increased number of nuclei and a remodelled collagen matrix (Figure 5.5). ECM arrangement appears more organised in Cells+MNPs+mag group compared to the other groups.

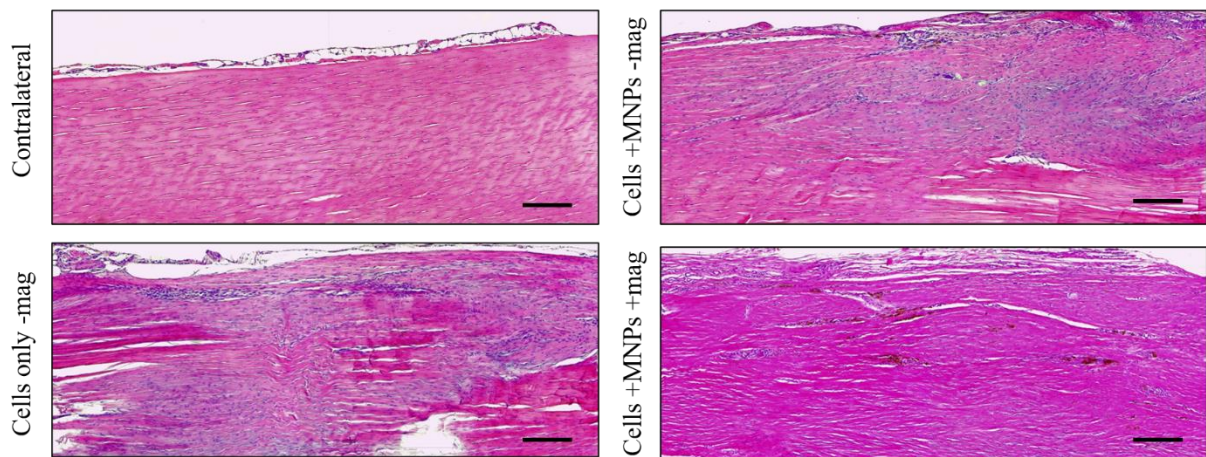


Figure 5.5 H&E staining of tendons sections, 4 weeks timepoint. Left, contralateral (top) and Cells only-mag group (bottom). Right, Cells+MNPs-mag (top) and Cells+MNPs+mag (bottom). Scale bar= 200 μ m. Slides were imaged with Cytation5 microscope (BioTek).

Tendons sections were further stained with Picrosirius Red and imaged under polarised light, in order to analyse the collagen matrix arrangement. As shown in Figure 5.6, stained collagen fibres appear in different birefringence colours under a polarized light, indicating the orientation of collagen fibres³⁰⁷. Different colours are also associated with collagen fibres thickness, where thick fibrous fibres appear as yellow/orange, while thinner fibres colours in green.^{330,331} The

first ones are commonly associated to collagen type I, while the second are linked to collagen type III. The yellow/orange (Col I) and green (Col III) channels were analysed separately with Directionality plug-in for ImageJ to determine fibres alignment.^{237,332} The analysis of the ratio Col I/Col III fibres direction distribution showed similar results between experimental groups. (Figure 5.6). Likewise, the ratio of the refractive intensity signal, quantified separately for Col I and Col III, displayed similar results.

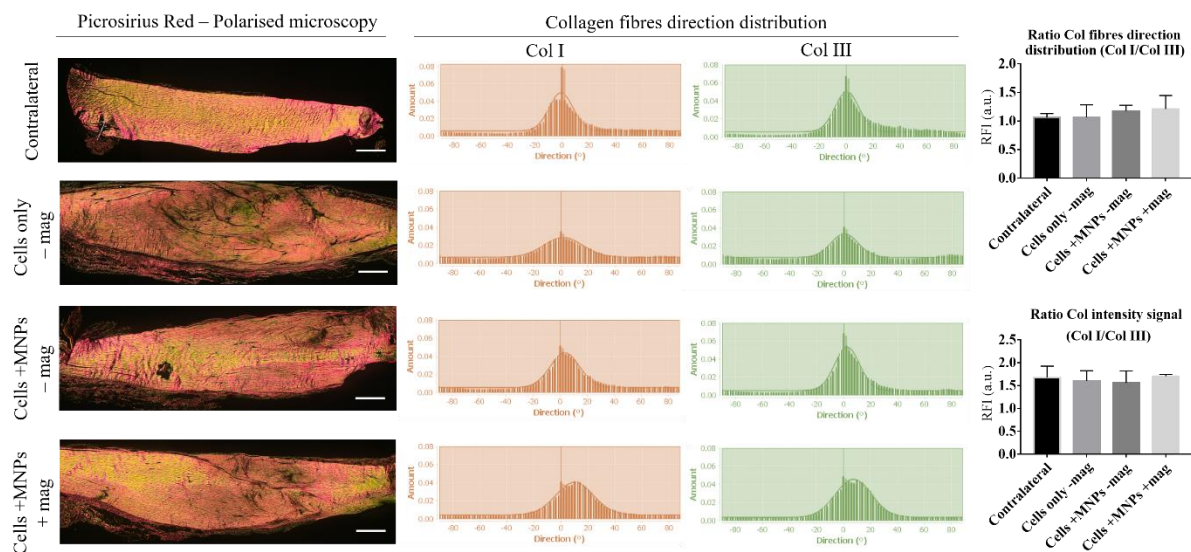


Figure 5.6 Picrosirius Red staining of tendon sections. Images were analysed with Directionality plug-in of ImageJ Software (middle), evaluating the red (Col I) and green (Col III) channels separately. Directionality distribution histograms were generated from each analysis. The fibres direction distribution and refractive signal intensity were quantified as ratio Col I/Col III. Directionality plug-in in Fourier spectrum analysis mode, trendline fitted to Gaussian function. Scale bar = 500 μ m. $n=3$. Sections were imaged at a Fluorescence Inverted Microscope under polarized light (Axio Observer 3851000135, Zeiss).

Moreover, to evaluate the tenogenic markers expression, TNMD presence was investigated via immunohistochemistry and quantified with ImageJ Software. As shown in Figure 5.7a, TNMD expression was detected in all experimental groups with a more aligned and organised pattern

in the Contralateral tendon. Levels of TNMD expression observed were not significantly different between experimental groups and controls (Figure 5.7b).

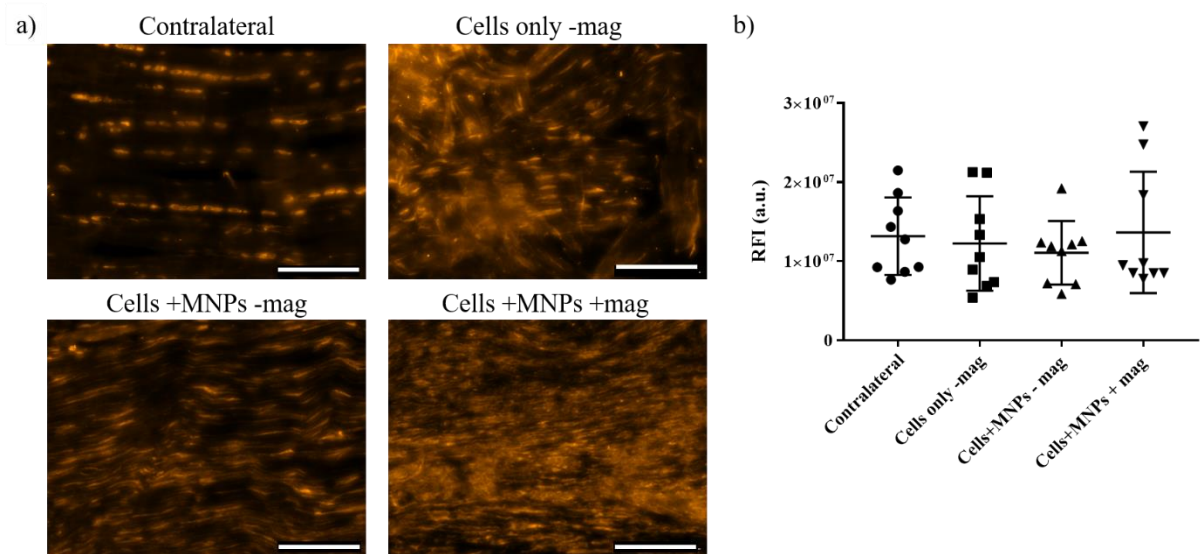


Figure 5.7 Tenomodulin immunohistochemistry analysis of tendon sections. a) Top, contralateral (left) and Cells only-mag (right); bottom, Cells+MNPs-mag (left) and Cells+MNPs+mag (right). Orange=TNMD. Scale bar= 100 μ m. $n=3$. Slides were imaged at Cytation5 microscope at x20 magnification (BioTek). b) Quantification of immunocytochemistry fluorescence for Tenomodulin. The quantification was performed with ImageJ software, analysing three pictures for each tendon for each animal in all the conditions. $n=9$.

5.4.3.2 Long term repair assessment

As performed on samples from 4 weeks timepoint, H&E staining was carried out on tendons sections from 8 weeks timepoint in order to evaluate the ECM and cell rearrangement following tendon defects creation also at later timepoints. Even though the matrix arrangement was similar in the level of organisation across experimental groups at 8 weeks (Figure 5.8), less cell nuclei and a more organised remodelling pattern can be found compared to tendon sections at earlier timepoints (Figure 5.5). Furthermore, collagen crimps can be identified in Cells+MNPs+mag

group which are typical of the native tendon tissue as they confer the elastic properties and mechanical stress resistance to the tissue.³¹

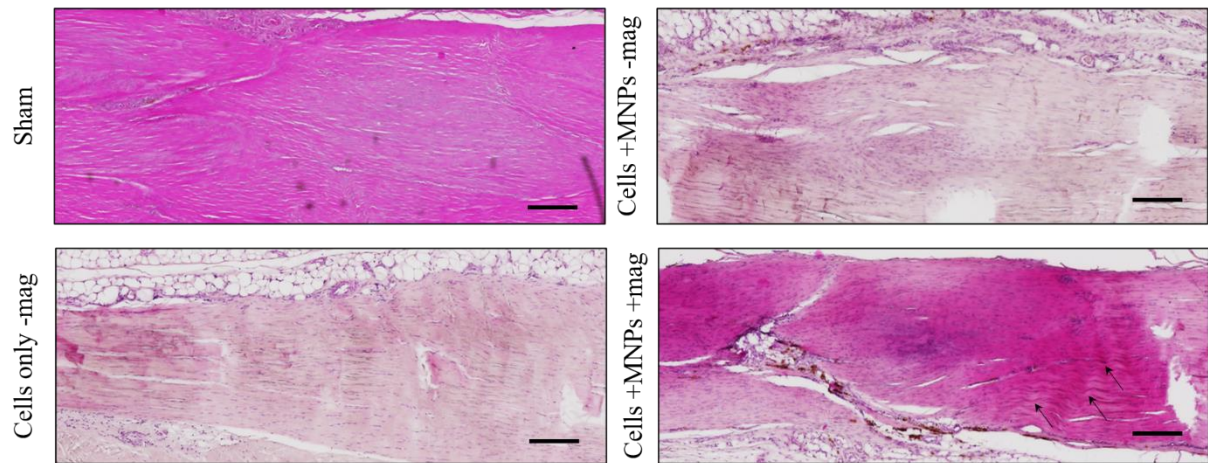


Figure 5.8 H&E staining of tendons sections, 8 weeks timepoint. Left, Sham (top) and Cells only-mag group (bottom). Right, Cells+MNPs-mag (top) and Cells+MNPs+mag (bottom). Arrows illustrate collagen crimps. Scale bar= 200 μ m. Slides were imaged with Cytation5 microscope (BioTek).

5.4.3.3 Calcific depositions

H&E staining revealed the presence of calcific depositions across all experimental groups at 8 weeks timepoint, while no such formations were found at 4 weeks. Figure 5.9 a, d shows how calcium deposits were found in all groups at 8 weeks including the Sham group. This finding was confirmed by μ CT analysis, showing calcific formations in the lower extremity of the tendons (Figure 5.9 b, c, e, f). To explore the cause of these formations, the presence of inflammatory infiltrate in the injured tendons was investigated. In particular, the presence of leukocyte markers (CD45 and CD163) was studied via immunohistochemistry. A positive inflammation signal for both markers was identified in all injured tendons and, interestingly, the inflammatory markers distribution was compatible with the calcium depositions (Figure 5.10).

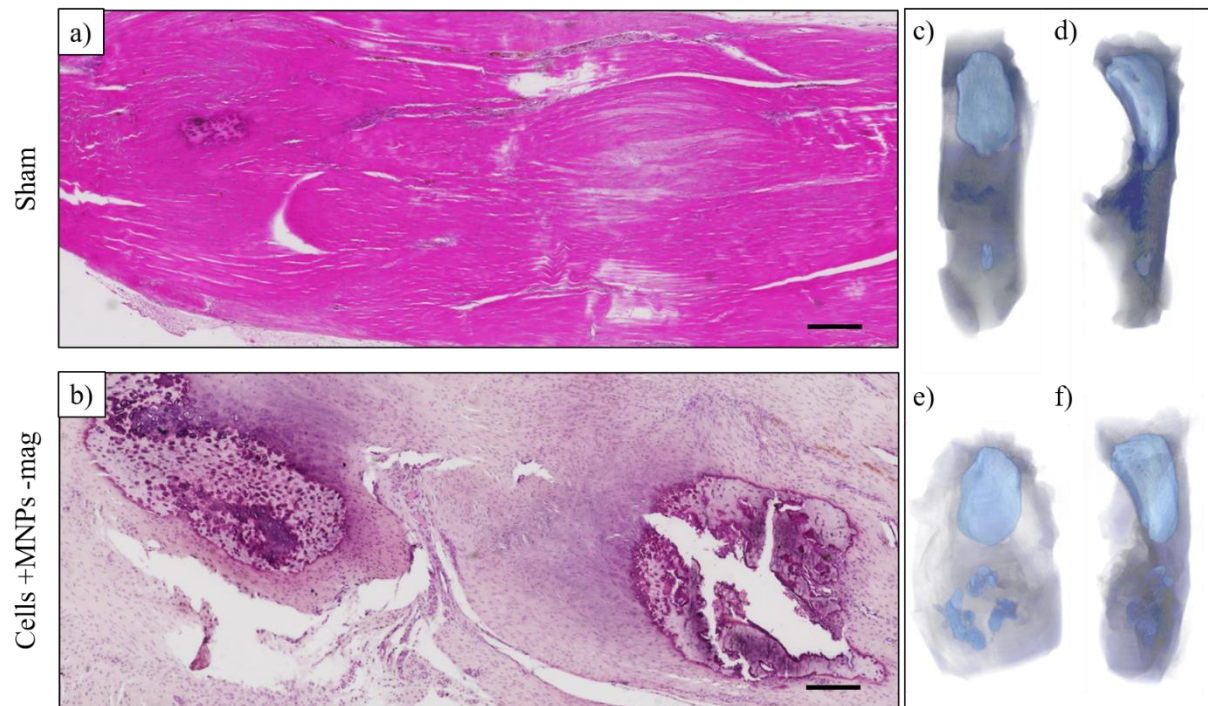


Figure 5.9 Calcific depositions in 8 weeks tendon sections. Left, H&E staining of tendons sections of a) Sham group and b) Cells+MNPs-mag group. Right, μ CT reconstruction of formalin-fixed tendon samples: c) frontal and d) lateral view of Sham group tendons, e) frontal and f) lateral view of Cells+MNPs-mag group tendons. Scale bar= 200 μ m. H&E-stained slides were imaged with Cytation5 microscope (BioTek) while X-Ray Microtomography System Skyscan 1072/1272 scanner (Skyscan, Kontich, Belgium) was used for μ CT images acquisition.

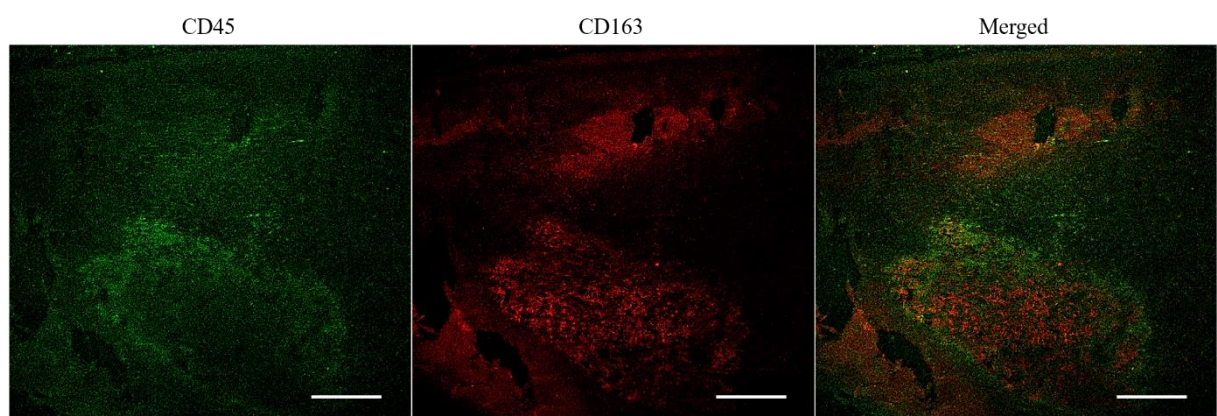


Figure 5.10 Immunohistochemistry for CD45 and CD163 inflammatory markers on Cells only-mag tendon section. Green: CD45; red: CD163. Scale bar=200 μ m. Images were acquired at an Inverted Confocal microscope (TCS SP8, Leica).

5.4.4 Iron accumulation and immune-response analysis

To assess MNPs safety and to investigate any potential iron accumulation as result of the degradation of implanted MNPs, iron presence was examined in some of the main detoxification organs. Liver, lungs and spleen sections were stained with Perls van Gieson staining (Figure 5.11) which detected iron only in spleen samples, while no iron deposits were found in any other organs. This was an expected result as spleen is a natural deposit of iron³³³ and similar iron deposits were found across all the groups, including Sham and Cells only-mag.

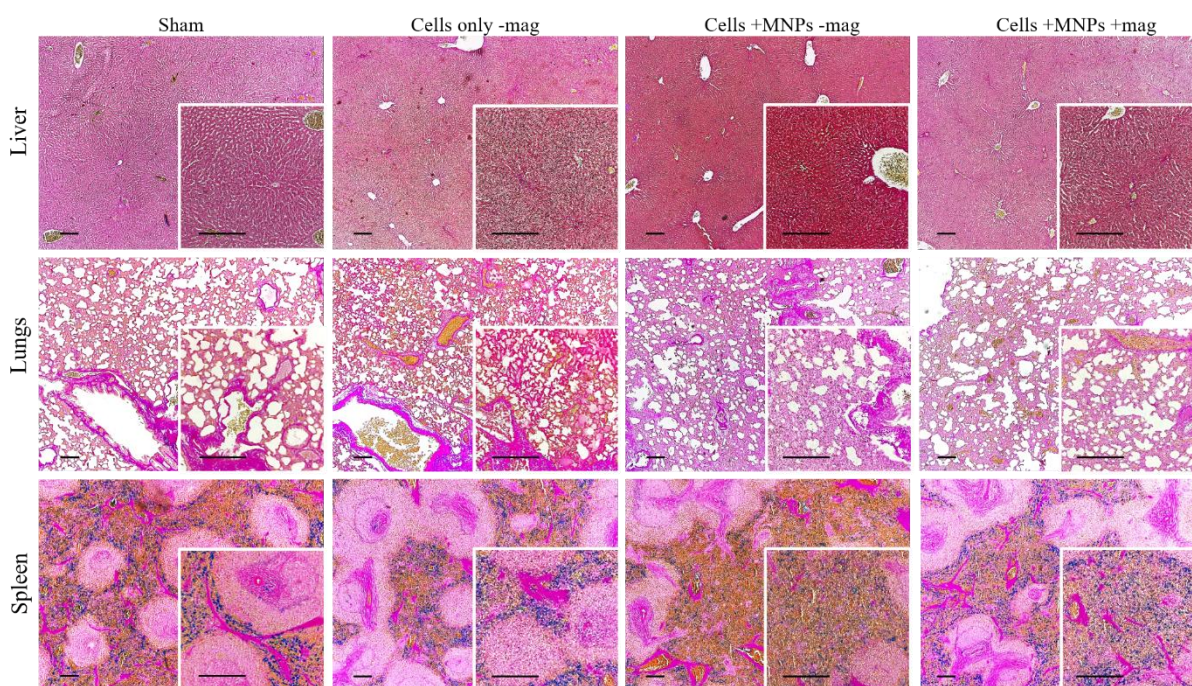


Figure 5.11 Perls van Gieson histological staining on liver, lungs and spleen samples. Blue: reactive ferric iron; purple/red: collagen; yellow: cytoplasm, muscle, corny layer of the skin, neuroglia and erythrocytes. Scale bar= 200 μ m. Sections were imaged at x4 and x10 with optic microscope (DM750, Leica).

For all the three rats from each experimental group the immune response was quantified after 4 weeks by measuring plasma levels of 14 cytokines (G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, TNF- α) via Multiplex assay. GM-CSF

was not detected in any of the plasma samples and therefore it was excluded from the analysis. According to literature,³³⁴⁻³³⁷ G-CSF, IFN- γ , IL-1 α , IL-1 β , TNF- α , IL-17A and IL-6 can be classified as pro-inflammatory cytokines, while IL-10 and IL-12p70 are known to have anti-inflammatory properties. IL-2, IL-4, IL-6 and IL-13 have regulatory properties towards the production and proliferation of other immunity components such as Lymphocytes T and B, or IgA, IgM and IgG production. The ratios Cells+MNPs-mag/Cells only-mag and Cells+MNPs+mag/Cells only-mag showed similar values for the tested cytokines, with a few exceptions. In fact, even though non-significant, IL-1 α , IL-6, IL-10 and IL13 ratios displayed higher values for Cells+MNPs+mag compared to the -mag group (Figure 5.12).

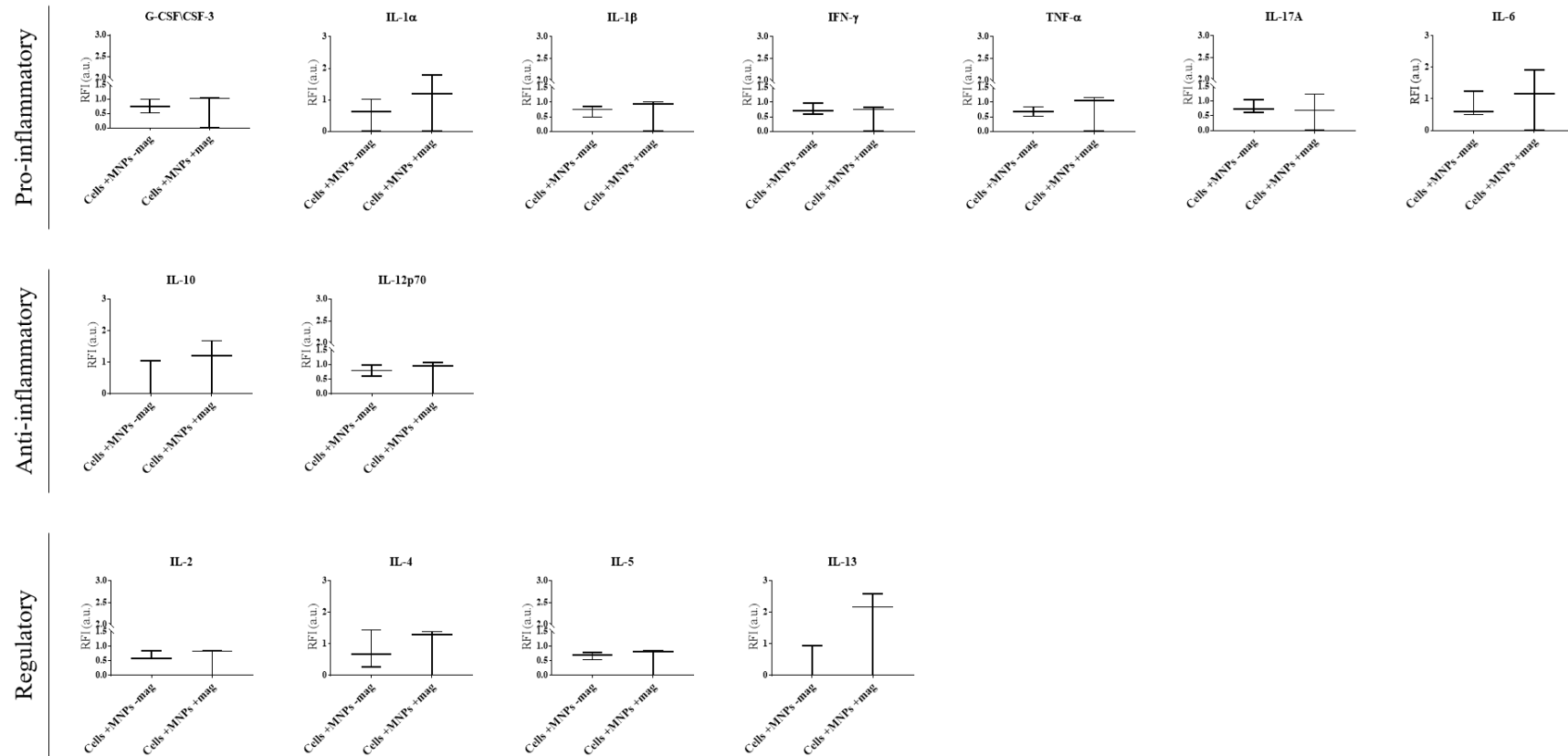


Figure 5.12 Cytokines panel analysis in rat plasma samples. Top, pro-inflammatory cytokines (G-CSF, IFN- γ , IL-1 α , IL-1 β , TNF- α , IL-17A and IL-6); middle, anti-inflammatory cytokines (IL-10 and IL-12p70); bottom, regulatory cytokines (IL-2, IL-4, IL-6 and IL-13). $n=3$. Cytokines levels were analysed via Multiplex Assay (ProcartaPlex, Thermo Fisher) and presented as ratios Cells+MNPs-mag/Cells only-mag and Cells+MNPs+mag/Cells only-mag. The fluorescence was measured using Luminex instrument (Luminex, MAGPIX, MAGPX20114721, Luminex BV).

5.5 Discussion

In this Chapter, the tenogenic regenerative potential of remotely-activated MNPs-hASCs was investigated *in vivo* in a rat patellar tendon defect. The remote bio-magnetic activation technology provided by MICA *in vitro* was translated *in vivo*, investigating an innovative way to remotely stimulate MNPs-labelled hASCs towards the tenogenic commitment.

A customised box was designed according to rat housing guidelines (Chapter 2.11.6) and assembled with permanent magnets carefully arranged in order to create a variable magnetic field throughout the box area. The generated magnetic field would be sensed as the rats walked in the box by the anti-Activin RIIA-MNPs tagged to the hASCs implanted in the rat patellar tendon, stimulating the activation of the mechano-receptor Activin RIIA (+mag group). A 3D computational model was created with COMSOL Software to predict the magnetic field within the box at 5 cm from the floor. This was an indicative value chosen by measuring the rat knees average distance from the floor and considering also a potential two-feet standing position. Taking into account the areas where most likely the rats would move, the magnetic field was calculated at 1 cm from the magnetic walls as representative of the peak magnetic field area. According to the predictions, the magnetic field at 1 cm from the wall had a peak strength of ~ 0.20 T at 5 cm height. This field profile showed lower values compared to 2D FEMM simulation. This was expected, since the 3D simulation provides a more complex profile than the one found in 2D, as it considers also the interactions of fields generated by neighbouring magnets. In fact, the field values predicted in the 3D simulation were confirmed by the empirical measurements of the field. The predicted magnetic field resulted to be higher than the one employed in clinic for Pulsed Electromagnetic Field (PEMF) therapy (≤ 20 mT) for elbows and rotator cuff tendinitis treatment.^{276,277,338,339} By using a higher magnetic field, we aimed to increase the magnetic forces that would act on the MNPs implanted in rats tendons,

enhancing the cellular mechano-transduction. The forces acting on a magnetic nanoparticle moving along a linear path in the X- plane were calculated with COMSOL Software at a fixed height of $y=5$ cm at 1 cm and 5 cm from the magnetic walls. The resulting forces profiles followed the same trend as the magnetic field gradient in particular, the magnetic force peaked at approximately 1.5 pN at 1 cm from the wall, where the magnetic field was stronger, and decayed at ~ 0.15 pN at 5 cm as the magnetic field decreased. Two additional boxes were prepared without magnets and were used to stimulate “-mag groups”, so that the rats were subjected to the same exercise regimen as the “+mag group” but without being exposed to a magnetic field. In fact, mechano-magnetic stimulation of stem cells-seeded constructs has shown to have a big positive impact in promoting tendons healing,⁸² as also testified by the physiotherapy regimens that are in most cases included in the clinical rehabilitation programs after a tendon injury.¹¹³ As negative control, the Sham group was left unstimulated in the cage, recreating the natural tendon healing environment in reduced-motility conditions. Sham group was sacrificed after 8 weeks from surgery in order to evaluate the self-healing and matrix natural reorganisation on the long period, as it is known that the remodelling stage of natural tendons healing begins after 1-2 months from the day of the injury.³⁵

The first step was to ensure the presence of hASCs in injured tendons, which were administered via injectable fibrin gels¹⁸² to investigate the tenogenic regenerative potential of remotely-activated Activin RIIA. The positive expression of human PolR2a and PPIB detected by the FISH analysis at 4 weeks and 8 weeks timepoints, testified the presence of the human cells several weeks after surgery. According to the FISH analysis, the strongest signal was detected in Cells+MNPs+mag group after 8 weeks from surgery suggesting that the MNPs labelling in conjunction with a variable magnetic field might help in the local retainment of the implanted hASCs at later timepoints.

Tendon repair and ECM remodelling were investigated after 4 weeks from surgery, the H&E analysis showed how the ECM rearrangement during tendon healing appeared more organised in all the rats from Cells+MNPs+mag group compared to the Sham group or Cells+MNPs-mag. This finding corroborates the importance of the magnetic stimulation in MNPs-labelled hASCs in improving ECM organisation and forming a more aligned pattern similar to natural tendon tissue.¹⁸

Interestingly, the ratio Col I/Col III fibres direction distribution provided similar results between experimental groups with values closer to 1 for Contralateral tendons (=1, identical Col I and Col III fibres orientation dispersion) and more dispersed values for the other groups (>1 Col I orientation more dispersed than Col III; vice-versa for values <1). As expected, this further supports a more reorganised matrix in injured tendons compared to intact tendons. In particular, according to tendons healing timeframe,³⁴ the deposition of Col I starts after about one month from injury, replacing the disorganised Col III initially deposited in the wound site to provide tissue stability. In line with this result, the ratio of the signal intensity of Col I/Col III was similar across all the groups, with values >1 suggesting higher levels of Col I compared to Col III. This ratio is legit in the Contralateral tendon sections, as Col I represents about the 97%¹⁸ of the ECM in native tendon tissue, and it is also consistent with the remodelling phase in injured tendons.³⁴ Moreover, an excessive deposition of Col III during tendon healing is associated with a fibrotic scar formation, which often leads to chronic tendinopathies.^{1,6} Remarkably, an increase of Col III has not been registered in any of the experimental groups after 4 weeks, suggesting a positive effect of hASCs for tendon healing improvement.¹⁸²

As part of the ECM tenogenic proteins, we investigated the TNMD expression after 4 weeks across the experimental groups. TNMD is an extracellular protein that is associated with mature tendon tissue²⁹⁶ and regulates the expression of other tenogenic proteins as well as collagen deposition.³⁴ TNMD was observed in all conditions and, even though no significant

difference was registered between groups, Cells only-mag showed higher TNMD values compared to the other groups, following fluorescence quantification with ImageJ. This suggests that MNPs has less impact in TNMD expression compared to hASCs. Interestingly, crimps-like structures can be appreciated in the ECM of tendons from +MNPs conditions. These crimps can be found in the ECM of native tendons and are essentials to provide the elasticity and mechanical stress resistance to the tendon.³¹ Similar crimps-like structures were also identified by H&E staining at 8 weeks timepoint in Cells+MNPs+mag condition, suggesting a positive impact of MNPs-labelled hASCs in ECM rearrangement during tendons healing.

At later timepoints, the H&E staining showed a generally more organised ECM compared to 4 weeks, as expected from the remodelling phase of tendons healing which begins 1-2 months after the initial injury and can last over a year.³⁴ Calcium deposits were detected by H&E staining in some 8 weeks tendon sections, across all the experimental groups including Sham animals. The affected tendons also showed a positive expression of CD45 and CD163 indicating a leukocytes infiltration which was not detected at earlier timepoints. In particular, immune cell location within the whole tendon corresponded to the calcific depositions that were identified by the H&E staining. In literature, the inflammatory infiltrate with macrophages has been described as part of the calcific deposition reabsorption process.^{44,52,53} The presence of calcifications even in the Sham group makes possible to exclude the MNPs/cells implant as major cause of the calcific formations, also considering the absence of calcifications in the 4 weeks groups. It is also unlikely that the calcific formations are a consequence of a collateral osteochondral differentiation of the implanted hASCs, as the FISH analysis did not co-localise any human content in the position where the calcific formations were detected, either at 4 weeks or 8 weeks. Calcifications are a common complication of

tendon healing even in humans, but further studies are needed to understand the underlying causes and find an adequate treatment.⁴⁶

Subsequently, MNPs safety was confirmed by investigating any potential iron accumulation in some of the main detoxification organs. Perls van Gieson staining of some of the main detoxification organs (liver, lungs and spleen), did not detect any iron accumulation except for spleen. As expected, being a natural iron deposit,³³³ spleen sections showed iron presence across all the groups with no difference between +/-MNP conditions.

As last step, plasma levels of cytokines were investigated by Multiplex assay after 4 weeks from surgery. The analysis did not show any significant difference between groups in any of the cytokines tested, even though IL-1 α , IL-6, IL-10 and IL-13 showed higher levels in +mag group. IL-1 α and IL-6 are pro-inflammatory cytokines but, while IL-1 α is mainly produced by monocyte lineage cells,³⁴⁰ IL-6 is produced by a wide range of cells throughout the body including T cells, B cells and fibroblasts.³³⁵ These two cytokines often cooperate in the production of acute phase proteins by the liver.³³⁵ On the contrary, IL-10 has strong anti-inflammatory properties, inhibiting the production of inflammatory cytokines such as IL-1 and IL-6 and TNF- α ³⁴¹ and repressing the cytokines production by natural killer cells and neutrophils.³⁴² Similarly, IL-13 is a regulatory cytokine which can inhibit the expression of TNF- α , IL-1 and IL-8.³⁴² It is important to specify that even though being nude rats, a basal inflammatory response is still physiological³⁴³ and it could be caused by several factors, including minor re-injuries like stitches dislodgment.³⁴⁴ Furthermore, the tendon's natural healing process implies a small inflammatory response around the wound site, which would not be surprising after 4 weeks from surgery³⁵ and an active exercise regimen. However, the differences were not significant between conditions.

This study has some limitations needing to be discussed. The first limitation was the setting of length and intensity of rat stimulation and exercise regimen. Since a widely-valid physiotherapy regimen is missing in clinical applications,¹¹³ choosing the right intensity and length of the stimulation was challenging. Being a relatively new and unexplored field, as starting point we translated *in vivo* the stimulation regimen used for *in vitro* studies, considering the achieved promising results. The timeframes were also compatible with some of the magneto-therapy regimens used in clinic (PEMF), which suggest three stimulations/week³³⁸ with 30 min-3 hours sessions.³⁴⁵ The results obtained might be useful to other studies investigating *in vivo* magnetic stimulations for tendon regeneration, as well as for follow up work carried out under the scope of this Thesis. For instance, it could be interesting to investigate different stimulation regimens in order to find the most appropriate to stimulate tendons healing. Mechanical testing of the repaired tendon would also give important insights on the efficacy of magnetic stimulation in the recovery of the original elasticity and resistance of the tissue. Lastly, some samples analysis is still ongoing and could provide additional relevant information about the study outcomes. For example, plasma cytokines levels should be analysed for the 8 weeks timepoint in order to screen any potential cytokines that could be associated with tendon healing resolution. Similarly, Col I, Col III, TNMD and SCXA expression need to be investigated at the later timepoint, so to explore tendons healing and matrix reorganisation on the long period.

5.6 Conclusion and future perspectives

This Chapter focused on the *in vivo* remote mechano-magnetic activation of MNPs-labelled hASCs for tendon tissue engineering applications. For this purpose, a box with fitted magnets was designed and assembled, in order to recreate a variable magnetic field as used for preliminary *in vitro* cells studies. Cells were successfully delivered by injectable fibrin

hydrogel and persist in the defect after 8 weeks from surgery. The developed magnetic fibrin construct could be considered a valid method for *in situ* cell delivery, as testified by the presence of implanted human cells after several weeks from surgery. The implanted magnetic construct proved to be safe as no increase in circulating inflammatory cytokines was registered, as well as no collateral iron deposition. Furthermore, the combination of MNPs and external magnetic field stimulation might improve the ECM organisation during tendon healing. Even though some analyses are still ongoing and they need completion before drawing any decisive conclusions, this study can contribute for new insights in magnetic associated strategies for tendon tissue engineering.

In conclusion, the main results reported in this Chapter can be summarised as follows:

- Remotely-activated MNPs-labelled hASCs might be beneficial in the deposition of a more organised collagen matrix during tendon healing,
- The magnetic implant has proven to be biocompatible under the tested conditions. In fact, implanted hASCs persisted in the defect for 8 weeks and no signs of collateral iron accumulation or inflammation could be detected after 4 or 8 weeks.

CHAPTER VI

SUMMATIVE DISCUSSION

Despite the significant frequency¹ of tendon injuries and tendinopathies, a globally-accepted therapy is still missing, creating a compelling clinical need for new treatment strategies.^{7,8} In the last years, regenerative medicine and tissue engineering have provided promising alternative approaches for tendon healing,¹⁴⁻¹⁶ offering a possible solution to this unmet clinical need. In this Thesis, it was investigated the remote mechanical activation of Activin RIIA towards tenogenic differentiation as a potential cell-based therapy for tendon injuries treatment. Differentiation, proliferation and ECM deposition of remotely activated stem cells were explored both *in vitro* and *in vivo*, investigating the efficacy of this novel integrated approach in promoting tendon regeneration.

In Chapter III of this Thesis, stem cells isolated from bone marrow and adipose tissue were investigated as potential cellular therapy for tendon regenerative approaches. In particular, following previous studies,²⁸⁵ we questioned the possibility to remotely mechanically stimulate the mechano-receptor Activin RIIA on different cell types by means of anti-Activin RIIA antibody-labelled MNPs and a magnetic bioreactor from MICA Technology.^{221,281,282} Three different antibody anti-Activin RIIA were employed, targeting different region of the receptor, with the aim to identify the mechano-responsive domain. The remote activation of the Smad2/3 tenogenic pathway was verified in tenocytes, hBMSCs and hASCs but, while in tenocytes the highest levels of pSmad2/3 were registered after 2 min magnetic stimulation, the highest levels of the complex were found after 10 min stimulation in hBMSCs suggesting a later activation of the tenogenic pathway in undifferentiated stem cells. One of the tested antibodies (Antibody 2) showed to better activate the Smad2/3 pathway both in tenocytes and in hBMSCs, possibly targeting the mechano-responsive region of the receptor. Proteins associated with the tenogenic phenotype^{27,106,296,346} such as TNMD, SCXA and Col I, revealed higher expression patterns in MICA-stimulated MNPs-labelled hBMSCs when compared to static control. At the same time, lower levels of Col III were registered for MICA

stimulated groups compared to static group, which might suggest a positive influence of mechanic stimulation in the reduction of fibrotic tissue formation.¹

Subsequently, different concentrations of MNPs were tested on hASCs, in order to investigate the dose-response relationship for the activation of the Activin RIIA. The optimal amount of MNPs for the activation of the Smad2/3 pathway in hASCs was found to be 25 µg MNPs/2x10⁵ seeded cells while the other MNPs concentrations tested were either too low or over-saturating the receptor. A 21-days differentiation experiment with MICA stimulation was performed on hASCs, investigating the expression levels of TNMD, SCXA, Col I and Col III. TNMD and Col I levels were higher in MNPs-labelled groups compared to unlabelled cells, even though no significant difference was registered between MICA and static groups. Similarly to hBMSCs, Col III levels were slightly lower in MICA-stimulated MNPs-labelled hASCs, while no increase of SCXA levels was achieved in MICA-stimulated groups compared to the static control. Interestingly, preliminary WB results on TNMD expression after a 7-days MICA differentiation assay showed a more marked difference between MICA-stimulated MNPs-labelled hASCs and the static groups, suggesting that earlier timepoints might be more crucial for tenogenic proteins expression rather than later timepoints. Nevertheless, this result needs to be further verified and the WB protocol optimised. It also needs to be considered that TNMD and SCXA are not univocally expressed by tenocytes⁵⁵⁻⁵⁸ and, so far, a precise tenogenic marker still needs to be identified. This was a big limitation in the choice of the most suitable stem cell source for tendon regeneration purposes, as bone marrow and adipose stem cells, showed similar results in terms of tenogenic markers expression and mechanical activation of the Activin RIIA. Nonetheless, considering the higher tissue-isolation efficacy, the higher proliferation rate and multipotency^{143,146} and previous studies published in literature,^{235,285,347} hASCs were selected for further 3D and *in vivo* models.

Once selected the most appropriate cell source, an effective cell delivery method was investigated. The development of biological construct is an important aspect of tissue engineering, as it represents a crucial step in the repair or replacement of a failing tissue.¹³ Tendon tissue has some mechanical characteristic,¹⁷ such as elasticity and stress-resistance, that are essential for the whole tissue functionality and they must be taken into consideration when developing a tissue engineering scaffold.¹⁵³ The scaffold material needs to be biocompatible, biodegradable and able to bear the mechanical stress tendons are subjected to.^{153,154} Among the biological materials, collagen and fibrin are two of the most investigated for tendon tissue engineering purposes.

Collagen I is the main component of tendons¹⁸ and its hierarchical organisation provides the resistance and elasticity in native tendon tissue.¹⁷ Therefore, several collagen-alignment strategies^{165–168} have been developed with the aim to recreate *in vitro* tendon highly organised collagen arrangement. In Chapter IV of this Thesis, a tendon-like³⁴⁸ oriented collagen 3D scaffold was developed by embedding Ø250 nm anti-Activin RIIA MNPs in a collagen I solution which was then solidified between two magnets in a constant magnetic field.²²⁶ 5×10^4 cells/mL and 3 mg/mL were found to be, respectively, the optimal cells seeding density and the ideal collagen concentration to minimise the gel contraction over the time^{311,312} and to guarantee a good cell viability up to 7 days. Furthermore, a possible relationship between MNPs size and collagen alignment was investigated by employing Ø1 µm MNPs for the preparation of the oriented collagen gels. The resulting gels were monitored at the microscope over the time up to 21 days and, even though the collagen alignment was maintained despite any potential ECM cellular reorganisation, Ø1 µm MNPs gels preserved a better collagen and MNPs orientation compared to Ø250 nm MNPs gels.

The organisation and alignment of the scaffold is essential in tendon tissue engineering as some cellular characteristics, like orientation and morphology,^{318,319} showed to be regulated

by pathways directly affected by topographical cues. In addition, stem cells proved to be more likely to differentiate when seeded on an aligned matrix rather than on a smooth surface.^{302,303} Being able to modulate the architecture and alignment of an engineered scaffold would enable a greater control over the cell behaviour in engineered tissues. With our model, we showed how remotely-aligned collagen gels provide topographical cues for *in vitro* hASCs alignment, as evidenced by actin filaments alignment along the collagen fibres. The developed tendon-like collagen gel could be used as *in vitro* tendon 3D model to investigate healing mechanisms and repair²⁹⁴ or it could be used in conjunction with synthetic materials, such as PHBHHx,¹⁶² for cell delivery in tendon tissue engineering.

Fibrin is another biological material that has been widely studied for tissue engineering applications and it is currently used in clinic at high concentrations as wound sealant.^{171–173} In fact, fibrin can be injected to the wound site where it solidifies adhering to the surfaces and adapting to the injury site.^{170,175} Furthermore, by regulating the ratio between fibrinogen and thrombin, the setting time and stiffness of the final gel can be adjusted according to the characteristics required.^{177,181} Fibrin has already been used as cell delivery method in a multitude of tissues including tendons, improving ECM deposition, cells distribution¹⁸² and overall tendons recovery during the first healing phases.¹⁸³ For these reasons and considering its well-established biocompatibility,^{171,173,177,180} fibrin was the second biological material to be investigated in Chapter IV as potential cell delivery method for tendon regeneration purposes. A fibrin gel formulation was developed by first assessing hASCs metabolic activity up to 14 days in gels at different fibrinogen and cells concentrations, as it is known that higher fibrinogen concentrations lead to stiffer gels with limited cells infiltration properties.^{177,181} In fact, gels at the highest fibrinogen concentration tested (50 mg/mL) lowered the cells proliferation rate compared to lower concentrations. 30 mg/mL was selected as ideal fibrinogen concentration, as a fair compromise between cells proliferation and gel stiffness.

The optimal thrombin concentration was investigated monitoring the gelation time at the spectrofluorometer.³⁰⁹ As expected, the gelation time decreased with the increase of thrombin concentration¹⁸¹ until the saturation concentration of 90 IU/mL at which the gelation time decreased to about 90 sec. Since a quick setting time is desired in preclinical and clinical applications in order to reduce the duration of the surgical procedure and to limit the risk of infections, 90 IU/mL thrombin concentration was selected for the final gel formulation. The degradation time of the definitive fibrin gel (3x10⁴ hASCs in fibrin gels 30 mg/mL fibrinogen and 90 IU/mL thrombin) was monitored via ICP, to ensure that the scaffold would not degrade faster than the cells ECM deposition speed, which would lead to ruptures and failure of the reconstructed tissue.¹⁶⁹ The analysis showed how the gels did not degrade until day 21 when cultured in media without aprotinin. The degradation rate of the gels was compatible with the healing and repair process in the native tendon tissue, which involves an initial amorphous collagen deposition after 2-4 days from the injury.³⁶ Cells within the fibrin matrix would benefit of the additional support provided by the gel mesh until the remodelling phase of tendon repair 1-2 months after the damage.³⁵

Following the *in vitro* findings presented in Chapters III and IV, further investigations were required to gain an understanding of the possibility to translate the remotely-activated tenogenic regeneration technology *in vivo* for future therapeutic treatments. In Chapter V, the tenogenic regenerative potential of remotely-activated MNPs-labelled hASCs was investigated *in vivo* in a rat patellar tendon injury model. For this study, fibrin gels were chosen over collagen gels as cell delivery method considering the *in vitro* results described in Chapter III. In particular, fibrin adhesive properties,^{171–173} quicker gelation time and wound-adapting shape were decisive characteristics for this choice. Furthermore, fibrin gels have already been used *in vivo* in some other studies for tendon tissue engineering applications,¹⁹⁴ including the repair of rabbit Achilles tendon¹⁸³ and rat patellar tendon¹⁸² defects, and showed to have a positive

impact on tendon's healing. In our study, the developed injectable fibrin gel was employed to deliver MNPs-labelled hASCs in a partial window defect in the patellar tendon in a rat model. The tendon location was chosen considering the tendon's advantageous location compared to others. In fact, the patellar tendon is more superficial compared to other tendons, such as the flexor tendon of the hand, and it requires only a minimum retraction of the surrounding soft tissue during surgery.¹⁹⁶ Furthermore, because of its size and accessibility, patellar tendon has been considered the best model for tendon defect reproduction consistency, as well as for the difference in the injured tendon compared to the native tissue.¹⁹⁶

In order to investigate *in vivo* the remote magnetic activation of MNPs-labelled hASCs towards the tenogenic commitment, a customized magnetic box was developed and assembled. The aim was to create an enclosed space where the rats would move freely, being exposed to a variable magnetic field at the same time. The arrangement of the permanent magnets fitted on the walls were carefully arranged in order to recreate the variable magnetic field generated *in vitro* by MICA Bioreactor. 2D²⁸⁶ and 3D²⁹¹ computational models were used to plan the magnetic arrangement and predict the magnetic field intensity the animals would be exposed to (≤ 0.20 T) as well as the magnetic forces acting on each implanted MNP ($0.15 \text{ pN} \leq F \leq 1.5 \text{ pN}$). By using a higher magnetic field compared to the one usually employed in clinic for PEMF therapy ($\leq 20 \text{ mT}$),^{276,277,338,339} we intended to increase the magnetic forces acting on the implanted MNPs-labelled hASCs, enhancing the mechano-activation of the Smad2/3 tenogenic pathway. Animals from “-mag” groups were allowed to move freely in identical non-magnetic boxes in order to provide them with the same exercise regimen as the “+mag” groups but without the magnetic field exposure. This was to ensure a mobilization of the injured tendon to promote the healing, as recommended in clinical therapy where controlled physical exercise post-surgery showed to increase tendon's strength and collagen fibres diameter.³⁵

The presence of hASCs after 4 weeks and 8 weeks from surgery was confirmed by FISH, as testified by the positive expression of human PolR2a and PPIB in the tendon samples. The FISH analysis showed the strongest signal in Cells+MNPs+mag group after 8 weeks, suggesting a possible positive effect of the joint use of MNPs and magnetic field in the retainment of the implanted cells at the tested timepoint.

Tendon's repair was monitored by assessing the ECM reorganisation as part of the tendon healing process.³⁵ After 4 weeks, the H&E staining showed a more organised ECM in Cells+MNPs+mag group compared to other groups, suggesting a positive effect of the joint use of MNPs-labelled hASCs and magnetic field in improving the ECM organisation in healing tendons. The ECM reorganisation was confirmed by the Picrosirius staining which highlighted a more reorganised matrix in injured tendons compared to contralateral tendons. Importantly, no Col III excessive deposition was registered in any of the groups, in line with the remodelling phase of tendons healing³⁴ and as desirable to prevent fibrotic scar formations.^{1,6}

TNMD expression was monitored after 4 weeks, as it is recognised as mature tendon tissue marker²⁹⁶ and it is an important regulator of the synthesis of other tenogenic proteins.³⁴ IHC analysis did not show any significant difference between the groups, suggesting that MNPs-driven stimulation of hASCs might have a limited impact in TNMD expression. Nevertheless, as also highlighted by H&E staining at 8 weeks timepoint, crimps-like structures were detected in Cells+MNPs+mag. Crimps are typical of the native tendon tissue and they are fundamental for the elastic properties and resistance of tendons.³¹ Their presence in +mag group corroborates the hypothesis of a positive impact of MNPs-labelled hASCs in ECM rearrangement.

The remodelling phase in tendon healing normally starts 1-2 months after the initial injury and it includes a replacement of the disorganised matrix initially deposited *in situ* to provide

stability, with a more organised Col I matrix.³⁴ In line with these considerations, H&E staining at 8 weeks timepoint showed a more organised matrix across all experimental groups, compared to 4 weeks timepoint. H&E staining revealed the presence of calcific depositions in tendon sections across all the groups, including Sham animals, along with a macrophages and leukocytes infiltration in the affected areas. Calcific depositions are a common complication of a defective tendon healing, whom underneath mechanisms are still to be clarified.⁴³ Although the presence of macrophages and leukocytes might testify an ongoing self-resolution of the deposits,^{44,52,53} this phenomenon needs to be further investigated in future studies to find the underlying causes and provide an adequate treatment.

Lastly, no iron accumulation attributable to MNPs degradation was detected in any of the detoxification organs tested,³³³ testifying the safety of MNPs for *in vivo* applications, as expected from the literature.^{206,207} Likewise, after 4 weeks from surgery, plasma levels of cytokines did not show any significant difference between groups. An increase in IL-1 α , IL-6, IL-10 and IL-13 was registered in +mag group, which could be attributable to the inflammatory response naturally occurring during tendon's healing process after 4 weeks from injury.³⁵

CHAPTER VII

CONCLUSION AND FUTURE PERSPECTIVES

7.1 Conclusion

The purpose of this Thesis was to investigate the efficacy of remotely activated stem cells-based therapy for tendon injuries treatment. The remote activation of the mechano-receptor Activin RIIA was explored *in vitro* on different cell types by means of antibody-labelled MNPs which were remotely stimulated through a vertical oscillating bioreactor (MICA). Moreover, by employing different anti-Activin RIIA antibodies targeting different regions of the receptor, we investigated the mechano-responsive domain of the receptor, contributing to the understanding of the pathways involved in the receptor mechanic activation towards the tenogenic commitment.

The achieved results suggested a synergistic effect of MNPs and magnetic stimulation in promoting stem cells tenogenic differentiation; these findings can be useful for future studies and follow-up works investigating the mechanisms involved in the mechanical activation of Activin RIIA. Subsequently, two 3D scaffolds were developed for *in vitro* and *in vivo* applications. In particular, through the combined use of MNPs and a permanent magnetic field, an oriented fibres collagen gel with aligned hASCs was achieved. This could be used as potential *in vitro* tendon model recapitulating in gross architecture the fibrillary structure of native tendons. We also developed an injectable fibrin gel which could be used as cell delivery method for tendon tissue engineering strategies, as it is biocompatible, adaptable to the wound site, quick-setting and stable *in vitro* for 21 days.

The developed fibrin gel was used as injectable delivery method for MNPs-tagged hASCs to investigate *in vivo* the remote mechano-activation of MNPs-labelled hASCs in the repair of a rat patellar tendon defect. The injected cells persisted in the defect after 8 weeks from surgery, and no collateral iron accumulation or adverse inflammatory response was

registered. The joint use of MNPs and external magnetic field might have a positive impact in improving ECM organisation during tendon healing.

7.2 Future perspectives

The presented Thesis provides the basis for further follow-up work under the scope of this study, suggesting several new avenues for investigation. For instance, the optimal stem cells source for tendon tissue engineering could be further investigated by repeating the differentiation assay with MICA including WB and PCR analysis to quantify the expression of tenogenic markers and RNA. Similarly, the identification of the mechanosensitive domain of Activin RIIA requires further investigation, potentially employing customised anti-Activin RIIA peptides instead of commercial antibodies, as this might lead to much stronger effects being seen in stem cells tenogenic differentiation.

The developed 3D oriented collagen scaffold requires further optimisation before a potential *in vivo* application. For example, different MNPs concentrations and magnetic field strengths could be tested in order to achieve an optimal fibres alignment. An important investigation would be to study the tenogenic markers expression in stem cells cultured on oriented and non-oriented collagen gels, in order to monitor any influence of the oriented scaffold on cells tenogenic differentiation. Both collagen and fibrin gels require an appropriate material characterisation in order to potentially improve the gels formulation and achieve better scaffold characteristics such as mechanical resistance, setting time and stability over the time.

Lastly, the *in vivo* study needs to be concluded and the remaining samples analysis to be performed, such as the tenogenic proteins expression at 8 weeks timepoint. Furthermore, the study could be repeated including a Sham group for the 4 weeks timepoint with the aim to investigate tendons self-healing potential at earlier timepoints. Different animal exercise

regimen post-surgery could also be tested in order to find the most suitable stimulation intensity to improve tendon healing.

Even though further work is required, the results presented in this Thesis can be considered as promising for the development of a remotely-activated cell therapy for tendon injuries.

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