Exploring cell sources and magnetic stimuli as tools for advancing tendon tissue engineering strategies

Ana Isabel Silva Gonçalves

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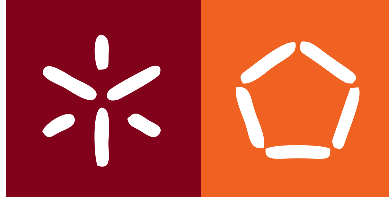

Universidade do Minho
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Exploring cell sources and magnetic stimuli as tools for advancing tendon tissue engineering strategies

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e da
Doutora Márcia T. Rodrigues

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Declaração

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I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of elaboration of the thesis. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 9th of October, 2017

Ana Isabel Silva Gonçalves

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ABSTRACT

Tendinopathy is a broad medical term to define pathology and dysfunction of tendons, including tendon rupture and chronic pain. The incidence of tendinopathies accounts for 50% of all musculoskeletal injuries and is increasing with the emergent worldwide prevalence of an increasingly active and ageing population. Tendinopathy pathophysiologies hinder the healing of tendons which rapidly evolve into chronic disorders. Current therapies focus on the surgical interventions using auto or allografts that can lead to infection, chronic pain in the harvest site, as well as donor site morbidity and are limited in restoring tendon functionality. Hence, there is an urgent need to find alternatives for an effective tendon regeneration.

In this thesis, we investigated the remarkable capacity of stem cell based regenerative medicine approaches as potential therapeutic tools combined with growth factors or combined with scaffold matrices prospecting tendon regeneration as opposed to simple tissue repair. The involvement of these approaches together with biochemical and magnetic tools were studied to target and direct tenogenic differentiation in order to assist *de novo* formation of tendon tissue toward improved tendon therapies. This was investigated following different strategies: search for ameliorated stem cell sources more prone to tenogenic commitment (Chapters 5 and 6) and search for a suitable tenogenic medium (Chapter 5) to induce stem cell differentiation towards tenogenic phenotype. The capacity of more complex architectures to stimulate tenogenic responses were explored in magnetic cell sheets (Chapter 7) and magnetic scaffolds (Chapter 8) to directly influence human adipose stem cells (hASCs). Finally, hASCs tenogenesis was triggered through mechanotransduction mechanisms via stem cell receptors activation (Chapter 9).

The results obtained provided insights on the morphological, biological and biophysical mechanisms that are pivotal in engineering cellular constructs towards tenogenic commitment. Furthermore, the search for cell populations and 3D matrices, as part of an integrated approach that targets tendon injury management, represent a promising approach to positively impact future tendon tissue engineering therapies, which seems to be determinant for tendon treatments that would fully restore tendon to its pre-injured state.

RESUMO

Tendinopatia é um amplo termo médico para definir a patologia e disfunção de tendões, incluindo a rutura e a dor crónica. A incidência de tendinopatias representa 50% das lesões músculo-esqueléticas e está a aumentar com a prevalência mundial emergente de uma população cada vez mais ativa e envelhecida. As fisiopatologias tendinopáticas impedem a cicatrização dos tendões, evoluindo rapidamente para distúrbios crónicos. As terapias atuais centram-se nas intervenções cirúrgicas usando auto ou aloenxertos que podem levar a infeção, dor crónica no local da recolha, bem como a morbidade do tecido dador, e são limitadas na restauração da funcionalidade dos tendões. Portanto, é necessário encontrar alternativas para uma regeneração efetiva dos tendões.

Nesta tese, investigamos a capacidade de abordagens regenerativas baseadas em células estaminais como possíveis ferramentas terapêuticas combinadas com fatores de crescimento ou combinadas com matrizes de suporte perspetivando a regeneração em alternativa à simples reparação do tecido. O envolvimento dessas abordagens juntamente com ferramentas bioquímicas e magnéticas foi estudado para direcionar a diferenciação tenogénica a fim de auxiliar a formação de tecido novo com vista ao melhoramento de terapias tendinosas. Isto foi investigado seguindo diferentes estratégias: procura de fontes celulares estaminais mais propensas ao compromisso tenogénico (Capitulos 5 e 6); procura de um meio tenogénico adequado (Capítulo 5) para induzir diferenciação de células estaminais em direção ao fenótipo tenogénico. A capacidade de arquiteturas mais complexas para estimular uma resposta tenogénica foi explorada em matrizes celulares magnéticas (Capítulo 7) e suportes 3D magnéticos (Capítulo 8) de modo a influenciar diretamente células estaminais adiposas humanas (hASCs). Finalmente, a diferenciação tenogénica de hASCs foi estudada através de mecanismos de mecanotransdução por ativação de receptores de células estaminais (Capítulo 9).

Os resultados obtidos forneceram informações sobre os mecanismos morfológicos, biológicos e biofísicos, os quais são fundamentais no desenvolvimento de matrizes celulares para o compromisso tenogénico. Além disso, a busca por populações de células e matrizes 3D, como parte de uma abordagem integrada que visa a manutenção de lesões no tendão, representa uma abordagem promissora para influenciar positivamente as terapias futuras de engenharia de tecidos do tendão, sendo determinante para tratamentos que restaurariam o tecido ao estado de pré-lesão.

TABLE OF CONTENTS

ACKNOWLEDGMENTS....v
ABSTRACT.... vii
RESUMO.... ix
TABLE OF CONTENTS.... xi
LIST OF ABBREVIATIONS xvii
LIST OF FIGURES....xxi
LIST OF TABLES xxv
SHORT CURRICULUM VITAE xxvii
THIS THESIS IS BASED ON THE FOLLOWING PUBLICATIONS xxix

SECTION I GENERAL INTRODUCTION 1

Chapter 1 Cell based approaches for tendon regeneration 3
1.1. Introduction 5
1.2. Tendon endogenous regeneration 5
1.2.1. Resident cell-mediated mechanisms for tendon regeneration 5
1.2.2. Tendon cells in cellular therapies 8
1.3. Isolation procedures of tendon resident cells 9
1.4. Alternative stem cells sources for cell based tendon tissue engineering 11
1.4.1. Embryonic stem cells (ESCs), Fetal fibroblasts (FFs) and Induced Pluripotent stem cells (iPSCs)....12
1.4.2. Mesenchymal stem cells (MSCs) originated from non-tendon tissues 13
1.5. Moving cell therapies into the clinics 16
1.6. Conclusion 18
1.7. References 18

Chapter 2 Bioengineered strategies for tendon regeneration 25
2.1. Introduction 27
2.2. Biological structure and ECM role, mechanical demands and bioactive molecules in tendon tissue.... 29
2.2.1. Tendon development and physiology.... 29
2.2.2. Tendon ECM: a critical participant in tendon functionality 30
2.2.3. Mechanical behavior of tendons.... 31
2.2.4. Bioactive molecules.... 32
2.3. Integrated approaches towards tendon repair 33
2.3.1. Stem cells and their role in tendon tissue engineering 34
2.3.2. Tendon tissue engineering strategies with and without exogenous scaffolds 35
2.3.3. Mechanotransduction and mechanical stimuli in tendon tissue engineering 37
2.3.4. Mechanical stimulation using magnetic force fields 42
2.4. Conclusions and future perspectives 44
2.5. References 45

Chapter 3 Bioreactors for tendon tissues engineering: challenging mechanical demands towards tendon regeneration 53
3.1. Introduction 55
3.2. Tendon structure-function and mechanobiology behaviour 56
3.2.1. Role of mechanical stimulation in tendon development and functionality 56
3.3. Biomechanics in homeostasis and tendinopathic tendons 59
3.3.1. Loading and overuse 60
3.4. Current therapies for the management of tendinopathies 64
3.5. Tendon tissue engineering (TTE) strategies 65
3.5.1. The role of cells in tendon tissue engineering 66
3.5.2. Bioactive molecules in tendon engineered substitutes 67
3.5.3. Recapitulating tendon matrix in functional substitutes 67
3.6. Bioreactors in Tissue Engineering 69
3.6.1. Designing bioreactors for tendon tissue engineering 69
3.6.2. The role of bioreactors in tendon/ligament tissue engineering 72
3.7. Mechanoregulation mechanisms 77
3.8. Concluding remarks 78
3.9. References 79

SECTION II EXPERIMENTAL SECTION 91

Chapter 4 Materials and Methods 93
4.1. Materials 95
4.1.1. Starch and poly(ε-caprolactone) (SPCL) polymeric blend 95
4.1.2. Magnetic nanoparticles (MNPs) 95
4.2. Scaffolds Fabrication 97
4.2.1. Rapid prototyping 97
4.3. Scaffolds and MNPs characterization 98
4.3.1. Micro-Computed Tomography 98
4.3.2. Scanning Electron Microscopy 98
4.3.3. SQUID Magnetometry 99
4.3.4. Zetasizer Measurements 99
4.4. *In vitro* cell culture studies 100
4.4.1. Cell isolation and expansion 100
4.4.2. Assessment of Tenogenic medium for stem cells differentiation 102
4.4.3. Selection of tenogenic prone subpopulations 102
4.4.4. Construction of magnetic cell sheets as tendon engineered patches 103
4.4.5. Functionalization of MNPs targeting hASCs mechano-responsive receptors for tenogenic commitment 104
4.4.6. Characterization and analysis techniques 104
4.5. *In vivo* studies 114
4.5.1. Subcutaneous model 114
4.6. Statistical Analysis 115

4.7. References 115

SECTION III EXPERIMENTAL STUDIES 119

Chapter 5 Understanding the Role of Growth Factors in Modulating Stem Cell Tenogenesis 121
5.1. Abstract 123
5.2. Introduction 123
5.3. Materials and Methods 126
5.3.1. Stem cell isolation and expansion 126
5.3.2. Morphological analysis 128
5.3.3. Immunolocalization of ECM Proteins: Collagen I, Collagen III and Tenascin C . 128
5.3.4. RNA isolation and Gene expression analysis 129
5.3.5. Statistical Analysis 130
5.4. Results and Discussion 130
5.4.1. Morphological analysis and cytoskeletal arrangement of tenogenic induced cells 130
5.4.2. Differential cytoskeleton alignment in distinct stem cell sources 130
5.4.3. Immunolocalization of ECM Proteins: Collagen I, Collagen III and Tenascin C . 131
5.4.4. Real time RT-PCR 136
5.5. General Discussion 139
5.6. Conclusion 141
5.7. References 142

Chapter 6 Human adipose tissue-derived tenomodulin positive subpopulation of stem cells: a promising source of tendon progenitor cells 145
6.1. Abstract 147
6.2. Introduction 147
6.3. Materials and Methods 149
6.3.1. Isolation of hASCs and subpopulation sorting using immunomagnetic bead separation 149
6.3.2. Pre-selection of the subpopulation most prone to tenogenic commitment 151
6.3.3. RT-PCR analysis of TNMD positive cells 152
6.3.4. Assessment of Collagen I and Collagen III deposition by TNMD positive cells .. 153
6.3.5. Statistical analysis 154
6.4. Results and Discussion 154
6.4.1. Isolation of hASCs subpopulations 154
6.4.2. Pre-selection of the subpopulation more prone to tenogenic commitment by flow cytometry 155
6.4.3. Tenogenic profile of TNMD positive cells 157
6.5. Conclusions 167
6.6. References 168

Chapter 7 Tissue-Engineered Magnetic Cell Sheet Patches For Advanced Strategies in Tendon Regeneration 173
7.1. Abstract 175
7.2. Introduction 175
7.3. Materials and Methods 178
7.3.1. Magnetic nanoparticles (MNPs) stability in the culture medium 178
7.3.2. Isolation of a Tnmd+ subpopulation of hASCs by IMS 178
7.3.3. Living patch construction using cell sheet technologies 179
7.3.4. Assessment of MNPs internalization in hASCs 180
7.3.5. Cellular viability and oxidative stress 182
7.3.6. Culture of magCSs under Magnet or Magnetic stimulating conditions 182
7.3.7. Statistical analysis 185
7.4. Results and Discussion 185
7.4.1. Living patch by Magnetic TNMD+ Cell Sheet Technologies 185
7.4.2. Intracellular Assessment of Magnetic Nanoparticles 189
7.4.3. Assessment of Tenogenic Potential and ECM production by Magnetic Cell Sheets 194
7.5. Conclusions 198
7.6. Supplementary material 199
7.7. References 200

Chapter 8 Exploring the Potential of Starch/Polycaprolactone Aligned Magnetic Responsive Scaffolds for Tendon Regeneration 207
8.1. Abstract 209
8.2. Introduction 209
8.3. Materials and Methods 210
8.3.1. Magnetic Scaffolds Development and Characterization 210
8.3.2. Cytotoxicity assessment 212
8.3.3. Cell Isolation and Expansion 213
8.3.4. Culture of hASCs on SPCL or magSPCL Scaffolds Under Magnetic Stimulating Conditions 213
8.3.5. MagScaffolds Subcutaneous Implantation 215
8.3.6. Statistics 216
8.4. Results and Discussion 217
8.4.1. Magnetic Scaffolds Development and Characterization 217
8.4.2. The Role of Magnetic Stimulus in the Tenogenic Differentiation 222
8.4.3. *In vivo* Subcutaneous Implants 226
8.5. Conclusion 227
8.6. References 228

Chapter 9 Triggering the activation of Activin A type II receptor in human adipose stem cells towards tenogenic commitment using mechanomagnetic stimulation 233
9.1. Abstract 235

9.2. Introduction 235
9.3. Materials and Methods 237
9.3.1. Magnetic nanoparticles (MNPs) conjugation with antibody 237
9.3.2. Cell Isolation and Expansion 238
9.3.3. Phosphorylation assays 238
9.3.4. hASCs culture 238
9.3.5. Dextran Immunolabelling 239
9.3.6. mRNA Extraction and Quantitative Polymerase Chain Reaction 240
9.3.7. Quantification of Collagen and Non-collagenous Proteins 241
9.3.8. Immunofluorescence 241
9.3.9. Statistics 242
9.4. Results and Discussion 242
9.4.1. Assessment of Smad2/Smad3 phosphorylation in hASCs triggered by TGF-β-like ligands 242
9.4.2. Development and characterization of functionalized MNPs complexes 243
9.4.3. Triggering tenogenesis in hASCs using MNPs-ActRIIA complex 246
9.5. Conclusions 251
9.6. Supplementary material 251
9.7. References 252

SECTION IV GENERAL CONCLUSIONS 257

Chapter 10 Concluding Remarks 259

2D – Two-dimensional

3D – Three-dimensional

A

A/A – Antibiotic/antimycotic

ACL - Anterior cruciate ligament

B

BGN – Byglican

C

CL – Cruciate ligaments

CO_2 Carbon dioxide

COL1A1 - Collagen type I, alpha 1

COL3A1 - Collagen type III, alpha 1

D

DAPI – 4',6-diamidino-2-phenylindole

DGAV – Direcção Geral de Alimentação e Veterinária

DMEM – Dulbecco's modified eagle medium

DCN – Decorin

DMA - Dynamic mechanical analysis

DNA - Deoxyribonucleic acid

dsDNA - Double-stranded DNA

E

ECM – Extracellular matrix

EDS - Energy dispersive spectrometer

EGF – Epidermal growth factor

ELISA – Enzyme-linked immunosorbent assay

ELAC - Electrochemically aligned collagen

F

FACS – Fluorescence-activated cell sorting

FACITs - Fibril-associated collagens with interrupted triple-helices

FBS – Fetal bovine serum

FDA - Food and Drug Administration

Fe – Iron

Fe_3O_4 – Iron oxide

FGF – Fibroblast growth factor

FITC – Fluorescein isothiocyanate

FMOD - Fibromodulin

G

g – g force

G′ – Storage modulus

G′′ – Loss modulus

GFs – Growth factors

GDF-5 - Growth and differentiation factor 5

H

h – hour

H&E – Hematoxylin and Eosin

H_2O_2 – Hydrogen peroxide

hASCs – Human adipose stem cells

hAFSCs – Human amniotic fluid stem cells

Hz – Hertz

I

ICP - Inductively coupled plasma

IGF-1 - Insulin-like growth factor 1

L

LDCs – Ligament derived cells

L929 – Mouse C3H/An connective tissue fibroblast like cell line

M

Min – minute

MEM – Minimum essential medium

Micro-BCA™ – Micro-bicinchoninic acid™

Micro-CT – Micro-computed tomography

MMPs - Matrix metalloproteinases

MNPs – Magnetic nanoparticles

MSC – Mesenchymal stem cell

MTS – Tetrazolium

MRI - Magnetic resonance imaging

Mkx - Mohawk

N

N/A – Not applicable, Not available

NO – Nitric oxide

P

PBS – Phosphate-buffered saline

PCL - Poly(ε-caprolactone)

PCL – Posterior cruciate ligament

PDL - Periodontal ligament

PDGF – Platelet-derived growth factor

PEMF - Pulsed electromagnetic fields

PI – Propidium iodide

PRP – Platelet-rich plasma

PT – Patellar tendon

R

ROS – Reactive oxygen species

RNA - Ribonucleic acid

RP – Rapid prototyping

RPM – Rotations per minute

RT – Room temperature

S

SCXA - Scleraxis

SD – Standard deviation

SEM – Scanning electron microscopy

SPCL - Starch poly(ε-caprolactone)

SVF – Stromal vascular fraction

T

TDCs – Tendon derived cells

TE – Tissue engineering

TSPCs - Tendon stem/progenitor cells

TTE – Tendon tissue engineering

TEM – Transmission electron microscopy

TERM - Tissue engineering and regenerative medicine

TGF-β – Transforming growth factor β

TNC – Tenascin

TNMD – Tenomodulin

U

UV – Ultraviolet

V

VEGF – Vascular endothelial growth factor

Figure 1-1. Schematic representation of basic elements present in tendon cell niche. ____ 6
Figure 1-2. Growth factors role in modulating stem cells differentiation towards tendon lineage. ____ 15
Figure 2-1. Schematic representation of cell-ECM interactions. ____ 31
Figure 2-2. Magnetic polymer scaffolds developed by rapid prototyping technology. ____ 43
Figure 3-1. Diagram on the development of a successful tissue engineered substitute towards clinical translation using a traditional TE approach with the actuation of bioreactors. ____ 65
Figure 3-2. Images of some commercially available bioreactors used in TTE strategies. ____ 72
Figure 5-1. Collagen I immunolocation in hAFSCs and hASCs cultured up to 28 days in different supplemented media. ____ 133
Figure 5-2. Tenascin C immunolocation in hAFSCs and hASCs cultured up to 28 days in different supplemented media. ____ 135
Figure 5-3. Real time RT-PCR analysis. ____ 137
Figure 6-1. Schematic representation of the experimental setup of the work. ____ 151
Figure 6-2. Gene expression of SCXA, TNMD, TNC and DCN in hASCs (TNMD+ subpopulation and CTR hASCs) cultured up to 28 days in α-MEM or GFs supplemented media. ____ 160
Figure 6-3. Collagen type I immunolocation and gene expression in control hASCs (CTR) and TNMD+ subpopulation cultured up to 28 days in different supplemented media. ____ 162
Figure 6-4. Collagen type III immunolocation and gene expression in control hASCs (CTR) and TNMD+ subpopulation cultured up to 28 days in different supplemented media. ____ 165
Figure 6-5. Expression of tenogenic markers determined by flow cytometry analysis of unsorted crude (CTR) and TNMD+ cells cultured for 28 days in basic medium (α-MEM) or in media supplemented with different GFs. ____ 166
Figure 7-1. Schematic representation of the magnetic patch construction employing cell sheet technology with Tenomodulin positive (TNMD+) adipose derived stem cells (hASCs) magnetized with iron oxide nanoparticles. ____ 177
Figure 7-2. Magnetic cell sheets (magCSs) after 7 days in αMEM culture. (A1, A2) Photographs of the harvesting process with a permanent magnet. ____ 187

Figure 7-3. Stability and cohesiveness of magCSs. 189
Figure 7-4 Internalization assessment and intracellular location of MNPs within TNMD+ hASCs. 191
Figure 7-5. Cellular reactive oxygen species and viability assays of TNMD+ hASCs cultured with red fluorescent MNPs for 16h, by flow cytometry analysis. 193
Figure 7-6. TNMD+ hASCs viability analysis (A) and proliferation (B) assembled in magCSs cultured for up to 7 days in αMEM medium under a magnet or magnetic stimulation conditions. 194
Figure 7-7. Real time RT-PCR analysis of collagen type I (COL1A1), collagen type III (COL3A1), tenascin C (TNC), scleraxis (SCXA), decorin (DCN) and tenomodulin (TNMD) gene expression of magCSs. 196
Figure 7-8. Quantification of collagen and non-collagenous proteins by Sirius Red/Fast Green Collagen staining kit, and Immunocytochemistry of Collagen type I, Collagen type III, Decorin and Tenascin C proteins deposited by TNMD+ cells in magCSs. 198
Supplementary Figure 7-1. Iron oxide nanoparticles characterization. 199
Supplementary Figure 7-2. Microscopy analysis of hASCs and TNMD+ hASCs subpopulation 3 days after IMS sorting. 200
Figure 8-1. Representative images of the developed magnetic scaffolds. 218
Figure 8-2. Iron oxide nanoparticles and scaffolds characterization. 219
Figure 8-3. (A) Relative viability of L929 cells in contact with magSPCL scaffold leachables after 24, 48, 72h and 7 days, data obtained by MTS assay, and (B) ICP analysis for iron quantification released to the culture medium. 221
Figure 8-4. Collagen I immunolocation in hASCs-mag/bare SPCL constructs, cultured for 7 days under magnetic stimulation or static culture. 223
Figure 8-5. Tenascin C immunolocation in hASCs constructs, cultured for 7 days under magnetic stimulation or static culture. 225
Figure 8-6. Quantification of collagen (A) and non-collagen proteins (B) secreted by hASCs cultured on magnetic scaffolds (magSPCL) under static or magnetic stimulation for 7 days, by Sirius Red/Fast Green Collagen staining kit. 226
Figure 8-7. Micrographs of histological sections stained with H&E. 227

Figure 9-1. Schematic representation of cellular Activin A receptor targeting with MNPs functionalized with Anti-Activin type IIA antibody (MNPs-ActRIIA) for tenogenic differentiation. 237
Figure 9-2. Phosphorylation assessment of Smad2/Smad3 in hASCs. 243
Figure 9-3. Development and characterization of functionalized MNPs complexes. 244
Figure 9-4. Phospho-Smad2/Smad3 assay of hASCs cultured in Activin A (positive control) and with MNPs-ActRIIA under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). 245
Figure 9-5. Real time RT-PCR analysis of tenascin C (TNC), tenomodulin (TNMD), scleraxis (SCXA), decorin (DCN), collagen type I (COL1A1) and collagen type III (COL3A1) gene expression of hASCs. 247
Figure 9-6. Quantification of collagen and non-collagenous proteins by Sirius Red/Fast Green Collagen staining kit in hASCs. 248
Figure 9-7. Immunocytochemistry of A) Tenomodulin and of B) Fibromodulin proteins (green) deposited by hASCs. 250
Supplementary Figure 9-1. Real time RT-PCR analysis of tenascin C (TNC), tenomodulin (TNMD), scleraxis (SCXA), decorin (DCN), collagen type I (COL1A1) and collagen type III (COL3A1) gene expression. 251

LIST OF TABLES

Table 1-1. Procedures focused on the application of stem cells in current clinical trials for tendon tissue. 17

Table 2-1. Summary of *in vitro* and *in vivo* studies on TTE approaches, from 2010. 39

Table 3-1. Summary of main characteristics of a bioreactor system. 70

Table 5-1. Description of the culture medium composition to induce the tenogenic potential of hAFSCs and hASCs. 127

Table 5-2. Primers used for quantitative RT-PCR analysis. 130

Table 6-1. Primers used for quantitative RT-PCR analysis. 153

Table 6-2. Expression of stemness and tendon related markers of unsorted hASCs and selected subpopulations, at passage 3, assessed after isolation in basal culture. 155

Table 6-3. Response of unsorted hASCs and hASCs subpopulations cultured in the presence of different growth factors on the expression of tenogenic markers determined by flow cytometry analysis after 28 days in culture. 156

Table 7-1. Primers used for quantitative real time RT-PCR analysis. 184

Table 9-1. Primers used for quantitative RT-PCR analysis. 240

SHORT CURRICULUM VITAE

Ana Isabel Silva Gonçalves was born in Braga, Portugal, in 1987. Regarding her previous education background, she obtained her MSc degree in Biological Engineering from School of Engineering at University of Minho in 2010. Ana I. Gonçalves joined the 3B's Research Group at the University of Minho in 2011 under supervision of Prof. Manuela E. Gomes, being actively involved in an emerging research line in the Group focused on Tendon Tissue Engineering and on the use of magnetically assisted tools for tendon regeneration. She worked as an assistant researcher in several projects, mainly in the isolation, manipulation, culture and differentiation of stem cells, dynamic cell culturing systems, and magnetic force based tissue engineering approaches. In 2013, she was awarded with a PhD grant from the Doctoral Program in Tissue Engineering, Regenerative Medicine and Stem Cells (TERM&SC), being the main focus of her research the development of tendon tissue engineering strategies exploring magnetic stimulation tools.

As a researcher in 3B's Research Group and during her PhD enrollment, Ana I. Gonçalves was actively involved in the preparation of proposals for National and European funding. She was part of the organizing committee of TermStem conferences and recently was co-chair of the session "Regenerative Medicine and Tissue Engineering I" at the European Orthopaedic Research Society (EORS) 2017 conference. Moreover, she co-supervises several trainees/MSc students and collaborates with other researchers at the 3B's Research Group. Ana I. Gonçalves shares the responsibility of the Bioreactors Laboratory and of some lab equipments, including the training of real time RT-PCR technique to other researchers. She supports the Biocompatibility and Tissue Engineering Laboratories classes of the Biomedical Engineering Master degree of University of Minho since 2013, and also the "Best students in UM" Program since 2015. She has reviewed manuscripts from the FASEB Journal, Journal of the Mechanical Behavior of Biomedical Materials, Connective Tissue Research, Scientific Reports, and Journal of Tissue Engineering and Regenerative Medicine.

In March 2014, she was a visiting PhD student at the Institute for Biomedical Technology and Medicine in University of Twente, The Netherlands, under co-supervision of Prof. Lorenzo Moroni. This trainingship focused on exploring rapid prototyping technologies as alternative and sophisticated tools to

fabricate scaffolds with precise orientation and alignment for tissue engineering and regenerative medicine. More specifically, to develop oriented fiber scaffolds of a blend of starch and polycaprolactone to be applied in tendon repair strategies.

More recently, she moved to the Institute for Science & Technology in Medicine in Keele University, UK, where she spent 4 months. The developed work focused on the cell response to magnetic stimuli through the actuation of vertical oscillating magnetic fields to magnetic nanoparticles' tagged cells for receptors activation and triggering of tendon differentiation, under co-supervision of Prof. Alicia El Haj.

As result of her research and collaborative work, Ana I. Gonçalves is currently author of 11 published/in press/under revision papers in international referee journals, 7 book chapters, and her work resulted in several communications presented in important international meetings (13 oral and 27 poster communications, including 2 invited presentations in the field of tissue engineering and regenerative medicine).

THIS THESIS IS BASED ON THE FOLLOWING PUBLICATIONS

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Gonçalves AI, Rotherham M, Markides H, Rodrigues MT, Reis RL, Gomes ME, Haj, AE. Triggering the activation of Activin A type II receptor in human adipose stem cells towards tenogenic commitment using mechanomagnetic stimulation, 2017, *submitted*

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Section I

General Introduction

Chapter 1

Cell based approaches for tendon regeneration

This chapter is based on the following publication:

Gonçalves AI, Costa-Almeida R, Gershovich P, Rodrigues MT, Reis RL, Gomes ME. Cell based approaches for tendon regeneration, in Tendon Regeneration: understanding tissue physiology and development to engineer functional substitutes, ME Gomes, MT Rodrigues and RL Reis (Eds), Elsevier Publisher ISBN-13: 978-0128015902, pp. 187–203, doi:10.1016/B978-0-12-801590-2.00006-5, 2015

1. CELL BASED APPROACHES FOR TENDON REGENERATION

1.1. Introduction

Cell based therapies have risen a particular interest in regenerative strategies as a biological mean to overcome limitations of current clinical therapies and improve natural healing response.

In tendons, cells are mainly surrounded by ECM components [1] due to the hypocellular nature of these tissues. Nevertheless, different cells exist, constituting a heterogeneous population of tenocytes, as well as tendon stem and progenitor cells (herein called tendon derived stem cells, TDSCs). Both stem cells and mature cells reside within an instructive microenvironment or niche that is responsible for providing physical/mechanical, biochemical and biological cues necessary for tendon homeostasis.

Cell niches constitute specialized dynamic microenvironments that vary according to their anatomic location and regulate cell biology within tissues and organs. The interactions between resident cells and their biological context define cell fate and function. Thus, cellular approaches in combination with tissue engineering strategies have sought to augment the regenerative potential of injured tendons aiming at potential therapeutic applications.

Cell-mediated strategies aim at repairing damage locally, through the delivery or injection of cells as main regenerative agent or in conjunction with biomaterials acting as cell carriers and bioactive molecules that mediate and modulate cell reparative processes at the site of injury.

Beyond tendon resident cells, several stem cell populations, that are natural endogenous modulators of tissue regeneration, have been described to be capable of differentiating into tenocytes with *in vitro* and *in vivo* effectiveness in treating tendon disorders. Other strategies focus on the potential use of cells from embryonic and fetal origins as alternative sources to stimulate tendon repair.

Recent works reporting cell based studies towards tendon regenerative therapeutics including approaches under a tissue engineering perspective will be discussed with more detail in the following sections. Although the translation of stem cell therapies into medical practices is still at an early stage, clinical studies on the use of stem cell based approach will be also reviewed in this chapter.

1.2. Tendon endogenous regeneration

1.2.1. Resident cell-mediated mechanisms for tendon regeneration

As active players in tendon milieu, tendon resident cells together with the extracellular matrix (ECM), orchestrate the necessary stimuli required in promoting damage repair.

Tendon tissues are initially very cellular but become less so throughout growth to adulthood as matrix elements are synthesized [2]. The hypocellular and hypovascular nature of adult tendons limits native cell mediated regenerative response. Tendon vascular blood supplies are confined to endotenon and epitenon, and it is likely that stem cell recruiting through the vascular system may be restricted to the surrounding areas of these layers. Thus, the resident cell populations, tenocytes and stem cells, have a critical role in physiological homeostasis and regulation of the tendon matrix (Figure 1-1) in both normal and pathological conditions [3].

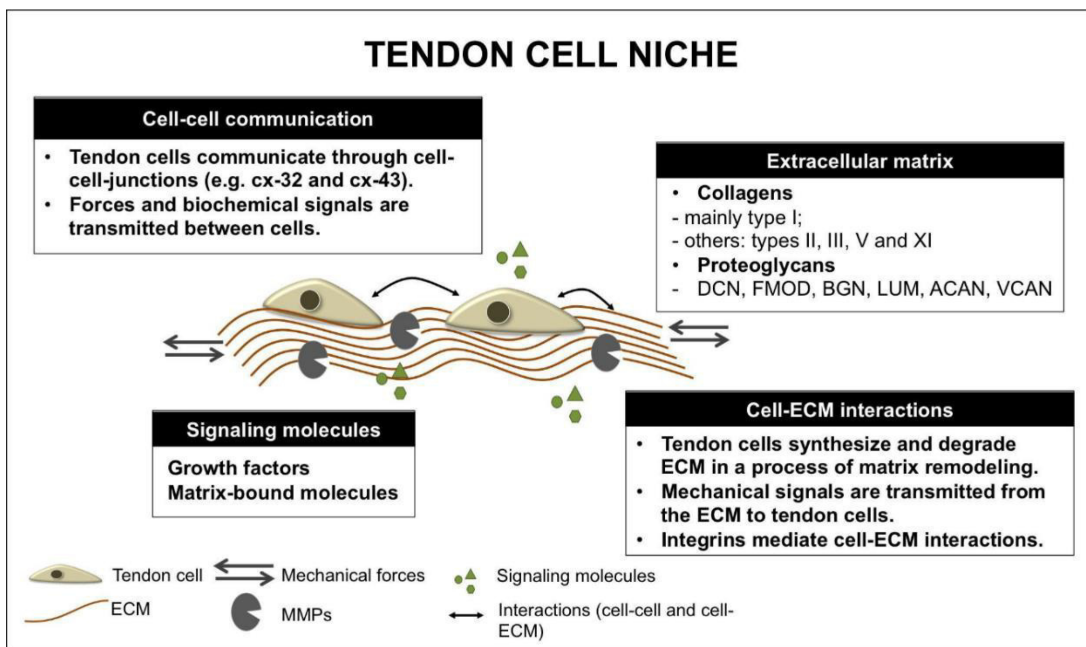

Figure 1-1. Schematic representation of basic elements present in tendon cell niche. Tendon tissue is mainly composed of extracellular matrix, particularly of collagens, as well as proteoglycans. The ECM is synthesized and degraded by cells, which communicate both among each other (cell-cell communication) and with the ECM (cell-ECM interactions). Mechanical forces act on the ECM, modifying it. Tendon functionality is achieved through specific signaling, including the release of growth factors and matrix-bound molecules through the activity of MMPs.

Abbreviations: cx-32, connexin-32; cx-43, connexin-43; DCN, Decorin; FMOD, Fibromodulin; BGN, Biglycan; LUM, Lumican; ACAN, Aggrecan; VCAN, Versican; ECM, Extracellular matrix; MMPs, Matrix metalloproteinases

Tenocytes are very elongated cells ranging from 80 to 300 μm [4], that are typically arranged in longitudinal rows, having sheet-like extensions that surround ECM fibers. These cells are mainly responsible for the secretion of the ECM, and thus collagen assembly and turnover [5]. Despite their intrinsic role in tendon niches, tenocytes are scarce cells, and harvesting them from native tendon could increase local tissue morbidity, thus impairing tendon mechanical properties, which ultimately would affect tissue function.

Growing evidence supports tendon derived stem cells, rather than tenocytes, as the main responsible for the initial healing response in acute injuries. Beyond the self-renewal capacity, proliferation and multi-lineage potential, stem cells secrete trophic paracrine factors to support the growth and differentiation of local stem and progenitor cells (e.g. SCF, SDF-1, angiopoietin-1), or with angiogenic (e.g. VEGF, IGF-1, bFGF, IL-6), chemotatic (several CCL and CXCL cytokines), anti-apoptotic (e.g. VEGF, HGF, IGF-I, TGF-b), anti-scarring (e.g. HGF, bFGF) or immunomodulatory (PGE-2, HLA-G5, HGF, iNOS, IL-10) action [6], that have a profound influence in local cell dynamics. The secretion of a broad range of bioactive molecules with paracrine effects is believed to be the main mechanism by which mesenchymal stem cells achieve their therapeutic effect [6]. This effect also includes the enhancement on cell survival and the activation of other resident stem cells and ECM remodeling.

Tendon injuries and degenerative conditions are commonly caused by misuse, over-use and/or aging [7]. Pathological tendon niches have been associated to an active, cell-mediated process that failures to regulate specific inflammatory cytokines and extracellular proteolytic activity [7, 8]. It is also known that degenerative tendon undergo structural and biomechanical alterations that interfere with the healing process, resulting in significant changes of the tendon niche. Thus, it is not surprising that tendon cells may be influenced by these changes provided by the surrounding environment. The changes induced in cell behaviour will, in turn, affect ECM maintenance and remodelling with concomitant changes at the morphological and composition levels, ultimately resulting in failure to achieve a regenerative process and impaired tendon function.

In summary, tendon resident cells, especially tendon derived stem cells (TDSCs), are promising tools for the treatment of different types of conditions because they mediate the repair process by secreting a multitude of bioactive molecules that ultimately lead to reformation of tissues at sites of injury [6].

Moreover, the knowledge on these cellular mechanisms could provide relevant information to induce a regenerative tendon niche upon injury with potential for cell based therapies.

1.2.2. Tendon cells in cellular therapies

Tendon derived stem cells (TDSCs) have been primarily identified in human and mouse tendons as a heterogeneous population that includes both stem and progenitor cells [1, 9]. These cells of mesenchymal origin have been reported to differentiate into tenocytes, chondrocytes, osteocytes and adipocytes after being induced *in vitro*; and to assist in the regeneration of tendon, cartilage, bone tissues as well as of tendon-to-bone junction in animal models [1, 10-13]. Thus, TDSCs constitute a potential cell source for tendon regeneration, owing to their stem cell characteristics, including clonogenicity, multipotency and self-renewal. Furthermore, they have also been reported to exhibit low immunogenicity. In fact, TDSCs did not express surface markers required for T-cell activation and transplant rejection, including major histocompatibility complex (MHC)-II, CD80 and CD86 [14].

Studies on the establishment and characterization of tendon cell lines [15] suggested the presence of a special cell population within tendons that possesses multiple differentiation capacity. Moreover, Zhang and Wang [16] reported that TDSCs from patellar and Achilles tendons of rabbits were able to differentiate into adipocytes, chondrocytes, and osteocytes *in vitro*, and form tendon-like, cartilage-like, and bone-like tissues *in vivo*.

Although TDSCs express high levels of tenogenic markers, including Scleraxis (SCX), Tenomodulin (TNMD), Collagen type I (Col1A1) and Decorin (DCN) [17], TDSCs differ from tenocytes in morphology, proliferative potential and expression of stem cell markers (Oct-4, SSEA-4, and nucleostemin).

Thus, understanding the activity of TDSCs in modulating tendon niches and consequently tendon regeneration is critical to harnessing their clinical therapeutic potential.

The allogeneic transplantation of TDSCs isolated from green fluorescent protein (GFP) rats, was described to be involved in tendon healing, with improved fiber arrangement and cell alignment observed up to 16 weeks, together with weak immunoreactions and anti-inflammatory effects after transplantation in a rat patellar model [18]. Hence, the use of allogeneic TDSCs in cell therapies is feasible and supported by their immunomodulatory effects, since the isolation of autologous TDSCs would otherwise result in donor site morbidity. Indeed, these cells can be isolated from other individuals during reconstructive

surgeries, or from cadavers. Altogether, TDSCs are a promising source of cells aiming at understanding tendon homeostasis and regeneration.

In order to enhance tendon regeneration outcomes of TDSCs, several studies report the combination of bioactive molecules or 3D matrices with TDSCs. Platelet-rich plasma (PRP) treated rabbit TDSCs were described to be beneficial in tendon healing by promoting the differentiation of TDSCs into active tenocytes [19] with enhanced tenocyte-related gene and protein expression, and total collagen production.

The application of a decellularized matrix, as a supportive 3D substract for tendon strategies can be of interest to better recreate tendon niche. Decellularized tendon tissues stimulate TDSCs proliferation and better preserve the stemness of TSDCs than monolayer cultures. *In vivo* implantation of decellularized matrices, promoted tendon-like tissue formation in a subcutaneous nude rat model [20]. The tendon-like tissue formed was increased when compared to the implantation of TDSCs alone. Related results were obtained in a similar study with decellularized fibroblast-derived matrix (dFM) obtained from dermal fibroblast in a patellar tendon injury model, the implantation of decellularized fibroblast-derived matrix significantly improved the histologic and mechanical properties of injured tendon [21]. Hence, the recent reports on TDSCs [19-21] provide some basis on tendon intrinsic mechanisms and TDSCs potential use as a feasible cell source envisioning to restore the normal structure and function to injury tendons.

1.3. Isolation procedures of tendon resident cells

The need to isolate individual tendon cells arises from the desire to understand the contribution of cells on the development and functionality of the tissue as a complex unit. Moreover, studies on the cellular level can unveil the essential yet complex cell interactions and intrinsic mechanisms involved in scarless repair and restore of function.

The isolation of tendon cells date as early as 1971 [22] when Dehm and Prockop digested tendon samples from chicks with trypsin and bacterial collagenase. Other works followed with chick embryo tendons [23-25], and protocols for isolation and *in vitro* proliferation of the isolated tendon cells [26].

The isolation procedure has been evolving ever since with slight modifications supported by the increasing knowledge on cell culture methodologies. Tendon cells have been isolated and expanded from

tendons of various anatomical regions, and from several species, including rabbit [16, 27], dog [28], mouse [1], rat [29] and human [1]. The technological advances associated to cell culture procedures enabled the use of cell based TE strategies for tissue injury treatment, including dense fibrous connective tissues such as tendons and ligaments.

As previously mentioned, adult tendons are hypocellular and the limited number of cells is often entrapped within a highly dense collagenous matrix. Due to an abundant ECM, enzymatic digestion is necessary to dissociate the fibrous tissue and release the resident cells [26]. The comprehensive culture systems are based on mechanical and/or enzymatic dis-aggregation of the tissue to disrupt the ECM and release the individual cells. The successful culture of primary tendon cells requires a maximum yield of viable and functional resident cells from the whole tissue.

Enzymes have traditionally been used for the isolation of tendon cells for primary cell culture. Widely accepted enzymatic protocols consist of several key steps and can be scaled depending on the size of the sample. As in any other cell culture procedure, isolation should be performed in aseptic conditions to avoid contamination with environmental agents as bacteria and fungi. The literature indicates that dissected tendon tissue should be kept at 4 °C and (if it is possible) be dis-aggregated within 2-4 hours after harvesting in order to avoid tissue deteoration and consequently a decrease in cell viability followed by cell death.

Before enzymatic digestion of the ECM, mechanical processing is often applied to reduce the tissue sample to smaller sections by cutting and mincing the tissue to facilitate the process of enzymatic digestion.

The most common and frequently used enzyme is collagenase, derived from *Clostridium histolyticum*. Collagenases from *Clostridium histolyticum* were firstly prepared by Mandl *et al* [30] and are commercialized by several research and development companies. Clostridial collagenase is a crude complex containing a collagenase more accurately referred to as clostridiopeptidase A which is a protease with a specificity for the X-Gly bond in the sequence Pro-X-Gly-Pro, where X is most frequently a neutral amino acid. Collagenase cleaves all 3 alpha helical chains of native types I, II and III collagens at a single locus by hydrolyzing the peptide bond following the Gly residue of the sequence: Gly 775-Ile (or Leu) 776 located approximately three-fourths of the chain length from each N-terminus [31]. Some isolation protocols include other enzymes, such as neutral protease (dispase) or trypsin to provide

overlapping substrate specificities for cleaving ECM components such as collagen and proteoglycans. Dispase, a bacterial enzyme produced by Bacillus polymyxa hydrolyses N-terminal peptide bonds of non-polar amino acid residues and is classified as an amino-endopeptidase. Dispase is frequently used as a secondary enzyme in conjunction with collagenase in tendon primary cell isolation and tissue dissociation applications [28, 29]. Trypsin is a pancreatic serine protease with specificity for peptide bonds involving the carboxyl group of the basic amino acids, arginine and lysine. Trypsin is one of the most highly specific proteases known, although it also exhibits some esterase and amidase activity. Purified trypsin alone was shown to be ineffective for tissue dissociation [22] since it shows limited selectivity for extracellular proteins, and because of this limitation, trypsin has been used in combination with collagenase [22-25].

The tissue to be digested is then incubated with the enzymatic solution, whose concentration and incubation time will depend on the dimensions and type of the tissue sample. The digestion product is then centrifuged and the isolated cells are re-suspended in cell expansion culture medium.

Besides the enzymatic digestion, there is at least one referred attempt to isolate tendon cells by directly incubating tissue specimens in cell culture medium and allowing cells to migrate from the tissue explant and adhere to the culture plastic substrate [28]. However, the feasibility of this protocol is questionable due to the low yield of cells and extended time of isolation and expansion.

1.4. Alternative stem cells sources for cell based tendon tissue engineering

Autologous cells are likely to provide an optimal approach to address a structural and functional replacement, avoiding immune reactions or disease transfer caused by allogeneic cells or grafts [32]. On the other hand, isolating local cells from the patient would increase tissue morbidity at the harvesting site, creating an undesirable side-defect [32]. Moreover, the last scenario of autologous cell isolation has the disadvantage of leaving the patient in standby during the period of cell expansion until a sufficient number of cells is achieved for implantation. Thus, a universal system, with heterologous cells that do not incite an immune or adverse health response, is preferable in restoring joint function and preventing further degeneration.

Tendon stem cells have not yet been fully explored for tendon TE applications and their use in regenerative approaches is limited by the availability and eventual donor site morbidity of the tissue where these cells are harvested from. Also, tenocyte phenotype may drift in longer culture periods [27,

33], which may become an obstacle towards therapeutic applications in tendon regeneration, apart from the fact of tendon naturally low cellularity.

Therefore, alternative cell types should be considered in finding the optimal conditions to induce other cell sources more widely available into tendon forming cells. The subsequent sections describe the current developments on the use of other stem cell sources for the treatment of tendon injuries and disorders in both human and veterinary medicine.

1.4.1. Embryonic stem cells (ESCs), Fetal fibroblasts (FFs) and Induced Pluripotent stem cells (iPSCs)

Embryonic stem cells (ESC) are pluripotent stem cells mainly responsible for embryonic development and growth. Embryonic stem cells are also characterized for their normal karyotype, high telomerase activity, and a long-term proliferative potential. All together, these properties make ESCs an interesting subject in studies aiming at cell based therapies. Nonetheless, there are still ethical constraints associated to their use. In addition, the risk of hyperproliferation and teratomas occurrence might be observed as well as immune rejection of the transplanted cells [34, 35].

Chen and colleagues [36] reported hESC-derived MSCs (hESC–MSCs) sheet with potential to regenerate tendon tissues *in vitro* and *in vivo* with a hESC–MSCs- fibrin gel construct in rat patellar tendon model. Histological staining of explants revealed bands of collagen and spindle-shaped cells morphology, also expressing SCX, six1 (SIX homeobox 1), epha4 (Eph receptor A4), and eya2 (EYA transcriptional coactivator and phosphatase 2) genes [36]. hESC–MSCs within a knitted silk-collagen sponge scaffold also proved to promote tendon regeneration in a rat Achilles tendon injury model [37].

In a horse model, the *in vitro* exposure to TGF-β3 was able to drive tenocyte differentiation of equine ESCs [38], and also on a three-dimensional (3D) collagen gel environment [39]. Intra-lesional injections of male fetal derived embryonic-like stem cells (fdESCs) in the digital flexor tendon also lead to significantly better histological scores and a strong trend for improved MRI parameters compared to placebo control [40].

Fibroblasts derived from fetal tissues also have been shown promising cells to form neo-tendon tissue. In a mouse Achilles tendon, fetal fibroblasts, isolated from mouse embryos, lead to enhanced tendon regeneration and better biomechanical properties relative to adult fibroblasts derived from the dorsal dermis of mice, *in vitro*, as well as *in vivo* within an injury model [41]. A recent work also underlines the

use of fetal instead of adult fibroblasts as a more promising source of cells for tendon repair, as fetal fibroblasts had less spontaneous osteogenic differentiation and reduced ectopic ossification *in vivo* [42]. Moreover, hESC-derived engineered grafts were used to repair injured Achilles tendons in mice [43]. Connective tissue progenitors derived from hESC lines and fetal tissues were induced to generate tendon tissues *in vitro*. The connective tissue progenitors-derived tissue engineered tendon grafts were able to replace injured Achilles tendons, to restore the ankle joint extension movement, and to clinically improve the animals motion [43].

Another potential source of cells for the envisioned therapies is the induced pluripotent stem cells (iPSCs) which can be reprogrammed from many adult somatic tissues, generating autologous cells for cell-replacement therapy. Although some technical limitations are to be addressed in iPSCs cultures, recent studies have shown that iPSCs derived from mesenchymal stem cells (hiPSC-MSCs) [44, 45] or from neural crest stem cells (iPSC-NCSCs) can be used for tenogenic differentiation [46]. An advantage of using iPSCs is the fact that as they do not derive from embryonic tissues, does not raise the ethical issues normally associated to ESCs.

1.4.2. Mesenchymal stem cells (MSCs) originated from non-tendon tissues

On the other hand, adult stem cells, autologous or allogenic, present themselves as good candidates for cell based tendon regeneration [47], including mesenchymal stem cells (MSCs) from bone marrow (BMSCs) and adipose tissue (ASCs), providing more reliable options as cell sources without ethical issues constraints or potential teratoma development. Moreover, adult stem cells also have multipotent capacity, self-renewal and clonogenicity properties and seem an advantageous choice since they may be isolated from autologous sources.

MSCs show evidence to improve healing and regeneration of injured tendon tissues. In fact, human MSCs-treated rats improved ECM structure, larger amount of collagen type I and III and increased neovascularization after 2 and 4 weeks of Achilles tendon injury [48], and the implantation of synovial MSCs accelerated early remodeling of tendon-bone healing in rats [49]. Veterinary clinical studies also report the use of autologous equine MSCs therapy in superficial digital flexor tendinopathy [50]. hMSCs treated with insulin were referred to be a promising option for augmentation of rotator cuff repairs in humans [51]. Regarding TE, several works revealed hMSCs [52-55] successful outcomes in terms of

tenogenic differentiation. Furthermore, the promising behavior of these cells for tendon has been assessed through different approaches as scaffold topography and alignment, mechanical stimulation or growth factors supplementation to the medium, improving the knowledge on the potential of these cells as a source for tendon TE strategies.

With respect to bone marrow as a therapeutic cell source for tendon repair, the transplantation of bone marrow cells has been used to treat Achilles tendon ruptures in a rat model [56] and can accelerate tendon-healing after primary repair of a similar injury in a rabbit model [57]. Also, intralesional autologous bone marrow-derived MSCs injection proved efficacy in the treatment of superficial digital flexor tendon (SDFT) tendinopathy in racehorses [58]. Autologous equine BMSC implantation into the core lesion was performed in horses affected by tendonitis [59].

TE studies report the use of BMSCs, which can be differentiated into tendon cells, seeded onto different substrate systems with different gradients of stiffness, mechanical compliances and ligand chemistry regions [60]. Overall, the presence of tenoblast markers was detected on collagen substrates within a narrow range of stiffness, specifically the gene level expression of SCX was altered as was TNMD, TNC, and Collagen type III.

Teh *et al* [61] recently reported the use of bone marrow derived MSCs from rabbits which were statically and mechanically cultured in a 3D aligned silk fibroin hybrid scaffold enhanced tenogenesis by the upregulated expression of tendon-related proteins [61].

Finally, adipose tissue may also represent such a source of stem cells for tendon cell based repairs. Beyond the typical characteristics of stem cells, multipotential capacity, high proliferation and renewal rates, adipose derived stem cells (ASCs) are harvested using minimally invasive procedures and are available in greater quantities. Moreover, ASCs enhance tendon healing in rabbit tendon models [62-64] and have shown effectiveness in the treatment of tendinitis of the superficial digital flexor tendon in horses [65, 66].

Tissue engineering investigations in the differentiation capacity of ASCs to tenocytes have been developed [67-72] based on scaffolding developments, mechanical stimulation and/or growth factor delivery. Cheng and colleagues [73] promoted the tenogenic differentiation of ASCs from topographical and chemical cues, while in a work developed by Yang *et al* [74] hASCs were seeded in a tendon ECM-

supplemented scaffold, exhibiting significantly increased proliferation and tenogenic differentiation than in pure collagen scaffolds or 2D tissue culture plates.

Resort the optimal conditions that will induce the foremost stem cells source into tendon forming cells with less detrimental effects in healthy tissues, is very important for the development of cell based approaches for tendon regeneration. To our knowledge, the optimal conditions to guide tendon differentiation have not been established yet, but several studies suggest [71, 75] that it may require, apart from a suitable stem cell source, an adequate combination of biochemical and mechanical signals.

In a previous work, we investigated the tenogenic differentiation potential of human amniotic fluid stem cells (hAFSCs) and adipose-derived stem cells (hASCs) [71]. As potential agents to target specific cell responses, growth factors were supplemented in the culture medium, namely, EGF, bFGF, PDGF-BB and TGF-β1, and the expression of tendon- related markers and the synthesis of tendon-like ECM evaluated. Overall results indicate that EGF and bFGF influenced the expression of tendon-related genes of hAFSCs, while EGF and PDGF-BB showed a more prominent effect in the genetic expression of hASCs [71] (Figure 1-2).

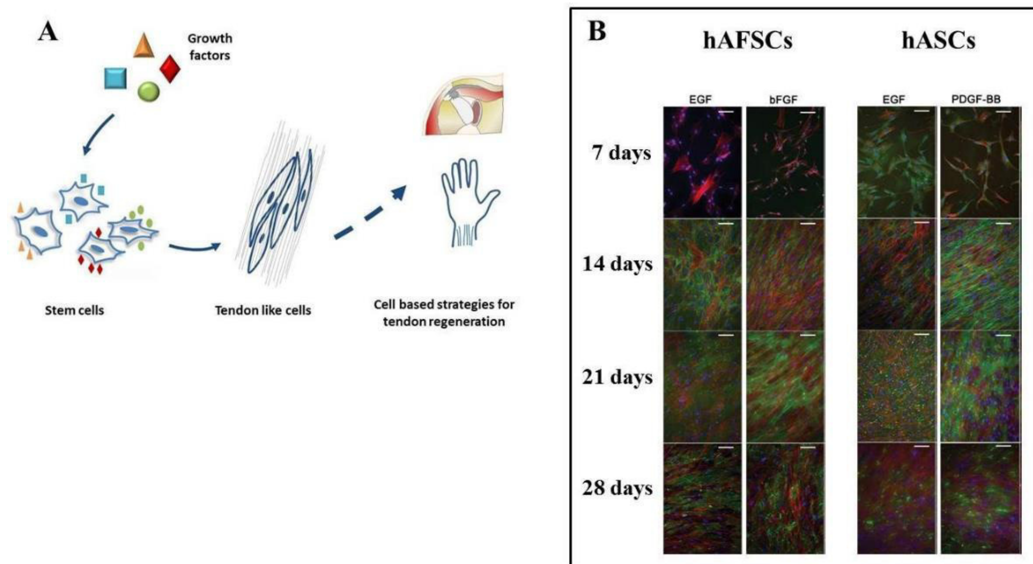

Figure 1-2. Growth factors role in modulating stem cells differentiation towards tendon lineage. (A) Schematic representation of a cell based approach for tendon tissue regeneration. (B) Tenascin C immunolocation in hAFSCs cultured up to 28 days in EGF/bFGF supplemented media and hASCs cultured up to 28 days in EGF/PDGF-BB

supplemented media. DAPI (blue) and phalloidin-conjugate (red) stain cell nucleus and cytoskeleton, respectively. Tenascin C is stained in green and represents a tendon ECM protein. Scale bar represents 100 μm.

Abbreviations: hASCs, human amniotic fluid stem cells; hASCs, human adipose-derived stem cells; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PDGF-BB, platelet-derived growth factor BB

1.5. Moving cell therapies into the clinics

In a clinical perspective, the routing of cell therapy to the medical routines must consider isolating, expanding, administering and eventually storing cells using clean rooms and good manufacturing practise (cGMP) processes that involve high costs and long processes for regulatory authorities approval. The translation issues of cell based therapy were recently reviewed by Ratcliffe *et al* [76], including scalable bioprocessing concerns and automation protocols, pointing out the limitations that still exist to achieve the requirements for commercialization as regulatory certainty and manufacturing guidelines. Indeed, cost-effectiveness, efficacy, reimbursement, and regulation are the most significant barriers to cellular therapy in clinical development [77]. Also, the major risks related to a cell based product are microbiological contamination, dedifferentiation/loss of cell function, cell transformation malignancies, immunogenicity and ectopic engraftment of cells to non target tissues [78].

The gold standard procedure in clinical treatments for tendon repair is the replacement of injured tissue with grafts, either from autologous or allogeneic origins. Cellular therapies have the potential to become successful treatments for tendon disorders, as suggested by the numerous *in vitro* studies and animal model assessments but few clinical studies reported the ideal cell source, the route or vehicle to successfully administer these therapeutic agents or the optimal bioscaffold to assist effective tissue regeneration for clinical use. Nevertheless, clinical trials involving the application of stem cells for the treatment of tendon injuries are currently being performed. Searching the database ClinicalTrials.gov (U.S. National Institutes of Health clinical trials web site) using the keywords “Tendon” and “Stem cells” retrieved four clinical trials indicated in Table 1-1. These trials focus on the treatment of tendon injuries using stem cells, or stem cells in combination with platelet rich plasma (PRP). On the other hand, when the keyword “Tendon” is searched alone, the retrieved search results bursts into a total of 421 studies. In addition to those previously mentioned these results englobe as different approaches and procedures as injection of PRP or of collagenase, drugs administration, surgical suture techniques, arthroscopic

repairs, stitches or fibrin-glue procedures. Moreover, commercial tendon substitutes as GraftJacket, Clarix™ graft or Artelon® are also part of these clinical studies.

Table 1-1. Procedures focused on the application of stem cells in current clinical trials for tendon tissue. (Source: ClinicalTrials.gov (accessed date:1/12/2014)).

ClinicalTrials.gov Identifier	Condition	Intervention	Phase
NCT02298023	Rotator Cuff Tear	Allogenic adipose-derived mesenchymal stem cells treatment	Phase 2
NCT01856140	Lateral Epicondylitis	ALLO-ASC (allogeneic adipose derived mesenchymal stem cells) injection	Phase 0
NCT02064062	Achilles Tendinitis	Autologous mesenchymal stem cells	Phase 2
NCT01687777	Rotator Cuff Tears	Autologous bone marrow mensenchymal stem cell transplantation and OrthADAPT (Membrane of collagen type I)	Phase 2

Phase 0 regards to exploratory screening studies involving very limited human exposure to the drug, with no therapeutic or diagnostic goals; **Phase 2** refers to studies that gather preliminary data on effectiveness and are compared to a placebo group of participants.

In spite of the promising stem cells effects on tendon healing prominent in a number of animal studies, as discussed previously in this chapter, up to date, the few clinical trials performed do not provide clear evidences on the efficacy and safety of applying stem cells locally for human tendon repair. Safety issues associated with cell therapies are tumorigenicity, heterogeneity, senescence and limited expansion. An example of a randomized controlled trial was reported by Clarke *et al* [79], in which the injection of autologous skin-derived tendon-like cells effectiveness was investigated on the treatment of refractory patellar tendinosis. This study considered a total of 60 patellar tendons from 46 patients. Only one cell- or control- (plasma suspension without cells) injection was administered per injected knee in each patient. Overall results indicated that both groups showed a treatment effect; however, the cell injection group showed a significantly greater improvement in tendon function than with the group treated with a plasma injection alone [79].

1.6. Conclusion

Clinically relevant cell based therapies for tendon repair and regeneration were addressed. The existence of a particular cell population within tendons that possesses multiple differentiation capacity and the reported studies with TDSCs opened new possibilities to treat damaged tendon. However, are compelling the associated drawbacks regarding its use in clinical therapies, namely phenotype drift and function loss during *in vitro* expansion and also tendons limited cellularity and morbidity problems that may be associated.

Thereby, alternative and multiple potential sources of adult stem cells have been suggested and all merit further evaluation. The studies discussed above propose that stem cell based technology may have a role in tendon grafting and repair. However, the success in cellular therapies may require recapitulating the events of tendon development for better strategies with efficient outcomes in the regeneration of tendon injuries. Recent studies suggest that stem cell based TE offers the potential to replace injured/damaged tissue with healthy, new living tissue [80].

In short, the application and selection of the most promising cell source is of crucial importance for the development of stem cell based tendon regenerative strategies. The cell source with more potential will guide functional therapies towards tendon repair.

Recreating an artificial *in vivo* tendon milieu by engineering functional *in vitro* cell microenvironments is a research priority. Cell based therapies per se or as a component of scaffold based approaches, this is TE strategies, have been proposed as a potential solution in restoring tendon with promising therapeutic frameworks as participants of physiological homeostasis and healing response.

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Chapter 2

Bioengineered strategies for tendon regeneration

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2. BIOENGINEERED STRATEGIES FOR TENDON REGENERATION

2.1. Introduction

The important mechanical function predisposes tendons to injury and rupture, having become a worldwide clinical problem. Commonly injured tendons include the rotator cuff, finger flexors, patellar, and Achilles tendon [1]. As poorly cellular and vascular tissues [2], tendons present some healing constrains, typically requiring surgical intervention. Current reparative surgeries rely on tissue replacement with auto- or allo-grafts [3] which are often accompanied with donor site morbidity, pain, inferior functionalities and eventually graft failure.

Due to the market demand, commercial substitutes for tendon repair have been developed as biological and synthetic scaffolds. Despite being Food and Drug Administration (FDA) approved, both positive and negative results have been reported in clinical applications. Among biological devices, Restore®, TissueMend® and OrthoMend® and GraftJacket® are some of the most used products for tendon repair. Restore® is composed of porcine small intestine submucosa (SIS), predominantly Collagen type I and III and controversial outcomes in rotator cuff tendon repair [4, 5]. The devices TissueMend® and OrthoMend® Soft Tissue Repair Matrix both made of collagen matrix derived from bovine skin, are thin sheets used to wrap around and reinforce the repaired area during healing of Achilles tendon [6]. GraftJacket® is another commercial scaffold obtained from cadaver human skin that has been recently used in rotator cuff [7, 8] and Achilles tendon rupture [6, 9].

Alternatively to biological scaffolds, synthetic ones are also available in the surgical market. Surgi-Wrap MAST Tendon Sheet® is a polymeric surgical mesh for tendon augmentation that function as a resorbable implant in a sheet form manufactured from poly (L-lactide-co-D,L-lactide) 70:30 (PLA). Although less reported in clinical trials Sportmesh® (ARTELON fibers (polycaprolactone based poly(urethane urea) material) is also used in tendon reparative surgeries [10, 11]. Independently of the origin, biological and synthetic scaffolds can cause adverse events such as non-infectious effusion and synovitis that ultimately may result in the failure of surgery [12].

These surgical approaches are not successful to consistently restore the original properties of a functional tissue. TTE strategies might overcome these limitations, providing the ultimate solution to restore the damaged tendon to its pre-injured state, improving the functionality outcomes whilst reducing healing time and medical complications due to surgery and follow-up care. TTE approaches have sought

to augment the injured tissue with cells, scaffolds, bioactive agents and mechanical stimulation to stimulate an efficient natural healing response. Furthermore, due to the particular nature of tendon as mechano-responsive tissues and the limited cell and vascular density in these tissues to promote healing, the key tools for restoring full tendon functions following injury will likely include an optimal combination of these agents.

One of the major difficulties of recreating tendon functional substitutes is to mimic the highly organized matrix that is the main responsible for its mechanical functions. Thus, the biomaterial fabrication technology should envision the development of biomimetic scaffolds for supporting and directing cell behavior, as well as for accommodating other predefined and reproducible features enabling multiple functionalities to synergistically enhance tissue regeneration capacity. Hence, the scaffold should guide cell proliferation and differentiation towards a specialized type of cells, -the tenocyte-, via physical and biochemical cues similar to the ones available in native cellular environment. The differentiation into tendon cells will allow the cells to respond to the surrounding milieu similarly to tenocytes in native tendons. To achieve this, TTE constructs should be able to simultaneously induce cellular alignment and orientation, and respond accordingly towards stimulated environments. Because tendons are mechano-responsive tissues dynamic loading modulates the synthesis and remodeling of the extracellular matrix (ECM) through mechanotransduction signaling, promoting biochemical signals responsible for cell proliferation, differentiation, and ECM synthesis [13].

It is widely accepted that environmental biochemical factors such as growth factors (GFs) are potential agents to target specific tissue responses because of their regulatory roles in cellular functions, including adhesion, proliferation, migration, matrix synthesis, and cell differentiation [14, 15]. Due to GFs role in cellular behavior, they have been attracting attention in tendon studies as a mean to promote tenogenic differentiation and ultimately guide injured tissue regeneration.

Nanotechnology has also arised instrumental as a tool for future developments in tissue engineering and regenerative medicine (TERM) approaches. In particular, there is an increasing interest regarding the use of magnetic nanoparticles (MNPs) in TE and regenerative medicine applications, which will be further debated in this review. MNPs may enable multifunctionality of TE strategies, as they can be used in the tagging, tracking and activation of stem cells both *in vitro* and *in vivo* [16].

In this review, we discuss recent efforts to identify topographical, mechanical and chemical factors in guiding stem cells to recapitulate critical aspects of tenogenesis and discuss the role these works may have in the future of TTE.

2.2. Biological structure and ECM role, mechanical demands and bioactive molecules in tendon tissue

2.2.1. Tendon development and physiology

Healthy tendons are strong bands of brilliant white and fibroelastic tissue, connecting muscles to bones, allowing the anatomic alignment of the skeleton and transmission of tensile forces with great resistance to applied mechanical loads [17], while providing connective flexibility, which permit body locomotion and joint stability/movement [2, 18].

Distinctly to other skeletal tissues as bone, muscle or cartilage, tendon morphogenesis is less known and understood. Human tendons appear in the mesenchyme of the limb bud at 6 to 8 weeks of fetal life and join with the muscles originated from the somites. Embryonic tendons are highly cellular and begin with a sparse, disorganized collagen fiber matrix. Throughout growth to adulthood, as matrix elements are synthesized [19], mature functional tendons become highly aligned structurally closely packed and composed of collagen fibers that are integral to its uniquely high tensile strength and function [20], Collagen fibers are arranged in bundles and primarily aligned in the direction of the long axis of the tendon. Bundles of fibers form tendon fascicles which, in turn, bound together by a thin layer of connective tissue named endotenon. Several fiber bundles and endotenon are encompassed by the epitenon, which is covered by another layer of connective tissue named paratenon. Together, the paratenon and the epitenon are called peritenon [2]. Besides the dense network of collagen type I fibers, the tendons are comprised of few blood vessels, a limited number of spindle-shaped cells (tenoblasts/tenocytes) found in rows between the collagen bundles, and water. Of the tendon dry weight, 60-85% is collagen, predominantly collagen type I (±60%) arranged in tensile-resistant fibers [21]. Collagen type III copolymerizes with collagen I, and despite its low amount in the ECM, it provides tissue elasticity and is essential for normal fibrilogenesis. Besides collagen, tendons also contain proteoglycans, among them aggrecan and decorin (DCN), and other glycoproteins, such as collagen oligomeric matrix protein (COMP), lubricin and tenascin-C. The primary role of proteoglycans and glycoproteins is to guide and maintain the organization of fibrous connective tissue. Aggrecan holds water and resists

compression while DCN, the most abundant tendon proteoglycan, facilitates fibrillar slippage, and regulates tendon structure by stabilizing and aligning collagen fibrils [22, 23], having a mediator role in the process of collagen fibrillogenesis and is involved in the development of a tendon-like fibril profile. COMP is involved in the repair and remodeling processes of the tendon [24, 25], as collagen III, and is particularly synthesized in response to, and to resist to loading [26]. Lubricin, another glycoprotein found in tendon matrix, enables sliding and recoil between adjacent fascicles playing an important role in interfascicular lubrication [27] and tendon gliding function [28]. Tenascin-C is an ECM protein present in developing and mature tendons [29] and the transcription of its gene is directly associated to mechanical loading [30-32]. The relaxation of the cytoskeleton was found to suppresses the induction of the tenascin-C gene by cyclic stretch [30]. Also, scleraxis (Scx) has been described as a specific molecular marker of tendon and ligaments [33], a basic helix-loop-helix (bHLH) transcription factor which regulates tendon formation and collagen type I, decorin, fibromodulin, and tenomodulin in tendon ECM.

2.2.2. Tendon ECM: a critical participant in tendon functionality

Tendons functional capacity is directly related to the composition of this unique ECM arranged in a highly organized hierarchy of parallel, crosslinked collagen fibrils assembled from nano to macro structures. This organization allows tendon to handle high unidirectional tensile loads and regulates cell behavior [34] (Figure 2-1).

Tenocytes are responsible to maintain tendon homeostasis [35] and in response to mechanical loads initiate controlled responses that particularly result in the production of biomolecules, mainly glycosaminoglycans and glycoproteins that play specific yet interactive roles in tendon adaptation to the tensile forces applied.

Tenascin-C, fibronectin, and elastin enhance mechanical stability allowing tendons to return to their pre-stretched lengths after physiological loading [35]. Additionally, elastin (2% of the dry weight) forms cross-linked sheets permitting the tissue to stretch and coil committing recovery after stimulus. ECM reorganization generates functional tissue with enhanced mechanical properties to withstand loading [13]. The direction of loading, intensity, frequency and type of stimuli also influences the synthesis and organization of the ECM niche.

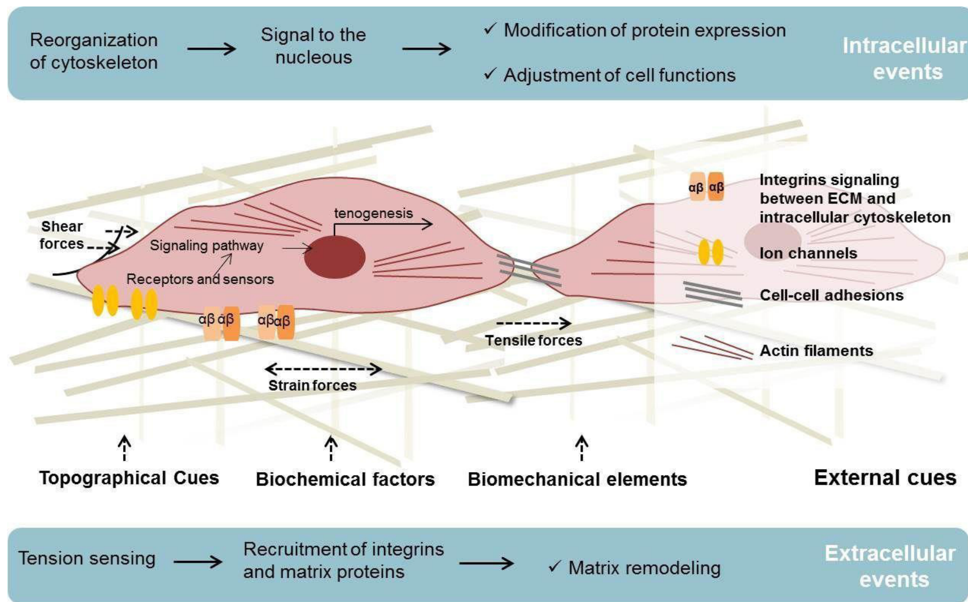

Figure 2-1. Schematic representation of cell-ECM interactions. Cell responses via the application of external cues from the extracellular environment, regulates a series of intracellular signalings achieving tenogenic differentiation.

2.2.3. Mechanical behavior of tendons

Tendon fiber patterns and viscoelastic characteristics contribute to their unique mechanical behavior [35] when subjected to dynamic mechanical forces, making tendon tissues highly efficient in response to constant loadings of variable transmittal forces in daily routine activities. Collagens and proteoglycans are primarily responsible for the viscoelasticity, while the collagen fibers resist tension forces to the tissue. Structural integrity and normal mechanical function of the tendon depends on precise alignment of collagen type I fibrils to confer longitudinal deformation and a high tensile strength to tendons but also on proteoglycans that regulate collagen fibrilogenesis and thus, indirectly, tendon function [23]. Proteoglycans such as aggrecan and versican, members of large modular proteoglycans or lecticans, provide tendon tissues with a high capacity to resist high compressive and tensile forces associated with loading and mobilization [23]. As distinct tendons have different functions by responding to different loading stresses, they differ in matrix composition and mechanical properties related to their location in the body. So, it is not surprisingly that tendons exhibit strain rate values according to the anatomical distribution. The Achilles tendon is the thickest and the strongest tendon in the human body, as it sustains the body weight and has a major impact in postural orientation. With approximately 15 cm long,

human Achilles experiences higher *in vivo* stresses than any other tendons, with a failure load is 5098 ± 1199 N and a modulus value of 819 to 870 MPa [36, 37]. In contrast, the dynamic modulus of the supraspinatus was found to be the stiffest (17.3 ± 8.9 MPa), of the rotator cuff tendons followed by the subscapularis (15.2 ± 6.9 MPa) and infraspinatus (12.4 ± 8.2 MPa) [38, 39]. These tendons are active participants in shoulder movement and in maintaining the shoulder joint (glenohumeral) stability. Load bearing tendons such as patellar tendon (PT) that connects the patella to the tibia and is involved in the ability of the knee to flex and extend, showed values of 3,855 ± 550 N (max. stress 40.6 MPa) [40] and modulus values were found between 300 and 660 MPa [41, 42].

Despite the similar location of flexor and extensor tendons of the hand, their specific function influences the pressure these tendons withstand. Human flexor digitorum profundus present elastic modulus values of 1000 MPa while human extensor digitorum longus exhibit 1600 MPa values [42].

Independently of the anatomical location, tendons are mechano-responsive tissues that require specific stimulation to be healthy and thus maintain their intricate functionality. Among the various types of mechanical stimulation, tensile loading seems to be the most appropriate for subjecting constructs aimed at tendon approaches as native tendon's primary function is to resist and transmit tension forces caused by muscle contractions.

2.2.4. Bioactive molecules

Unraveling the regulation mechanisms of tendon tissue differentiation remains a critical question required to develop efficient therapies [43]. Some studies propose the use of embryonic signalling factors as cues to promote tendon regeneration [20, 44, 45]. These environmental biomolecules that are present during embryonic tendon formation will induce a developmental response from progenitor cells guiding them to regenerate new functional tissue [46]. Although the limited knowledge on the precise mechanisms of action of signaling molecules numerous GFs are described to participate in both tendon development and healing. These biomolecules powerfully regulate the cell response; their exogenous addition can further stimulate stem cells into the tenogenic lineage. Among GFs studied, FGF4 (fibroblastic growth factor) and TGF-β2 (transforming growth factor beta 2) appear to be important tenogenic factors [44] upregulating tendon progenitor marker Scx expression in tendon mesenchymal progenitor cells. Moreover, TGF-β signaling was reported to be a potent inducer of Scx [33, 47, 48] and its disruption or receptor inactivation resulted in the loss of most tendons and ligaments in the limbs,

trunk, tail and head [48] of mice embryos. Interestingly, after short-term exposure to bone morphogenetic proteins (BMP), as BMP4, BMP2 or BMP7, scleraxis expression was shown to be downregulated in manipulated limbs [33]. Although TGF-β1 wasn't detected in tendon on days 13-16, TGF-β2 and -β3 were present in patterns that were dynamic with time, and distinct from one another, suggesting independent roles of TGF-β isoforms in tendon development [45]. Not only are the TGF-β involved in tenogenesis as growth and differentiation factors (GDFs) -5, -6, and -7, were described to induce neotendon/ligament formation when implanted at ectopic sites in a rat model [49] highlighting the critical role of TGF-β superfamily in tendon strategies. Despite the knowledge gathered in the last few years, the use of GFs in clinical tendon repair remains challenging. Little is known about the synergistic and antagonist interactions, or the optimal spatial and temporal distribution of GFs that could produce optimal results [50]. Nevertheless, a few clinical studies report the application of cocktails of GFs, mainly on the form of platelet rich plasma or platelet lysates to stimulate tendon regeneration [51-55].

2.3. Integrated approaches towards tendon repair

Tendon tissue engineering strategies often address the challenge to explore stem cell commitment towards the tenogenic lineage, which can be induced through the provision of cellular cues and stimulation. Therefore, biological [56-58] and mechanical stimuli [59-61] have been employed for the differentiation of stem cells towards tendon cells. TE protocols are well defined and established for investigating skeletal tissues, as bone or cartilage. A very good example is the standardization of differentiation culture media for bone and cartilage supplemented with regulatory factors that became part of the routine in worldwide cell culture labs. These include TGF-β for chondrogenic differentiation, and dexamethasone, ascorbic acid and β-glycerophosphate for inducing the osteogenic phenotype. However, concerning tendon approaches this basic information remains elusive. Studies on differentiation cocktails are dispersed, and the outcomes are not requiring additional validation before obtaining the consensus of the scientific community. Moreover, the fact that tendon specific markers are also not fully established is another critical factor resulting in debatable and apprehensible outcomes. A more detailed description on potential tenogenic differentiation culture media will be discussed in section "Stem Cells and Their Role in Tendon Tissue Engineering". Thus, considerable challenges remain to be addressed regarding the innate complexity of natural tendon tissue and the limited research in this area.

The necessary steps in developing functional TE substitutes include: cells which can maintain good proliferation and differentiation toward tenogenic lineage; a scaffold which can provide a three-dimensional environment for cell growth; and appropriate mechanical and/or chemical stimulation providing appropriate behavioral instructions for stimulating the new tissue formation. Since 1990's, a significant expansion and maturation of the TERM field has occurred, translated into promising tissue-engineered and cell-based products. Nevertheless, TERM continues to grow and designing innovative multifunctional biomaterials with complex features and controlled biological activity that more closely recapitulate tendon morphogenesis. Firstly, by developing biomimetic materials recreating the delicate microenvironment of tendons, in which mechanical properties and efficient gliding are pivotal elements to completely restore the damaged tissue. Second, efforts on engineering smart surfaces with which cells interact and simultaneously being stimuli-responsive structures are key functionalities to evoke targeted biological responses, enhancing better tissue regeneration capacity. Based on their biomechanical demands, native geometry and anatomical properties, tendon regenerative investigation may enable the orchestration of coordinated strategies that would nourish tenogenic achievement.

2.3.1. Stem cells and their role in tendon tissue engineering

The human body presents a number of endogenous mechanisms for the repair and regeneration of damaged tissues in which stem cells play a major role.

The discovery of tendon stem cells with regenerative capabilities [62] opened new possibilities to treat injured tendon tissues. In an attempt to answer if tendon derived cells (TDCs) possess multiple differentiation capacities, Yanming Bi and coworkers [62] investigated the commonly used criteria that define stem cells (clonogenicity, multipotency and self-renewal) in adult tendon cells and pursued to identify critical components of the tendon stem-cell niche. Outcomes revealed that the isolated cell population from both human and mouse tendons possess clonogenicity, self-renewal and multidifferentiation potentials. Also, tendon stem/progenitor cells (TSPCs) could regenerate tendon-like tissues after extended expansion *in vitro* and transplantation *in vivo*, residing in a unique niche predominantly comprised of an ECM being mainly organized by two proteoglycans, biglycan (Bgn) and fibromodulin (Fmod). The depletion of these molecules affected the differentiation of TSPCs by modulating BMP signaling and impaired tendon formation *in vivo*. On completion, an ECM-rich niche may control the self-renewal and differentiation of TSPCs and its alteration in terms of composition leads

to tendon malformation and pathologic ossification [62]. This is in agreement that ECM directly influences the proliferation and differentiation of stem cells, playing an important role in stem cell niche [34]. Although stem/progenitor tendon cells have not been fully explored for TTE applications, their application in regenerative approaches is limited by the associated morbidity of healthy tendon tissue, where these cells are harvested from. Therefore, finding the optimal conditions that will induce other sources of stem cells, with less detrimental effects in healthy tissues, such as the adipose tissue, into tendon forming cells is crucial for the development of stem cell based tendon regeneration. To date, these conditions have not been established, but several studies suggest [63, 64] that it may require (i) biochemical signals, (ii) mechanical signals or (iii) an adequate combination of both provided by either the culturing environment and/or scaffold material. Moreover, tendon associated markers are barely explored, and difficult to establish beyond developmental biology. Nevertheless, scleraxis [33, 65, 66], tenascin-C [31, 66], decorin [31, 67] and tenomodulin [66, 68-70] are regarded as generally accepted tendon specific markers.

Moreover, and despite the effort, no differentiation media with specific supplementation towards the tenogenic lineage has been standardized or established but some attempts are being carried out [56, 63]. Combining the exogenous addition of GFs to the cellular microenvironment could provide a trigger to assist the differentiation of multipotent cells into a tenogenic lineage, enhancing the expression of tendon related markers and the synthesis of tendon-like ECM [63]. Recent advances in basic biology have shown the enormous potential of stem cell technologies for therapeutic approaches in the clinics [71]. The application of stem cells in tendon regenerative strategies is of key importance to restore tissue functionality as these cells stimulate the repair, maintain homeostasis and, in some cases, reduce the inflammation process. Cell based therapies have been employed in veterinary medicine specifically by direct cell applications for the treatment of equine tendon injuries [72-74].

2.3.2. Tendon tissue engineering strategies with and without exogenous scaffolds

In order to meet the tendon requirements from a cellular, physical and biomechanical point of view, strategies are being developed where biomimetic concepts are being investigated as well as the cellular response to environmental cues (Table 2-1).

Being highly organized and mechanically responsive tissues, the envisioned biomimetic systems must induce cellular alignment and orientation, and simultaneously respond in a precise manner to defined

environment stimuli, considering the anatomical region and type of tendon to regenerate. Hypothesizing that tensile stimulation in combination with a biomimetic alignment would direct human mesenchymal stem cells (hMSCs) differentiation towards a ligament fibroblast-like phenotype, Subramony and coworkers [59] developed an aligned nanofiber matrix seeded with hMSCs and cultured into a custom high-throughput bioreactor which applied uniaxial tensile strain. The importance of coupled effects was verified, as unaligned constructs cultured with mechanical loading only modulate cell attachment morphology without subsequent induction of cell differentiation.

Also, the structural characteristics of the connective tissue, such as collagen fibril diameter and organization are crucial design parameters for biomimetic scaffolds intended for tendon or ligament regeneration [75]. A study on cell–material interactions was carried out by Erisken *et al* [75] which focused on scaffold fiber diameter effect on the response of human tendon cells. PLGA (Poly(lactic-co-glycolic acid) scaffolds with different fiber diameters were produced by electrospinning and used to test the response of human rotator cuff tendon fibroblasts. Cells were significantly more aligned on scaffolds with larger fibers (1.80 μm) and could more readily spread on nanofiber scaffolds as compared to microfiber ones due to smaller depth of grooves with a higher fiber density. Also, fibroblasts synthesized more collagen and proteoglycans on scaffolds with a smaller fiber diameter (320 nm). They hypothesized that the scaffolds with a smaller fiber diameter resemble a matrix in the state of injury and the cells, therefore, initiated a healing response by producing more matrix components. While in scaffolds with larger fibers likely approximate the fiber diameter of a healthy tendon matrix, the cells would, therefore, minimize excess matrix deposition [75]. Moreover, the largest fiber diameter scaffolds favored the formation of a more tendon-like matrix. Therefore, it is probable that the interaction between microfiber scaffolds and fibroblasts more closely resembles native environment, better mimicking the collagen fibers of the native tendon.

In order to achieve a multiscale architecture evolving to meet the biomechanical, biochemical and topographical requirements, favorable topographical cues with either biochemical cues (e.g. growth factors) or mechanically forces would reinforce the performance of constructs in successful TTE strategies. The importance of topographical control and incorporation of GFs in biomaterials for stem cell proliferation and differentiation was demonstrated in the work of Cheng and colleagues [76], which focused on the development of PDGF-containing PLGA-m-PEG nanoparticles inserted into aligned collagen fiber scaffolds. This combination promoted the tenogenic differentiation of ADSCs from two

different cues, topographical and chemical [76]. Likewise, the development of a mechano-variant platform which created localized regions of tenogenic cell differentiation mediated through the combination of substrate mechanics and ligand gradients (fibronectin and collagen type I, as ECM representatives), was reported by Sharma *et al* [77].

Although scaffold mechanics and ECM cues are important factors to be considered when designing a biomimetic system, alternative scaffold-free approaches have been also investigated. A bioengineered strategy using sheets of hMSCs to revitalize cryopreserved tendon grafts was studied by Ouyang and colleagues [78]. The hMSCs sheets were well-incorporated within the peritenon, around the tendon, and adopted the characteristic spindle-shaped morphology of tenocyte like cells. Another study reported neo-tendon formation in a nude mouse model using an engineered tendon composed by tendon derived stem cells and connective tissue growth factor without exogenous scaffolds [79]. Cell-sheet approaches also resulted in the development of a functional periosteal progenitor cell (PPC) sheets, using polymerized fibrin-coated dishes for osteo-tendon interface [80]. The bioengineered PPC sheets were evaluated in an extra-articular bone tunnel model in rabbit, aiming at enhancing tendon-bone healing. The results demonstrated improvement of healing by applying the PPC sheet at the tendon-bone interface. It was also observed an increased amount of glycosaminoglycans and perpendicular collagen fibers at the insertion as well as fibrocartilage tissue that was formed by PPC sheet between the tendon and bone 4 weeks post-implantation. After 8 weeks, the PPC sheet treatment revealed fibrocartilage and new bone regeneration. The PPC sheet showed to possess a powerful inductive ability between tendon and bone to incorporate healing, as the periosteal progenitor cell monolayer maintains the differentiated capacity and the osteochondral potential in order to promote fibrocartilage formation [80]. Also, these studies evidenced that cell sheets may be an interesting alternative approach for engineering tendon and tendon interfaces.

2.3.3. Mechanotransduction and mechanical stimuli in tendon tissue engineering

It is known that loading modulates ECM synthesis and tissue remodeling through mechanotransduction mechanisms, promoting biochemical signals responsible for cell proliferation, differentiation, and ECM synthesis [13]. In recent years, mechanical cues are being successfully employed to promote stem cell differentiation and functional tissue formation in musculoskeletal engineered strategies. The ability of cells to sense and respond to their mechanical environment is critical

for cell and tissue rearrangement. As neotissue forms in TE constructs, mechanical loading modulates ECM synthesis and remodeling through mechanotransduction. Mechanotransduction defines cellular mechanisms that convert mechanical stimuli into biochemical signals responsible for cell proliferation, differentiation, and ECM synthesis [13]. Mechanical stimuli in the extracellular environment activate mechano-sensitive receptors and sensors at the surface of the cells, namely ion channels, caveolae, integrins, cadherins, growth factor receptors, and others that have a contribution in the mechanotransduction response by triggering intracellular signaling mechanisms (Figure 2-1). The major cell-cell and cell-ECM sites of mechanotransduction may be integrin-dependent. Integrins are adhesion receptor proteins and transmembrane components involved in cells sensing and response to external forces of their surroundings, therefore establish the connection and communication between the extracellular environment to the intracellular cytoskeleton (Figure 2-1). The activation of signaling events result in cytoskeletal reorganization, cell shape, mechanotransduction, and ultimately tenogenic differentiation. In this sense, tenogenic differentiation might be achieved by activating the mitogen-activated protein kinase (MAPK) signaling pathway [81]. Moreover, a recent study by Havis and coworkers demonstrated that TGF-β and MAPK are two important signalling pathways involved in tendon gene expression in late limb explants during tendon differentiation [82]. Also, RhoA/ROCK (small G-protein) and focal adhesion kinase (FAK) are signaling events that sense mechanical stretching and drives tenogenic differentiation [61].

Since tendon cells use force as a mechanical signal to sense and respond to the surrounding microenvironment, the application of mechanical stimuli becomes imperative in TTE approaches. In a recent study, Yuan Xu and coworkers [31] assessed the efficacy of tendon derived stem cells (TDSCs) in a poly(L-lactide-co-ε-caprolactone)/collagen (P(LLA-CL)/Col) scaffold under mechanical stimulation both *in vitro* and *in vivo*, and evaluated the efficacy of the transplanted construct to promote rabbit patellar tendon defect regeneration. TDSCs upregulated the expression of tenogenic differentiation-related mRNA while down-regulated the expression of osteogenic and chondrogenic differentiation-related mRNA under a mechanical traction stimulation system.

As tensile strain is one of the major mechanical stimulus that tendons are subjected to, an investigation performed by Bayer and colleagues [83] proposed to observe the response of human tendon cells to release of tension. The loss of tension leads to a decrease in phenotypical markers for

tendon, normal tendon architecture was disturbed and expression of pro-inflammatory mediators was induced.

In addition, the mechanical parameters must also be considered when designing a material support for cells. An *in situ* cell–matrix strains study was carried out by Duncan and colleagues [84] in which they applied tension in tendon fascicles and contrasted with cell–collagen gel constructs. This work suggests that the collagen type I gel strings did not replicate the non-uniform strain pattern of native tendon fascicles. Houssen *et al* [85] continuously monitor tendon microstructure during multiple loading cycles, stating that mechanical preconditioning mostly arises from sliding and is reversible on long time-scales, and thus, supporting the notion that the mechanical behavior of a tendon fascicle relies on its microstructure remodeling. Loading was also verified to act on tendon gene expression. Legerlotz *et al* [86] showed significantly higher expression of COL1A1 and IL6 in fatigue-loaded specimens of isolated tendon fascicles compared with controls.

In summary, all studies using stimulation highlight the requirement for mechanical loading of tendon substitutes in order to maintain normal tendon structure and biochemical phenotype.

Table 2-1. Summary of *in vitro* and *in vivo* studies on TTE approaches, from 2010.

Material **Approach**	**- Cells** **- Culture method**	**Main outcomes**	**Ref.**
-PLGA-m-PEG NPs -Collagen-NPs composite fibers loaded with PDGF Topographical (aligned collagen fiber) and chemical (PDGF release) cues investigation on tenogenic differentiation of ADSCs.	ADSCs Static *In vitro*	Controlled and sustained release of PDGF from biocompatible PDGF-containing collagen–NP fibers that enhanced the proliferation and promoted the tenogenic differentiation of ADSCs from two different cues.	[76]
-PHBHHx tubes -PHBHHx fibers -PHBHHx/collagen gel hybrids Collagen gels reinforced by polymer fibers inserted into the	Rat tenocytes *In vivo* rat Achilles tendon repair model	PHBHHx in conjunction with collagen and tenocytes showed an accelerated return of function when compared to control, with no prolonged immunological or inflammatory response in the rat model, over 40 days. Cells migrate into the PHBHHx-collagen	[96]

lumen of scaffold tubes for implantation to repair an induced Achilles tendon defect.		core and become organized along the fibre surface within the PHBHHx fibre-collagen core *in vivo*.	
-Collagen ELAC threads, mimicking native tendon, used to study the effect of collagen orientation on cell morphology, proliferation and tenogenic differentiation.	hMSCs Static culture	The anisotropically oriented dense collagen matrix (substrate densely packed and uniformly aligned) promoted tenogenic differentiation of hMSCs, even in the absence of bioinductive cues.	[97]
-Silk fibroin (SF) Investigation of a synergistic effect of 3D mechanical conditioning and aligned contact guidance.	MSCs Static and dynamic culture	Hybrid SF scaffold showed favorable topographical and surface chemistry for cellular alignment. High amounts of ligament-related proteins indicated ligament fibroblast differentiation.	[98]
-RGD-coupled alginate Co-delivery system based on TGF-β3-loaded RGD-coupled alginate microspheres encapsulating periodontal ligament and gingival stem cells.	Human PDLSCs, GMSCs and hBMMSCs *In vitro* and *in vivo* (subcutaneoustransplant intoimmunocompromised mice)	The MSC and alginate constructs were shown to effectively differentiate and organize their ECM into tendon tissue. Important role of the microenvironment as well as of inductive signals (TGF-β3) for the viability and differentiation of dental MSCs into tendon-like tissue.	[99]
-Poly(l-lactic acid) Aligned braided bundles of electrospun PLLA nanofibers capable of supporting both stem cell expansion and tenogenic differentiation.	hMSCs Cyclic tensile strain	The mechanical properties of braided nanofibrous scaffolds mimicked the tri-phasic mechanical behavior of native T/L when loaded. Seeded with hMSCs, the scaffolds were able to support cell attachment, proliferation, and tenogenic differentiation when mechanically stimulated in a bioreactor.	[100]
-Poly(lactide-co-glycolide) Investigation on the role of nanofiber matrix alignment and mechanical stimulation on MSC differentiation.	hMSCs Bioreactor (uniaxial tensile strain)	MSCs differentiate into ligament fibroblast-like cells when cultured on aligned nanofibers and subjected to tensile loading. On unaligned nanofibers, mechanical loading only modulates cell attachment	[59]

		morphology without subsequent induction of fibroblastic differentiation.	
-Poly(e-caprolactone) Multilayered electrospun scaffolds coated with tendon-derived ECM, potential for rotator cuff TE.	hASCs Static culture	TDM-coated scaffolds showed increased levels of collagen accumulation by hASCs as compared to FN- or PBS-coated scaffolds. Gene expression of type I collagen, decorin, and tenascin-C increased over time, with no effect of scaffold coating, as well as sulfated glycosaminoglycan and dsDNA.	[101]
-Silk fibers and type I collagen Knitted silk-collagen sponge scaffold-hESC-derived mesenchymal stem cells construct in promoting tendon regeneration.	hESCs-MSCs *In vitro* dynamic mechanical stress and *in vivo* mechanical loading	hESCs-MSCs differentiated into the tenogenic lineage, evaluated both *in vitro* and in a rat Achilles tendon injury model. In ectopic transplantation, the TE tendon under mechanical stimulus displayed more regularly aligned cells and larger collagen fibers. Dynamic mechanical stimulation is beneficial to TE tendons.	[102]
-Poly(ethylene glycol)-based hydrogel material oligo(poly(ethylene glycol) fumarate) OPF-hydrogels as hMSCs carriers under cyclic tensile strain bioreactor in promoting tendon/ligament fibroblast phenotype.	hMSCs Custom tensile culture bioreactor	Cyclic strain significantly upregulated all tendon/ligament fibroblastic genes examined by day 21, whereas genes for other pathways (osteogenic, chondrogenic and adipogenic) did not increase. Novel system in examining cellular differentiation under tensile loading in response to controlled physicochemical changes in the extracellular environment.	[103]
-Acrylamide (4-12%) and bisacrylamide (3.8-5%) gels Substrate system with a gradient of stiffness, mechanical compliances and ligand chemistry, creating local regions	hBMSCs Static culture	Increased osteogenic fate was observed on FN substrates and tenogenic differentiation observed on collagen substrates; and these responses further depended on the stiffness of the substrate. Mechanovariant substrates offer a platform to engineer cell differentiation into targeted lineages without the necessary use of exogenous	[77]

of tenogenic differentiation on a single substrate.		biochemical stimulation apart from cell culture growth medium.	

PLGA-m-PEG: Poly(lactic-co-glycolic acid)-monomethoxy-poly(ethylene glycol); **NPs:** nanoparticles; **PDGF:** platelet-derived growth factor; **ADSCs:** adipose-derived stem cells; **PHBHHx:** poly(3-hydroxybutyrate-co-3-hydroxyhexanoate); **hMSCs:** human mesenchymal stem cells; **ELAC:** electrochemically aligned collagen; **RGD:** arginine-glycine-aspartic acid tripeptide; **PDLSCs:** periodontal ligament stem cells; **GMSCs:** gingival mesenchymal stem cells; **hBMMSCs:** human bone marrow mesenchymal stem cells; **ECM:** extracellular matrix; **TGF-β:** transforming growth factor-beta; **hMSCs:** human mesenchymal stem cells; **PLLA:** poly(L-lactic acid); **T/L:** tendon and ligament; **hASCs:** human adipose stem cells; **TDM:** tendon derived matrix; **FN:** fibronectin; **PBS:** phosphate-buffered saline; **dsDNA:** double-stranded DNA; **TE:** tissue-engineered; **hESCs:** human embryonic stem cells; **OPF:** oligo(poly(ethylene glycol) fumarate; **hBMSCs:** human bone marrow stromal cells.

2.3.4. Mechanical stimulation using magnetic force fields

Recently a growing interest in another distinct field is emerging, resultant from the combination of nanotechnology and magnetic nanoparticles (MNPs) in tissue engineering strategies.

MNPs biomedical applications include (i) imaging contrast agents for magnetic resonance imaging (MRI), (ii) localization of therapy/targeted drug release, and more recently, (iii) utilization of nanoparticles for cell labeling/internalization and the development of micro-nano engineered scaffolds.

Furthermore, the idea of using magnetic fields to remotely control magnetic responsive systems has recently been examined in general TE field with likewise high potential for tendon approaches, including (i) 3D magnetic scaffolds which can provide unique capabilities not available with other methods and materials [87]; (ii) magnetic hydrogels with a magnetic component embedded in the matrix [88]; and (iii) assembling of magnetically-labeled cells into larger tissue prototypes, through magnetic force appliance.

An example of an *in vitro* loading system using an applied magnetic field to produce time varying loading in rat tail tendon fascicles was investigated by Adekanmbi *et al* [89]. The effect of high frequency low magnitude (HFLM) loads on tendon mechanobiology was studied [89]. HFLM *in vitro* loading was found to maintain the elastic moduli of cultured tendon fascicles after 7 days, while the moduli of unloaded tendons decreased two-fold. HFLM loaded tendon fascicles showed higher tensile strength than unloaded and fresh fascicles, and differences between groups were not statistically significant. Further, although HFLM loaded tendons displayed higher modulus and tensile strength than fresh controls, static loaded tendons did not, suggesting that HFLM loading provides greater stimulation.

Furthermore, the incorporation of MNPs within 3D scaffolds or hydrogels may constitute an attractive approach to achieve magnetically responsive systems enabling remote control over TE constructs *in vitro*

and *in vivo* [90]. The functionalization of inert scaffolds with MNPs influences the creation of magnetic forces capable of attracting and retaining cells within the material through a rather simplistic, fast and cost-effective approach [90].

In a previous work, we developed magnetic polymer scaffolds with aligned architecture aimed at applications in tendon tissue engineering (Figure 2-2). Aligned fibrous scaffolds were obtained from a biodegradable blend of 30 % starch and 70 % polycaprolactone (SPCL) by rapid prototyping through the incorporation of iron oxide particles in SPCL matrix in a 0.018:1 weight ratio. Tenascin C protein was detected by immunolocation assays in scaffolds seeded with human adipose stem cells (hASCs) for 7 days and exposed to a magnetic field, as shown in figure 2. Main results suggest that the effect of the magnetic aligned scaffolds structure combined with magnetic stimulation influences stem cell behavior towards the tenogenic phenotype, and consequently with potential to impact the field of TTE.

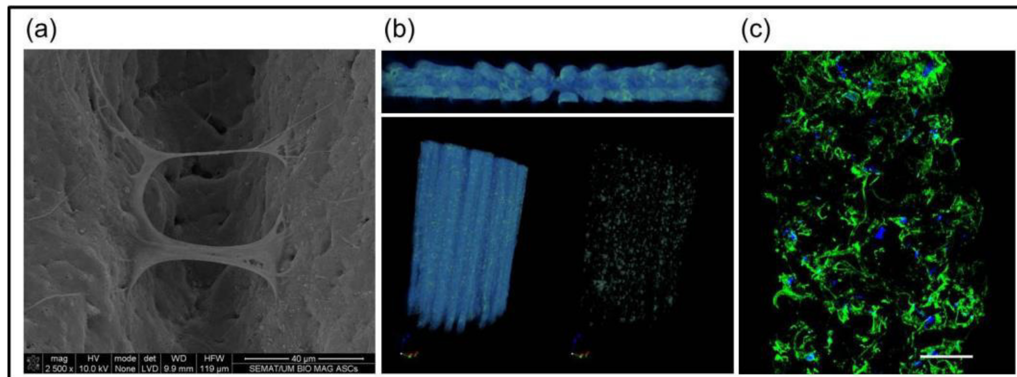

Figure 2-2. Magnetic polymer scaffolds developed by rapid prototyping technology. (a) SEM micrograph of an aligned magnetic scaffold cultured with hASCs in basal medium for 7 days under magnetic stimulation; (b) 3D representation of the developed magnetic scaffolds by micro-CT analysis. The polymeric matrix is represented in blue (SPCL) and MNPs are observed in grey; (c) Tenascin C immunolocation in hASCs-magSPCL constructs after 1 week under magnetic stimulation. DAPI (blue) stains cell nuclei and tenascin C (green) is a typical protein present in tendon ECM. Scale bar represents 100 μm.

The magnetic force can also contribute for a uniform distribution of the cells within the 3D template [90]. Various methods have been developed to fabricate magnetic hydrogels, including a blending method, an *in situ* precipitation method and the grafting-onto method [88]. Also, MNPs can be associated with stem cells presenting an advantage as cells naturally respond to magnetic forces, and consequently, the application of a magnetic field may enhance cell biological performance, and ultimately stimulate

cell proliferation, migration and differentiation. Moreover, MNPs have been used for controlled cell assembly through magnetic force appliance, in order to achieve 3D multilayer constructs [91].

In fact, magnetic responsive materials have already been investigated for *in vitro* and *in vivo* bone TE applications [92-94]. Another novel technique that makes use of magnetic force and functionalized MNPs is the magnetic forced-based tissue engineering (Mag-TE) methodology proposed by Ito *et al* [95] for production of magnetic cell sheets. Herein, cells are labeled with ferrous based nanoparticles being then manipulated using magnets. Up to date, no adequate system proposed to study the application of simultaneous topographical and magnetic stimuli on stem cells behavior towards the tenogenic phenotype, but all these magnetically based approaches can also be envisioned to engineer tendon substitutes.

2.4. Conclusions and future perspectives

Efforts to differentiate stem cells towards the highly specialized tendon cells have been challenged by paucity of tenogenic cues as well as a lack of benchmarks to assess tenogenic lineage commitment. Future directions on tendon tissue engineering (TTE) should conceive highly efficient and multifunctional tendon constructs fulfilling the currently unmet requirements for tendon regeneration.

An envisioned functionalized cell-scaffold construct would be stimuli-responsive where incorporated cells could be governed by their ability to respond to scaffold surface, sensing and responding to their surroundings, and guided into the tenogenic phenotype. Also, not less important is the function and characteristics of the ECM in cueing stem cell behavior. Beyond that, as load bearing tissues, mechano-signaling is a critical component in new tissue formation and ECM organization of engineered tissue substitutes, underlying the importance of bioreactors in TTE strategies. Despite the many described methodologies to fabricate tendon oriented scaffolds, a functional and translational TE construct is not available yet. Thus, novel approaches are required to fill the gap in tendon tissue replacement.

The ability to tailor the microenvironment at a nano- and micro-scale, thus spatially controlling the localization of cells and other biological factors, would assist on the development of tailored structures with precise architectures. The utilization of MNPs and application of magnetic stimulation are currently regarded as an advanced technique to drive tissue regeneration influencing new biological and engineering solutions. Thus, these magnetic strategies envision greater mechanical stimulation of the seeded cells, improved cell seeding and the means of assembling a scaffold in the desired configuration.

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Chapter 3

Bioreactors for tendon tissue engineering: challenging mechanical demands towards tendon regeneration

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3. BIOREACTORS FOR TENDON TISSUE ENGINEERING: CHALLENGING MECHANICAL DEMANDS TOWARDS TENDON REGENERATION

3.1. Introduction

Tendons are unique connective tissues with the vital role to store and return elastic energy, resist damage, provide mechanical feedback and amplify or attenuate muscle power and transmit forces from muscle to bone. Although tendon relevance in joint biomechanics and overall human body is often misunderstood and disregarded to other tissues such as bone or cartilage, in recent years a growing interest on tendon mechanical properties has highlighted potential studies towards improved therapeutic strategies in the orthopedic field.

The term "tendon" comes from the latin word tendere meaning to stretch [1]. Despite the high tensile strength, tendons have limited intrinsic healing capabilities. It is estimated that approximately 50% of all musculoskeletal injuries are tendon-related. Upon injury, tendon undergoes degeneration and morphohistological misalignment of the collagen fibres. Ultimately, severe damage will result in pain and disability. Thus, a major challenge in tissue engineering (TE) and regenerative medicine is to recreate tendon niche and replicate biomechanical forces involved in tendon functionality including stretching, loading, compression and torsion.

One potential approach to artificially generate the biomechanical demands of tendons is using complex advanced systems such as bioreactors. Bioreactors are designed considering the specific parameters of the replacing tissue or organ, especially in what concerns to tissue biomechanics and maintenance of a desired phenotype prior to implantation. Since the birth of TE in the early 90s, bioreactors have been primarily used for studying basic pathways, expand and grow tissue/organ substitutes, maintain *ex vivo* organ vitality and priming therapeutic cells before implantation. The use of suitable biomechanical and biophysical environments in which cells could synthesize a functional matrix results in a closer mimic of tendon tissue, leading to maturation of cell-laden constructs prior to implantation. This way, the utilization of bioreactors as *in vitro* models are expected to minimize the number of animal experiments as the implantation step only occurs when the morphological, biological and biomechanical properties of the engineered construct match those of the natural tissue.

In tendon scenario, bioreactors have been used to culture tendon engineered substitutes and to investigate suitable *in vitro* conditions for establishing benchmarks and protocols for effective cell

programming toward the tenogenic phenotype. Thus, bioreactors are promising tools for developing and culturing *in vitro* generated tendon substitutes, as potential alternatives to pharmacological therapies and to fulfill the current need for tissue substitutes to treat tendinopathies.

This chapter will outline state of the art TE strategies on cell culture or cell laden 3D matrices using mechanically active environments provided by bioreactor systems for tendon regeneration as a potential means to obtain functional tendon substitutes. For a better understanding on the performance of these systems and their role in strategies applied to tendon TE, the intrinsic properties and requirements of tendons will be explored with a particular emphasis on the role of biomechanical stimulation in tendon development and maturation as well as the biomechanics-tissue functionality relationship.

3.2. Tendon structure-function and mechanobiology behaviour

3.2.1. Role of mechanical stimulation in tendon development and functionality

Tendons are specialized connective tissues that serve to transmit forces between muscles and bones, and thus allow body motion. Their crucial role in musculoskeletal functionality implies distinct mechanical properties which are assured by tissue-specific structure and molecular composition, namely highly organized collagen fibres arranged parallel to the tendon axis. The smallest structural unit of tendon is fibril composed of collagen molecules assembled in quarter-staggered D-periodic pattern [2]. Fibrils form fibres, which group together to form fibre bundles or fascicles, enveloped by thin layer of connective tissue called endotenon. Fascicles bundles are in turn enclosed by another layer of loose connective tissue sheath, the epitenon, that provides vasculature, lymphatics and innervation to the tendon unit. Tendons may be eventually enveloped by aleoral sheath of paratenon that serves to reduce friction with adjacent tissues, thus enabling free tendon movement against its surroundings [2].

Tendon fibroblasts- tenocytes- are found longitudinally aligned in the rows between collagen fibres and are mainly responsible for synthesis and maintenance of the extracellular matrix (ECM). Interestingly, a population of cells with universal stem cell characteristics, named tendon stem/progenitor cells (TSPCs) has been identified in both human and murine tendons [3].

The major component of tendon is collagen type I that represents approximately 95% of the total collagen content and around 60% of the tissue dry mass [4]. Tendon-specific mechanical integrity and function is acquired via multistep process of collagen fibrillogenesis during tendon development [5-7]. In

the first stage, collagen molecules assemble in the extracellular space to form immature fibril intermediates. Fibril intermediates associate subsequently end-to-end forming longer and mechanically functional fibrils. The linear growth is then followed by lateral growth step, where fibrils associate laterally generating fully mature fibrils with larger diameters [7]. The process of fibril assembly is regulated by heterotypic interactions between fibril-forming and fibril-associated collagens, and fibril-associated proteoglycans. For example, interaction between two fibrillar collagens- type I and type III plays a role in initial fibril assembly and control of fibril diameters [8]. The ratio of collagen type III to collagen type I exerts spatial and temporal variations throughout tendon development and in the mature tendon collagen type III is present mainly in the endotenon and epitenon. Similarly, collagen type V may assemble with collagen type I and has been implicated in fibril nucleation and diameter regulation [9, 10]. Type XII and type XIV collagens which represent Fibril-Associated Collagens with Interrupted Triple-helices (FACITs) are localized near the surface of fibrils and may contribute to fibrillogenesis regulation by providing molecular bridges between collagen fibrils and other components of the ECM. Although their role in fibril assembly is not well understood yet, it was hypothesized that collagen type XII may stabilize fibril structure during tendon development [11], while type XIV limits fibril diameter [12]. Beside the collagen class, molecules that belong to small-leucine-rich proteoglycans (SLRPs) family, such as decorin, biglycan, fibromodulin and lumican are believed to actively regulate tendon fibrillogenesis, as their targeted disruption in mouse models lead to abnormal fibril phenotypes [13-15]. Interestingly, biglycan and fibromodulin have been recognized as critical components of tendon stem cell niche regulating TSPCs differentiation and tendon formation *in vivo* [3].

Noteworthy, the cellular composition and collagen organization are not uniform along the tendon length and demonstrate regional differences in the myotendinous junction, which is the interface between tendon and muscle and in the tendon to bone attachment site, called enthesis [16]. These molecular and cellular variations are translated in different mechanical properties of specific tendon regions that reflect nonhomogenous mechanical loading requirements in different anatomical sites [17].

The molecular mechanism governing the synthesis and spatial organization of collagen in developing tendons has not been fully elucidated. Since collagen is the main component of various connective tissues, it is impossible to trace tendon development by mapping its expression. In fact, no marker unique for tendon has been identified to date. The basic helix-loop-helix transcription factor scleraxis

(Scx) has been described as an early tendon marker, whereas a type II transmembrane glycoprotein tenomodulin (Tnmd) is considered a late tendon marker [18]. Though not specific to tendon, two other transcription factors are involved in tendon development, the homebox protein Mohawk (Mkx) and a member of zinc finger transcription factor family, early growth response factor 1 (Egr1). Mkx null mice presented wavy-tail phenotype and hypoplastic and less vibrant tendons throughout the body with reduced fibril diameters and down-regulation of type I collagen expression, when compared to the wild type (WT) counterparts. In addition to disruption of postnatal collagen fibrillogenesis, mutant mice exhibited abnormal tendon sheats [19, 20]. Similarly, Egr1 has been shown to positively regulate collagen transcription in postnatal tendons. Egr1-/- mutant mice demonstrated a deficiency in expression of Scx, Col1a1 and Col1a2 genes, reduced fibril diameter and packing density, resulting in mechanically weaker tendons, compared with their WT littermates [21, 22]. Interestingly, ectopic expression of either Mkx or Egr1 promoted tenogenic differentiation of mesenchymal stem cells via activation of TGF-β signalling pathway [22, 23].

Tendon embryogenesis has not been fully investigated and most of the data comes from developmental studies in invertebrates and chick and mouse models. The vertebrate tendons originate from mesoderm or mesectoderm, more specifically, the craniofacial tendons are derived from neural crest cells, axial tendons originate from syndeotome, whereas limb tendons come from limb lateral plate. Noteworthy, tendons share same embryological origins with cartilage and bone, but not with skeletal muscles, which originate from dermomyotome (axial), mesoderm (head), or somites (limb). Despite these distinctions, the development of various components of the musculoskeletal system progresses in their close spatial and temporal association. It has been demonstrated that depending on anatomic location, tendon development requires the presence of muscle. In chick somites surgical ablation of dermomyotome prior to myotome formation results in absence of Scx expression, indicating that muscle is required for initiation of development of axial tendons [24]. Similarly, in Myf5-/- ; MyoD-/- double mutant embryos Scx expression is undetectable in mouse somites what further supports that myotome specification is indispensable for axial tendon progenitor formation [25]. Contrarily, limb and head tendon development is initiated independently of muscle in mouse, chick and zebrafish embryos. Scx expression is induced normally in muscleless limbs of Pax3 mutant mice [26] and myod1-myf5 deficient zebrafish embryos, as well as in murine and zebrafish craniofacial tendons [27], however the absence of muscles results eventually in tendon development arrest and loss of Scx expression [26, 28]. Hence, muscle is

crucial for Scx induction in axial tendons, as well as for the maintenance of its expression in cranial and limb tendons. Since this pattern has been conserved across different species, it may indicate a requirement for mechanical forces provided by muscle during tendon morphogenesis. The two main signalling pathways identified as being involved in tendon development are TGFβ-SMAD2/3 and FGF-ERK/MAPK pathways [29]. FGF signalling from the myotome was firstly associated to induction of Scx-expressing tendon progenitors in adjacent somatic subcompartment of developing axial tendons in chick [24]. Disruption of TGFβ signalling in Tgfb2-/- and Tgfb3-/- double mutant embryos leads to the loss of most tendons and ligaments [30]. Since Scx expression is disrupted only at E12.5, it has been suggested that TGFβ is required for tendon progenitor maintenance. Interestingly, in pharmacologically immobilised chick embryos both FGF and TGFβ signalling cascades were downregulated, suggesting that FGF and TGFβ ligands regulate tendon differentiation acting downstream to mechanical forces present in developing embryo [31]. Additionally, growth differentiation factors (GDFs) that belong to the bone morphogenetic protein (BMP) family have been implicated in tendon development. GDF-5 deficient mice exhibited altered ultrastructure and composition, and inferior mechanical properties of Achilles tendon, when compared with control littermates [32]. Similarly, GDF-6 deficiency in mice was associated with reduction in tail tendon collagen content and compromised tail material properties [33]. Beside those mentioned so far, some other signalling pathways, such as highly conserved Wnt pathway or calcium signalling might be involved in tendon morphogenesis.

3.3. Biomechanics in homeostasis and tendinopathic tendons

Being subjected to dynamic mechanical forces *in vivo*, tendons exhibit a unique crimp pattern and viscoelastic properties akin to a spring that enable tendon to effectively store and subsequently release mechanical energy. In a typical tendon stress-strain curve, four different regions can be distinguished. The initial toe region, where tendon is strained up to 2%, corresponds to stretching-out of the characteristic crimp pattern. In the linear region of the curve where stretching does not exceed 4%, collagen fibres lose their crimp pattern and the slope of the linear region defines the Young's modulus of the tendon. Stretching over 4% results in microscopic tearing, whereas strain beyond 8-10% leads to macroscopic failure and tendon rupture [34]. Studies of force-length relationship revealed that with increasing forces, tendons lengthen to a certain degree (ascending limb)- after a certain point application of force results in tendon failure (descending limb) [35]. Viscoelastic properties of tendon are defined by

creep, that indicates increasing deformation under constant load, stress relaxation upon deformation, as well as hysteresis, or energy dissipation, which implies that an amount of energy is lost during loading, consequently the loading and unloading curves look differently. Mechanical properties vary depending on tendon anatomical site and specific function, and are therefore dictated by the level of mechanical load to which particular tendon is subjected [36]. These mechanical forces placed on tendon are in turn determined by type of activity, passive or active mobilization, joint position, level of muscle contraction, tendon relative size, etc. Additionally, variations in the rate and frequency of mechanical loading would result in different tendon forces [34].

3.3.1. Loading and overuse

Tendons are metabolically active tissues and tendon-resident fibroblasts respond to dynamic mechanical loading by alterations in synthesis of ECM components and matrix degrading enzymes. A growing body of evidence supports the key role of mechanical stress in promotion and maintenance of tendon-specific phenotype. While mechanical forces are essential for tendon development and homeostasis, both complete unloading, and contrarily excessive loading beyond physiological range might have detrimental effects on tendon functionality.

Hannafin *et al*. investigated the effect of stress deprivation and cyclic tensile loading on histological and mechanical characteristics of canine flexor digitorum profundus tendon [37]. Stress deprivation resulted in significant changes in cell morphology and number, collagen fibre alignment and progressive decrease in the tensile modulus over 8-week period. However, tendons subjected to cyclic tensile loading for 4 weeks demonstrated increased Young's modulus (93% of the control) when compared to stress-deprived tendons (68% of the control), as well as maintained normal histological patterns [37]. Surgical release of tensile strain in an engineered human tendon model resulted in disruption of tendon architecture, downregulation of tendon related markers and induction of pro-inflammatory mediators [38]. To determine if loss of tensile tension could induce apoptosis in tendon cells Egerbacher *et al*. cultured rat tail tendons for 24h under cyclic loading or stress-deprived conditions. Upregulated caspase-3 expression and increased number of apoptotic cells in stress-deprived tendons, when compared with the loaded group, indicated that loss of homeostatic tension induces programmed cell death [39]. Employing a transgenic mouse model, where GFP expression is driven by Scx promoter, Maeda *et al*. demonstrated that gradual and temporary loss of transmittal forces from skeletal muscles by application

of botulinum toxin A resulted in reversible loss of Scx expression and a decline in tendon mechanical properties. Acute loss of tensile loading by complete tendon transection led in turn to destabilization of ECM structure, excessive release of active TGF-β and massive tenocyte death [40].

Wang *et al*. investigated the effect of different mechanical stimulation regimes on rabbit Achilles tendon integrity in a bioreactor system [41]. In the absence of loading, gradual loss of collagen fibre organization, increased cellularity and cell roundness were observed, indicating a progressive divergence from the native tendon phenotype. Tendons stimulated with 3% cyclic tensile strain demonstrated moderate ECM disruption and elevated expression levels of matrix metalloproteinases (MMPs), MMP-1, -3 and -12, whereas excessive loading of 9% resulted in partial tendon ruptures. However, tendons stimulated with 6% cyclic tensile strain maintained their structural integrity and cellular function, what suggests that there is a narrow range of tensile loading promoting an anabolic effect and tendon tissue homeostasis [41]. In a follow up study, the model was extended to characterization of degenerative changes observed in tendon under loading-deprived conditions. When unloaded for 6 and 12 days, tendons exhibited progressive degenerative alterations, abnormal collagen type III production, increased cell apoptosis and impaired mechanical properties. However, application of 6% cyclic tensile strain at day 7 for another 6 days was able to reverse morphological degenerative changes and partially restore mechanical properties of the unloaded tendon to the levels characteristic for the healthy tissue [42]. Although mechanical stimulation is crucial for tendon-specific phenotype maintenance, excessive mechanical loading has been implicated as the major causative factor of tendon overuse injuries, collectively referred to as tendinopathies.

Histopathological presentation of painful tendons may comprise increased or decreased cellularity, cell rounding, increased vascularity and innervation, increased collagen type III expression and proteoglycans content, and collagen fibril disorganization. Molecular changes in tendinopathy include elevated expression of collagen type I and III, biglycan, fibromodulin, aggrecan, fibronectin, tenascin C and alterations in expression levels (both upregulation and downregulation) of MMPs and tissue inhibitor of metalloproteinases (TIMPs) that regulate ECM turnover [43, 44]. However, the etiology of tendon injuries has not been fully elucidated yet and especially the role of inflammation in tendon pathology and healing process remains the subject of debate and ongoing controversy [45-49]. Several factors have been postulated to be implicated in tendon disease occurrence, including age, gender, body weight,

vascular perfusion, nutrition, joint laxity, systemic diseases, muscle weakness, physical load, repetitive loading, abnormal movement, poor technique and training errors, fast progression and high intensity, environmental conditions, running surface, etc. Moreover, genetic predisposition (e.g. variants within COL5A1, tenascin C and MMP3 genes in Achilles tendinopathy), treatment with corticosteroids or fluoroquinolones, oral contraceptives uptake, as well as existing comorbidities such as obesity, diabetes or hyperlipidaemia have been proposed as risk factors in tendon pathology development, as reviewed in [49-52].

Soslowsy *et al* employed an intensive running regime for 4, 8 and 16 weeks to induce an overuse injury in a rat model. Compared to the control group, which was allowed normal cage activity, the supraspinatus tendons in the excercised animals demonstrated increased cellularity and collagen fibre disorganization, the features that are normally observed in human tendinopathy. The tendons from the running group exhibited enlarged cross-sectional area and decreased mechanical properties, when compared to the control group [53]. In an *in vitro* study by Thrope *et al* application of cyclic loading mimicking high intensity exercise resulted in matrix damage and cell rounding in equine superficial digital flexor tendon explants. Those morphological changes were accompanied by increased expression of inflammatory mediators and MMPs in the loaded samples, when compared to the control group [54]. Similar ECM damage and inflammatory response was observed in bovine flexor tendon overloading model [55].

Due to low cellularity, poor vascularization and innervation, tendon demonstrates restricted intrinsic healing capacity. Repaired tendon never regains the mechanical properties and hence full functionality of the pre-injured tissue- indeed, final tensile strength of healed tendon might be reduced by up to 30% [56] or not restored two years after surgical repair [57]. After an acute injury, tendon healing process normally follows a course of distinct yet overlapping stages of early inflammation, proliferation and remodeling, each orchestrated by a specific set of cellular and biochemical components. In the initial inflammatory phase, erythrocytes, platelets and inflammatory cells (e.g. neutrophils, monocytes, macrophages) infiltrate the wound site and release vasoactive and chemotactic agents to promote angiogenesis and fibroblasts recruitment. During the proliferative phase, tendon fibroblasts synthesize collagen and other ECM components leading to granulation tissue formation around the wound site. After 6-8 weeks, final remodeling phase commences. This stage is characterized by decreasing cellularity and reorganization of collagen architecture where collagen type III is replaced by collagen type I. As the scar

matures, covalent bonding between collagen fibres increases which leads to higher tendon stiffness and tensile strength, yet healed tendon never matches characteristics of intact tissue. During tendon healing upregulation of several growth factors and cytokines, such as insulin-like growth factor-1 (IGF-1), platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) stimulates cell migration, proliferation, angiogenesis and synthesis of collagen and other ECM components [48, 58].

Tendon stem/progenitor cells role in tendon homeostasis and disease is not well understood yet, however it was suggested that TSPC malfunction may contribute to impaired healing and repair, or tendon pathology development. Especially, age- related depletion of stem cell pool and/or a decline in stem cell function associated with entrance of senescence state might be implicated in pathology onset and progression. Mechanoresponse of TSPCs have been studied both *in vitro* and *in vivo* indicating critical role of mechanical loading in tendon stem cell fate and function. Mechanical loading at physiological level (4% stretching) promoted TSPCs proliferation and differentiation into tenocytes, whereas at excessive stretching (8%) TSPCs differentiated in non-tenocytes such as adipocytes, chondrocytes and osteocytes, in addition to tenocytes [59]. In a follow up *in vivo* study employing mouse treadmill running model it was found that tendons subjected to repetitive, strenuous mechanical loading produced high levels of PGE2, which in turn was associated with decreased proliferation of TSPCs and TSPC differentiation into adipocytes and osteocytes [60]. Such non-tenocyte differentiation of TSPCs under abnormal mechanical loading may explain some pathological features of late tendinopathy such as lipid accumulation, mucoid formation and tissue calcification.

Management of tendon injuries includes surgical procedures and nonsurgical modalities such as physiotherapy. Application of mechanical stimulation may be beneficial for the proper organization of collagen fibres and prevention of adhesion formation during tendon healing. In injured canine flexor tendons, active mobilization increased their tensile strength and restored gliding surfaces while reducing intrasynovial adhesion formation [61, 62]. In a rabbit model of Achilles tendon healing, early mobilization after tenotomy favoured more rapid restoration of tissue functionality, when compared to the group subjected to continuous immobilization [63]. Study of 64 human patients with Achilles tendon ruptures treated surgically and with early mobilization indicated that application of an early mobilization rehabilitation program reduces range of motion loss and muscle atrophy, increases blood supply, as well

as improves strength of calf muscles and ankle movement [64]. 12 weeks of eccentric resistance training in elite soccer players increased peritendinous type I collagen synthesis in individuals suffering from Achilles tendinosis, whereas collagen metabolism was not affected in healthy control group [65]. However, a 10-year follow up study of postoperative regimes of Achilles tendon ruptures showed that early mobilization and immobilization in tension resulted in similar clinical outcomes and isokinetic strengths [66]. Although some conflicting data exists and the optimal rehabilitation protocol and precise molecular mechanism underlying the beneficial effects of mobilization remain to be determined, controlled tendon loading and motion plays crucial role in tendinopathy management [67].

3.4. Current therapies for the management of tendinopathies

Conservative treatments and/or grafting surgeries are the gold standards for the treatment of tendon injuries. The treatment of choice is influenced by tendon location, type and severity of lesion as well as on the symptoms and clinical evidence of injury.

Independently of the treatment selected, the mid to long term outcomes are not completely satisfactory with a risk of recurrence of symptoms that include pain, instability and degradation of mechanical function.

In the particular case of tissue grafting, besides the morbidity of donor tissue and the risk to (re)rupture of the inflicted tendon, both tendons may experience long term consequences as loss of mechanical competence, functional disability and degeneration that may progress into nearby tissues.

Alternatively to tissue grafting from autologous or cadaveric sources, biological augmentation matrices of decellularized mammalian-origin tissues mainly human (GraftJacket®), porcine (Restore™), equine (OrthADAPT®) or bovine (TissueMend®) have been investigated and presented to the clinical field, revised by Chen *et al* [68]. The main reasons for a lack of compliance on the medical use of these devices may be caused by the decelularization process that may be insufficient to remove all the resident cells and there is a potential risk of immune-rejection and for zoonose transmission.

Artificial augmentation devices constitute an alternative to tissue grafting procedures and to biological augmentation devices [69]. Commercially available devices as LARS™, Kennedy ligament augmentation device, Dacron®, Gore-Tex and Trevira, revised by Batty el al [70], were described to avoid and provide improved knee stability [69] and full weight bearing. However, artificial devices have shown controversial

outcomes on the long term follow up, concerning mechanical failure or mechanical mismatch with native tissues, instability, synovitis, chronic effusions and progression to early osteoarthritis.

3.5. Tendon tissue engineering (TTE) strategies

Tendons require a unique combination of cells within an abundant hierarchically organized ECM coordinated by mechanical, biochemical and architectural sensing and signalling. A failure to this balance results in significant non-functional modifications and/or disease.

A traditional TE strategy is inspired by the natural elements within a tissue niche, namely cells, a 3D structure and their highly orchestrated biochemical signalling in different combinatorial approaches with the final goal of stimulating and inducing new tendon formation with restored function (Figure 3-1). As mechano-responsive tissues, mechanical conditioning in tendons is essential and a critical parameter of the native environment for tissue development and maturation, which ultimately will translate into successful 3D tissue equivalents and improved clinical therapies (Figure 3-1).

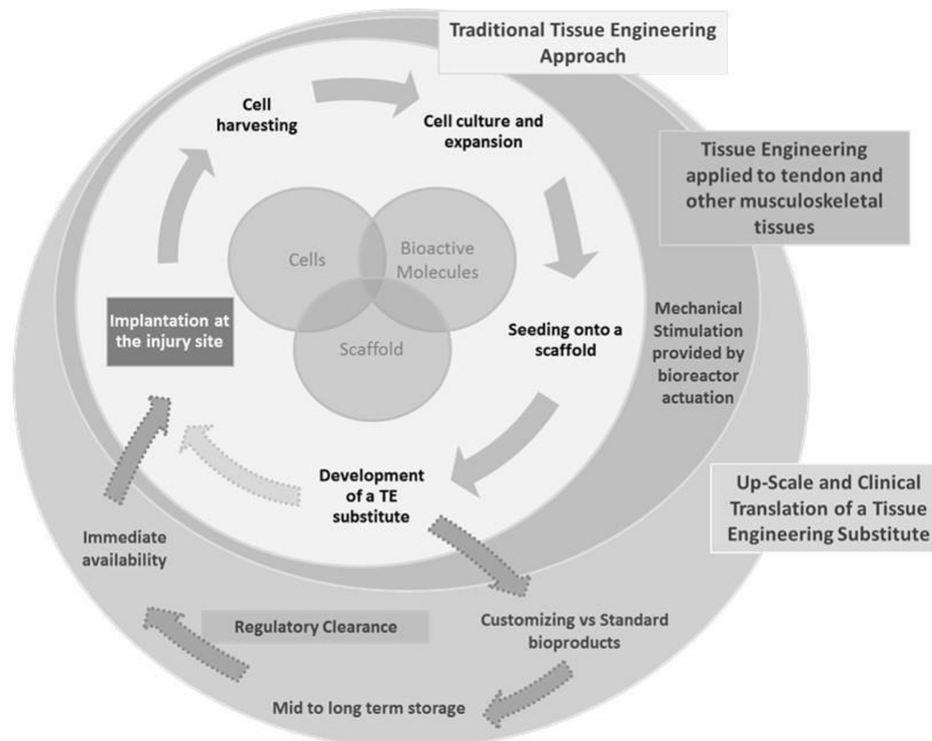

Figure 3-1. Diagram on the development of a successful tissue engineered substitute towards clinical translation using a traditional TE approach with the actuation of bioreactors.

3.5.1. The role of cells in tendon tissue engineering

Tendon resident cells, mainly tenoblasts and tenocytes, are scarce and are responsible for the maintenance of ECM. These elongated cells stretched between the collagen fibres of the tendon, synthesize collagen and other macromolecules, assemble these molecules into a cohesive unit, and organize the fibre phase in parallel with the direction of tensile load, being capable of transducing and responding to mechanical stimuli [71]. This organization makes extremely difficult to repair damage to tendinous structures. Natural tendon healing usually leads to the formation of fibrotic tissue, impairing tendon mechanical properties and resulting in a poor quality of life. More recently, a tendon stem cell population was identified [3] and described to participate in the endogenous regeneration process of tendons.

The hypocellular and hypovascular nature of tendons can relate to significant limitations in the process of repair and regeneration, especially upon tears and ruptures. Cells, especially the resident cell populations, have a critical role in the regulation of the tendon matrix in both normal and pathological conditions [72] being an important parameter to consider to engineer functional tendon inspired substitutes.

Several cell sources have been investigated for tendon strategies [73]. Tendon cells are often the first choice despite the donor tissue morbidity and the infliction of a secondary defect to heal the damaged tendon. However, they are still a great source to study mechanisms and predict native biological responses. Other sources as dermal fibroblasts or muscle derived cells [74] have been also approached as cell alternatives for tendon approaches.

Stem cells are also a promising source due to their high self-renewal potential and ability to differentiate into tissue oriented lineages, with evidence to commit toward tenogenic cues [75, 76]. The secretion of a broad range of bioactive molecules with paracrine effects is believed to be the main mechanism by which mesenchymal stem cells achieve their therapeutic effect [77]. The fact that tendons have their own local stem cell population may indicate that stem cells are likely to participate and even mediate the renewal and remodelling of tissues but also influence fibrous scarring due to abnormal or irregular signalling mechanisms and influence tissue recovery upon injury.

More recently, embryonic stem cells (ESCs) [78, 79] and induced pluripotent stem cells (iPSCs) technology [80, 81] have also been proposed for TTE. Ethical considerations associated to human

embryos research and the risk of *in vivo* teratoma formation presents significant drawbacks for the clinical application of ESCs. A potential strategy to overcome these limitations is using iPSCs, which are highly available cells obtained from multiple sources of autologous cells and can be reprogrammed into a wide range of cell types. The very few studies on human iPSCs for tendon applications [80, 81] show controversial outcomes and their potential for tendon fundamental studies and clinical approaches should be more deeply investigated.

Apart from cell sources, cell-based therapies offer the potential to induce a regenerative response by stimulating local cells and inspiring the synthesis of a structural matrix to ensure remodelling of damaged tendon tissues.

3.5.2. Bioactive molecules in tendon engineered substitutes

The lack of understanding on cell to cell and cell-matrix communication in tendon niches results in limited knowledge to guide biological responses for effective treatment. Moreover, cell secretome and signalling interactions are envisioned to constitute cells most biologically significant role towards repair mechanisms.

Several biomolecules, some identified in developmental biology studies and described earlier in this chapter, have been described to participate in tenogenic commitment, namely EGF, FGF, PDGF, IGF-1, GDF-5, TGF-β [75, 82-84], however their precise spatio-temporal distribution requires further research to promote *in vitro* tenogenic differentiation. Thus, progress towards the recognition of the molecular players favouring homeostasis and regeneration will assist the establishment of tendon benchmarks and methodologies paving the way for clinical translation of cell based therapies.

3.5.3. Recapitulating tendon matrix in functional substitutes

The matrix of tendon tissues is quite unique and complex. Not only complies with a supportive function of cells as its organization and structure relates to tendon mechanical properties and function adapted to the anatomical location.

Thus, another main challenge in the successful design of a tendon substitute is to mimic the intrinsic alignment of tendons and recapitulate their complex hierarchical architecture while remaining mechanically competent towards achieving proper biomechanical functionality to support a complete regeneration of damaged tissues.

Leading edge biomaterial advances have featured fibre fabrication technologies for the production of architecturally aligned scaffolds aiming at tendon replacement strategies. These technologies include 3D printing [85], a combination of polyelectrolyte complexation and microfluidics [86, 87], electrospinning [88, 89] and electrochemical alignment technique [90, 91] combined with textile techniques.

A magnetic responsive scaffold based on a polymeric blend of starch and PCL fibres (SPCL) with aligned structural features incorporating magnetic nanoparticles by 3D printing was shown to assist the tenogenic differentiation of human adipose-derived stem cells upon the actuation of an external magnetic field, and evidenced good biocompatibility and integration within the surrounding tissues upon implantation in an ectopic rat model [85].

Instructive tenogenic matrices with microscaled parallel aligned fibrils were developed combining polyelectrolyte complexation and microfluidics [86, 87]. These fibrous photocrosslinkable hydrogels were fabricated with chitosan (positively charged) and methacrylated gellan gum (negatively charged) [92] or a combination of alginate (ALG) with methacrylated hyaluronic acid (MeHA, negatively charged) or chondroitin sulfate [87]. The MeHA-ALG fibres could be manipulated using textile technologies, allowing the fabrication of 3D constructs with increasing complexity and functionality.

Electrospinning is a familiar technique to TE approaches. It allows the production of long and continuous fibres with controlled diameter mimicking the extracellular matrix of tendon at the nanoscale. Nano-scaled fibres are expected to provide topographical cues at the cell level and stimulate cell response by contact. Accordingly, several studies report that aligned nanofibers can stimulate different cell sources, including dermal fibroblasts [93] and iPSCs [94] to commit towards the tenogenic phenotype [93] in both synthetic and natural based scaffolds [93-96] and to enhance tendon regeneration *in vivo* [93]. However, nanofiber scaffolds produced by electrospinning are mainly 2D systems and their scaling up into 3D scaffolds is limited unless combined with other scaffolding fabrication techniques.

The advancement of medical textiles has created a new generation of biomimetic scaffolding fabrics ranging from simple gauze or bandage materials to scaffolds for tissue culturing and a large variety of prostheses for permanent body implants.

Textile technologies are powerful tools for producing complex and hierarchical 3D constructs using bio-inspired fibre-based materials as building blocks. Textile platforms, including weaving, twisting, braiding and knitting, offer unique advantages, such as versatility to fine-tune the properties of a scaffold such as size, shape, porosity, and mechanical properties by varying the assemble parameters, namely

fibre diameter, fibre number or braiding angle [97] with potential to develop improved 3D constructs with biomimetic properties to achieve tenogenic differentiation [97]. Moreover, with textile technologies it is expected constructs to grow in 3D and in a hierarchical architecture mimicking tendon native structure.

A novel biofabrication modality, ELAC, allows the production of continuous electrochemically aligned collagen threads through a pH gradient process between two parallel electrodes [98] and combines textile techniques to fabricate complex 3D scaffolds. Since the development of this technique in 2008, several works report their potential for tendon and ligament based approaches by mimicking the native tendon's structure and mechanical properties. ELAC scaffolds were shown to induce MSCs tenogenic differentiation [90, 99] with the production of a tenomodulin positive matrix. Moreover, in a rabbit patellar tendon model, ELAC braided scaffold was shown to be biocompatible and biodegradable and assisted the increase in volume fraction of the tendon fascicle compared with the control [100].

Although these fabrication technologies assist the alignment of collagen fibres and mimic tendon structure, the topographical and mechanical stimulation of cells is still limited in these scaffolds. Thus, the application of bioreactors to culture and stimulate biological processes in cells laden scaffolds may more effectively recreate tissue dynamic environment, improving the biofunctionality of these constructs aiming to mimic *in vivo* physiological conditions.

3.6. Bioreactors in Tissue Engineering

3.6.1. Designing bioreactors for tendon tissue engineering

Bioreactors have been widely investigated as advanced tools for tissue engineering of musculoskeletal tissues. These dynamic systems are designed to provide different mechanical conditioning to cells or cell laden 3D matrices in order to resemble the physical forces experienced in the native tissue environment, such as shear stress, hydrostatic pressure, flow perfusion, microgravity or mechano-magnetic stimulation. For example, in bone tissues nutrients and wastes are transported within a lacunocanalicular network, whose circulation has been recreated by shear stress and flow perfusion systems [101, 102]. Bioreactors applying perfusion have also been shown to have promising results in cartilage studies as well as hydrostatic pressure bioreactors described to simulate the main forces which cartilage is subjected to in the articular joints [103]. Bioreactors offer controllable and reproducible dynamic environments with enhanced access of the cells to nutrient supply from the culture medium, improved

oxygen diffusion and a more efficient metabolic waste removal (Table 3-1). Moreover, bioreactors allow homogeneous and long term cultures with the possibility for standardization and automation procedures. Depending on the system, external factors such as pH, temperature, and cell metabolite concentration can be monitored and adjusted, enabling higher cell proliferation rates and decreasing the number of cells that must be initially seeded, while favoring desirable cell responses. With the appropriate stimulus, it is also envisioned a reduction/elimination of media supplementation including serum requirements. It is also expected that cultured cells subjected to the mechanical conditioning will be able to synthetize native tissue-like extracellular matrix in shorter periods of time, and following a more controlled and organized distribution as the mechanical forces will likely better mimic the native niches. Thus, bioreactor application in tissue engineering strategies offer great advantages over 2D cultures and cell laden 3D matrices cultured in static conditions. These include i) biomechanical conditioning of 3D constructs, providing adequate loading regimes according to the type of stimulation required; ii) increase of mass transport, as the supply of oxygen and soluble nutrients is a critical concern when culturing 3D constructs *in vitro*; 3) controlled culture conditions enabling the systematic study of tissue-specific physiological requirements; 4) computer monitoring and programmable options to control and adjust environmental parameters; reducing the limitations associated to a human operator; 5) reproducible cycles of stimulation/standardization.

Table 3-1. Summary of main characteristics of a bioreactor system.

Cultures in bioreactors

- Controlled over *in vitro* environment/ reproducible environment:
    - pH
    - temperature
    - humidity
    - oxygen tension
    - nutrient supply / waste exchange
    - cell metabolite quantification
- Improved oxygen diffusion
- Homogeneous and longevity cultures (non-stop up to several months)
- Standardization of protocols
- Biochemical conditioning:
    - Single or multi dosage without interrupting with the experimental setup
- Biomechanical conditioning:
    - Stretching (mostly cyclic)
    - Loading

- Tension/compression
  - Mechano-magnetic
- Closer environment to a tissue niche than static 3D cultures

Bioreactor equipment:

- Non-toxic, especially the parts in direct contact with the cultures
- Suitable for aseptic conditions
- Quick and easily assembled
- Sterilizable if reusable
- Preferably low-cost
- Easy to clean and store
- Portable, to fit cell culture incubators
- Multiparameter / Tunability of parameters
- Computer control of parameters
- Real time monitoring using imaging techniques as microscopy, MRI or micro computed tomography
- Possibility for computer assisted automation
- Possibility for up-scaling strategies

The development of bioengineered products is a time-consuming task and, thus, approaches to potentially accelerate their clinical use are needed. Bioreactor design can be more or less complex depending on the final application and monitoring parameters. However, all bioreactors are composed of a driving system, a control box and connection cables. The driving system is the motor or pump responsible for impelling mechanical stimulation or medium circulation through perfusion forces into the samples, often located in culture chambers. The control box or computer aided software allows controlling the system, including fluid velocity and biochemical parameters. The tubing cables, often made of materials permeable to gases, are necessary to connect the different parts of the bioreactor and to the power socket.

The bioreactors are designed to operate under aseptic conditions and inside a standard CO_2 incubator at 37°C. Thus, the assemble of the bioreactor and the positioning of the samples in the beginning of the experiment as well as the handling of the system for medium exchange or collection of the samples for analysis are performed within biosafety hoods. The bioreactor materials should be non-toxic, especially the ones in contact with the constructs or tendon tissue samples, and bioreactor parts should be sterilisable if re-usable. Linking parts such as tubes, nuts, o-rings, lids and luer adaptors keep the system closed, and have to be well tightened to prevent malfunctioning, fluid leakages and consequent

contaminations. In order to assure gas interchange and pressure compensation, a 0.22µm filter is normally used.

3.6.2. The role of bioreactors in tendon/ligament tissue engineering

The combination of multiple factors known to exist in tendon niches in a tridimensional and complex environment may enable to generate predictive models relating to cellular responses towards scaffold design parameters and ultimately to recapitulate the alignment, the hierarchical architecture and tendon tissue formation. Commercial bioreactors systems for tendon tissue engineering have been designed to meet these requirements proposing a solution to the limitations of static cultures. Generally, these systems allow the researcher to control and manipulate the deformation cycles as well as the strain and rate levels applied on the sample. Hereafter, we will address some of these systems with different complexity which may be used for biologic tendon samples or TTE constructs.

Mechanical stimulation

The bioreactors that have been used so far for TTE greatly differ in terms of complexity and multiparameter analysis (Figure 3-2).

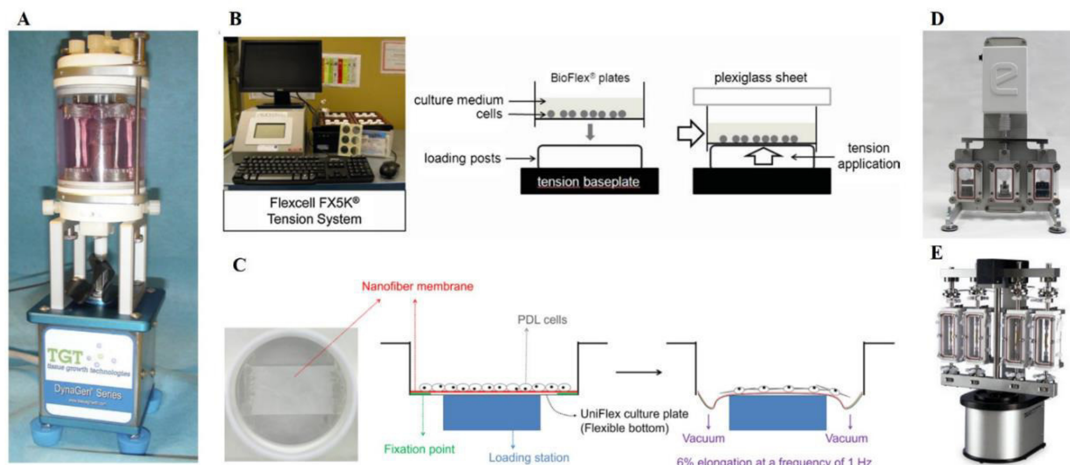

Figure 3-2. Images of some commercially available bioreactors used in TTE strategies. A) LigaGen model L30-4C, DynaGen series, Tissue Growth Technologies. (Reprinted with permission); B) The Flexcell system equipped with the PDL cells supported on nanofiber matrix, where the dynamic mechanical tensional force was applied to the matrix/cell through equipment vacuum. (Reprinted with permission); C) Schematic diagram of the FX5K® Tension System. (Reprinted with permission); D) TC-3 load bioreactor system (Adapted from

http://www.ebersmedical.com); E) ElectroForce® multi-specimen BioDynamic 5200 (Adapted from http://www.tainstruments.com).

One of the simplest systems is the Cell Stretching System from STREX designed for stretching cells in culture, applying a stress load to cells up to 20% of stretching ratio. Morita *et al* have been working with this device to investigate the optimal uniaxial cyclic stretching stimulation to BMSCs [104-107] towards tenogenic differentiation. These works suggest optimal normal strains between 7.9 and 8.5 % for assisting the production of Col I and TNC proteins, respectively.

Flexcell International Corporation developed Flexcell® Tension Systems which are computer-regulated bioreactors that use vacuum pressure to apply cyclic or static strain to cells growing *in vitro* with control over the magnitude and frequency of the stretching. Depending on the model, some systems are able to check and analyze real time cellular biochemical changes in response to strain. In a work developed by Kim *et al* [108], the FX-5000 tension system from Flexcell® was used to apply mechanical stress (strain of 6% elongation at a frequency of 1Hz) to rat periodontal ligament (PDL) cells seeded onto nanofiber-equipped culture plates with random or aligned topography. The cells cultured on the oriented nanofibers combined with the mechanical stress produced PDL specific markers, including periostin and tenascin, undergoing ligamentogenesis, with simultaneous down-regulation of osteogenesis. Moreover, the cell/nanofiber constructs engineered under mechanical stress showed sound integration into tissue defects with significantly enhanced new bone volume and area, in a rat premaxillary periodontal defect model [108]. In another recent study from Sun *et al* [109], rabbit fibroblasts from ligament tissues and bone marrow MSCs were mechanically tested under Uniflex/Bioflex culture system from Flexcell®, as a mean to mimic mechanical strain in ligament tissue. Results showed that uniaxial stretch (15% at 0.5Hz; 10% at 1.0Hz) stimulated fibroblast proliferation and collagen production while uniaxial strains (5%, 10%, and 15%) at 0.5Hz and 10% strain at 1.0Hz were favorable for MSCs. Similar results on the increment production of total collagen by human fibroblasts from the rotator cuff with cyclic strain (Flexcell FX5K™ Tension System; 10% elongation and 0.5Hz frequency) were achieved by Lohberger and colleagues [110]. Also, increasing levels of the matrix metalloproteinases MMP1, MMP3, MMP13, and MMP14, analysed by RT-qPCR, were observed in stimulated conditions as well as tenascin-C and scleraxis.

The LigaGen® Ligament and Tendon Bioreactor from BISS TGT was also designed to provide mechanical stimulation, imparting axial stress or strain to 3D tissue engineered constructs or decellularized tissues to recreate physiological conditions *in vitro*, with studies aiming at hand tendons and ACL regenerative medicine. The chambers of this bioreactor deliver oscillatory axial stimulation and the stress/strain profiles, a simple harmonic (sinusoidal) or a physiological waveform, can be defined by the operator. The bioreactor can be complemented with a perfusion system to provide convective media transport around the samples.

The TC-3 load bioreactor from EBERS Medical Technology SL is a computer controlled system designed to enclose tissue samples or cell laden scaffolds under mechanical tension and compression axial loading.

Herein, the tension grips apply tension loads on samples as different as sheet-like, membrane substrates or 3D-like samples. This specific bioreactor can operate in two different working modes: horizontal or vertical configuration depending on the type of experiment to be developed as the requirement for immersion or air liquid interface, for example. TC-3 load bioreactor allows simultaneous flow and deformation conditions but hydrostatic pressure conditions can also be simulated.

ElectroForce® BioDynamic® systems from TA Instruments can be used to simulate *in vivo* conditions and provide accurate characterization of biomaterials and biological specimens under tension and perfusion flow regimes. Also, an integrated digital video extensometer can be added to the system for primary, secondary and shear strain measurements. Ligaments, tendons or other thin and elongated specimens are attached to the tensile grips while the chamber is perfused with nutrients. The great advantage of this bioreactor in comparison to the above described is the possibility of integrating a Dynamic Mechanical Analysis (DMA) software, allowing determination of the viscoelastic properties at the same time of culture/stimulation.

Apart from the model used, the significant cost of commercial bioreactors and the limited number of samples the operator can handle per experimental setup are the main disadvantages pointed out. Therefore, several research groups have custom designed bioreactors developing new systems to meet more accurately the specific parameters of a tissue or tissue substitute to be screened and evaluated.

One of the most relevant parameters for tendon substitute development is the application of cyclic strain [111-114], and thus a major consideration to the customization of bioreactors.

Wang *et al* [41] developed a bioreactor system, which applies pre-programmed uniaxial stimulation, to study different cyclic tensile strain (0.25Hz for 8h/day, 0–9% for 6 days) on rabbit Achilles tendons. Overall results showed that 3% cyclic tensile strain did not prevent matrix deterioration (gene expression of MMP1, 3, and 12 were highly upregulated by 3% strain stimulation compared to the other groups), whilst at 6% cyclic tensile strain structural integrity and cellular function of the tendons was maintained. Moreover, at 9%, massive rupture of the collagen bundles was also verified.

Youngstrom *et al* [115] studied the influence of cyclic mechanical conditioning (0%, 3%, or 5% strain at 0.33Hz for up to 1h daily for 11 days) provided by a custom bioreactor on the maturation and cellular phenotype of decellularized tendons obtained from four equine sources seeded with bone marrow-derived mesenchymal stem cells. Cultured cells at 3% and 0.33Hz integrated within these tissue derived scaffolds, exhibited higher elastic modulus and higher expression of tenogenic genes.

Magnetic stimulation

In recent years, magnetic driven actuation has been investigated as an alternative form of bio-stimulation in TE strategies. It is known that magnetic forces influence biological processes and magnetotherapy protocols have been proposed in tissue regeneration and inflammation control after injury. Furthermore, magnetic stimulus may act synergistically with magnetizable nanoparticles internalized by cells in culture or embedded within 3D scaffolds creating local forces that can be physically sensed by cells assisting mechanotransduction processes that will ultimately lead to an *in vitro* maturation of the cell-laden constructs prior to implantation. This approach has been previously hypothesized and reported by our group on the use of magnetic bioreactors in the stimulation of stem cells towards tenogenic, osteogenic or chondrogenic differentiation [85, 116, 117] and by others for osteogenesis [118, 119], cardiac TE [120] and neuronal regeneration [121].

3D printed magnetic scaffolds cultured with hASCs cells exposed to oscillation frequency conditions provided by a magnefect nano device (nanoTherics Ltd, UK) showed that magnetic stimulation tend to accelerate the production of collagen and noncollagenous proteins by cells after 7days [85]. This device was initially set up for magnetofection purposes but the magnetic properties of the system showed potential for applications in magnetic force-based tissue engineering.

On the other hand, magnetic responsive membranes implanted subcutaneously in rats exposed to a PEMF waveform with a magnetic field intensity peak of 0.01 T, a duty cycle of 6.3 ms and a frequency

of 75 Hz for 2h a day, 5 days a week (Magnum XL Pro, Globus) showed to modulate tissue inflammatory response, translated by a decrease in the number of mast cells infiltration and reduction of the thickness of the fibrous capsule [116]. The coils that provided the mechano-magnetic stimulation within a therapeutic mat were placed under the animals´ cage. Magnum devices from Globus, are commercially available magnetotherapy devices which provide low-frequency pulsed magnetic fields, being composed of solenoids that permit both the superimposition and the opposition of the magnetic field to treat surface or deep pathologies. These instruments are generally used in human clinical procedures, mostly physiotherapy centers, for applications in muscle, bone-tendon and anti-ageing treatments. The use of magnetic forces in tissue healing is quite recent and some pioneer studies suggest the influence of magnetic field in modulating tendon injury recovery after rat Achilles transection [122]. Besides pain relief [123] and stimulation of blood circulation, magnetotherapy has been reported to stimulate tendon cell proliferation [124, 125] in the promotion of the healing process.

Bioreactors that generate pulsed electromagnetic field (PEMF) have also been investigated. Recently, Liu *et al* [126] and Tucker *et al* [127] described the use of a commercial device, Physio-Stim® PEMF system from Orthofix Inc, to promote gene expression of human tenocytes and to improve early tendon healing in a rat rotator cuff model, respectively. The FDA approved Orthofix stimulators claim to generate a uniform, low-level PEMF shown to be safe in clinical studies for the healing of nonunion fractures [128].

The portable SomaPulse® is another non-invasive PEMF system. It applies a sequence of magnetic pulses programmed to introduce a magnetic field into musculoskeletal tissues. Despite the multiple devices available for magnetic stimulation, the application of electromagnetic fields is still not properly understood, nor how the exposure to PEMF influences tendon resident cells or tendon tissue responses. Despite the recent scientific interest on the magnetic force impact over biological tissues, the wide range of magnetic properties such as intensity, time of exposure or frequency has to be more deeply explored and optimized to individual conditions, tendon anatomical location and associated pathologies. Electromagnetic fields are expected to influence cells response ate the molecular levels or to act on mediators of inflammation. Girolamo *et al* [129] reported that a PEMF (1.5mT, 75Hz) enhances tendon cell proliferation and the release of anti-inflammatory cytokines and angiogenic factor (IL-1B, IL-6, IL-8 and TGF-β). Herein, the PEMF was generated by a pair of rectangular horizontal coils placed opposite to each other.

3.7. Mechanoregulation mechanisms

Physiological responses to mechanical loading are initiated by a process called mechanotransduction, in which cells detect physical changes in their microenvironment through specialized machinery and then translate the information into an appropriate biological response [130]. This mechanosensitive feedback mechanism modulates cellular functions as proliferation, differentiation, migration and apoptosis, and is crucial for organ development and homeostasis [131].

Tendon tissues, physiologically adapted to transmit mechanical forces in a daily basis, are the perfect model to study the mechanisms involved in the translation of mechanical forces into a functional response. Growing evidence suggests that mechanical forces regulate the expression of the bHLH transcription factor Scx through activation of the TGF-β/Smad2/3 pathway in adult tenocyte cultures, which, in turn, is required for maintenance of tendon-specific ECM [31, 40, 132].

Furthermore, it is accepted that these forces can be at least partially mimicked by the stimuli provided by bioreactors. Mohawk (Mkx) and the downstream tendon-associated genes Tenomodulin (Tnmd), Collagen type I (Col1a1 and Col1a2), but not Scleraxis (Scx), showed an increased expression in Achilles tendon-derived rat tenocytes subjected to stretching at 2% and 0.25 Hz for 6 h in a FX-5000 tension system (Flexcell International) [133].

Moreover, magnetic-mechano actuation directed to cell surface receptors is a good example to remotely deliver mechanical stimuli into individual cells. Studies reported that magnetic field of variable frequency may influence cellular response and intracellular signaling favoring the differentiation into desired phenotypes and higher proliferation rates in a shorter culture time and in a more reproducible manner [129, 134-136]. 3D scaffolds may also be used for tendon mechanobiology studies, in which the actuation of mechanical loads provided by bioreactors may be combined with the stimulation from the topographical and physical-chemistry properties of the scaffold to the seeded cells. An example is the fibre composite hydrogels developed by Screen and coworkers [137] envisioned to be a mechanotransduction research platform. Collagen type I gene expression was upregulated in NIH/3T3 fibroblasts laden in the hydrogels and subjected to cyclic tensile loading of 5% dynamic tensile strain, at 1 Hz for 24h. Also, Jones *et al* [138] showed that TGFβ activation plays an important role in mechanotransduction, specifically in the regulation of MMP genes of human tenocytes isolated from tissues with tendinopathic conditions. These tenocytes were seeded onto 3D collagen gels and a 5%

cyclic uniaxial strain at 1 Hz for 48h was applied over these constructs using the Flexcell FX-4000™ device. Treatments with TGFβ1/TGFβRI inhibitor were compared to mechanical strain regimes, and the outcomes with strain or TGFβ treatment were similar. Overall, there was a decrease in MMP1, -3, -11, -13 and -17 and an increase in collagen at the mRNA level [138].

3.8. Concluding remarks

Since the first investigation with tendon/ligament bioreactors published in the 90s [37], these devices have evolved into more complex systems able to test more specimens simultaneously and control/programme several parameters. Despite the advances in recent years and the awareness for mimicking the different fundamental aspects of tendons, which are intrinsically associated to tissue function and activity, currently available tendon substitutes are not biomechanically competent as artificial replacements of tendons. Nevertheless, bioreactors can fulfill this functional gap offering a powerful solution for improving and assisting the development of new tissue engineering equivalents as they provide a controlled, dynamic and monitoring environment that more closely resembles native tissues, with potential toward scale up strategies.

Moreover, bioreactors provide the possibility for testing a variety of different cell laden 3D structures, including scaffolds, membranes, tissue explants, etc., can be investigated and assessed in systematic and reproducible conditions as predictive tool of tissue substitute performance in similar physiological conditions, resembling the native environment. However, the optimal *in vitro* conditions and the optimal 3D tissue substitute have not been established and the challenge stands for an accurate time spatial recapitulation of physical and chemical signals that cells may experience in tendon niches, as well as cell response to such potential stimuli, providing important insights into the long-term capability of engineered constructs to maintain tissue proper functionality. Cellular mechano-sensing mechanisms and the information exchange in biomechanical regulatory signals between the cell and its surroundings also have an important role in determining the potential outcomes of bioreactor microenvironments towards pre-clinical models. These issues need to be thoroughly addressed in forthcoming years in order to achieve bioreactor designs that fully comprise biological and biomechanical demands of tendon tissue.

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Section II

Experimental Section

Chapter 4

Materials and Methods

4. MATERIALS AND METHODS

The aim of this chapter is to describe the experimental procedures used throughout this thesis. Although each chapter contains a materials and methods section providing details for each experimental setup, herein this chapter comprises a general perspective complemented with the rationale for the selection of the materials, cells and techniques used.

4.1. Materials

4.1.1. Starch and poly(ε-caprolactone) (SPCL) polymeric blend

Polycaprolactone (PCL) is a biodegradable polyester, and it is produced using the caprolactone monomer and a suitable catalyst. Starch is a polysaccharide highly available in nature, renewable and inherently biodegradable. Starch is synthesized in amyloplasts of green plants and deposited in the major depots of seeds, tubers and roots in the form of granules [1, 2]. Natural starch consists of a mixture of glycans synthesized composed of 10-20 % α-amylose and 80-90 % amylopectin [3]. Amylose molecules are made up of 200 to 20 000 glucose units, forming a helix structure due to the bond angles between the glucose unit. Amylopectin molecules are made up of about two million glucose units. The side chain branches of amylopectin are made up of about 30 glucose units attached with $1\alpha \rightarrow 6$ linkages approximately every 20 to 30 glucose units along the chain [4]. Starch can be blended with synthetic polymers to improve its mechanical weakness and also to increase biodegradability, for example with PCL yielding in SPCL blends. Therefore, SPCL is a polymeric blend resultant from the combination of polycaprolactone and starch [5]. The scaffolds produced from this blend have previously showed promising results towards *in vitro* and *in vivo* musculoskeletal strategies [6-11].

The SPCL blend used for the fabrication of the scaffolds in this thesis (Chapter 8) was purchased from Novamont, Italy, at 30/70 wt%.

4.1.2. Magnetic nanoparticles (MNPs)

Magnetic nanoparticles (MNPs) are characterized by an iron oxide core of magnetite (Fe_3O_4) or its oxidized form maghemite (γ-Fe_2O_3), and have been used for a wide range of biomedical applications from magnetic resonance imaging (MRI) to cell/biomolecule tracking/separation [12] with growing

potential for therapeutic strategies. Moreover, iron oxide particles have been reported not to evidence a cytotoxic behavior [13, 14].

Recently MNPs have been exploited as remotely controlled multidimensional tools [15, 16] for the development of complex systems in magnetic tissue engineering (magTE).

In this thesis, MNPs and magnetic force-based approaches were explored achieving complex magnetic TE systems aiming at tendon regeneration strategies. The magnetic TE systems were investigated as magnetic 3D scaffolds, magnetic cell sheets or magnetically labeled stem cells. Thereby, MNPs were used for fabricating magnetic responsive scaffolds (magSPCL), magnetic cell sheets (magCSs), and for the activation of cell surface receptors. MNPs were plain surfaced, chitosan or dextran coated or dextran red fluorescent, considering their final application and as described in detail in each experimental chapter. All types of MNPs used in this thesis were purchased from Micromod Partikeltechnologie GmbH.

Iron oxide particles

Plain iron oxide particles were used for the production of the magSPCL scaffolds in Chapter 8. The chemical nature of selected iron oxide particles is: iron (II/III) oxide (Fe_3O_4, magnetite) [CAS: 1317-61-9] with a 250 nm diameter (45-00-252, Micromod).

Chitosan iron oxide particles

Chitosan iron oxide composite particles (nanomag®-C) of 150 nm in size were used for the production of the magCSs described in Chapter 7. Chitosan coated MNPs are characterized by an iron oxide [CAS: 1317-61-9] 80-85 wt% and chitosan [CAS. 9012-76-4] (04-00-152, Micromod).

Dextran iron oxide particles

Dextran iron oxide composite particles (nanomag®-D) of 250 nm in size functionalized with COOH groups in the surface were explored in Chapter 9. These particles were used for receptor’s targeting taking advantage of COOH chemistry for covalent binding of antibodies. These nanoparticles are characterized by an iron oxide core [CAS: 1317-61-9] 85 wt%, dextran [CAS: 9004-54-0] and COOH modified (09-02-252, Micromod).

Cross-linked dextran red fluorescent labelled particles

Cross-linked dextran iron oxide particles (nanomag®-CLD-redF) are red fluorescent and have 100 nm in size. These particles were used in Chapter 7 for the internalization studies with TNMD+ hASCs. The chemical nature of these particles is iron oxide [CAS: 1317-61-9] 75-80 wt%, cross-linked dextran, and red fluorescent labelled (23-00-102, Micromod).

4.2. Scaffolds Fabrication

Tissue engineered scaffolds must provide cell anchorage sites, mechanical stability and structural guidance, to further promote *in vitro* cell colonization and differentiation if desired, and integration with the surrounding native tissue upon implantation [17]. In this thesis, SPCL-based scaffolds were fabricated by rapid prototyping for tendon TE approaches taking advantage of magTE strategies and incorporating MNPs as remotely controlled tools.

4.2.1. Rapid prototyping

In this thesis, bioprinting technology was employed to produce aligned 3D layered scaffolds from a natural based polymer blend of SPCL, already presented above. The Bioplotter (Envisiontec GmbH B10) and a 3D-Bioplotter® system (Manufacturer Series, 4th Generation) were used to fabricate SPCL and magSPCL scaffolds made of SPCL and SPCL with MNPs, respectively, in Chapter 8.

Rapid prototyping (RP) is a versatile tool for advanced TE biofabrication of a great variety of biomaterials. The main advantage of RP technique is the ability to produce complex and fine designed products rapidly and directly from a 3D computer model [18]. These properties highlight the translational and personalized medicine potential of obtaining customized scaffolds to precisely treat specific tissue defects [19].

Both SPCL and magSPCL scaffolds were obtained from a biodegradable blend of SPCL (30/70 wt%, Novamont, Italy), and in the case of magSPCL, commercially available iron oxide particles (45-00-252, Micromod) were incorporated in the SPCL powder (0.018:1 ratio, w/w) before processing. SPCL and SPCL iron oxide MNPs mixtures were placed in a stainless steel syringe and processed at 120 °C (PCL melting temperature) through a high temperature print head cartridge unit. Then, the melted mixture was extruded and plotted through a 22-gauge needle from a 3-axis system to create a 3D object by air pressure control. Two layered scaffolds were plotted along the 0° direction creating a longitudinal

parallelly arranged scaffold mimicking tendon aligned structure. All printing parameters: temperature, pressure, speed, etc, are fine controlled by the user and the printed patterns designed using the 3D-Bioplotter® software. All samples were cut into 5 mm × 5 mm scaffolds and sterilized using ethylene oxide. Samples fabricated only with SPCL (without iron oxide MNPs) were used as experimental control.

4.3. Scaffolds and MNPs characterization

The scaffolds developed under this thesis, namely SPCL and magSPCL scaffolds, were characterized in terms of physic-chemical properties. In the following sections, technical information about the procedures selected for the scaffolds evaluation and MNPs characterization in terms of magnetic properties, size and particle stability is provided.

4.3.1. Micro-Computed Tomography

The microarchitecture of the scaffolds, namely SPCL and magSPCL scaffolds, developed in Chapter 8, was analyzed using a high-resolution X-Ray Microtomography System Skyscan 1072 scanner (Skyscan, Kontich, Belgium) and a microtomography System1272 scanner (Skyscan, Belgium), respectively.

Micro-Computed Tomography (micro-CT) is a non-destructive imaging technique that provides a high resolution 3D construction of a sample, resultant from the digital projection of two-dimensional (2D) trans-axial images of x-rays passing through a sample. This technique provides relevant qualitative and quantitative information regarding sample density and microstructure. Data sets from the scaffolds acquisition were reconstructed using NRecon (version 1.6.6.0, SkyScan) software and DataViewer (version 1.4.4, Skyscan) and CT Vox (version 2.3.0, Skyscan) software were used to build 3D virtual models of the scaffolds from acquired data sets.

4.3.2. Scanning Electron Microscopy

The microarchitecture of the scaffolds, namely SPCL and magSPCL scaffolds, developed in Chapter 8, was analyzed by Scanning Electron Microscopy (SEM). SEM is an imaging technique used to analyze the surface of a solid specimen. Instead of using light to produce an image, the SEM creates a beam of electrons and sweeps it over the surface of the specimen. The incoming electron beam is condensed through a set of lenses before it encounters the sample, where the energy of beam electrons is

transferred to the electrons in the sample. The secondary electrons emitted from the sample are collected and used to produce an image [20]. SEM provides qualitative information regarding sample's surface morphology, including microstructure, porosity, pore size, lumen thickness, topography and composition. Samples are then surface coated either with a carbon layer if a compositional analysis is required, which is assessed by a coupled Energy dispersive X-ray analyzer (EDS) or with gold or metal alloys as gold/palladium, to favor the conductivity of samples and obtain a better image [20]. NanoSEM, FEI Nova 200, was used to analyze the microarchitecture of the scaffolds.

Energy dispersive X-ray analyzer

The elemental characterization of the SPCL and magSPCL scaffolds was performed with an energy dispersive spectrometer (EDS, Pegasus X4M) coupled to the SEM, and an INCAx-Act, PentaFET Precision, Oxford Instruments, to confirm the presence of iron (Fe) element in magnetic structures. The Energy dispersive X-ray analyzer (EDS) quantitatively calculates the relative percentage of the atomic elements present in a specimen and allows the construction of elemental maps showing the distribution of elements.

4.3.3. SQUID Magnetometry

Magnetization (M) measurements of the magSPCL scaffolds were performed in a superconducting quantum interference device (SQUID-VSM) magnetometer from Quantum Design at several temperatures between 300K and 77K and under an applied magnetic field (B) up to 5 T. The SQUID based magnetometry system allows magnetic moment measurements with a sensitivity down to $1x10^{-8}$ emu (low fields), in a temperature range from 1.8K to 400K, and under a magnetic field ranging up to ±70 kOe. The main module is liquid Helium-free, meaning that it works using a closed-cycle refrigeration system able to provide the initial liquid He required for cool down from a gas source, and to maintain the system operational at the required temperature and field ranges.

4.3.4. Zetasizer Measurements

Iron oxide particles, dextran iron oxide and chitosan iron oxide MNPs were characterized in terms of size and stability using a Zetasizer Nano ZS (Malvern Instruments, United Kingdom) in water dispersant.

4.4. *In vitro* cell culture studies

In this thesis, cell-based systems were also investigated as building blocks in TE paradigm. Thus, stem cell biology and cell sources, as well as some aspects of cell culture including harvesting, selection, expansion, and differentiation, are described next.

Human primary cell lines, namely adipose stem cells and tendon/ligament derived cells, were obtained from samples from Hospital da Prelada (Porto, Portugal), with patient's informed consent in accordance to Helsinquia declaration, and under a protocol with the 3B's Research Group, approved by the ethics committees of both institutions. Human adipose tissue samples were harvested from adult patients, during esthetical/plastic surgical procedures. Tendon and ligament tissues were collected from discarded surgical pieces of adult patients undergoing programmed reconstructive/orthopedic surgeries. All research performed with human data (anonymous or coded) and/or samples not specifically collected for research purposes (including leftover material from surgeries) was processed according to the Portuguese legislation and EC standards of data protection, quality and security, as established in the European directives 95/46/CE, 2004/23/CE, 2006/17/CE.

Human amniotic fluid stem cells (hAFSCs) were obtained from human amniotic fluid specimens collected during amniocentesis procedures of male fetuses, using backup cultured cells that would otherwise be discarded. These cells were kindly provided by Prof. James J. Yoo from Wake Forest Institute for Regenerative Medicine (WFIRM), NC, USA.

To perform the cytotoxicity assessment of the scaffolds in Chapter 8, mouse C3H/An connective tissue fibroblast like cell line (L929) used was purchased from European Collection of Cell Cultures (ECACC, UK).

4.4.1. Cell isolation and expansion

Isolation of human adipose stem cells from the SVF of adipose tissue

Human adipose-derived stem cells (hASCs) were used in all experimental Chapters of this Thesis. hASCs were obtained from lipoaspirate samples from the abdominal region of donors undergoing abdominoplasties. The tissue was digested with 0.05 % w/v of Collagenase from *Clostridium histolyticum* (C6885, Sigma) under agitation for 45 min at 37°C. Stromal vascular fraction was obtained after filtration and centrifugation (800 rcf, 10 min, 4°C). SVF pellet was re-suspended in PBS and centrifuged (350 rcf, 5 min, RT). The supernatant was discarded and the cell pellet was re-suspended and cultured in α-MEM

medium (12000-063, Alfagene) supplemented with 10 % v/v of fetal bovine serum (FBS, 10270, Gibco) and 1 % v/v of antibiotic/antimycotic solution (15240062, Alfagene) and maintained at 37°C in a humidified tissue culture incubator with 5 % CO_2 atmosphere.

hASCs were selected by plastic adherence and then expanded in monolayer culture until achieving a sufficient cell number for the experimental setup. The phenotype of hASCs has been extensively investigated in previous studies using tissue samples from the same anatomical region site, harvested with similar technique and isolation following similar procedures by [21, 22].

Isolation of human amniotic fluid stem cells

Human amniotic fluid stem cells (hAFSCs) were used in the studies described in Chapter 5. As previously mentioned, hAFSCs were kindly provided by Prof. James J. Yoo from WFIRM. Briefly, after collecting and plating human AFSCs in Petri dish cultures, hAFSCs were immunoselected as c-kit or CD117 positive, as described in detail elsewhere [23] and expanded in α-MEM medium (Gibco) containing 15 % embryonic stem cell screened FBS (ES-FBS, Hyclone), 1 % glutamine (Sigma) and 1 % antibiotic (Gibco), supplemented with 18% Chang B (Izasa/C101) and 2% Chang C (Izasa/C108) at 37 °C with 5 % CO_2 atmosphere.

Mouse Fibroblast-like cell line

Mouse C3H/An connective tissue fibroblast like cell line (L929) used for the cytotoxicity assessment of the scaffolds in Chapter 8 was purchased from European Collection of Cell Cultures (ECACC, UK). Cells were cultured in α-MEM medium (12000-063, Alfagene) supplemented with 10 % v/v of fetal bovine serum (FBS, 10270, Gibco) and 1 % v/v of antibiotic/antimycotic solution (15240062, Alfagene) and maintained at 37°C in a humidified tissue culture incubator with 5 % CO_2 atmosphere.

Cryopreservation

The cryopreservation process of the cells used throughout the development of this thesis was based on the following protocol: cell suspensions of 1×10^6 cells per ml of cryopreservation solution: 10 % (v/v) DMSO - Dimethyl Sulfoxide (N182, VWR) in FBS, were transferred into 1.5 ml cryovials (479-6841, VWR). Cell suspensions were sequentially cooled down, to -20 °C and then moved to -80 °C freezer. After a minimum of 12 h, samples were stored until usage at -176 °C in the gas nitrogen phase using a cryopreservation tank (Biosystem 24, Statebourne).

4.4.2. Assessment of Tenogenic medium for stem cells differentiation

Efforts to differentiate stem cells towards tendon-like cells have been challenged by the paucity of tenogenic cues as well as a lack of benchmarks to assess tenogenic lineage commitment [24, 25]. Moreover, given that the identification of adequate growth factors (GFs) as biochemical supplementation for tenogenesis still remains elusive, the work developed in Chapter 6 of this thesis proposed to examine the individual influence of four GFs on the tenocytic potential of human amniotic fluid- and adipose-derived stem cells. The GFs associated to tendon development and healing, namely EGF, bFGF, PDGF-BB and TGF-β1 were selected as an attempt to establish a culture medium for tenogenic differentiation of adult stem cells. Herein, a weekly evaluation up to 28 days was performed by the assessment of tendon related markers using real-time RT-PCR analysis and immunocytochemistry. These techniques are described in subsequent sections.

4.4.3. Selection of tenogenic prone subpopulations

Stem cell enrichment strategies, particularly exploring hASCs subpopulations, open new prospects for cell based and TERM applications, which may take advantage of a potentially autologous cell source, predisposed to differentiate into specific lineages. In this thesis, the tenogenic potential of hASCs subpopulations investigated and screened for tenogenic associated markers was based on their positive expression for Tenomodulin (TNMD), STRO-1, CD29, or SSEA-4 markers assessed by flow cytometry. Among the subpopulations studied, the TNMD positive subpopulation of hASCs was shown to be more susceptible to commit towards the tenogenic lineage, in Chapter 6 and was further investigated as cellular units for the construction of magnetic CS in Chapter 7. The process of hASCs subpopulation sorting is described in the next section.

Immunomagnetic sorting (IMS)

This technique was used for cell sorting in Chapter 6 and 7. Immunomagnetic separation methods are based on the cell labeling with small magnetizable particles (beads) via antibodies or lectins. When the mixed population of cells is placed in a magnetic field, bead attached cells will be attracted to the magnet and may thus be separated from the unlabeled cells [26]. Immunomagnetic beads are uniform, polymer particles coated with a polystyrene shell that provides a smooth hydrophobic surface to facilitate physical absorption of molecules, such as antibodies, and surface hydroxyl groups that allow covalent

chemical binding of other bioreactive molecules, such as streptavidin, lectins, and peptides [27]. Iron (III) oxide (Fe_2O_3) deposited in the core gives the beads superparamagnetic properties that lead to consistent and reproducible reactions to a magnetic field without permanent magnetization of the particles. These are the two properties on which immunomagnetic separation (IMS) depends [27].

Briefly, the Dynabeads® (M-450 Epoxy, 14011, Invitrogen) were conjugated with the primary antibodies of interest following the manufacturer's instructions. For this purpose, $2x10^7$ beads were washed in the coupling buffer (0.1M PBS, pH 7.4–8.0) using a Dynal MPC magnet (MPC®-S, Dynal Biotech), re-suspended in each antibody at a final concentration of 200 µg/mL, and incubated overnight at room temperature under gentle stirring. After this period, beads coupled with the antibody were separated from the supernatant with the Dynal MPC magnet, mixed with 1 mL of a Ca^{2+} and Mg^{2+} free PBS (Sigma) supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich) buffer at pH 7.4, and incubated for 5 minutes under gentle stirring. This step was repeated three times. Then, the antibody-coated beads were mixed with the hASCs (previously isolated from SVF and expanded in 2D culture) and 45 minutes incubated at 4°C under gentle stirring, in order to select the hASCs subpopulation of interest. Subsequently, the supernatant was discarded and the bead-bound cells washed three times with 0.1% PBS/BSA buffer using the magnet as previously described.

4.4.4. Construction of magnetic cell sheets as tendon engineered patches

In Chapter 7 of this thesis, magnetic cell sheets (magCSs) were developed with a tenogenic prone subpopulation of hASCs (TNMD+ hASCs). Briefly, TNMD+ hASCs were seeded onto 6 well plates (Falcon) at a density of 600,000 cells per well and left to adhere for 2h at 37°C. Then, chitosan coated iron oxide MNPs were cultured with the TNMD+ hASCs at 400µg/mL (corresponding to 200pg/cell) followed by overnight incubation. After 16h, MNPs labeled TNMD+ hASCs were washed with D-PBS (Alfagene), harvested from the 6 well plates using TrypLE™ Express (12605-028, Alfagene) and seeded into 24 well plates. Cells were cultured in basic α-MEM medium under a 24-well magnet array plate (magnetic induction of 350mT per magnet per well) for up to 7 days. After 24h and every two days, cells were washed with D-PBS and the culture medium replaced by fresh α-MEM basic medium.

4.4.5. Functionalization of MNPs targeting hASCs mechano-responsive receptors for tenogenic commitment

The objective of the work developed in Chapter 9 was to activate the mechanosensitive membrane receptor Activin receptor type IIA (ActRIIA) via functionalized MNPs through the use of magnetic actuation and towards tenogenic commitment. For this, carboxyl functionalized magnetic nanoparticles (nanomag®-D, 09-02-252, Micromod) were covalently coated with Anti-ActRIIA antibody (ab135634) „MNPs-ActRIIA", or with Anti-Rabbit-IgG Fc antibody (ab97196) „MNPs-IgG", by carbodiimide activation. Briefly, particles were activated using EDAC (03449, Sigma) and NHS (130672, Sigma) dissolved in 0.5M MES buffer pH6.3 (Sigma) for 1h at room temperature under continuous mixing. The particle suspension was washed and re-suspended in 0.1M MES buffer containing 60µg of anti-rabbit secondary antibody (ab97196). The particle suspension was continuously mixed overnight at 4°C and then washed and re-suspended in 0.1mL MES buffer containing 10µg of Anti-Activin Receptor type IIA antibody. Particle suspensions were mixed for 3h at room temperature and then blocked with 25mM Glycine (Sigma) for 30 minutes before final washing and re-suspension in distilled water. Functionalized nanoparticles were then incubated for 30min with hASCs which were previously seeded at 10,000 cells/well in 24-well plates. Magnetically stimulated groups were placed in a commercially available vertical oscillating magnetic bioreactor (MICA Biosystems Ltd), at oscillation frequency of 1Hz and 65mm of displacement, provided every other day for 1h sessions. Non-stimulated groups were kept in identical conditions but without magnetic field.

4.4.6. Characterization and analysis techniques

Cytotoxicity screening of magnetic scaffolds

In order to establish the possible toxic effect of leachables and or MNPs eventually released from the developed magnetic scaffolds described in Chapter 8, an indirect cytotoxicity test was performed using L929 cell cultures with extracts of the materials, following the ISO/EN 109935-5 guidelines.

The magSPCL scaffolds and latex rubber discs were extracted in standard culture medium for 24 h at 37 °C and agitation (60 rpm). The ratio of material to extract fluid was constant and latex rubber discs were used as positive control for cell death. The medium containing magSPCL scaffold- or latex- extracts were incubated for 24h was filtered through a 0.22 µm filter and placed in contact with the monolayer of the L929 cell line. Standard culture medium was used as negative control for cell death (or positive

control for cell viability) representing the ideal environment for cell growth. The MTS assay was performed to assess the possible cytotoxicity of scaffolds extracts determining the metabolic activity of the cells for up to 7 days in contact with extract culture medium.

Cell viability assays

MTS assay

MTS assay was used in the studies described in Chapters 6, 7 and 8 to evaluate the metabolic activity of cells. MTS is s a colorimetric assay that quantifies cell metabolic activity and is based on the bioreduction of the substrate (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) (Cell Titer 96 Aqueous Solution Cell Proliferation Assay, Promega) into a brown formazan product. The intensity of formazan color reflects the number of viable cells, as dying cells lose the capacity to reduce MTS.

Briefly, samples were washed in PBS and incubated in a mixture containing serum-free cell culture medium DMEM (without phenol red) and MTS reagent at a 5:1 ratio for 3 h at 37°C in a humidified incubator with 5 % CO_2 atmosphere. The optical density was measured at 490 nm using a microplate reader (Synergy HTTM, Biotek, USA).

7-AAD Viability staining

7-AAD (7-amino-actinomycin D) has a high DNA binding constant and is efficiently excluded by intact cells, and it was used in Chapter 7. It is useful for DNA analysis and dead cell discrimination during flow cytometric analysis. When excited by 488 laser light, 7-AAD fluorescence is detected in the far red range of the spectrum. It is known as a viability dye or as a dead cell exclusion dye.

TNMD+ hASCs suspensions were incubated at room temperature for 10 min with 7- AAD reagent. A minimum of 30,000 cells/tube were acquired and analysed on a FACSAriaIII cytometer (BD Biosciences) using FACS DIVA 7.0 software. PerCPCy5.5 channel was used for 7-AAD detection.

Proliferation/Cell content assay

The proliferation of TNMD+ hASCs, at specific culture conditions in Chapter 7, was assessed by DNA quantification using the PicoGreen® dsDNA quantification assay. PicoGreen® dsDNA quantification assay is a colorimetric assay that employs fluorescence to evaluate cellular proliferation through the measurement of DNA. After the PicoGreen® fluorescent marker is added to the solution, it specifically

binds to double-stranded DNA emitting fluorescence, read at 480 nm (excitation) and 520 (emission). DNA content is determined by comparison with a predetermined DNA standard curve.

After cell culture, growth medium was removed, cells were washed with PBS and kept in 1 mL of ultra-pure water for 1 h prior freezing at -80°C for cell membrane disruption, and then thawed to RT for DNA quantification. Briefly, DNA quantification was performed by adding 28.7 µL of sample or standard to a well of a 96-well white polystyrene plate, mixed with 100 µL of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and 71.3 µL of 1X Quant-iT™ PicoGreen® reagent, in a total volume of 200 µL, all reagents from Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies), and incubated for 10 min at RT. Fluorescence was read at 480 nm (excitation) and 520 (emission) in the microplate reader.

Histological analysis of cells and tissue engineered substitutes

Histology analysis was performed in Chapter 7 and 8. Histology analysis provides increased contrast to better visualize the morpho-structural detail of a particular biological sample. The goal of histological preparation is to create a section of the sample that can be easily imaged, comprising three important steps: 1) fixation of the specimen to maintain the structures found within the sample, 2) dehydration in a graded series of alcohol baths and embedding in paraffin and 3) sectioning the embedded specimen into thin sections using a microtome. In the particular case of magCSs developed in Chapter 7 and due to their delicate nature, magCSs were included in an 4% agarose/PBS solution (Seakem LE, Lonza) previously to paraffin embedding, and 4 µm thickness sections were obtained using a microtome (HM355S, Microm, Thermo scientific).

Hematoxylin-Eosin Staining

The most commonly used stains in histology are hematoxylin and eosin (H&E). HE staining was performed in magCSs and SPCL and magSPCL scaffolds, in Chapter 7 and 8, respectively. Hematoxylin is a base that preferentially colors the acidic components of the cell a bluish tint. Because the most acidic components are DNA and RNA, the nucleus and regions of the cytoplasm rich in ribosomes stain dark blue. Eosin is an acid that dyes the basic components of the cell a pinkish color. Representative sections were stained with H&E, observed under a microscope (Leica, DM750) and images acquired and processed with LAS V4.6 software.

Sirius Red/Fast Green Staining

Sirius Red/Fast Green collagen staining kit (9046, Chondrex) is a semi-quantitative assay that was used in Chapters 7, 8 and 9 of this thesis for detection and quantification of collagen and non-collagenous proteins in cultured cells and cellular constructs, according to the manufacturer protocol. Sirius red dye specifically binds to the helical structure on fibrillar collagen while Fast green binds to non-collagenous proteins in cultured cell layers. Briefly, the dye solution was added and incubated for 30min in order to completely immerse the samples. A dye extraction buffer was then mixed and the OD values read in a spectrophotometer (Synergy HT, Biotek Instruments) at 540 nm and 605 nm. In Chapter 7, data of collagen and non-collagenous proteins was normalized with total protein (technique described in a subsequent section) results.

Prussian Blue staining for Iron

Prussian blue staining was performed in studies developed in Chapter 7. Prussian blue (PB) reaction involves the treatment of samples with acid solutions of ferrocyanides. Any ferric ion (+3) present in the tissue combines with the ferrocyanide and results in the formation of a bright blue pigment called Prussian blue, or ferric ferrocyanide. This is a sensitive histochemical test that detects small amounts of ferric iron. A solution of equal parts of 20% hydrochloric acid (VWR) and 10% of potassium ferrocyanide is used to stain the cells, which are then counterstained with Eosin-Y Alcoholic solution (71204, Thermo Scientific) for cytoplasmic detection and visualized under a transmitted and reflected light microscope (Axio Imager Z1m, Zeiss).

Immunocytochemistry

Immunocytochemistry (ICC) uses fluoresceinated antibodies and anti-antibodies to provide more precise intracellular and extracellular localization of macromolecules, and was used in Chapters, 5, 6, 7, 8, 9. The principle of this technique relies in the specific binding of a primary antibody to an antigen, followed by binding of a fluorescent-labeled secondary antibody to the primary antibody and visualization under a fluorescence microscope. This technique involves preliminary steps after cell culture: a fixation step that preserves the chemical and structural state of the cell components; a cell membrane permeabilization step that enables the antibody to enter into the cell when the antigen is intracellular, by removing the lipids from the membranes; a blocking step that blocks unspecific binding of antibodies; these steps are intercalated with washing steps to remove unbound antibodies.

After cell culture, cells or cell constructs were fixed with 10 % v/v of formalin for 30-60 min, permeabilized with 0.025 % v/v of Triton-X 100/PBS (Sigma) for 10 min and blocked with 2.5 % w/v of horse serum (Vector Laboratories) for 40 min. Cells were incubated with primary antibodies overnight at 4°C and with secondary antibodies Alexa Fluor 488 or 594 for 1 h at RT, according to the host of the primary antibody. Between antibodies incubations, cells were washed with PBS 3 times for 10min.

Flow cytometry

Flow cytometry (FC) is a method for detection and quantification of specific antigens. The principle of this technique relies likewise on antigen-antibody binding but instead of analyzing the antigen under a fluorescence microscope, the antigen is quantified using a flow cytometer. The optical detectors of a flow cytometer convert all detected light into electronic signals that are subsequently converted into digital data. After immunostaining of cells in suspension, single cells pass through a liquid stream, and the fluorophore-labeled antigens are excited by a laser inducing the emission of light at specific wavelengths which is detected and recorded on an optical-to-electronic coupling system.

After cell culture, cells were re-suspended in medium and centrifuged (400 g, 5 min). Cells were immunostained with fluorescent-labeled antibodies for 1 h or with primary antibodies for 1 h followed by incubation with the secondary antibodies Alexa Fluor 488 or 594 for 45 min, according to the host of the primary antibody, all at RT. After washing with PBS, cells were centrifuged (400 g, 5 min) and fixed with 1 % v/v of paraformaldehyde. Samples were analyzed in a Fluorescence-activated cell sorting (FACS) Calibur Flow Cytometer (BD Biosciences) using the CELLQuest software V3.3 or in a FACSAriaIII cytometer (BD Biosciences) using FACS DIVA 7.0 software.

A specialized type of flow cytometry is Fluorescence-activated cell sorting (FACS), which can be used to retrieve cell populations of interest from a heterogeneous population for further study. If a cell or particle can be specifically identified by its physical or chemical characteristics, it can be separated using a cell sorter. In the studies described in Chapter 7, a double positive hASCs cells for CD90 and MNPs were sorted using a Cell Sorter (FACSAriaIII™, BD Biosciences and FACS DIVA 7.0 software), and collected into D-PBS with 2% of FBS, for further assessment of MNPs cellular internalization.

Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assay (ELISA) is another method for detection and quantification of specific antigens. The principle of this technique relies likewise on antigen-antibody binding, in which the antigen is quantified using a solid-phase enzyme immunoassay.

In the study described in Chapter 9, a Sandwich ELISA kit was used to detect phosphorylated Smad2 and Smad3 proteins in lysed hASCs. PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA Kit (12001, Cell signaling technology) is a solid phase sandwich enzyme-linked immunosorbent assay that recognizes endogenous levels of phospho-Smad2 (Ser465/467) and Smad3 (Ser423/425) proteins. The Smad2/3 mouse antibody is coated on the surface of the microwells. After incubation with cell lysates, Smad2/3 are captured by the coated antibody. Following extensive washing, a Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) detection antibody is added to detect captured phospho-Smad2 (Ser465/467) and phospho-Smad3 (Ser423/425) proteins. Anti-rabbit IgG, HRP-linked antibody is then used to recognize the bound detection antibody and then TMB is added to develop color. The magnitude of the absorbance for this developed color is proportional to the quantity of phospho-Smad2 (Ser465/467) and phospho-Smad3 (Ser423/425) proteins, and measured using a spectrometer. Briefly, magnetically stimulated and non-stimulated groups were subjected to different incubation periods of TGF-β-like ligands or MNPs complexes. Subsequently, cells were immediately lysed with cell lysis buffer (9803, Cell Signaling Technology) and assessed for phospho-Smad2/3 proteins by ELISA assay, following the manufacturer's instructions. hASCs cultured without MNPs in αMEM medium supplemented with Recombinant Human/Mouse/Rat Activin A Protein (338-AC-010, R&D systems) or TGF-β3 at 10 and 20 ng/mL were investigated as positive controls of ActRIIA activation, whereas the MNPs conjugated only with secondary IgG antibody constituted the negative control of the ActIIRA activation.

Micro-Computed Tomography

The microarchitecture of the scaffold-free constructs, developed in Chapter 7, was assessed by mCT as a regular 3D structure, as previously described for the magSPCL and SPCL scaffolds. The analysis from acquired data sets and the 3D virtual models were built using DataViewer (version 1.4.4, Skyscan) and CT Vox (version 2.3.0, Skyscan) software.

Scanning Electron Microscopy and EDS

SEM and EDS technique, already described above, were used to characterize the structure and morphological aspects of the magCSs in Chapter 7. Typically, cells or cell constructs require a drying step similar to the preparation needed for conventional histology, which were performed with a serious of grading ethanol concentrations and air dried overnight. Afterwards, samples were sputter coated before analysis by a JSM-6010LV equipment (JEOL).

The elemental characterization of the magCSs was performed with an energy dispersive spectrometer (EDS, Pegasus X4M) coupled to the SEM, and an INCAx-Act, PentaFET Precision, Oxford Instruments, to confirm the presence of iron (Fe) element in magnetic structures.

Inductively Coupled Plasma

Inductively coupled plasma optical emission spectrometry (ICP) is a technique that can determine concentrations of trace to major elements, detecting most elements in the periodic table.

For ICP analysis, the culture medium samples were collected from the magnetic scaffolds cultured for 3 and 7 d, and after 4 and 16h of hASCs incubation with MNPs, respectively in Chapter 7 and 8. Samples were filtered with a 0.22µm filter (VWR) and analysed in a JY2000-2, Horiba Jobin, Yvon. An iron (Fe, 1000µg/mL) standard solution (13830, Specpure) was used to prepare the standard concentration solutions.

Dynamic Mechanical analysis

Dynamic Mechanical Analysis (DMA) analysis of magCSs from Chapter 7 was performed using a TRITEC 2000 model from TRITON manufacturer and tension cycles of increasing frequency (0.1-10 Hz) were applied. DMA is a technique that is widely used to characterize a material's properties as a function of temperature, time, frequency, stress, atmosphere or a combination of these parameters. DMA equipment applies a sinusoidal deformation to a sample of known geometry, which can be subjected by a controlled stress or a controlled strain. The deformation suffered by the sample is directly related to its stiffness. DMA measures stiffness and damping properties reported as modulus (E') and tan delta (tan δ). Because DMA applies a sinusoidal force, the modulus can be expressed as an in-phase component, the storage modulus, and an out of phase component, the loss modulus. The storage modulus is the measure of the sample's elastic behavior. The ratio of the loss to the storage is the tan delta and is often called damping. It is a measure of the energy dissipation of a material.

Transmission Electron Microscopy

A JEOL JEM 1400 TEM (Tokyo, Japan) was used in the study described in Chapter 7 to analyze MNPs internalization into the cells. Transmission Electron Microscopy (TEM) is a destructive imaging technique that gives high resolution 2D images of a sample. An electron beam passes through the sample and interacts with the atoms at various depths and the transmitted electrons produce diverse type of signals which are detectable by the TEM equipment and transformed into a 2D image. TEM uses much thinner sections compared with light microscopy and requires heavy metal precipitation techniques rather than water soluble stains to stain samples. In practice, the resolution of TEM is about 0.2 nm, which is more than a thousand-fold greater than the resolution of the compound light microscope. Briefly, samples were fixed in a solution of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in cacodylate Buffer, 0.1M (pH 7.4). Then, samples were dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin sections (40–60nm thickness) were prepared on a RMC Ultramicrotome (PowerTome, USA) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on a 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate and examined. Images were digitally recorded using a CCD digital camera (Orious 1100W Tokyo, Japan).

Fluorescence and Confocal Microscopy

Fluorescence microscopy is a non-destructive imaging technique that gives high resolution 2D or 3D images of fluorescent samples, by projecting light at a specific wavelength into the sample which is then absorbed by the fluorophore and reflected at different wavelengths. Confocal microscopy is based on the same fluorescence microscopy principle, but in contrast to fluorescence microscopy, confocal microscopy has a spatial pinhole in the detector system that eliminate out-of-focus (background) light.

The fluorescence microscope used for visualizing fluorescently stained cells was an AxioImager Z1m fluorescence microscope (Zeiss) or Axio Observer (Zeiss). Confocal images of constructs were acquired using a laser scanning confocal microscopy (LSCM), using a Leica TCS SP8 confocal microscope (Leica, Germany).

Cells were counterstained with DAPI and Phalloidin. DAPI (4,6-Diamidino-2-phenyindole dilactate) is a nuclear and chromosome counterstain that emits blue fluorescence upon biding to AT regions of DNA. DAPI (40009, VWR) solution was used for 10 min incubation periods for cell nuclei staining. Phalloidin-

Tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC, red) staining is a method to visualize the cytoskeleton of cells. Phalloidin is a rigid bicyclic heptapeptide lethal toxin which binds and stabilizes actin filaments (F-actin) and prevents the depolymerization of actin fibers. Visualization of actin can be attained by incubating previously fixed cells with fluorescent Phalloidin-TRITC. Accordingly, cell actin filaments were visualized after fixation of cells in 10 % v/v of formalin, followed by incubation with Phalloidin-TRITC (0.1 mg/mL, P1951, Sigma) for 20 min at RT. In part of the work described in Chapter 7, a Fluorescein Phalloidin Isothiocyanate Labeled (green) (P5282, Sigma) was used to stain the cells cytoskeleton.

Reactive oxygen species (ROS) assay

In Chapter 7, DCFDA - Cellular ROS Detection Assay (ab113851, abcam), was performed according to the manufacturer's instructions. Cells constantly generate (ROS) during aerobic metabolism. The ROS generation plays an important protective and functional role in the immune system. The cell is armed with a powerful antioxidant defense system to combat excessive production of ROS. Oxidative stress occurs in cells when the generation of ROS overwhelms the cells' natural antioxidant defenses [29]. The most straightforward techniques use cell permeable fluorescent and chemiluminescent probes. 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used techniques for directly measuring the redox state of a cell [29].

Briefly, TNMD+ hASCs were stained with 20μM of 2',7'-dichlorofluorescin diacetate (DCFDA) or left unstained (non-stained control cells) followed a 30min incubation at 37°C before being treated for 4h with Tert-butyl hydroperoxide (TBHP, the positive control). DCFDA is oxidized by ROS into 2', 7'-dichlorofluorescein (DCF) which is a highly fluorescent compound (FL1); TBHP mimics ROS activity oxidizing DCFDA to fluorescent DCF, being considered the positive control of ROS measurement. A minimum of 30,000 cells/tube were acquired and analysed on a FACSAriaIII cytometer (BD Biosciences) using FACS DIVA 7.0 software. FL1 channel (FITC) was used for DCF detection.

Protein Content Assay

Micro-BCA assay was used to determine the amount of total protein in magCSs developed in Chapter 7, according to manufacturers' instructions (Fisher Scientific). Micro Bicinchoninic Acid Protein (Micro BCATM) is a colorimetric assay that quantifies protein content in a sample. This assay is based in the

reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline environment and the chelation of Cu+1 by two molecules of BCA creates a purple color that is linear with increasing protein concentrations.

Briefly, cellular proteins were extracted from samples using radioimmunoprecipitation assay (RIPA buffer, R0278, Sigma), combined with a protease inhibitor cocktail (P8340, Sigma), at 1:100 proportion. After cell lysis, samples were incubated on ice for 5min and stored at -80°C. Albumin (BSA) was diluted at different concentrations between 0 and 200 μg/mL to prepare the standards of a calibration curve. Working Reagent was prepared by mixing Micro BCA Reagent MA, MB and MC at the proportion of 25:24:1. Following, 150 μL of the sample (including blank, control and standards) and Working Reagent were mixed in a multi-well plate and incubated at 37°C for 2 h. After, the plate was cooled to RT and the absorbance was read at 562 nm in a Synergy HT multi-mode microplate reader (Synergy HT, BioTek, USA).

Assessment of the genetic expression of tenogenic markers

Real time RT-PCR was used in the works described in Chapters 5, 6, 7 and 9. Reverse transcriptase polymerase chain reaction (RT-PCR) is a technique for detection of the amount of a specific RNA transcript in a specimen [30] and starts with the reverse transcription of RNA into cDNA using a reverse transcriptase (RT). This technique allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in “real time”. Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The measured fluorescence reflects the amount of amplified product in each cycle. There are three major steps that make up each cycle in a real-time PCR reaction, which are generally run for 40 cycles: 1) denaturation of double-stranded DNA into single strands by DNA polymerase; 2) annealing of complementary sequences (primers); and 3) primer extension by DNA polymerase activity, which occurs at rates of up to 100 bases per second.

Total RNA was extracted using TRI reagent (T9424, Sigma) according to the manufacturer’s instructions. RNA quantity and purity were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), followed by cDNA synthesis performed with the qScript cDNA Synthesis kit (Quanta Biosciences) and using the Mastercycler® ep realplex gradient S machine (Eppendorf) using an initial amount of total RNA of 1μg in a total volume of 20μL. The quantification of the transcripts was carried out using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences) following the

manufacturer's protocol, in a Real-Time Mastercycler ep realplex thermocycler (Eppendorf). The primers were pre-designed with Primer 3 software and synthesized by MWG Biotech. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene, and the $2^{-\Delta\Delta Ct}$ method was selected to evaluate the relative expression level for each target gene [31]. All values were first normalized against GAPDH values, and then to a calibrator sample.

4.5. *In vivo* studies

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.

4.5.1. Subcutaneous model

A subcutaneous model was used to determine *in vivo* host response to the developed scaffolds in Chapter 8. Adult Wistar male rats (16-week-old Wistar male rat with an average weight between 400 and 475 g) were used in this experiment, and four full thickness skin longitudinal incisions (about 1 cm) containing the subcutis and the panniculus carnosus (skin and smooth muscle) were performed in the dorsum of each animal (anterior and posterior incisions). The scaffolds were inserted into these pockets, one scaffold per pocket and a total of four scaffolds per animal, and the incisions were carefully closed using surgical staples. Surgeries were performed under general anesthesia with an intraperitoneal (IP) administration of a combination of medetomidine (Dormitor-0.5 mg kg^{-1}) with ketamine (Imalgene-75 mg kg^{-1}). After surgery, animals were monitored daily to confirm full recovery from surgery and to check for signs of infection until the end of the experiment. One administration of Carprofen (Rimadyl, 2.5–5 mg kg^{-1}) was subcutaneously administered to each animal at the end of the surgery to minimize any postsurgical pain or distress.

After recovery, animals were fed ad libitum and allowed unrestricted cage activity in individual cages. None of the animals used showed clinical symptoms of pain or distress during the experiment.

After 7 days of implantation, animals were anesthetized with isofluorane and euthanized with an intraperitoneal injection with an overdose of pentobarbital sodium (Eutasil from Ceva Saúde Animal). Afterwards, pockets were retrieved for further characterization and evaluation.

Histological analysis

Explants were subjected to standard histological tissue processing and stained with hematoxylin and eosin (H&E, 05-12011/L, 05-M10003, Biooptica), already described above. Stained sections were observed under a transmitted and reflected light microscope with apotome 2 (Axio Imager Z1m, Zeiss). Explants embedded in paraffin were visualized under a stereo microscope (Schott KL 200, Stemi 1000, Zeiss), and images were captured by a digital camera (Power-Shot G11, Canon).

4.6. Statistical Analysis

GraphPad Prism 6 software was used to perform statistical analysis. Data with normal distribution was analyzed using Two-way ANOVA and non-parametric data analyzed by One-way ANOVA, following multiple comparison tests. Significance was set to *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and results were presented as mean ± S.D.

4.7. References

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Section III

Experimental Studies

Chapter 5

Understanding the Role of Growth Factors in Modulating Stem Cell Tenogenesis

This chapter is based on the following publication:

Gonçalves AI, Rodrigues MT, Lee SJ, Atala A, Yoo JJ, Reis RL, Gomes ME. Understanding the role of growth factors in modulating stem cell tenogenesis, PLoS One 2013; 8:e83734. doi: 10.1371/journal.pone.0083734

5. UNDERSTANDING THE ROLE OF GROWTH FACTORS IN MODULATING STEM CELL TENOGENESIS

5.1. Abstract

Current treatments for tendon injuries often fail to fully restore joint biomechanics leading to the recurrence of symptoms, and thus resulting in a significant health problem with a relevant social impact worldwide.

Cell-based approaches involving the use of stem cells might enable tailoring a successful tendon regeneration outcome. As growth factors (GFs) powerfully regulate the cell biological response, their exogenous addition can further stimulate stem cells into the tenogenic lineage, which might eventually depend on stem cells source. In the present study we investigate the tenogenic differentiation potential of human- amniotic fluid stem cells (hAFSCs) and adipose-derived stem cells (hASCs) with several GFs associated to tendon development and healing; namely, EGF, bFGF, PDGF-BB and TGF-β1. Stem cells response to biochemical stimuli was studied by screening of tendon-related genes (collagen type I, III, decorin, tenascin C and scleraxis) and proteins found in tendon extracellular matrix (ECM) (Collagen I, III, and Tenascin C).

Despite the fact that GFs did not seem to influence the synthesis of tendon ECM proteins, EGF and bFGF influenced the expression of tendon-related genes in hAFSCs, while EGF and PDGF-BB stimulated the genetic expression in hASCs. Overall results on cellular alignment morphology, immunolocalization and PCR analysis indicated that both stem cell source can be biochemically induced towards tenogenic commitment, validating the potential of hASCs and hAFSCs for tendon regeneration strategies.

5.2. Introduction

Tendons are highly prone to injury and their intrinsic hypocellularity and hypovascularity makes their natural healing extremely slow and inefficient when severely damaged. Surgical repair with grafts is common but unsuccessful in a long term basis as the biochemical and mechanical properties of healed tendon tissue never match those of intact tendon, ultimately resulting in the progression of degenerative diseases, such as osteoarthritis [1].

The regenerative mechanism underneath the unique organization of collagen fibers and resident cell alignment in between the fibers is still unknown. Thus, the limited ability of tendon to self-repair and the

limitation of treatment regimens have hastened the motivation to develop stem cell-based strategies that explore the natural endogenous system of tissue regeneration.

Amniotic fluid stem cells (AFSCs) have shown to be highly proliferative, exhibiting high self-renewal capability and potential to differentiate into several lineages [2]. In addition, human AFSCs are easy to obtain, representing an almost unlimited stem cell source with immunosuppressive properties [3].

Adipose tissue is also a promising source of stem cells as adipose-derived stem cells (ASCs) have been explored for therapeutic applications, and may represent a potential choice for tendon repair and regeneration [4]. Tissue availability, easy and minimally invasive access to adipose sources place these cells in a unique position relative to other MSCs in the tissue engineering and regenerative medicine (TERM) field. Moreover, human ASCs (hASCs) isolation is a simple and relatively easy enzyme-based methodology, and evidences suggest an immune-privileged behavior [5].

We and others have demonstrated that under appropriate inductive conditions human AFSCs [2,6,7] and hASCs can be directed into several skeletal tissue-related lineages, such as bone [2,6-8] and cartilage [2,6,8].

It is widely accepted that several different environmental factors contribute to the overall control of stem cell activity [9]. Growth factors (GFs) are potential agents to target specific tissue reactions because of their regulatory roles in cellular functions, including adhesion, proliferation, migration, matrix synthesis, and cell differentiation [10]. For instance, fibroblast- (FGF), platelet derived- (PDGF) and transforming-(TGF-β) growth factors are markedly upregulated throughout tendon repair mechanisms [11].

Since growth factors such as epidermal-(EGF), FGF, PDGF and TGF-β have been described to play a role in tendon development and tendon healing, they are to be investigated in this study.

EGF is a potent mitogen that participates in MSCs and fibroblast proliferation [12,13], and is also involved in the initial phase of tendon healing. Besides MSCs proliferation, EGF treatment also preserves early progenitors within a MSC population [12], and increases the paracrine activity of stem cells.

bFGF was recently described to maintain an undifferentiated state of ligament stem cells (LSCs) [14]. Also, LSCs proliferate faster with bFGF treatment [12,14]. FGF signaling is required for the early stages of differentiation in a number of lineages and is also an essential mediator of self-renewal in human stem

cells [14]. Additionally, bFGF stimulates the production of collagenous and non-collagenous ECM [15], thus evidencing a role in proliferation and tendon commitment. *In vitro* studies suggest that bFGF and PDGF not only stimulate tendon fibroblast proliferation but promote changes in the expression of matrix genes showing promise for improving tendon healing [16]. PDGF also plays a role in the migration and proliferation of the tenocytes, fibroblasts, and MSCs responsible for tissue homeostasis. Furthermore, PDGF modulates the synthesis of ECM [17] and supports the formation of a vascular network, which sustains biofunctional and physiological integrity [18] of the tissue. Its biological action highlights the PDGF potential to treat and enhance the biologic response of injured tendons and ligaments [19].

TGF-β has also been attracting attention in the tenogenic regenerative field as TGF-β participates in all three phases of tendon healing process: inflammation, proliferation and remodeling [20]. Moreover, TGF-β has been described to be involved in tendon formation [21], to induce tendon markers in mesenchymal cells [22] and to stimulate upregulation of gene expression and production of ECM in LSCs [14], playing a role in tendon cell fate. The three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3) were shown to participate on collagen production and cell viability [23]. In particular, TGF-β1 increased the production of collagenous and non-collagenous extracellular matrix protein in LSCs [16].

Expanding and culturing cells while maintaining a tenogenic phenotype would be useful in producing a more efficient tendon bioengineered substitute. Although EGF, FGF, PDGF and TGF-β were described to contribute to tendon and ligament development and healing [1,21], the exact nature of tendon regeneration remains unknown. Furthermore, the exogenous addition of GFs to the cellular microenvironment could provide a trigger to assist the differentiation of multipotent cells into a tenogenic lineage, and establish a biochemical link between cells and native tissue, thus participating in the process of restoring tendon functionality.

In this study we propose to assess the tenogenic potential of human amniotic fluid-derived stem cells (hAFSCs) and human adipose-derived stem cells (hASCs) in the presence of specific biochemical culture conditions that might be used in cell-based strategies for tendon repair.

We hypothesize that the exposure to the proper biochemical cues, that is, GFs that participate in tendon formation and ECM synthesis, would potentially stimulate tenogenic differentiation of stem cells. Furthermore, the inclusion of these GFs in the culture medium would enhance the expression of tendon-

related markers and the synthesis of tendon-like ECM. The successful tenogenic differentiation of stem cells also outcomes for cell-laden scaffolding strategies towards assisting and/or improving regeneration in locus. An accelerated proliferation and remodeling process could improve gliding and strength enhancement at the injury site and simultaneously reduce the risk of fibrosis and tendon failure during the repair/regenerative process.

5.3. Materials and Methods

5.3.1. Stem cell isolation and expansion

Human amniotic fluid stem cells (hAFSCs) were obtained from human amniotic fluid specimens collected during amniocentesis procedures. The amniotic fluid was obtained under an IRB protocol approved by Wake Forest School of Medicine. Back-up human amniocentesis cultures, that would otherwise be discarded, were harvested by trypsinization. Within the pool of hAFSCs the c-Kit (CD117) positive population was immunoselected with magnetic microspheres, whose protocol has been described in detail elsewhere [2]. hAFSCs were assessed for several markers by flow cytometry and showed to be negative for CD45, CD33 and CD133, and positive for CD73, CD90, CD105, CD29 and CD44 [2]. Then, hAFSCs were expanded and cryopreserved. The basic amniotic fluid cell (BAFC) medium was composed by α-MEM (Invitrogen) plus 15 % embryonic screened FBS (ES-FBS, Fisher Scientific), 1 % glutamine (Sigma), 1 % antibiotic/antimycotic (A/A) solution (Gibco), 18 % Chang B (Izasa/C101) and 2 % Chang C (Izasa/C108) at 37 °C with 5 % CO_2 atmosphere.

Human ASCs were obtained from lipoaspirate samples of the abdominal region, under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee.

Cells were isolated from tissue samples and cultured as described before [22], and have been previously characterized by RT-PCR for CD44, STRO-1, CD105 and CD90 markers [22]. Briefly, the tissue was rinsed in phosphate-buffered saline (PBS, Sigma-Aldrich) containing 10 % of an antibiotic-antimycotic solution (Gibco). The fat solution was immersed in a 0.05 % collagenase type II (Sigma/C6885) solution for 45 minutes at 37 °C under mild agitation. The digested tissue was

centrifuged at 304 g for 10 minutes at 4 °C, after which the supernatant was eliminated. Lysis buffer was used to dissolve the pellet followed by a centrifugation at 304 g for 5 minutes. Cells were expanded in basic medium composed of α-MEM (Invitrogen) supplemented with 10 % FBS (Alfagene), and 1 % A/A solution (Alfagene).

The data obtained from amniocentesis back-up cultures and from lipoaspirate samples was analyzed anonymously. After reaching a sufficient cell number (approximately 380,000 cells), hAFSCs and hASCs were cultured in media conditioned with different growth factors known to participate in tendon healing mechanisms [1], as described in Table 5-1.

The growth factor's concentration of 10 ng/mL was selected with basis on previously published reports on tendon and ligament regeneration strategies [14,24-26]. Thus, in this work we considered 10 ng/mL studied as the minimum concentration of growth factor to likely influence cellular differentiation. The inclusion of ascorbic acid in the culture medium has been associated with an increased MSCs proliferation and human collagen synthesis, thus a positive and promising factor aiming at a successful tenogenic medium.

Tenogenic differentiation was weekly evaluated up to 28 days based on cell morphology and on the presence of Tenascin C, Collagen I and Collagen III proteins, as well as on PCR analysis for tendon-related markers (scleraxis, tenascin C, decorin, collagen type I and collagen type III), as described in detail below.

Table 5-1. Description of the culture medium composition to induce the tenogenic potential of hAFSCs and hASCs.

Medium	Description
A)	**Basic medium:** α-MEM, FBS (10%), A/A (1%)
B)	**AFSCs expansion medium (BAFC):** α-MEM + 15 % ES-FBS , 1 % glutamine, 1 % antibiotic, 18 % Chang B and 2 % Chang C
C)	**hASCs:** basic medium A + glutamine (2mM) and ascorbic acid (0.2mM)
D)	**Basic medium A** + glutamine (2mM) + ascorbic acid (0.2mM) + EGF (10ng/ml)
E)	**Basic medium A** + glutamine (2mM) + ascorbic acid (0.2mM) + bFGF (10ng/ml)
F)	**Basic medium A** + glutamine (2mM) + ascorbic acid (0.2mM) + PDGF-BB (10ng/ml)

G)	**Basic medium A +** glutamine (2mM) + ascorbic acid (0.2mM) **+** TGF-β1 (10ng/ml)

α-MEM: Minimum Essential Medium Eagle - Alpha Modification; FBS: fetal bovine serum; A/A: antibiotic/antimicotic solution; FBS-ES: fetal bovine serum embryonic screened; EGF: endothelial growth factor (Peprotech/100-15); bFGF: basic-fibroblast growth factor (Peprotech/100-18B); PDGF-BB: platelet-derived growth factor (eBioscience/14-8501); TGF-β1: transforming growth factor-β1 (eBioscience/14-8348).

5.3.2. Morphological analysis

Human AFSCs and hASCs were monitored daily and photographs were obtained from live cells collected weekly using a phase contrast microscope (Axiovert 40 CFL, Zeiss). Multiple regions within each sample well were observed and representative sections captured by a digital camera (PowerShot G11, Canon).

5.3.3. Immunolocalization of ECM Proteins: Collagen I, Collagen III and Tenascin C

Samples from each culture condition were rinsed in PBS, fixed in a 10 % buffered formalin solution (43.05-k01009, INOPAT) overnight and kept in PBS at 4°C until usage.

Collagen I (Rabbit polyclonal Anti-Collagen I, ab292, Abcam), Collagen III (Monoclonal Anti-Collagen, Type III, C7805, Sigma-Aldrich) and Tenascin C (Mouse monoclonal Anti-Tenascin C antibody [BC-24], ab6393, Abcam) expression was assessed on cells cultured onto tissue culture treated 6-well plates (Falcon). After cell permeabilization with 0.025 % Triton-X100 (Sigma/X100)/PBS solution, the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector). Then, cells were incubated overnight with the primary antibodies above mentioned, diluted in antibody diluent with background reducing components from Dako (Dako) at 4 °C. The dilution ratio was optimized to 1:3000, 1:500, 1:3000 for Tenascin C, Collagen I and Collagen III antibodies, respectively.

Afterwards, samples were rinsed in PBS, following inactivation of endogenous peroxidase activity with hydrogen peroxide solution (0.3 % w/v, Panreac). The samples were incubated for 1 hour at room temperature with the respective fluorescent secondary antibody (rabbit anti-mouse Alexa Fluor 488/A11059, or donkey anti-mouse Alexa Fluor 594/A21203, Invitrogen; dilution 1:200), considering the host species of the primary antibodies. After the incubation with secondary antibodies, samples were rinsed in PBS and stained with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μl, D9564, Sigma)

for 10 minutes. Negative controls assessed for immunofluorescence detection were incubated in Dako diluent in the absence of the primary antibody. Finally, samples were incubated with a Phalloidin–Tetramethylrhodamine B isothiocyanate (Phalloidin) solution, which was prepared accordingly to manufacturer's instructions (P1951, Sigma; dilution 1:200). All samples were observed under a microscope (Imager Z1m, Zeiss) and images were acquired using a digital camera (AxioCam MRm5). The total growth surface area (9.6 cm^2) of each well was screened under the microscope. A minimum of 2 wells per sample, condition and endpoint were analyzed. Also, a minimum of 2 samples per independent experiment (n=3) were investigated for protein detection by immunofluorescence.

5.3.4. RNA isolation and Gene expression analysis

Total RNA was extracted using TRI Reagent® RNA Isolation Reagent (T9424, Sigma) following the manufacture's instruction. RNA was quantified on a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific) and first-strand complementary DNA was synthesized from 1 µg of RNA of each sample (qScript™ cDNA Synthesis Kit, Quanta Biosciences) in a 20 µL reaction using a Mastercycler® ep realplex gradient S machine (Eppendorf). Reverse transcription followed by the polymerase chain reaction (RT-PCR) was the technique selected to analyze mRNA expression derived from cells cultured in different media. RT-PCR was performed to assess the gene expression of typical markers for tenogenic differentiation, namely collagen type I, collagen type III, tenascin C, decorin and scleraxis. The transcript expression of target genes was analyzed and normalized to the expression of endogenous housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (n=3). The primers were designed with Primer 3 software (Table 5-2) and synthesized by MWG Biotech.

A $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative expression level for each target gene. ΔCt values were obtained by the difference between the Ct values of target genes and the GAPDH gene. These values were then normalized by subtracting the ΔCt value of the calibrator sample, their respective Ct value in basic medium condition, to obtain ΔΔCt values. Results are represented as relative gene expression in comparison to calibrator sample that is equal to 1.

5.3.5. Statistical Analysis

All quantitative results are expressed as the mean ± standard deviation. Two-Way ANOVA followed by Bonferroni's Multiple Comparison test were assessed to determine whether differences between sample groups were significant. Differences were considered significant when the p value was <0.05.

Table 5-2. Primers used for quantitative RT-PCR analysis.

Gene Abbreviation	Forward Primer	Reverse Primer	Accession number
GAPDH	tgtaccaccaactgcttagc	ggcatggactgtggtcatgag	MN 002046.4
COL1A1	agccagcagatcgagaacat	acacaggtctcaccggtttc	NM_000088.3
COL3A1	gggaacatcctccttcaaca	gcagggaacaacttgatggt	NM_000090.3
TNC	gttaacgccctgactgtggt	ccacaatggcagatccttct	NM_002160.3
DCN	gccattgtcaacagcagaga	cgagtggtccagtgttctga	NM_001920.3
SCXA	tgccttgcagcctcttactt	ctcccagtggagtgtggagt	BK_000280.1

5.4. Results and Discussion

5.4.1. Morphological analysis and cytoskeletal arrangement of tenogenic induced cells

The typical morphology of tendon cells corresponds to a spindle–like shape longitudinally oriented to tendon axis [21]. Since the morphologic features are important for achieving a functional tissue, we investigated stem cell morphology when cultured in the different conditioned media. To reveal the cytoskeleton organization in response to the various GFs supplemented media, cells were stained with phalloidin.

5.4.2. Differential cytoskeleton alignment in distinct stem cell sources

Overall, both hAFSCs and hASCs developed an extensive network of actin fibers by 14 days in culture. Nevertheless, cell alignment was more evident in hAFSCs than in hASCs cultures. By 21 days, both hAFSCs and hASCs evidenced an alignment pattern in culture medium with PDGF-BB and TGF-β1. This pattern was also observed in basic medium (A) and in medium with bFGF in hAFSCs, but slightly faded in hASCs cultured in basic medium (C). After 28 days in culture, hASCs alignment could be detected in

all culturing media, although in medium with PDGF-BB the phalloidin stain was less intensive. For hAFSCs the evidence of aligned distribution was only observed in medium supplemented with EGF and PDGF-BB. Interestingly, hAFSCs and hASCs evidenced an aligned distribution in basic medium by 14 days and 28 days in culture, respectively, indicating that these GFs are important but not essential in achieving an aligned morphology of these cells.

Phalloidin selectively binds cell actin filaments and the actin cytoskeleton has been suggested to have a relevant participation in the alignment and organization of the collagenous ECM in embryonic tendon [27]. Moreover, the longitudinal organization of actin fibers within cell rows is a promising feature for sensing the tensile loads naturally exerted by the muscle to the bone, transmitted to the tendon tissue. Since actin fibers and cytoskeletal tension are often associated, the synthesis of cytoplasmic mechanical fibers (resultant from cytoskeletal rearrangement to meet the extracellular environmental conditions) will ultimately be translated into biochemical signals, which would trigger cell differentiation mechanisms.

5.4.3. Immunolocalization of ECM Proteins: Collagen I, Collagen III and Tenascin C

The resident cells of mechanically functional tissues are often responsible for the production and maintenance of the ECM, including the collagen fibers. Collagen type I, Collagen type III and Tenascin C are present and play a role in the ECM of native tendon tissues. Thus, the immunolocalization of these proteins was assessed as a tool to characterize the tendon-like matrix synthesized by stem cells and stimulated into the tenogenic lineage. Stem cells develop a collagen I rich extracellular matrix in different supplement media. During tendon development, collagen fibrillogenesis generates a tendon-specific ECM that determines the functional intrinsic mechanic properties of the tissue through cellular deposition of parallel arrays of collagen fibrils.

The immunofluorescence analysis revealed that both hAFSCs and hASCs developed a Collagen I-rich matrix in a timeline sequence. The synthesis of collagen fibrils occurred first as an intracellular step with assembly and secretion of procollagen [28]. By 7 days in culture, Collagen I fluorescence signal was concentrated around the nuclei, which likely corresponds to the synthesized pro-collagen chains. Despite variations in the fluorescence signal, it occurred in both hAFSCs and hASCs and in all conditioned media. Beyond 14 days, Collagen I was detected outside hASCs in basic medium (C) and in media supplemented with EGF, bFGF and PDGF-BB. Only in GFs supplemented media the extracellular collagen

synthesized by hAFSCs was observed, and seemed to have an aligned fibrillar-like shape. This process can be associated to the extracellular step of collagen matrix production, where the pro-collagen is converted into collagen and subsequent incorporation into stable cross-linked collagen fibrils [28]. Extracellular collagen showed two distinct distribution patterns, corresponding to a either aligned or randomly oriented matrix network. The aligned pattern of Collagen I in hASCs was widely observed in supplemented culture media with the exception of medium with TGF-β1 after 14 days in culture. In basic media (A and C) the collagen matrix was more randomly dispersed. In hAFSCs the collagen distribution was more randomly oriented after 21 days in culture, despite the saturation of collagen outside the cells.

Overall, Collagen I was detected in both cell types and in all conditioned media throughout the experimental timeline and the collagenous matrix seemed to increase throughout the time in culture.

Collagen III

Besides Collagen I, Collagen III has also an important role in fibril formation. Collagen III copolymerizes with Collagen I, and despite its low amount in the ECM, it provides tissue elasticity. Although the immunolocalization of Collagen III was assessed in this study, in general its fluorescence signal was very mild (data was not shown).

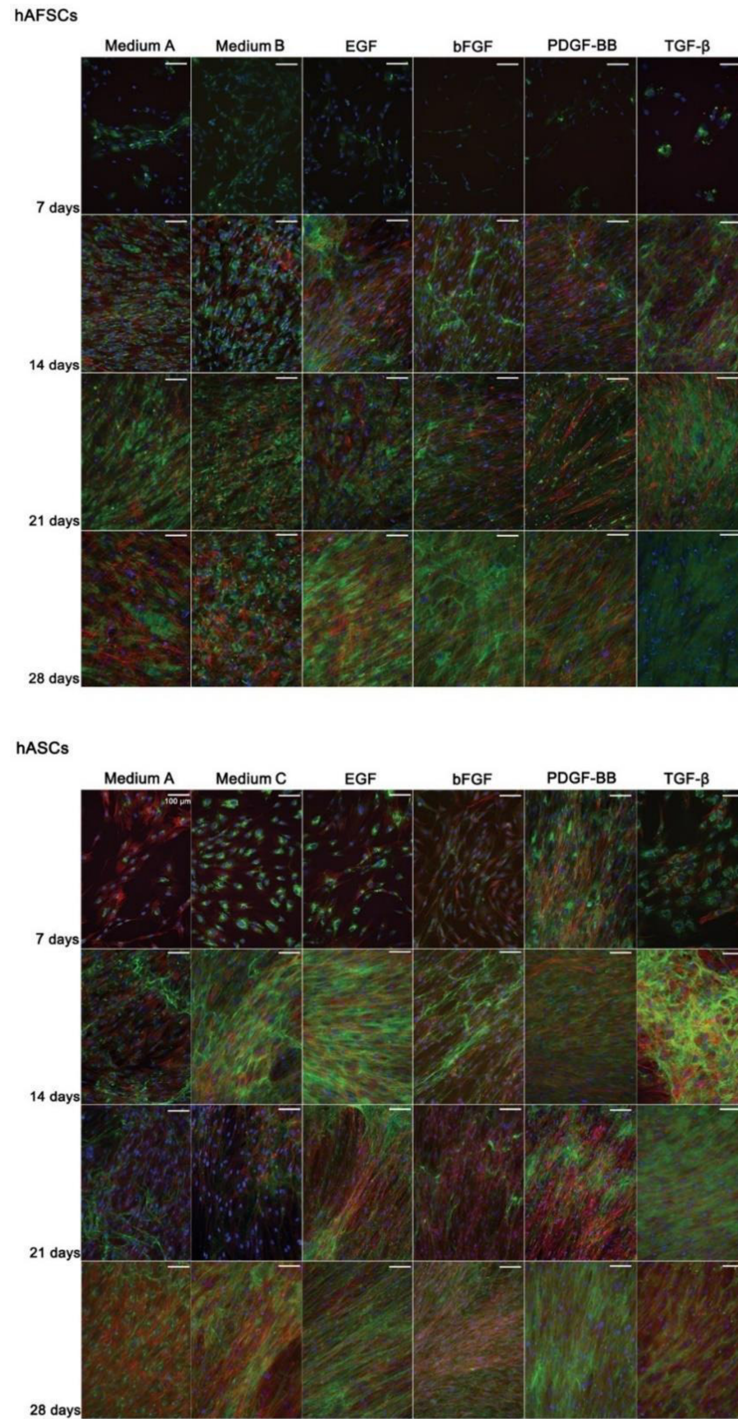

Figure 5-1. Collagen I immunolocation in hAFSCs and hASCs cultured up to 28 days in different supplemented media. DAPI (blue)and phalloidin-conjugate (red) stain cell nucleus and cytoskeleton, respectively. Collagen I is

stained in green and represents the major tendon ECM protein. Scale bar represents 100 μm. Magnification: 200 x.

Tenascin C

Tenascin C is an ECM protein highly regulated by the tissue microenvironment [29]. Although rarely present in most adult tissues, Tenascin C is upregulated in embryonic and developing tissues, or in tissues experiencing a fast rate of growth, and influences cell adhesion and migration.

The fluorescent signal of Tenascin C was neglectable in hAFSCs by 7 days in culture. After 2 weeks, the expression increased in all culture conditions except in the presence of bFGF. After 21 days, Tenascin C was not detected in basic media (A and B) or in TGF-β1 supplemented medium. Thus, medium supplemented with EGF, bFGF or PDGF-BB seemed to influence Tenascin C production in hAFSCs for longer periods of culture. Similarly, Tenascin C was residually detected in hASCs after 7 days in the conditioned cultures. Nevertheless, the protein detection tended to increase with the time in culture up to 21 days, being the strongest signal detected in EGF, PDGF-BB and TGF-β1 culture media.

The expression of Tenascin C did not seem to be associated to the alignment of cells per se. Another supportive data for this statement relied on the fact that the Tenascin C protein network is continuously synthesized, as observed by an increased fluorescence signal in later time points, especially in hAFSCs. Despite the fact that cells were proliferating, especially in longer culturing times, the confluence of the cells on did not show to arrest proliferation or detaching from the culture plate's surface, as it is commonly reported for other cell types.

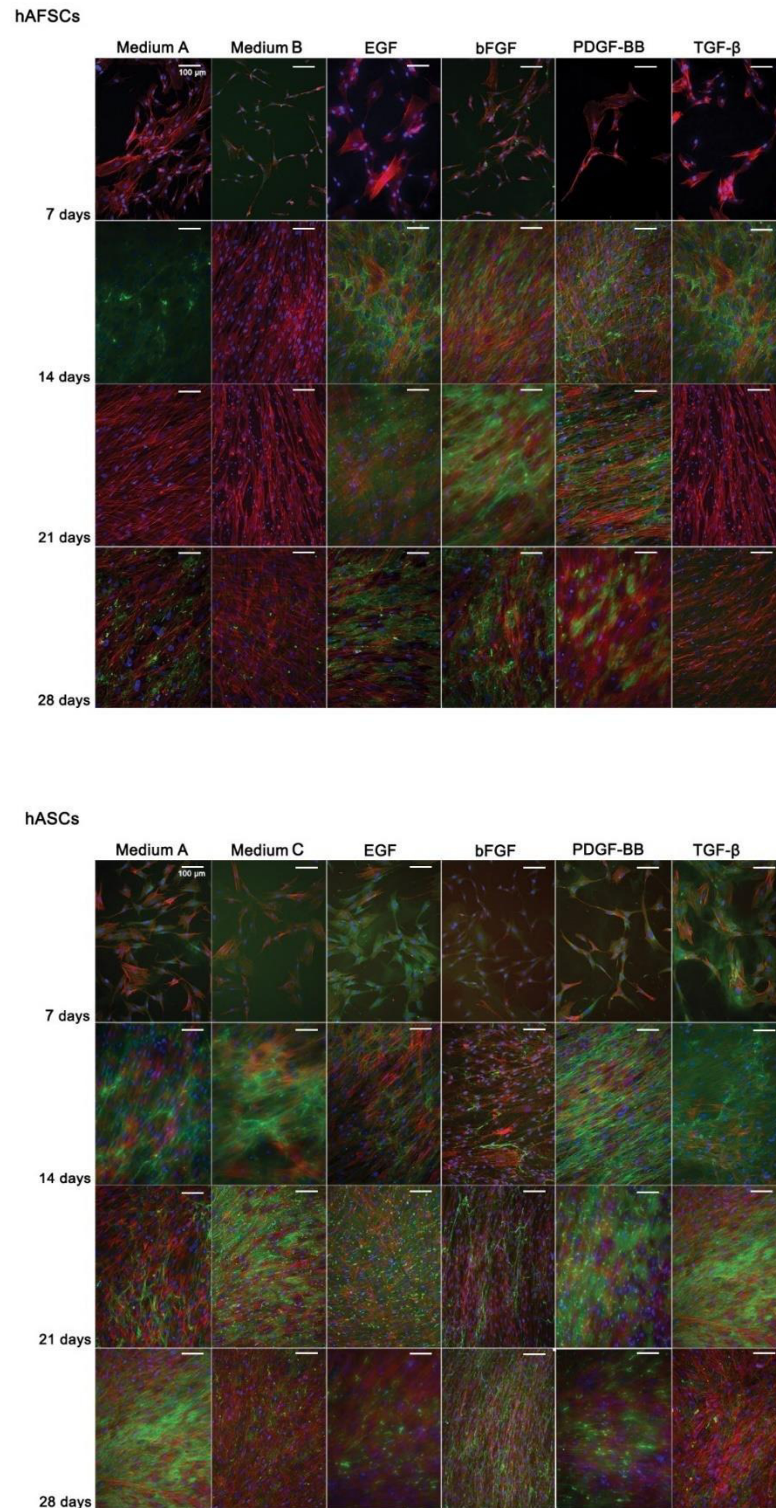

Figure 5-2. Tenascin C immunolocation in hAFSCs and hASCs cultured up to 28 days in different supplemented media. DAPI (blue)and phalloidin-conjugate (red) stain cell nucleus and cytoskeleton, respectively. Tenascin C is stained in green and represents a tendon ECM protein. Scale bar represents 100 μm. Magnification: 200 x.

5.4.4. Real time RT-PCR

The immunolocalization procedures for ECM detection were complemented with RT-PCR analysis of tendon-related markers in order to enhance information and eventually establish a timeline event for tenogenic differentiation on a molecular biology and protein basis.

Decorin

Decorin is a proteoglycan that regulates tendon structure by stabilizing and aligning collagen fibrils [30]. The decorin expression in hAFSCs was evident as early as 7 days in basic (A) and in EGF supplemented medium (Figure 5-3). By 14 days, decorin values were similar to the GAPDH's, and increased again by 21 days in EGF and bFGF media. Only in basic medium (A) hAFSCs expressed decorin after 4 weeks in culture. Conversely, decorin expression of hASCs only increased after 21 days in culture (Figure 5-3). The highest values were detected in PDGF-BB and basic (C) media, followed by TGF-β1, bFGF and EGF, respectively. Then, decorin expression decreased after 28 days for all studied media yet with increased values when compared to the 14 day time point. The influence of EGF medium can be explained by decorin involvement in signal transduction through the EGF receptor. Moreover, supplemented PDGF-BB is likely to participate in the ECM synthesis by increasing decorin and collagen type I expression in hASCs. PDGF-BB has been described to modulate both fibroblast proliferation and ECM synthesis [18]. Although TGF-β1 is known to interact with decorin, the functional significance of this interaction is still unclear [31].

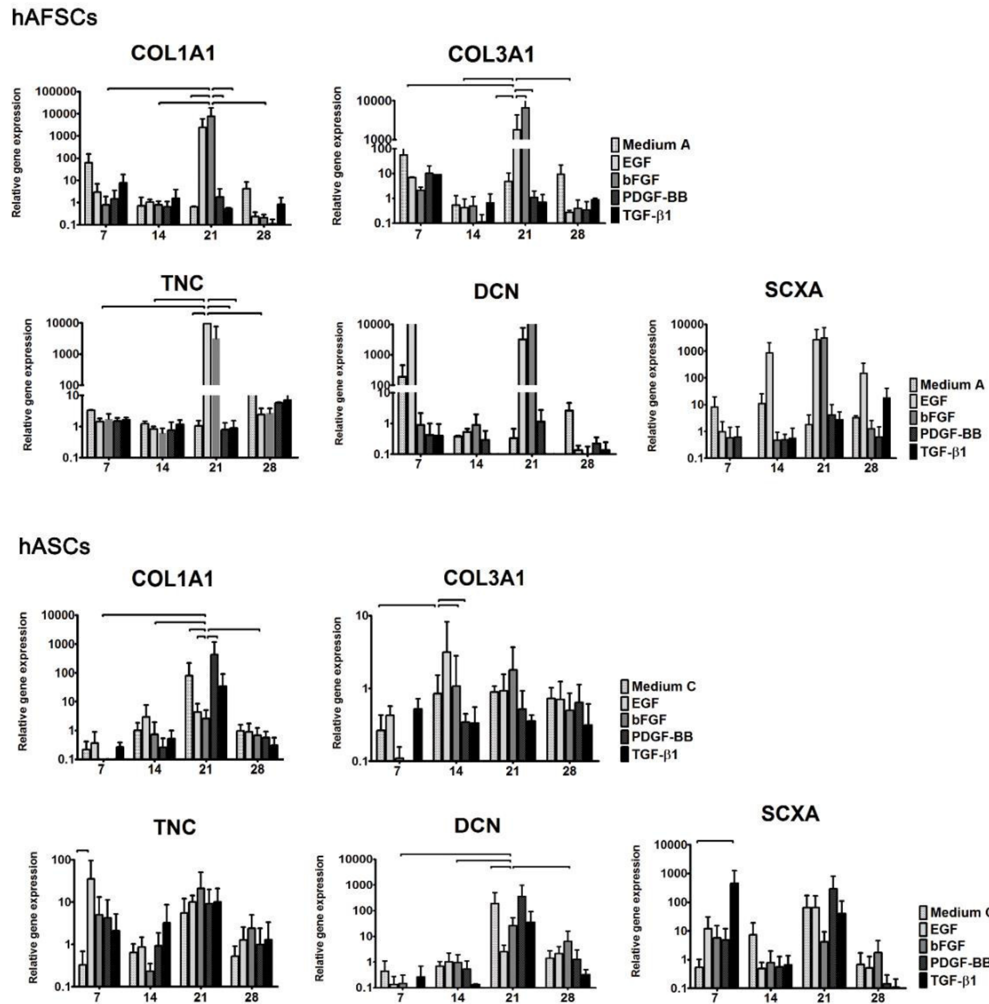

Figure 5-3. Real time RT-PCR analysis. Expression of tenascin C (*TNC*), decorin (*DCN*), collagen type I (*COL1A1*), collagen type III (*COL3A1*) and scleraxis (*SCXA*) genes in hAFSCs and hASCs cultured in different supplemented media. The x axis represents the culture time, namely 7, 14, 21 and 28 days. The relative gene expression is represented in the y axis. Horizontal lines represent differences statistically significant for $p < 0.05$.

Collagen type I and type III

The major fibrillar component of tendon is type I collagen [21], and the synthesis of collagen type I is the crucial step in determination of the tensile strength of tendons [32].

In hASCs, collagen type I has an increased expression around 21 days in culture (Figure 5-3). The highest values were found for cells cultured in PDGF-BB, basic (C) and TGF-β1 media, respectively. Furthermore, collagen type I expression in hAFSCs reached a peak by 21 days in EGF or bFGF media, although some expression was detectable as early as 7 days in basic (A) and in TGF-β1 conditioned

media. In both cell types, collagen type I expression correlated with the gene expression of decorin. Furthermore, decorin seemed to be upregulated due to growth factor supplementation, indicating a catabolic state to collagenous matrix production. These results confirm the mediator role of decorin in the process of collagen fibrilogenesis and its involvement in the development of a tendon-like fibril profile.

Besides collagen type I, collagen type III is essential for normal fibrilogenesis and its regulation despite its smaller amounts in tendon tissues.

In our study, the collagen type III expression showed high values at the first time point in all media (Figure 5-3). The highest levels were observed in basic medium (A), followed by culture medium supplemented with PDGF-BB, TGF-β 1, EGF and bFGF. Collagen type III relative expression decreased by 14 days, increasing again by 21 days for hAFSCs cultured in basic (A), EGF and bFGF media. Although these values were maintained in basic medium (A) at 28 days, they decreased in all other culture conditions. Conversely, collagen type III expression of hASCs maintained a basic level during the entire experiment, and was not particularly influenced by the cell culturing conditions.

Tenascin C

The expression of tenascin C was maintained low in hAFSCs up to 21 days in culture (Figure 5-3). Then, a pick in the expression level was observed in media supplemented with EGF and bFGF. Tenascin C relative expression in hASCs was high by 7 days in culture, registering the highest values found in EGF supplemented medium. The exception was made for cells cultured in basic medium (B). After 2 weeks in culture, the expression decreased for all but TGF-β 1 culture medium, and increased to the highest experimental values by 21 days in all conditioned media.

Overall, tenascin C was highly expressed after 21 days in culture, being more homogeneously expressed in hASCs. On the other hand, hAFSCs showed a peak of tenascin C only in EGF and bFGF supplemented media.

Scleraxis

Scleraxis is a transcription factor expressed in the progenitors and tendon tissue cells. In our study, scleraxis was upregulated by 7 days in basic (A) and EGF media and tended to increase in EGF supplemented medium by 14 and 21 days in hAFSCs. A peak was reached in bFGF medium by 21 days. Our results in hAFSCs are justified by Reed and Johnson work on adipose and umbilical cord blood stem

cells [26]. These studies indicate that FGF signal is necessary and sufficient for scleraxis expression of a tendon progenitor cell population. In hASCs, the scleraxis genetic expression was increased at day 7, especially in TGF-β1 medium, and overall culture media by day 21. These results are also supported by the literature since TGF-β signaling is a potent inducer of scleraxis in cultured cells [3].

Human AFSCs and hASCs expressed all tendon-associated genes studied, with increased expression values by 21 days in culture.

The expression of the genetic markers had a dissimilar pattern in hAFSCs and hASCs, indicating that these stem cell populations respond differently to the different GFs. The expression of tendon-related genes of hAFSCs seemed to be mainly influenced by EGF and bFGF media while hASCs were more influenced by EGF and PDGF-BB. Interesting is the fact that EGF participated in stimulating the genetic expression of both hAFSCs and hASCs but in a distinctive way; EGF influenced the expression of tenascin C in hAFSCs and collagen type III expression in hASCs. Thus, the genetic expression towards a tenogenic lineage commitment may be influenced by stem cell origin, as cell origin may condition the stem cell response to local biochemical environment, i.e. the media composition.

5.5. General Discussion

Stem cell differentiation in general and tenogenic differentiation in particular, are modulated by several biomolecules, including growth factors presented in a highly defined and tunable micro-environment. These biomolecules are expected to establish cell-to-cell contact and interact with intracellular signaling molecules as a responsive behavior to the extracellular milieu. The communication is likely to result in gene expression regulation, and ultimately on the synthesis of proteins to promote cell mechanisms that conduct to cell adjustments towards the external stimuli provided.

Since tendon associated markers are also found in other tissues and cells, a combination of these markers may provide some insight into the *in vitro* tenogenesis or tenogenic differentiation. The presence of tendon-related ECM proteins namely Tenascin C, Collagen I and Collagen III, described to have a key role in healthy functional tendons, were detected in both stem cells.

The growth factors (GFs) were selected based on recent publications, which associated these factors with tendon development and healing mechanisms [17]. The main goal of the present study was to understand how these GFs influence the tenogenic differentiation of the two stem cell sources, so as to

eventually help defining an appropriate cell culture medium for inducing cells into tenogenic lineage. Although the GFs studied clearly influenced the stem cell response in terms of ECM production, none of these GFs evidenced a distinctive action in the synthesis of tendon-related proteins. Interestingly, the fluorescence signals for Tenascin C and Collagen I protein are detected in culture conditions where the genetic expression is low. We hypothesize that this effect may occur due to the timeline translational determinants that take place intracellularly from the upregulation of the interest gene to the protein translation, and transport to the extracellular matrix being produced. These results emphasize the complex dynamic of the GFs in cell processes and environmental interactions that take place in promoting cell differentiation *in vitro*.

Both hAFSCs and hASCs showed a longitudinal organization of actin fibers within cell rows as well as alignment of collagen fibers, suggesting that these are important morphological features to investigate for a deeper understanding of the unique orientation of the collagen fibrils in tendons.

Despite variations in the genetic expression levels of tendon-associated markers, the GFs studied do not seem to be essential for the biochemical stimulation of *in vitro* tenogenesis, as Tenascin C and Collagen I were also observed in basic medium. It is likely that stem cells studied may respond more promptly to mechanical stimulation rather than to biochemical signals provided by the culture medium.

Although pronounced conclusions were not achieved, the study demonstrates that some growth factors have a greater effect in a particular cell source than another, despite the fact that all growth factors studied are associated to tendon healing mechanisms.

Since tendon is a mechanoresponsive tissue, appropriate mechanical loads at physiological levels are usually beneficial to tendons in terms of enhancing the mechanical properties of the tendon [33], and achieving a healthy and functional tissue. Tendon tissues require specific micro-environments according to their function and correspondent anatomical regions. Therefore, we speculate that tenogenic differentiation of hASCs and hAFSCs cultured in basal medium could be enhanced or accelerated in the presence of an external mechanical stimulus. The selection of the stem cell source seems to be relevant in designing a tendon regeneration strategy. Despite the fact that both hAFSCs and hASCs did follow a tenogenic pathway, the cell responses to exogenous GFs stimulation were distinct, especially the ones found at a molecular biology level. These results indicate that the interactions occurring between these cells and the biochemical milieu are complex and intrincated and may be

dependent on stem cell origin. These data point out the importance of understanding the cellular and molecular events induced by factors regulating stem cell proliferation and the consequences on their cellular influence.

In summary: results on cellular alignment morphology, immunolocalization and PCR analysis indicated that both stem cells can be biochemically induced towards tenogenic features. The stimulation into tenogenic lineage was demonstrated by the elongated and aligned cell morphology, the presence of a Tenascin C and Collagen I-rich ECM and the expression of gene markers typically associated to tendon tissues. Despite variations found between hAFSCs and hASCs responses to the GFs supplemented to the culture medium, the results further validate the potential of using adipose tissue and amniotic fluid as stem cell sources for tendon tissue engineering.

5.6. Conclusion

The concept of stem cell inclusion in tendon-related strategies increases the number of cells locally, stimulating tissue regeneration. Furthermore, the cellular commitment towards the tenogenic lineage will simultaneously supply the local milieu with growth factors and cytokines that could be important and lineage-oriented for tendon regeneration.

Despite the relevance already described in tendon healing mechanism, the growth factors evaluated in our study did not emphasize a particular outcome on the tenogenic process of hAFSCs and hASCs considering the synthesis of tenogenic ECM proteins. Nevertheless, EGF and bFGF as well as EGF and PDGF-BB do have a molecular influence on the tendon-related genetic expression of hAFSCs and hASCs, respectively, although their role in the process of tenogenic differentiation of stem cells is still unveiled. The fact that stem cells harvested and expanded from different sources showed a distinct behavior to the GFs supplemented in the culture medium may indicate that the origin of these cells may also have an effect on the process of differentiation. Thus, the selection of a source of stem cells should be considered as a potential contribute for injury/defect-directed approaches in the regenerative medicine field. These results also suggest that the tenogenic differentiation follow complex and elaborated pathways that may depend on multiple growth factors at precise chronological points and /or a combination of multifactorial stimuli from the highly specific and delicate tendon natural environment.

5.7. References

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Chapter 6

Human adipose tissue-derived tenomodulin positive subpopulation of stem cells: a promising source of tendon progenitor cells

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6. HUMAN ADIPOSE TISSUE-DERIVED TENOMODULIN POSITIVE SUBPOPULATION OF STEM CELLS: A PROMISING SOURCE OF TENDON PROGENITOR CELLS

6.1. Abstract

Cell-based therapies are of particular interest for tendon and ligament regeneration given the low regenerative potential of these tissues. Adipose tissue is an abundant source of stem cells, which may be employed for the healing of tendon lesions. However, human adult multipotent adipose-derived stem cells (hASCs) isolated from the stromal vascular fraction (SVF) of adipose tissue originate highly heterogeneous cell populations that hinder their use in specific tissue-oriented applications. In this study, distinct subpopulations of hASCs were immunomagnetic separated and their tenogenic differentiation capacity evaluated in the presence of several growth factors (GFs), namely EGF, bFGF, TGF-β1 and PDGF-BB, that are well-known regulators of tendon development, growth and healing. Among the screened hASCs subpopulations, tenomodulin (TNMD+) positive cells were shown to be more promising for tenogenic applications and therefore this subpopulation was further studied assessing tendon-related markers (scleraxis, tenomodulin, tenascin C and decorin) both at gene and protein level. Additionally, the ability for depositing collagen type I and III forming extracellular matrix structures were weekly assessed up to 28 days. The results obtained indicated that TNMD+ cells exhibit phenotypical features of tendon progenitor cells and can be biochemically induced towards tenogenic lineage, demonstrating that this subset of hASCs can provide a reliable source of progenitor cells for therapies targeting tendon regeneration.

6.2. Introduction

Acute or chronic tendon injuries are one of the leading causes of disability in athletes, active working people and elder population worldwide. Therapeutic strategies currently used namely autografts and allografts do not provide successful long-term outcomes and healed tendons do not completely recover original strength and functionality, especially in senior patients. Thus, the development of stem cell-based therapies and the search for alternative sources of progenitor cells for tendon tissue engineering (TTE) is of particular interest given the low natural regenerative potential together with the limited availability of native progenitor cells in tendons (3 to 4% of the total tendon cell population) [1, 2].

Stem cells derived from adipose tissue (ASCs) might potentially be employed for tendon and ligament repair and regeneration [3-5] and several attempts have been made to induce their *in vitro* tenogenic differentiation using growth factors (GFs) [6-8], mechanical cues of the substrate [9] or combination of mechanical and biochemical stimulation [10]. The main advantage of ASCs is that adipose tissue offers patient-derived autologous mesenchymal stem cells (MSCs), which can be easily harvested with lower morbidity, in contrast to MSCs derived from other sources [11]. The yield of ASCs per gram of tissue is 500-fold higher than that obtained for bone marrow MSCs (BM-MSCs) [12]. Withal, direct comparison between ASCs and BM-MSCs phenotypical properties gives high identity similarities [13]. Cultured ASCs exhibit favorable regenerative and immunotolerant profiles increasingly considered in cell therapy and tissue engineering (TE), being presently under investigation for a high number of clinical therapeutic applications [14, 15]. ASCs are typically isolated from the pool of cells available in the stromal-vascular fraction (SVF) of adipose tissue by methods based on enzymatic digestion followed by adherence and expansion of fibroblast-like cells to the plastic of the culture flask and removal of non-adherent cells [12, 13, 16]. However, the heterogeneity within ASCs population together with the innate tendency to differentiate towards adipose lineage may jeopardize ASCs proliferation and differentiation potential [17, 18] and consequently their therapeutic/regenerative potential in tissue-specific cell therapies.

In previous works from our group, viable, homogenous and phenotypically stable subpopulations of human and rat ASCs were successfully separated from crude populations [19-22] using the immunomagnetic sorting (IMS) method, enabling to select subpopulations that were found to be more prone to osteogenic [19, 20], chondrogenic [19] or endothelial differentiation [21]. Therefore, purification of ASC subpopulations opens new prospects for TE applications, which may take advantage of (potentially autologous) cell sources predisposed to differentiate into specific lineages. Based on these findings, we hypothesized that the separation of a human ASCs (hASCs) subpopulation susceptible to commit towards the tenogenic lineage represents an effective strategy for the detection of progenitor cells that might be used with higher efficiency and more effectively in tendon and ligament regeneration therapies. Therefore, the main aim of this study was to identify and characterize a subpopulation of hASCs prone to tenogenic differentiation. For this purpose, hASCs subpopulations were isolated from hASCs and sorted with IMS using CD29, STRO-1, and SSEA-4 antibodies based on our previous studies of hASCs

subpopulations [19-23], and tenomodulin (TNMD), a highly expressed marker in tendons and ligaments [24].

As a first approach, subpopulations of hASCs positive for TNMD, STRO-1, CD29, and SSEA-4 markers were isolated and their tenogenic differentiation capacity assessed for 28 days upon culturing cells with various growth factors (bFGF, TGF-β1, and PDGF-BB) that are well known regulators of the development and healing of tendon tissue [25-27] and described to participate in stem cell tenogenesis [7, 28, 29]. The results obtained using flow cytometry analysis suggested that TNMD positive cells could be the most prone to tenogenic commitment among the studied subpopulations. Therefore, we further examined the effects of growth factor (GFs) supplementation on gene expression and synthesis of proteins consistent with tenogenic phenotype on the TNMD+ subpopulation for 14, 21 and 28 days by quantifying the expression of the tendon-related markers Scleraxis (SCX), Tenomodulin (TNMD), Tenascin C (TNC), Decorin (DCN), Collagen type I and III (COLI and COLIII), and the ability of the cells to form tendon-like ECM. The increased expression of these markers observed both at the gene and protein level together with the production of a fibrillar collagenous ECM confirmed that TNMD+ cells commit to the tenogenic phenotype more efficiently than unsorted hASC population. Moreover, bFGF and TGF-β1 supplementation to TNMD+ cells contribute to the *in vitro* guidance of TNMD+ cells towards tenogenic lineage envisioning a pool of stem cells with increased therapeutic potential over hASCs crude population for tendon cell based therapies.

6.3. Materials and Methods

6.3.1. Isolation of hASCs and subpopulation sorting using immunomagnetic bead separation

Primary cultures of hASCs were enzymatically isolated from lipoaspirate samples obtained under protocols previously established between 3B's Research Group and Hospital da Prelada (Porto, Portugal) with an informed consent of the patients.

Cells were expanded in basic culture media composed of α-MEM (A-MEM, Invitrogen) supplemented with 10 % FBS (Alfagene) and 1 % A/A solution (Alfagene). The immunomagnetic beads (Dynabeads® M-450 Epoxy, 14011, Invitrogen) were conjugated with the i) anti-Tenomodulin- (C-20) (Santa Cruz Biotechnology, SC-49324), ii) anti-STRO-1- (Invitrogen, 398401), iii) anti-CD29- (BD Pharmingen,

555442), and iv) anti-SSEA-4- antibodies (Santa Cruz Biotechnology, SC-59368) following the manufacturer's instructions. For this purpose, 2x107 beads were washed in the coupling buffer (0.1M PBS, pH 7.4–8.0) using a Dynal MPC magnet (MPC®-S, Dynal Biotech), re-suspended in each antibody at a final concentration of 200 μg/mL, and incubated overnight at room temperature under gentle stirring. After this period, beads coupled with the antibody were separated from the supernatant with the Dynal MPC magnet, mixed with 1 mL of a Ca^{2+} and Mg^{2+} free PBS (Sigma) supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich) buffer at pH 7.4, and incubated for 5 minutes under gentle stirring. This step was repeated three times. Then, the antibody-coated beads were mixed with the hASCs (previously isolated from SVF and expanded in 2D culture) and 45 minutes incubated at 4°C under gentle stirring, in order to select the hASCs subpopulation of interest. The experimental setup of this work is outlined in (Figure 6-1). Subsequently, the supernatant was discarded and the bead-bound cells washed three times with 0.1% PBS/BSA buffer using the magnet as previously described. IMS of hASCs without antibody coupling was considered a cell isolation control and defined as unsorted hASCs. The bead-bound target was discarded and the remaining supernatant sample used for the downstream applications as a heterogeneous unsorted hASCs population. Sorted and unsorted hASCs were then re-suspended in basic α-MEM medium.

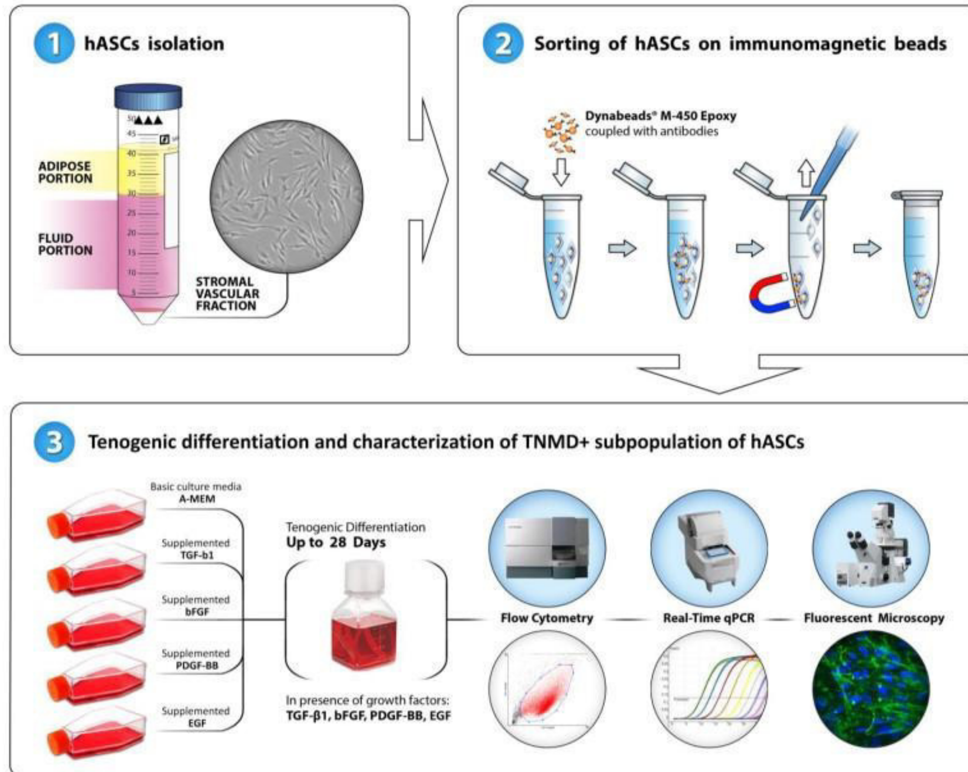

Figure 6-1. Schematic representation of the experimental setup of the work: (1) hASCs isolation from the stromal vascular fraction (SVF), (2) sorting of hASCs subpopulations using the immunomagnetic separation (IMS) method with Dynabeads® coupled with CD29, STRO-1, SSEA-4 or TNMD antibody, and (3) tenogenic differentiation and characterization of TNMD positive subpopulation of hASCs.

6.3.2. Pre-selection of the subpopulation most prone to tenogenic commitment

The expression of CD73, CD90 and CD105 of isolated hASCs subpopulations and unsorted hASCs was assessed by flow cytometry. Since this work aims at investigating precursor cells that may be more effectively applied for tendon regeneration therapies, the constitutive expression of tendon markers Scleraxis (SCXA), Tenomodulin (TNMD), Tenascin C (TNC), and Decorin (DCN) in each subpopulation was determined by flow cytometry analysis, and compared to unsorted hASCs. A minimum of 20,000 hASCs were acquired with a FACS- Calibur Flow Cytometer (BD Biosciences) and the results analyzed with CellQuest software V3.3 and expressed as a percentage of the total number of cells collected and acquired within the hASCs population.

Tenogenic differentiation of hASCs subpopulations using culture media supplemented with GFs

Unsorted hASCs and the subpopulations sorted with CD29, STRO-1, SSEA-4 and TNMD markers were seeded onto tissue culture polystyrene 13mm diameter coverslips (Sarstedt) (2,000 cells per coverslip), and onto 25 cm^2 culture flasks (1,000 cells/cm^2). The cells were cultured for 28 days in basic medium or in one of the four differentiation media, which were considered to potentially stimulate tenogenic differentiation of hASCs. The differentiation media were composed of α-MEM supplemented with L-alanyl-L-glutamine 2 mM (Sigma, G8541) and ascorbic acid 0.2 mM (Wako Chemicals, 013-1206) plus a GF: endothelial growth factor (EGF; Peprotech/100-15), basic-fibroblast growth factor (bFGF; Peprotech/100-18B), platelet-derived growth factor (PDGF-BB; eBioscience/14-8501), or transforming growth factor-β1 (TGF-β1; eBioscience/14-8348). The GFs concentration was set to be 10 ng/mL in accordance to published tendon and ligament regeneration reports [7, 30, 31].

The tenogenic differentiation of hASCs induced by the GFs in the culture media was evaluated by flow cytometry performed as previously described, and the expression of SCXA, TNMD, TNC, DCN was quantified in CD29, STRO-1, SSEA-4 and TNMD subpopulations, and compared to unsorted hASCs.

The preliminary evaluation based on flow cytometry analysis on the tendon markers assessed in the sorted subpopulations suggested that TNMD+ cells could have more potential for tendon engineering strategies considering the selected culture media. Subsequently, TNMD+ cell behavior was investigated after 14, 21 and 28 days of culture to better understand the influence of supplemented GF in the tenogenic commitment of TNMD+ over unsorted hASCs. After each time point, Scleraxis, Tenomodulin, Tenascin C, and Decorin were assessed by real-time qPCR and flow cytometry. The ability of TNMD positive cells to synthetize a collagen-rich ECM is an important parameter to successfully achieve a tenogenic phenotype and was also considered at both gene (*COL1A1*, *COL3A1*) and protein (COL I, COL III) level. Basic medium and unsorted hASCs were considered as experimental controls.

6.3.3. RT-PCR analysis of TNMD positive cells

Total RNA was extracted from hASCs (unsorted and TNMD subpopulation) before the addition of GFs to the culture media (day 0) as well as on day 14, 21 and 28 of tenogenic differentiation using TRI reagent (Sigma Aldrich) according to the manufacturer's instructions.

The cDNA synthesis was performed with the qScript cDNA Synthesis kit (Quanta Biosciences) and using 1 µg of total RNA in a volume of 20 µL. The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using 200 ng of cDNA and the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences) following the manufacturer's protocol, in a Real-Time Mastercycler ep realplex gradient S thermocycler (Eppendorf). The primers were pre-designed with Primer 3 software (Table 6-1) and synthesized by MWG Biotech. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was selected to evaluate the relative expression level for each target gene [32]. All values were firstly normalized against GAPDH values, and then to the unsorted hASCs (CTR) and TNMD+ cell samples collected before the induction of tenogenic differentiation (day 0).

Table 6-1. Primers used for quantitative RT-PCR analysis.

Target Gene	Primer sequence	NCBI reference
Human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F - AGCCTCAAGATCATCAGCAA	NM_002046.5
	R - GTCATGAGTCCTTCCACGAT	
Human Scleraxis (*SCXA*)	F - CGAGAACACCCAGCCCAAAC	XM_001717912
	R - CTCCGAATCGCAGTCTTTCTGTC	
Human Tenomodulin (*TNMD*)	F - GTCACATTCTAAATGCAGAAGC	NM_022144.2
	R - ATTGCTGTAGAAAGTGTGCTC	
Human Tenascin C (*TNC*)	F - ACTGCCAAGTTCACAACAGACC	NM_002160.3
	R - CCCACAATGACTTCCTTGACTG	
Human Decorin (*DCN*)	F - CAGCATTCCTCAAGGTCTTCCT	NM_001920.3
	R - GAGAGCCATTGTCAACAGCA	
Human Collagen, Type I, alpha 1 (*COL1A1*)	F - CGAAGACATCCCACCAATCAC	NM_000088.3
	R - GTCACAGATCACGTCATCGC	
Human Collagen, Type III, alpha 1 (*COL3A1*)	F - GCTGGCTACTTCTCGCTCTG	NM_000090.3
	R - TTGGCATGGTTCTGGCTTCC	

6.3.4. Assessment of Collagen I and Collagen III deposition by TNMD positive cells

Immunolabeling against COLI (Abcam, ab292) and COLIII (Abcam, ab7778) and cell nuclei staining with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 µg/µl, D9564, Sigma) was performed after 28 days in different supplemented media. The images were acquired under a microscope (Imager Z1m, Zeiss)

equipped with a digital camera (AxioCam MRm3) and under a confocal microscope TCS SP8 (Leica Microsystems).

6.3.5. Statistical analysis

Results are presented through the manuscript as mean ± standard deviation. The statistical analysis of data was performed using GraphPad Prism 6 software. The flow cytometry data as well as qPCR were analyzed using two-way analysis of variance (ANOVA) followed by Sidak's post-hoc test for multiple comparisons. The statistical degree of confidence was set as $p<0.05$ although lower degrees of significance were identified ($p<0.01$, $p<0.001$ and $p<0.0001$) and indicated accordingly.

6.4. Results and Discussion

6.4.1. Isolation of hASCs subpopulations

Human ASCs isolated from SVF constitute a heterogeneous population of potential progenitor cells for mesenchymal tissues. However, cells expressing tissue specific biomolecules as tendon markers are quite limited in crude hASCs population. Thus, in this study, we sought to identify a subpopulation of hASCs more prone to tenogenic differentiation using immunomagnetic beads. A tendon oriented subpopulation would result in a highly specific group of stem cells with potential added value over heterogeneous stem cell populations for application in tendon-based therapies.

Within the unsorted heterogeneous hASCs population, four distinct subpopulations were identified. The CD29+ hASCs was a considerable larger subpopulation (99.86%) than STRO-1+, SSEA-4+ or TNMD+ hASCs combined (8.35%) as assessed by flow cytometry. This may be related to the role of CD29 as stromal cell marker being expressed in stromal cells from adipose tissue, skin and bone marrow [33, 34], and consequently less specific to identify a progenitor subpopulation of cells with potential for *in vitro* tenogenesis. The second larger subpopulation was STRO-1+ cells: 4.64% followed by SSEA-4+ cells with 2.31% and TNMD+ cells that corresponded to 1.40% of the total hASCs population.

6.4.2. Pre-selection of the subpopulation more prone to tenogenic commitment by flow cytometry

The selection of hASCs based on TNMD, CD29, SSEA-4 and STRO-1 markers did not affect their stemness potential, as flow cytometry data indicated that both sorted and unsorted hASCs were positive for the stemness markers CD73, CD90 and CD105 (Table 6-2) but differently expressed SCXA, TNMD, TNC and DCN. Among the studied subpopulations and in comparison to the unsorted hASCs population, the TNMD+ subpopulation evidenced a higher percentage of positive cells for SCXA (3.6%), TNMD (8.3%), TNC (19.7%) and DCN (13.6%), suggesting that TNMD+ cells may have more potential for tenogenic differentiation.

Table 6-2. Expression of stemness and tendon related markers of unsorted hASCs and selected subpopulations, at passage 3, assessed after isolation in basal culture. The numbers represent percentage values.

Cell Populations	CD73	CD90	CD105	SCXA	TNMD	TNC	DCN
Unsorted hASCs	99.6	99.8	98.3	0.6	2.4	6.6	4.6
TNMD+	96.5	99.8	98.9	3.6	8.3	19.7	13.6
STRO-1+	97.8	99.6	98.9	2.6	2.6	-	7.6
CD29+	99.4	99.9	98.7	0.9	0.3	0.9	3.2
SSEA-4+	99.1	99.9	99.4	0.5	0.4	7.2	6.1

Nevertheless, hASCs sorted for CD29, STRO-1 or SSEA-4 were found in higher numbers within the crude hASCs population, which renders these cells more availability and easier expansion capability. Furthermore, in previous studies by our group, stem cells from different origins were observed to differently respond to GFs aiming at tenogenic differentiation [7]. Thus, we have also explored the influence of these GFs [7] on the behavior of hASCs subpopulations with a common origin in unsorted hASCs. GFs are important players in tissue homeostasis, repair and regeneration, having the ability to modulate tissue specific pathways or be involved in cell recruitment and often in stimulating mitogenic activity [29]. In the particular case of bFGF, TGF-β1 or PDGF-BB, these GFs were selected because they

are naturally available in the tendon milieu and described to be mediators of tendon repair and regenerative mechanisms [25, 34], with a potential role in MSCs tenogenic differentiation [7, 33]. Thus, hASCs subpopulations were cultured in medium supplemented with one of these GFs to better elucidate how the exogenous stimulation of bioactive molecules could influence the tenogenic commitment of sorted hASCs subpopulations. Table 6-3 shows the expression of SCXA, TNMD, TNC and DCN quantified by flow cytometry in studied subpopulations and in unsorted hASCs cultured in basal or supplemented GFs media for 28 days.

Table 6-3. Response of unsorted hASCs and hASCs subpopulations cultured in the presence of different growth factors on the expression of tenogenic markers determined by flow cytometry analysis after 28 days in culture. The numbers represent percentage of cells positive for SCX, TNMD, TNC or DCN markers.

	αMEM (without GFs)					bFGF				
	hASCs	TNMD+	STRO1+	CD29+	SSEA-4+	hASCs	TNMD+	STRO1+	CD29+	SSEA-4+
SCXA	3.47	5.12	2.21	1.62	2.32	1.6	6.42	11.24	1.00	2.09
TNMD	4.06	6.65	3.04	2.42	4.43	0.81	11.32	10.54	1.02	3.18
TNC	19.51	6.55	9.78	10.41	17.51	11.29	18.17	24.6	1.47	13.71
DCN	22.55	25.77	14.93	15.75	19.50	18.08	41.65	40.3	17.32	18.70
	TGF-β1					PDGF-BB				
	hASCs	TNMD+	STRO1+	CD29+	SSEA-4+	hASCs	TNMD+	STRO1+	CD29+	SSEA-4+
SCXA	4.58	10.27	8.66	3.94	5.06	2.1	4.68	6.86	1.87	1.94
TNMD	3.45	15.43	12.78	10.54	8.43	2.03	6.16	4.74	1.7	4.48
TNC	23.38	29.17	24.59	29.83	14.32	14.47	19.68	17.45	15.74	15.51
DCN	27.2	46.19	28.82	38.77	28.84	23.31	31.42	21.71	18.37	18.29

Legend: Growth factors (GFs): bFGF (Recombinant human fibroblast growth factor-basic), TGF-β1 (Recombinant human transforming growth factor-β1), PDGF-BB (Recombinant human platelet-derived growth factor BB); αMEM of hASCs (unsorted population) was supplemented with L-alanyl-L-glutamine (2 mM) and ascorbic acid (0.2 mM) and designated as basic medium. All GFs were supplemented at a concentration of 10 ng/mL.

In unsorted hASCs, the expression of tendon markers does not vary considerably with the GFs supplementation in the culture medium. Oppositely, TNC expression of CD29+ cells and DCN expression of CD29+ and SSEA-4+ cells seemed to be stimulated by TGF-β1. Among the studied subpopulations TNMD+ and STRO-1+ cells seemed to better respond to GFs, especially bFGF and TGF-β1, increasing significantly the number of cells with positive expression for all tendon markers analyzed.

Overall, the TNMD+ subpopulation showed the highest number of positive cells for tendon markers after 28 days in culture with the exception for TNC expression that is expressed by an increased number of unsorted hASCs and STRO-1+ cells under bFGF supplementation. This tendency was maintained in basal medium, confirming the outcomes suggested in Table 6-2, and when different GFs were supplemented to the medium. These results are supported by studies found in the literature that describe TNMD as a tendon-selective gene [35, 36] with relevance for the acquaintance of the tenogenic phenotype by MSCs.

Furthermore, the number of cells expressing tendon markers seemed to be enhanced in the presence of GFs, suggesting a potential role of GFs in the synthesis of tendon-related molecules and in particular in TNMD+ cells. Thus, TNMD+ subpopulation was further investigated to better understand the time frame evolution of their tenogenic profile under GFs differentiation environment up to 28 days and compared to the unsorted hASCs population.

6.4.3. Tenogenic profile of TNMD positive cells

The expression of scleraxis (*SCXA*), tenomodulin (*TNMD*), tenascin C (*TNC*) and decorin (*DCN*) was assessed by qPCR analysis in TNMD+ cells and unsorted hASCs population (CTR) after 14, 21 and 28 days in different supplemented media (Figure 6-2).

The results of genetic expression indicated a tendency for an increased expression of tenogenic genes in TNMD+ cells in comparison to unsorted hASCs. An exception is made for the *TNC* gene in which the trend is just the opposite. In particular, TNMD+ cells showed a significant up-regulation of *SCXA* after 14 days in bFGF supplemented medium while *TNMD* gene was predominantly expressed at 21 days in basic medium and media supplemented with bFGF and TGF-β1 as well as in EGF culture media at 28 days. Despite the higher levels of *DCN* detected in comparison to all other studied genes, *DCN* expression was also up-regulated in basal and bFGF media in TNMD+ cells after 4 weeks in culture.

One of the tendon markers assessed was TNC, a disulfide-linked hexamer glycoprotein and member of ECM proteoglycans [37] suggested to be involved in the regulation of tendon formation [38] and repair [39]. Low levels of *TNC* expression in tenocytes isolated from healthy adult tissue is one of the features of tensional tendon phenotype [40] that combined with a high expression of *COL 1* and *SCX* are most representative of the normal adult tendon phenotype [40]. Our results showed that *TNC* was up-regulated in the unsorted population (CTR) at 14 days in basal and TGF-β1 media, at 21 days in bFGF and PDGF-BB media and at 28 days in bFGF and TGF-β1 culture media. However, *TNC* was down-regulated in the TNMD+ subpopulation in unsupplemented medium as well as in presence of GFs, being particularly evident in bFGF medium. The low *TNC* expression and high *SCXA* expression may indicate that TNMD+ cells were achieving a normal tendon phenotype [40], that can also be influenced by the GFs in the culture media, specifically bFGF and TGF-β1. Studies describing the ability of bFGF to regulate *SCX* expression [41] and the involvement of TGF-β1 in the tendon phenotype/commitment of stem cells [42] support these results.

DCN is the most abundant proteoglycan in tendon that participates in the regulation of the collagen fibrillogenesis and cell proliferation as well as in the modulation of immune responses [43, 44]. Moreover, DCN is an important signaling molecule during joint and tendon formation in the developing limb at early stages of differentiation [45]. In our study, *DCN* overexpression was found in both TNMD+ and unsorted hASCs. This outcome is in agreement with the fact that DCN is relatively higher in hASCs than in MSCs from other sources and in native tendon [46]. *DCN* increases from 14 to 28 days in TNMD+ subpopulations cultured in basal and PDGF-BB media ($p<0.01$ and $p<0.001$ respectively). Although both DCN and TNMD are associated with tenocyte proliferation and that PDGF-BB and bFGF were described to accelerate fibroblast, tenocyte, and adipose-derived stem cell proliferation [47-49], bFGF down-regulated *TNMD* in the TNMD+ cells by 21 days. However, EGF, a biomolecule shown to increase non-collagenous protein synthesis by anterior cruciate and medial collateral ligament fibroblasts [50], stimulates the *TNMD* up-regulation by 28 days in culture. In control populations, bFGF stimulated the *TNC* expression but TGF-β1 inhibited *TNC* relative expression after 21 days in culture.

Overall, and despite differences found between control and TNMD+ subpopulation, results suggest that the cell response is differentially influenced by GFs supplementation in terms of expression upregulation of the studied genes. Moreover, analyzing the effect of each GF within a same population

(unsorted or TNMD+ subpopulation) along the experimental setup, it is clear that different GFs may differentially influence the genetic expression of the tendon markers investigated.

In the presence of bFGF not only *SCXA* was up-regulated in TNMD+ cells but the expression was increased in comparison to the *SCXA* values of cells cultured in TGF-β1 or EGF supplemented media as early as 14 days in culture. Studies in the literature support these results describing the bFGF role in the regulation of the *in vitro* expression of *SCX* in chondrocyte cell line, tendon progenitors and ASCs [51, 52]. Furthermore, an overexpression of *SCXA* was described to enhance tenogenic differentiation of human embryonic stem cells [53] and more recently the ectopic expression of *SCXA* was shown to promote hBM-MSCs differentiation to tendon progenitors [54].

The *DCN* was also increased in bFGF medium over all the other GFs supplemented media, the *TNMD* gene was significantly overexpressed in bFGF medium relatively to basal, TGF-β1, PDGF-BB and EGF supplemented media for 14 days of culture in TNMD+ cells whereas *TNC* was overexpressed in unsorted cells after 21 days in bFGF supplemented cultures. After 28 days of culture, *TNMD* expression was increased in TNMD+ cells cultured in bFGF, TGF-β1 and EGF media while *DCN* expression was found higher in basal and bFGF media. Despite the different contributions of studied GFs to the increased expression of tendon genes, the TNMD+ subpopulation is associated with an increased expression of *SCXA*, *TNMD* and *DCN*. Altogether, gene analysis indicates that by selecting the appropriate subpopulation it is possible to obtain cells that may be potentially closer to native tendon progenitor cells. This may be a promising regenerative value of TNMD+ cells over heterogeneous pools of hASCs for the treatment of tendon-associated pathologies.

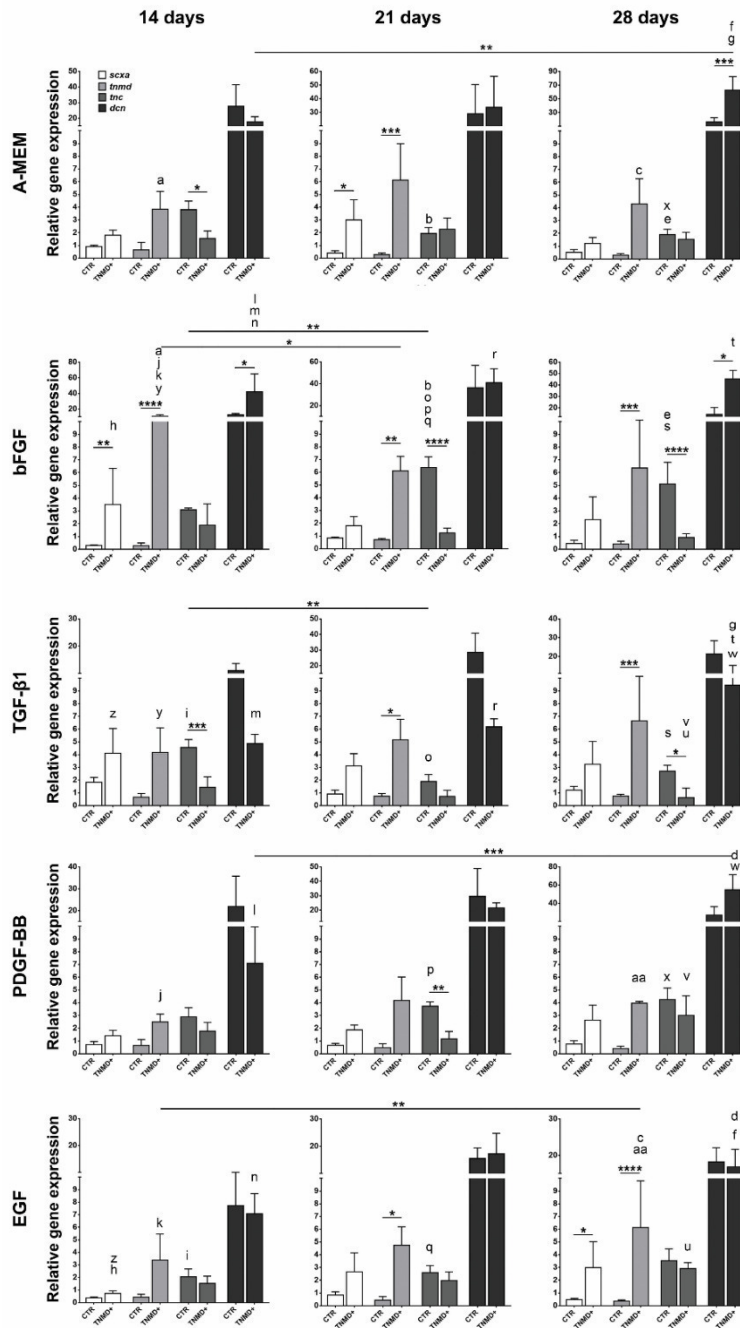

Figure 6-2. Gene expression of *SCXA*, *TNMD*, *TNC* and *DCN* in hASCs (TNMD+ subpopulation and CTR hASCs) cultured up to 28 days in α-MEM or GFs supplemented media. Data are represented as average of relative gene expression and error bars represent standard deviation. Symbols and letters denote statistically significant differences with different degrees of confidence. Two-Way ANOVA followed by Sidak's multiple comparisons test was performed and symbols *, **, ***, **** indicate statistically significant differences for $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively.

Formation of ECM: gene expression and Collagen I and III deposition

It is widely accepted that tissue microenvironment regulates development, functioning and repair of tendons and ligaments [55]. In healthy tendon, ECM is mostly composed of fibril-forming collagens namely COLI and COLIII [56]. Therefore, the ability of progenitor cells to express and deposit these proteins into a tendon-like ECM may ensure desirable tenogenic differentiation capacity and assist tendon regeneration.

Analysis of *COL1A1* expression obtained from unsorted and TNMD+ cells showed that cells cultured in unsupplemented medium presented higher values (Figure 6-3), suggesting that GFs may not be required for *COL1A1* upregulation. Although cells from mesenchymal origin may constitutively express and synthesize COLI, adipose tissue expresses COLI, COLIII and DCN at higher levels when compared to other tissues, namely bone marrow, umbilical cord blood, umbilical cord tissue or even from native tendon [57].

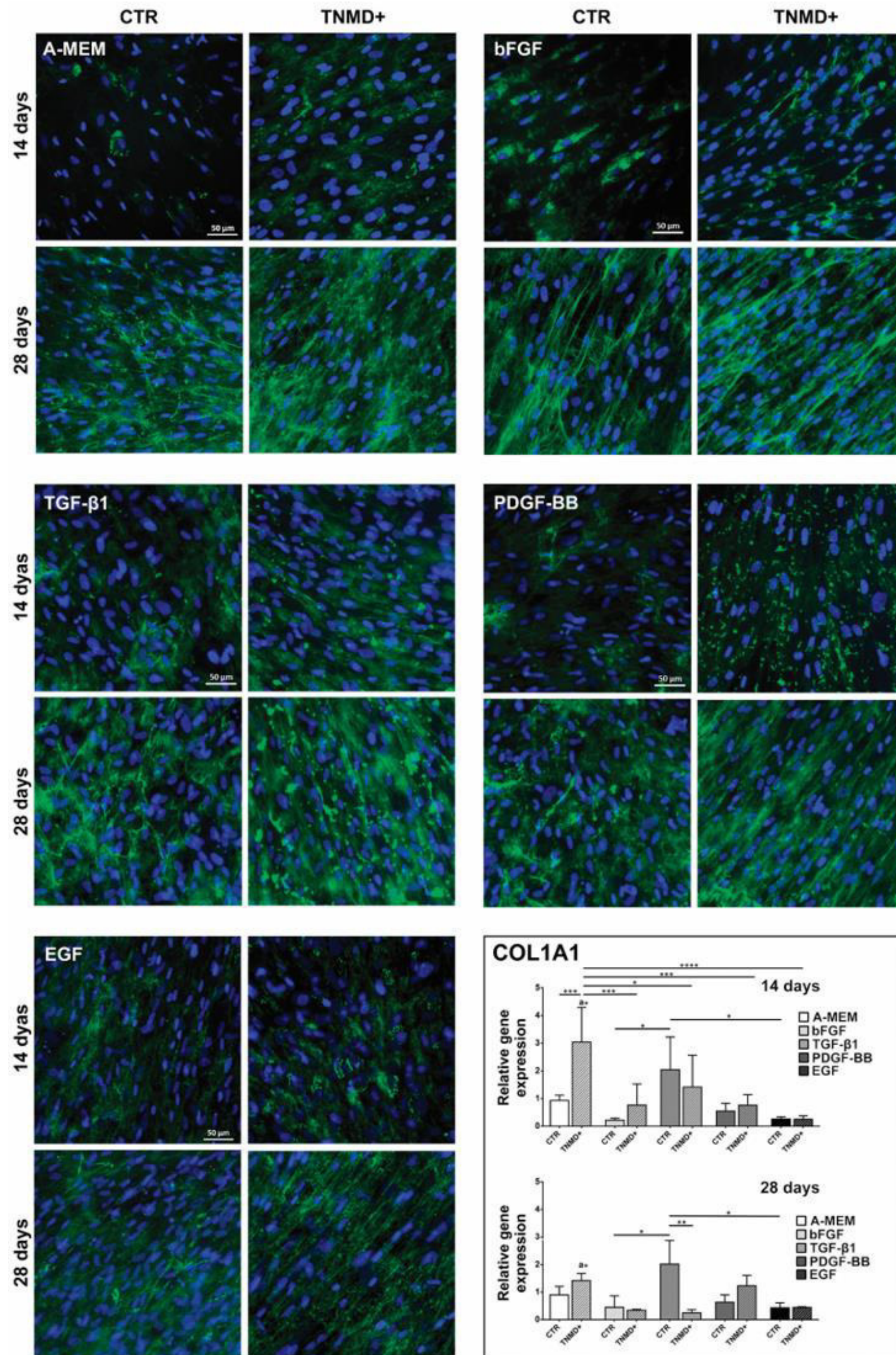

Figure 6-3. Collagen type I immunolocation and gene expression in control hASCs (CTR) and TNMD+ subpopulation cultured up to 28 days in different supplemented media. Collagen I is stained in green and nuclei stained with DAPI (blue). For qPCR data analysis symbols *, **, ***, **** indicate statistically significant differences for $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively. Letter "a*" denotes $p<0.05$ of significance. Two-Way ANOVA followed by Sidak's multiple comparisons test was used.

Both unsorted hASCs and TNMD+ subpopulation developed COLI and COLIII-rich networks in all medium conditions (Figure 6-3 and Figure 6-4). This is expected, as several works described the ability of unsorted hASCs populations to commit towards the tenogenic phenotype and synthesize a collagen-rich ECM [58]. After 28 days in culture, supplementation with bFGF, TGF-β1 and PDGF-BB resulted in the formation of a more aligned collagenous fibrillary network-COLI.

As for *COL1A1*, *COL3A1* was shown to be increased in basal medium cultured TNMD+ cells over unsorted (CTR) hASCs and over supplemented culture media after 14 days in culture. In unsorted hASCs, TGF-β1 impacts *COL3A1* expression over hASCs supplemented with bFGF and PDGF-BB. Despite the fact that *COL3A1* also decreases with time in TNMD+ cells in basal culture (b, $p<0.01$), there was a tendency for an increase in *COL3A1* in unsorted hASCs supplemented with TGF-β1 after 28 days in culture. The trend was similar when bFGF (especially by 14 days) and PDGF-BB was supplemented to the medium. In all other supplemented media, the gene values were quite alike. Among GFs studied, only TGF-β1 could induce an upregulation of *COL1A1* and *COL3A1*. This may be related to the fact that the TGF-β superfamily influences connective tissue formation, and TGF-β isoforms participate in tendon healing by regulation of collagen synthesis in tendon fibroblasts [59]. Moreover, studies with sheath fibroblasts, epitenon and endotenon tenocytes isolated from rabbit flexor tendons showed that the addition of TGF-β1 to culture media increased collagen I and III production [60].

Immunolocalization of COL III showed similar deposition in TNMD+ subpopulation and unsorted hASCs after 14 days in culture (Figure 6-4). The formation of a COL-III-rich network increased with the time in culture and seemed to be more evident in the TNMD+ subpopulation after 28 days. Furthermore, this dense fibrous-like structure tended to demonstrate alignment orientation to a certain degree in TNMD+ cells in comparison to unsorted hASCs. As a tendon oriented subpopulation, the production of an aligned collagen matrix by TNMD+ cells is a desirable result since tendons are highly organized tissues whose parallel orientation is essential for their functionality in force transmission mechanisms [61, 62].

Therefore, the better alignment of the collagen fibers produced by TNMD+ cells together with the tendency of these cells to align themselves following the typical aligned displacement and parallel orientation of tendon cells within the tendon matrix relate this subpopulation to a tenogenic predisposition, a potentially advantageous feature toward the acquaintance of a healthy tendon phenotype. Moreover, a successful and complete tenogenic commitment of tendon-prone stem cells will

favour local regenerative mechanisms, envisioning an improved therapeutic action over current cell-based applications.

Although both populations produce Collagen I and Collagen III in a dependent manner, TGF-β1 supplementation to the medium also guides cell tenogenic phenotype.

Our previous study on the influence of GFs as mediators of tenogenic differentiation has shown that EGF and PDGF-BB evidenced a more prominent effect in the genetic expression of tendon markers in a hASCs population than TGF-β1 and bFGF [7], and that result may be related to the stem cell origin, as stem cells derived from the amniotic fluid were more influenced by bFGF and PDGF-BB to express tendon genes. We speculate that although TNMD+ cells are originated from the hASCs pool, differences in the expression of tissue specific markers may be due to paracrine signaling noise that could mask biological responses of tissue oriented subpopulations.

In this work we compared the tenogenic potential between a crude population of hASCs and sorted TNMD+ cell subpopulation cultured with EGF, bFGF, TGF-β1, and PDGF-BB. It was clear that the cellular population has more impact in the gene and protein expression of tendon markers than GFs supplemented to the culture media with the exception of TGF-β1. Nevertheless, the exposure of TNMD+ cells to GFs should be deeper investigated in a time and concentration dependent manner, since GFs may be useful in the stimulation of cells (subpopulations) envisioning cell-based therapies. Since the synthesis and alignment of the collagen fibers within the ECM produced by TNMD+ cells is an important parameter to consider in tenogenic differentiation together with the gene expression of tendon markers, we decided to analyse the protein expression of non-collagenous markers (SCXA, TNMD, TNC and DCN) by flow cytometry in order to confirm the efficiency of tenogenic differentiation after 28 days in differentiation culture media.

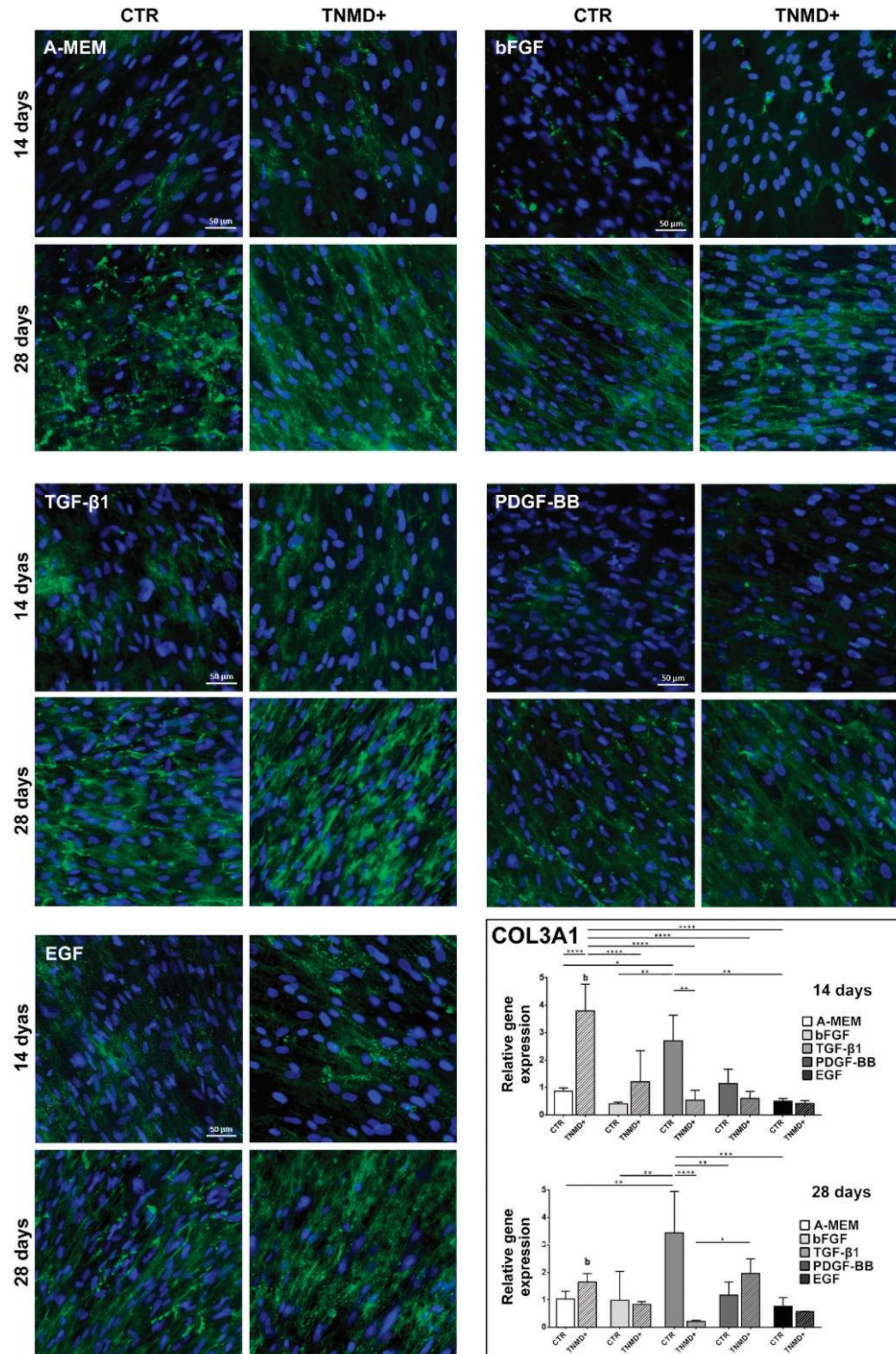

Figure 6-4. Collagen type III immunolocation and gene expression in control hASCs (CTR) and TNMD+ subpopulation cultured up to 28 days in different supplemented media. Collagen III is stained in green and nuclei stained with DAPI (blue). For qPCR data analysis symbols *, **, ***, **** indicate statistically significant differences for $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively. Letter "b**" denotes $p<0.01$ of significance. Two-Way ANOVA followed by Sidak's multiple comparisons test was used.

Expression of tendon specific markers by TNMD+ cells - Flow Cytometry

Figure 6-5 shows the results obtained for the number of cells the expressing SCXA, TNMD, TNC and DCN in unsorted hASCs (CTR) and TNMD+ hASCs subpopulation upon 28 days of culture by flow cytometry analysis. TGF-β1, and bFGF in a smaller extent, significantly influenced the number of cells expressing SCXA and TNMD within the TNMD+ subpopulation. Moreover, TNMD+ cells positive for these markers were increased over unsorted hASCs, which confirms the results on the increased TNMD expression in TNMD+ cells. The percentage of positive cells for TNMD in this subpopulation is significantly higher with TGF-β1 than all other conditions, highlighting the relevance of TGF-β1 in the synthesis of TNMD and follows published reports on TGF-β as a promotor of the expression of TNMD in Rhode Island chicken embryos [63]. Supplementation with TGF-β1 significantly increased the percentage of cells expressing SCXA in TNMD+ cells in α-MEM but also in media supplemented with PDGF-BB or EGF. TGF-β1 also showed a positive influence in TNC expression in TNMD+ cells versus control hASCs. Interestingly, the expression of DCN does not seem to depend on the cell population or the GF supplementation as no differences were observed among studied conditions.

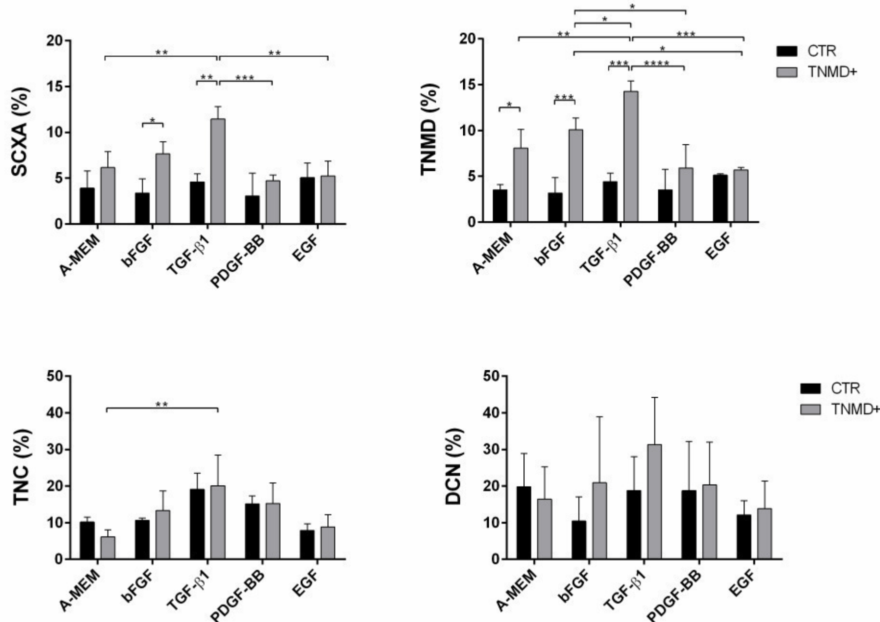

Figure 6-5. Expression of tenogenic markers determined by flow cytometry analysis of unsorted crude (CTR) and TNMD+ cells cultured for 28 days in basic medium (α-MEM) or in media supplemented with different GFs. Expression is represented as average percentage of positive cells for the different analyzed markers and error bars

represent standard deviation. Two-Way ANOVA followed by Sidak's multiple comparisons test was performed and symbols *, **, ***, **** indicate statistically significant differences for $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively.

This study was meant to be a starting point for cell based therapies, highlighting the differential role of stem cell subpopulations to potentially heal and regenerate tendon lesions. Although the yield of tenomodulin positive cells sorted from adipose tissue fractions is quite low, a subset of cells with more tenogenic action could be therapeutically more advantageous than a heterogenous population of hASCs harvested in higher quantity. We hypothesized that the tenogenic potential of TNMD+ cells may be constrained by cell to cell signalling provided by other cell subpopulations naturally available in the pool of hASCs. Despite variations found in the responses to the studied GFs, the influence of TGF-β1, and bFGF to some extent, seemed to be the most relevant in guiding tenogenesis. Nevertheless, a tenogenic differentiation medium may require a combination of these or other GFs, encouraging further investigations. Moreover, future studies of *in vitro* microenvironments for hASCs differentiation may also consider mechanical and physical factors, exploring synergistic effects on the stimulation of the tenogenic phenotype.

6.5. Conclusions

The present study shows that there are considerable differences associated to the expression of tendon markers and extracellular matrix components in a heterogeneous hASCs population isolated from SVF and its subpopulations sorted using specific markers. Among the hASCs subpopulations screened aiming at tendon applications, hASCs positive for TNMD evidenced a higher number of cells expressing surface tenogenic markers after 28 days in culture and their tenogenic differentiation capacity was further investigated in basal and differentiation culture media supplemented with GFs.

The findings of this study indicate that TNMD+ cells respond differently from the unsorted population, with an increased percentage of cells positive for SCX, TNMD and DCN assessed by flow cytometry, up-regulation of tendon genes, and the ability to synthesize a Collagen I and Collagen III rich ECM. Thus, TNMD+ cells have been identified as a subset of hASCs more prone to be induced into tenogenic lineage and explored for cell-based approaches aiming at tenogenic therapies. The fact that tendon markers were highly expressed in TNMD+ cells even in the absence of GFs supplementation, clearly demonstrates

a commitment of TNMD+ phenotype towards the tenogenic lineage. This work also highlights the relevance of identifying stem cells subpopulations and inquiries about the different roles these subpopulations may have in lineage commitment for regeneration of tissues. Given the wide availability of hASCs and their therapeutic features in cell-based therapies, TNMD+ subpopulation seems to be a promising source of precursor cells for tendon tissue engineering and regenerative medicine applications.

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Chapter 7

Tissue-Engineered Magnetic Cell Sheet Patches For Advanced Strategies in Tendon Regeneration

This chapter is based on the following publication:

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7. TISSUE-ENGINEERED MAGNETIC CELL SHEET PATCHES FOR ADVANCED STRATEGIES IN TENDON REGENERATION

7.1. Abstract

Tendons are powerful 3D biomechanically structures combining a few cells in an intrincated and highly hierarchical niche environment. When tendon homeostasis is compromised, restoration of functionality upon injury is limited and requires alternatives to current augmentation or replacement strategies. Cell sheet technologies are a powerful tool for the fabrication of living extracellular-rich patches towards regeneration of tenotopic defects. Thus, we originally propose the development of magnetically responsive tenogenic patches through magnetic cell sheet (magCSs) technology that enable the remote control upon implantation of the tendon-mimicking constructs. A Tenomodulin positive ($Tnmd^{+}$) subpopulation of cells sorted from a crude population of human adipose stem cells (hASCs) previously identified as being prone to tenogenesis was selected for the magCSs patch construction. We investigated the stability, the cellular co-location of the iron oxide nanoparticles (MNPs), as well as the morphology and mechanical properties of the developed magCSs. Moreover, the expression of tendon markers and collagenous tendon-like matrix were further assessed under the actuation of an external magnetic field.

Overall, this study confirms the potential to bioengineer tendon patches using a magnetic cell sheet construction with magnetic responsiveness, good mechanoelastic properties and a tenogenic prone stem cell population envisioning cell-based functional therapies towards tendon regeneration.

7.2. Introduction

Tendon lesions have a high incidence, representing a considerable share of musculoskeletal pathologies, and constituting a major cause of pain and disability in the adult population. Moreover, the socio-economic costs associated to tendon treatments and clinical procedures are an important argument for the development of new tendon repair and/or regeneration strategies. Besides tendon grafts, augmentation strategies are being pursued with synthetic or extracellular matrix (ECM)-rich patches. Commercial biological patches are mammalian tissue derived, and include human, porcine,

bovine and equine sources which may give rise to postoperative immune response complications [1-3], the risk of infection, long-term integrity loss, and, in some cases, inferior mechanical support.

Hence, this work explores the development of living autologous patches fabricated using a magnetic stem cell sheet template with improved tenogenic capacity aiming at promoting the natural healing and regeneration of rotator cuff tendons. Cells are active elements in tissue engineering strategies and local delivery of cells as regenerative agents have been developed with clinical significance. In the particular case of tendon disorders, clinical trials focusing on cell therapy rely on injections of stem cells or stem cells combined with platelet-rich plasma [4]. However, the direct application of stem cells hinders their therapeutic action due to the cell dispersion and spreading towards other tissues and the lack of support to guide the cells [5, 6]. Cell sheet technologies can locally delivery cells in a 3D ECM enriched context to support and guide the cells upon implantation as regenerative platforms with clinical market significance, following autologous approaches if desired. Being a living construct, cell sheets offer paracrine signaling while simultaneously recreate the structural complexity of the ECM of native tissues. Companies such as CellSeed or Cytograft Tissue Engineering, Inc. have considered the potential of repairing damaged tissues using cell sheet technologies for esophageal, cornea and periodontal tissue regeneration.

Ito *et al* proposed a new technology to obtain cell sheets based on the use of magnetic forces [7]. The potential of magnetic cell sheets has been reported in angiogenic [8-10], cardiac [11], bone [12], and skeletal muscle [13, 14], highlighting the role of this strategy as a potential alternative over artificial synthetic scaffolding materials for a wide range of tissues. More recently, scaffold-free constructs have been reported in stimulating neo-tendon formation with promising outcomes in *in vivo* tendon healing [15, 16]. However, tenogenic cell therapy resorting on magnetic cell sheets (magCSs) hasn't been investigated to date. Therefore, we hypothesized that the construction of magnetic cell sheets with a tenogenic prone subpopulation of hASCs (TNMD+ hASCs) [17] could be an effective approach for the fabrication of a living patch aiming at tendon regeneration.

Although tendon resident cells are an optimal approach to address a structural and functional replacement, isolating local and scarce cells would increase tissue morbidity at the harvesting site, creating an undesirable side-defect. Thus, the sorting of stem cell subpopulations from the stromal vascular fraction of adipose tissue has proven to be an efficient method for selecting precursor cells

predisposed into musculoskeletal tissue lineages including osteogenic [18-20], chondrogenic [18], endothelial [21, 22] or tenogenic [17]. Tenomodulin is a glycoprotein highly and specifically expressed in tendons and ligaments, with an extracellular C-terminal domain, thus a good target for cell sorting [23-25]. Thus, and based on the potential of tenogenically predispose TNMD+ stem cells assessed in a previous study from our group [17], this subpopulation was selected as the prefered cell source to fabricate our cell sheets. Additionally, the incorporation of magnetic nanoparticles (MNPs) results in magnetic responsiveness constructs with reinforced mechanical properties and a cell-directed biomechanical stimulation provided by MNPs [26, 27] that can further trigger a tendon specific ECM production. Moreover, we anticipate that the exogenous delivery of stem cells labeled with MNPs assisted by magnetic field therapy may also trigger the inflammation response, as suggested in a previous work [28], by modulating the inflammatory environment *in situ*. Therefore, the concept herein explored is the use of tenogenically predisposed stem cells in combination with MNPs stimulated by an external magnetic field (Figure 7-1) prospecting ECM production and reinforcement, and aiming at creating a specific patch that mimics tendon native milieu for achieving functional tendon regeneration.

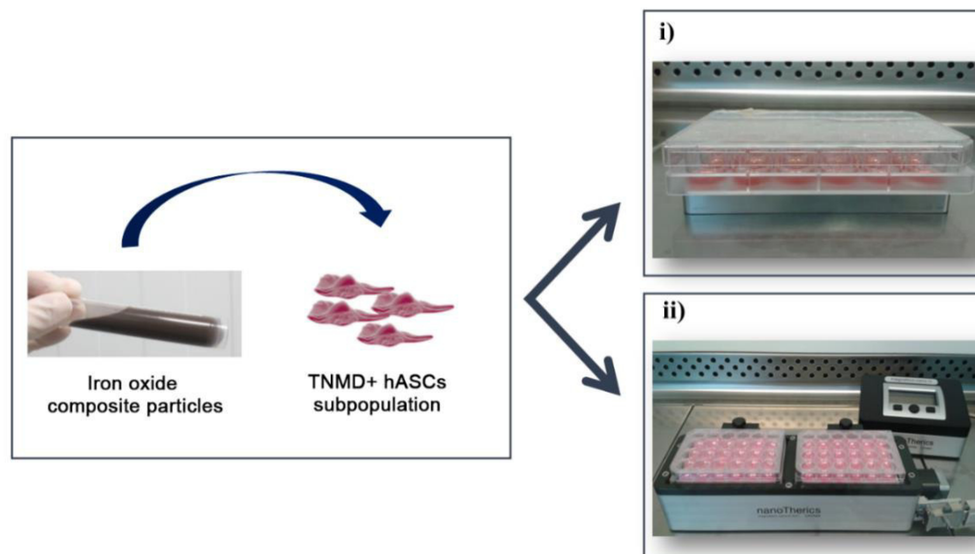


Figure 7-1. Schematic representation of the magnetic patch construction employing cell sheet technology with Tenomodulin positive (TNMD+) adipose derived stem cells (hASCs) magnetized with iron oxide nanoparticles: TNMD+ cell sheets were cultured under a permanent well-array magnet (i) or under magnetic stimulated conditions using a low frequency oscillating magnetic field (ii) up to 7 days in basic culture medium.

7.3. Materials and Methods

7.3.1. Magnetic nanoparticles (MNPs) stability in the culture medium

MNPs used in the present work are commercially available (nanomag®-C, 04-00-152, Micromod). Chitosan iron oxide MNPs (chemical nature: iron oxide [CAS:1317-61-9] 80-85 wt%, chitosan [CAS. 9012-76-4]) are ~150nm in size and have a polydispersity index < 0.2. Moreover, MNPs saturation magnetization is > 75emu/g iron (H > 10.000 Oe), and are referred to be stable in aqueous buffers pH>4. These MNPs were characterized for water dispersant stability using a Zetasizer Nano ZS equipment (Malvern Instruments, UK). Moreover, to confirm the stability of MNPs in culture medium, a turbidity assay was performed at 450nm [29], and monitored over a period up to 24h with a MNP concentration of 400μg/ml using a microplate reader (Bio-Tek, synergie HT). MNPs suspended in αMEM culture medium were analysed in quadruplicates and MNP free culture medium served as experimental control (Supplementary Figure 7-1).

7.3.2. Isolation of a Tnmd+ subpopulation of hASCs by IMS

Human adipose-derived stem cells (hASCs) were obtained from lipoaspirate samples from the abdominal region, under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee.

Human ASCs were enzymatically isolated from lipoaspirate samples and characterized as described elsewhere [30, 31]. hASCs were expanded in basic culture medium composed of α-MEM (A-MEM, Invitrogen) supplemented with 10 % FBS (Alfagene) and 1 % A/A solution (Alfagene), prior to immunomagnetic sorting (IMS) of the TNMD+ hASCs subpopulation of interest. This subpopulation was selected based on a study previously performed in our group, in which a TNMD+ subpopulation of hASCs showed improved tenogenic potential over other subpopulations isolated from hASCs [17]. IMS was performed by coupling commercially available magnetic beads (Dynabeads® M-450 Epoxy, Invitrogen) with anti-Tenomodulin (C-20) antibody (Santa Cruz Biotechnology, SC-49324) following the manufacturer's instructions and as previously described [17]. The TNMD+ subpopulation was screened by flow cytometry for stemness markers as CD73, CD90, and CD105 [30] and for tenogenic markers,

including TNMD, TNC, DCN and SCXA [17]. Immunocytochemistry to confirm the presence of Tenomodulin protein in TNMD+ subpopulation was also performed immediately before the construction of magCSs as described in section 2.6.5 (Supplementary Figure 7-2).

7.3.3. Living patch construction using cell sheet technologies

Tnmd$^+$ hASCs were seeded into 6 well plates (Falcon) at a density of 600,000 cells per well and left to adhere for 2h at 37°C. Then, chitosan coated iron oxide MNPs were cultured with the Tnmd$^+$ hASCs at 400μg/mL (corresponding to 200pg/cell) followed by overnight incubation. After 16h, MNPs labeled Tnmd+ hASCs were washed with D-PBS (Alfagene), harvested from the 6 well plates using TrypLE™ Express (12605-028, Alfagene) and seeded into 24 well plates. Cells were cultured in basic α-MEM medium under a 24-well magnet array plate (magnetic induction of 350mT per magnet per well) for up to 7 days, and defined as "magnet" condition. After 24h and every two days, cells were washed with D-PBS and the culture medium replaced by fresh α-MEM basic medium. MagCSs were characterized for the presence of MNPs and morphological analysis (SEM, Micro-CT, Hematoxilyn & Eosin, Masson Trichrome, Sirius Red/Fast Green stains, and Dynamic mechanical analysis).

SEM and Micro-CT analysis

After 7 days in culture, magCSs were fixed in 10% buffered formalin (5701, Thermo Scientific) for 1h at room temperature and dehydrated in a series of increasing ethanol concentrations. Their structure and morphological characterization was analysed by scanning electron microscopy (SEM: JSM-6010LV, JEOL) and a high-resolution X-ray Microtomography (micro-CT) System1272 scanner (Skyscan, Belgium). The elemental characterization was also performed using an energy dispersive spectroscope (EDS: INCAx-Act, PentaFET Precision, Oxford Instruments) to confirm the presence of iron (Fe) element.

The micro-CT scans were obtained in a SkyScan1272 equipment (Bruker) with a resolution of pixel size set at 18.09μm, a camera binning of 4x4 and an exposure time of 260ms. The X-ray source was set at 45kV and 200μA. Representative data sets of 736 projections were acquired with a rotation range of 360° and a rotation step of 0.2°. Data sets from the magCSs acquisition were reconstructed using NRecon (vs1.6.6.0, SkyScan) software. DataViewer (version 1.4.4, Skyscan) and CT Vox (version 2.3.0, Skyscan) software were used to build 3D virtual models of the magCSs from acquired data sets.

Hematoxilyn and Eosin (H&E), Masson Trichrome, and Sirius Red/Fast Green Staining

MagCSs were included in an 4% agarose/PBS solution (Seakem LE, Lonza) and paraffin embedded. Representative sections were stained with HE, Masson trichrome (04-010802, Bio-optica) and Sirius Red/Fast Green collagen staining kit (9046, Chondrex), according to the manufacturer's protocols. Sections were observed under a microscope (Leica, DM750) and images acquired with LAS V4.6 software.

Dynamic mechanical analysis (DMA)

The mechanical properties of the developed magCSs were characterized using a DMA equipment, equipped with the tensile mode. Tension cycles of increasing frequency (0.1-10 Hz) were applied and the measurements carried out under simulated physiologic conditions at 37°C in αMEM medium placed in a Teflon reservoir. The thickness of magCSs calculated as 52.06μm was assessed by confocal laser scanning microscopy (TCS SP8, Leica) using LASX software vs8. The storage (elastic) modulus, E', and the loss modulus, E'', were considered as main parameters to assess magCSs mechanical properties. The distance between compression plates was the same for all samples tested. A minimum of 5 samples per condition were assessed (n=5).

7.3.4. Assessment of MNPs internalization in hASCs

Cells were seeded onto 24 well plates at a density of 60,000 cells per well, followed by incubation with red fluorescence MNPs (Micromod) at 20, 200 and 400μg/mL under static (St) or dynamically magnetic stimulation (Dy) provided by magnefect nano device (nanoTherics Ltd, UK) with an oscillation frequency of 2Hz and 0.2mm of displacement for 4 and 16h before being characterized by fluorescence microscopy, Prussian blue staining, metabolic activity by MTS assay and ICP analysis.

The co-location of MNPs in Tnmd+ hASCs was assessed by seeding the cells onto 24 well plates (60,000 cells/well), followed by incubation with red fluorescence MNPs at 400μg/mL (corresponding to 200pg/cell). The cell suspension was immunostained for 30min with anti-human CD90 (Thy-1) FITC (eBioscience), washed with D-PBS and centrifuged at 400g for 6min. The double positive cells for CD90 and MNPs were sorted using a Cell Sorter (FACSAriaIII™, BD Biosciences and FACS DIVA 7.0 software), and collected into D-PBS with 2% of FBS. Sorted cells were then cultured in 13mm tissue culture

coverslips (Sarstedt) in αMEM medium for 3 days and then fixed in 10% buffered formalin (5701, Thermo Scientific) before iron detection (Prussian blue) and LAMP-1 immunolocation.

Prussian blue staining

For iron detection, Tnmd+ cells incorporating MNPs were stained with a solution of equal parts of hydrochloric acid (20%, VWR) and potassium ferrocyanide (10%, P3289, Sigma) for 20 min before rinsed 3 times with distilled water. Cells were counterstained with Eosin-Y Alcoholic solution (71204, Thermo Scientific) for cytoplasmic detection and visualized under a transmitted and reflected light microscope (Axio Imager Z1m, Zeiss).

LAMP-1 immunolabelling

Fixed Tnmd+ cells undergoing LAMP-1 immunolabelling were permeabilized with 0.025% Triton-X100 (Sigma/X100)/PBS solution, blocked with Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector) and incubated overnight with mouse monoclonal [H4A3] to LAMP-1 antibody (ab25630, Abcam) in diluent with background reducing components (S3022, Dako) at 4°C. Cells were rinsed in 0.025% Triton-X100 (Sigma/X100)/PBS solution, following incubation for 1h at room temperature with Alexa Fluor® 488 donkey anti-mouse IgG (H+L) (A21202, Alfagene). Then, samples were rinsed in PBS (P4417, Sigma) and stained with 4,6-Diamidino-2-phenyindole dilactate (DAPI, 40009, VWR) for 10min. The images were acquired under a confocal laser scanning microscope (TCS SP8, Leica Microsystems CMS).

Inductively Coupled Plasma (ICP) spectrometer analysis

Culture medium samples were collected after 4 and 16h of incubation, filtered with a 0.22μm filter (VWR) and frozen at -80°C until analysis. The incorporation efficiency of MNPs within cells was analysed and measured by subtracting the initial concentration of MNPs added to the culture medium to the obtained ICP values (JY2000-2, Horiba Jobin Yvon). An iron (Fe, 1000μg/mL) standard solution (13830, Specpure) was used to prepare the standard concentration solutions.

Transmission electron microscopy (TEM) analysis

Tnmd+ hASCs were fixed in a solution of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in cacodylate Buffer, 0.1M (pH 7.4). Then, samples were dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin sections (40–

60nm thickness) were prepared on a RMC Ultramicrotome (PowerTome, USA) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on a 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate, and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera (Orious 1100W Tokyo, Japan).

7.3.5. Cellular viability and oxidative stress

Tnmd+ hASCs incorporating MNPs were tripsinised and analysed by flow cytometry for i) cell viability using a 7-Aminoactinomycin D (7-AAD) staining solution (YC2.420404, BioLegend) and ii) for oxidative stress using the DCFDA - Cellular Reactive Oxygen Species (ROS) Detection Assay (ab113851, abcam), both performed according to the manufacturer's instructions.

Briefly, Tnmd+ hASCs suspensions were incubated at room temperature for 10min with 7-AAD, while for ROS detection assay, Tnmd+ hASCs were stained with 20µM of 2',7'-dichlorofluorescin diacetate (DCFDA) or left unstained, (non-stained control cells) followed a 30min incubation at 37°C before being treated for 4h with Tert-butyl hydroperoxide (TBHP, the positive control). In both assays, a minimum of 30,000 cells/tube were acquired and analysed on a FACSAriaIII cytometer (BD Biosciences) using FACS DIVA 7.0 software. FL1 channel (FITC) and PerCPCy5.5 were used for DCF and 7-AAD detection, respectively. Three independent cell suspensions were considered.

7.3.6. Culture of magCSs under Magnet or Magnetic stimulating conditions

MagCSs constructed by magnetic force-based TE were cultured under permanent magnet well array („Magnet") or under magnetic stimulating conditions using a low frequency oscillating magnetic field bioreactor („Mag stimulation"). Both conditions had an actuation of 350mT per magnet per well, however in magnetic stimulating conditions samples were subjected to an oscillation frequency of 2Hz and 0.2mm of displacement for 1h/day, provided by a magnefect nano device (nanoTherics Ltd, UK). magCSs were characterized for cell viability, proliferation and tenogenic phenotype 3 and 7 days after magCSs construction in both conditions.

Metabolic activity and proliferation

The metabolic activity of magCSs was evaluated by MTS assay (Cell Titer 96® Aqueous Solution Cell Proliferation Assay, Promega). MagCSs were rinsed in PBS before being 3h incubated in a mixture of FBS-free and phenol red free medium and MTS solution (5:1 ratio) at 37°C and 5% CO_2 atmosphere. Then, 100μl of each sample was transferred to a new 96-well plate and the absorbance read at 490nm (Synergy HT, Bio-TeK Instruments). Triplicates were made of each sample and a blank reading was performed. Cell content and proliferation of magCSs were assessed by Quant-iT PicoGreen dsDNA Assay Kit (P7589, Invitrogen). Samples were collected into microtubes with 1mL of ultrapure water and stored at -80°C. Then, samples were thawed, sonicated (VCX-130PB-220, Sonics) and the supernatant containing the dsDNA was collected to a new microtube upon magnetic separation (MPC®-S, Dynal Biotech). The supernatant was analysed using a microplate reader with an excitation of 485/20nm and an emission of 528/20nm. Samples and standards were made in triplicate.

mRNA Extraction and Real-time RT-PCR

Total RNA was extracted from the magCSs using TRI reagent (Sigma Aldrich) according to the manufacturer's instructions. RNA quantity and purity were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The cDNA synthesis was performed with the qScript cDNA Synthesis kit (Quanta Biosciences) and using the Mastercycler® ep realplex gradient S machine (Eppendorf) with an initial amount of total RNA of 1μg in 20μL. The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences) following the manufacturer's protocol, in a Real-Time Mastercycler ep realplex thermocycler (Eppendorf). The primers were pre-designed with Primer 3 software (Table 7-1) and synthesized by MWG Biotech. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was selected to evaluate the relative expression level for each target gene [32]. All values were first normalized against GAPDH values, and then to the samples collected at day 1 of culture.

Table 7-1. Primers used for quantitative real time RT-PCR analysis.

Gene	Sequence	NCBI reference
Human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F- AGCCTCAAGATCATCAGCAA R- GTCATGAGTCCTTCCACGAT	NM_002046.5
Collagen, Type I, alpha 1 (*COL1A1*)	F- CGAAGACATCCCACCAATCAC R- GTCACAGATCACGTCATCGC	NM_000088.3
Collagen, Type III, alpha 1 (*COL3A1*)	F- GCTGGCTACTTCTCGCTCTG R- TTGGCATGGTTCTGGCTTCC	NM_000090.3
Tenascin C (*TNC*)	F- ACTGCCAAGTTCACAACAGACC R- CCCACAATGACTTCCTTGACTG	NM_002160.3
Scleraxis (*SCXA*)	F- CGAGAACACCCAGCCCAAAC R- CTCCGAATCGCAGTCTTTCTGTC	XM_001717912
Decorin (*DCN*)	F- CAGCATTCCTCAAGGTCTTCCT R- GAGAGCCATTGTCAACAGCA	NM_001920.3
Tenomodulin (*TNMD*)	F- CCGCGTCTGTGAACCTTTAC R- CACCCACCAGTTACAAGGCA	NM_022144.2

Total Protein quantification

Cellular proteins were extracted from magCSs after day 1, 3 and 7, using radio-immunoprecipitation assay (RIPA buffer, R0278, Sigma), combined with a protease inhibitor cocktail (P8340, Sigma), at 1:100 proportion. After cell lysis, samples were incubated on ice for 5min and stored at -80°C. Then, samples were thawed, sonicated and the supernatant collected to a new microtube upon magnetic separation (MPC®-S, Dynal Biotech). The supernatant containing the soluble protein was centrifuged at 8,000g for 10min at 4°C before being transferred into a new tube on ice immediately before total protein quantification. Diluted albumin standards from 0 to 200µg/mL concentrations and samples were prepared in triplicates, whose absorbance was measured at 562nm.

Quantification of Collagen and Non-collagenous Proteins

A Sirius Red/Fast Green Collagen Staining Kit (9046, Chondrex) was used to quantify the amount of these proteins in magCSs. Cells were fixed in 95% ethanol and 5% glacial acetic acid for 10min at RT. Then, magCSs were completely immersed into the dye solution and incubated for 30min. A dye

extraction buffer was then mixed and the OD values read in a microplate reader at 540nm and 605nm. Data of collagen and non-collagenous proteins was normalized with total protein results.

Immunofluorescence

Immunolabeling of the magCSs prior to detachment was performed after fixation with 10% buffered formalin (5701, Thermo Scientific). The primary antibodies, Tenomodulin (sc-49324, SantaCruz Biotechnology), Vinculin (V9131, Sigma), Collagen type I (ab292, Abcam), Collagen type III (ab7778, Abcam), Decorin (ab54728, Abcam), and Tenascin C (ab6393, Abcam) were used. The procedure was similar to the one described for lysosomal immunolabeling. In the case of Tenomodulin and Vinculin immunolocation, a 20min staining with Phalloidin Tetramethylrhodamine B isothiocyanate (P1951, Sigma) was performed before observation with a fluorescence inverted microscope (Axio Observer, Zeiss). Semi-quantification of immunocytochemistry was performed using ImageJ software, based on Area fraction measurement of positively stained area.

7.3.7. Statistical analysis

Results are presented as mean ± standard deviation (SD) and analysed using two-way analysis of variance (ANOVA) or Unpaired t test analysis, for significant differences among studied conditions, GraphPad Prism 6 software. A minimum of 95% confidence interval was considered for all measurements ($p<0.05$) and denoted by brackets represented in the graphs.

7.4. Results and Discussion

7.4.1. Living patch by Magnetic TNMD+ Cell Sheet Technologies

Scaffold free strategies may be alternative tools to physiologically and functionally recapitulate native tissues integrating a bio-instructive milieu in which cells respond to. The application of MNPs has shown interesting outcomes as diagnosis and theranostic tools with increasing potential for clinically envisioned approaches [33, 34] in the years to come.

Since MNPs intrinsic properties such as size, surface charge and composition have a crucial role in cell interaction [35, 36], we investigated the stability of water soluble chitosan iron oxide MNPs used in the magnetic cell sheets (magCSs) construction measuring the average zeta potential which was found

to be +40.02±5.93mV (Supplementary Figure 7-1 A). This is an expected result as MNPs have a magnetite core and a positive charged shell of chitosan. Moreover, the positive charge of these MNPs allows electrostatically interactions with the cell membrane, resulting in magnetically functionalized cells. It has been reported that MNPs should be internalized by cells to some extent, as it is an important step in the feasibility of magCSs construction [7]. Turbidity measurements were also performed to confirm the MNPs stability in culture medium. The MNPs were easily dispersed and colloidally stable over a period of 24h (Supplementary Figure 7-1 B).

Development and characterization of magCSs with improved tenogenic potential

Magnetic cell sheets were successfully prepared combining MNPs with a TNMD+ subpopulation of hASCs. Moreover, these cell sheets can be easily recovered and manipulated using a 300mT magnet (Figure 7-2 A1, A2). The magCSs formed a dense TNMD+ cell layer (Figure 7-2 F) incorporating MNPs along a thickness of ~52μm estimated by confocal microscopy analysis after 7 days of culture (Figure 7-2 H) and as observed by histological stains and by SEM coupled with EDS analysis (Figure 7-2 B1, B2, G) with iron represented in red. SEM analysis also evidenced TNMD+ cells forming aligned columns of parallel fusiform cells with the co-location of the MNPs. HE stain (Figure 7-2 C1, C2) was applied to assess overall morphology and cell and MNPs distribution, while Masson's trichrome (Figure 7-2 D) and Sirius red/Fast green stains were employed to detect collagen fibers within the surrounding non-collagenous proteins and MNPs. Overall, the histologic stains demonstrated the presence of collagen and non-collagen proteins in the developed magCSs, as observed in a typical tendon ECM, in a consistent ECM distribution.

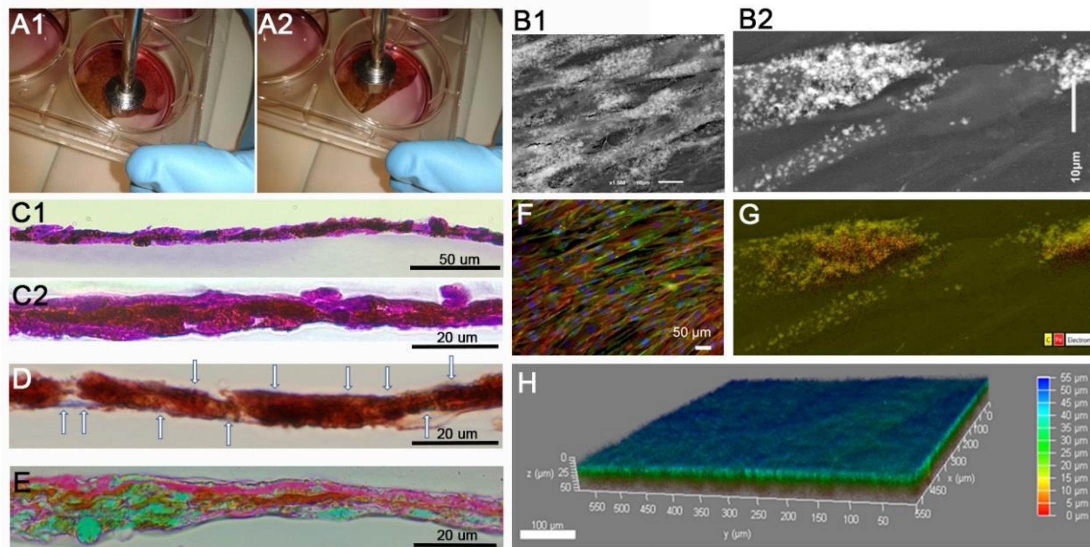


Figure 7-2. Magnetic cell sheets (magCSs) after 7 days in αMEM culture. (A1, A2) Photographs of the harvesting process with a permanent magnet. (B1, B2) SEM micrographs show the presence of iron oxide MNPs in bright white and by EDS mapping: iron (Fe) in red (G). (C1, C2) H&E staining of the magCSs trans-section. (D) Masson trichrome stain of the magCSs trans-section. (E) Sirius red/Fast green collagen staining of the magCSs trans-section. (F) Micrograph of Tenomodulin immunolocation (green) in the magCSs; Cell nuclei (blue) and cytoskeleton (red) were counterstained with DAPI and phalloidin, respectively. (H) Confocal laser scanning micrograph of the magCSs tridimensionality.

The fabrication of biological matrices should consider the biomechanical requirements needed for tendon functionality. Thus, the mechanical/viscoelastic properties of developed magCSs as a tendon patch were tensile tested by dynamic mechanical analysis (DMA). The storage modulus (E‘) and loss factor (tan δ) values are shown as a function of frequency (Figure 7-3 A). MagCSs E‘ values were within 34.83–37.19MPa ± 0.73, suggesting a high elastic behaviour and a recover potential. Human dynamic loads were described to occur between 1.7 and 2.3Hz [37], corresponding to a slow or fast walk respectively, being the standard walk of 2.0Hz. Despite the frequency spectrum tested, magCSs elastic potential was not affected by the frequency applied. Comparison between DMA results with the literature is challenging due to the lack of information and divergencies associated to the method of analysis, namely whether the entire cell sheet is evaluated in a tensional deformation cycle (DMA) or only a local elasticity measurement by indentation with atomic force microscopy (AFM).

Nevertheless, taking as a reference human ASCs stiffness and human tendon mechanical values, some comparisons may be devised. The storage modulus found for magCSs (36.34MPa ± 5.60 at 2Hz) are considerably increased over hASCs exhibiting an elastic modulus of ~ 3 - 5.2kPa as determined by AFM [38, 39]. Additionally, values for human tendon rotator cuff were reported between 12.4 - 17.3MPa [40, 41]. The higher E' values obtained for magCSs, are likely associated to a biomechanical reinforcement due to the incorporation of MNPs highlighting the capacity of these constructs to transmit loads between tendons and bone. This outcome is even more interesting considering a scaffold-free approach. The tan δ provides information about the intrinsic damping properties of viscoelastic materials [42]. Unlike elastic modulus values, magCSs will likely dissipate more energy with increasing frequency values, which can be translated into a higher damping capability.

The native ECM provides structural support for cell layers as basement membranes, and for individual cells as substrates for cell motility [43]. The high viscoelastic properties found in the magCSs may also be associated to the ECM produced by the TNMD+ hASCs. This matrix provided sufficient strength and cohesiveness to enable the magCSs to be detached as a contiguous cell membrane evidencing high elastic response towards tensional deformation.

The micro-CT analysis also supports the organization, biomechanical strength and cohesiveness of the ECM formed by the TNMD+ hASCs. Micro-CT images (Figure 7-3 B) of magCSs evidenced topographical roughness and a relatively homogeneous distribution of cells and MNPs throughout the magCSs.

Vinculin, a cytoskeletal protein associated with cell-cell and cell-matrix junctions, was also detected in the fabricated magCSs (Figure 7-3 C, D) confirming DMA and micro-CT results.

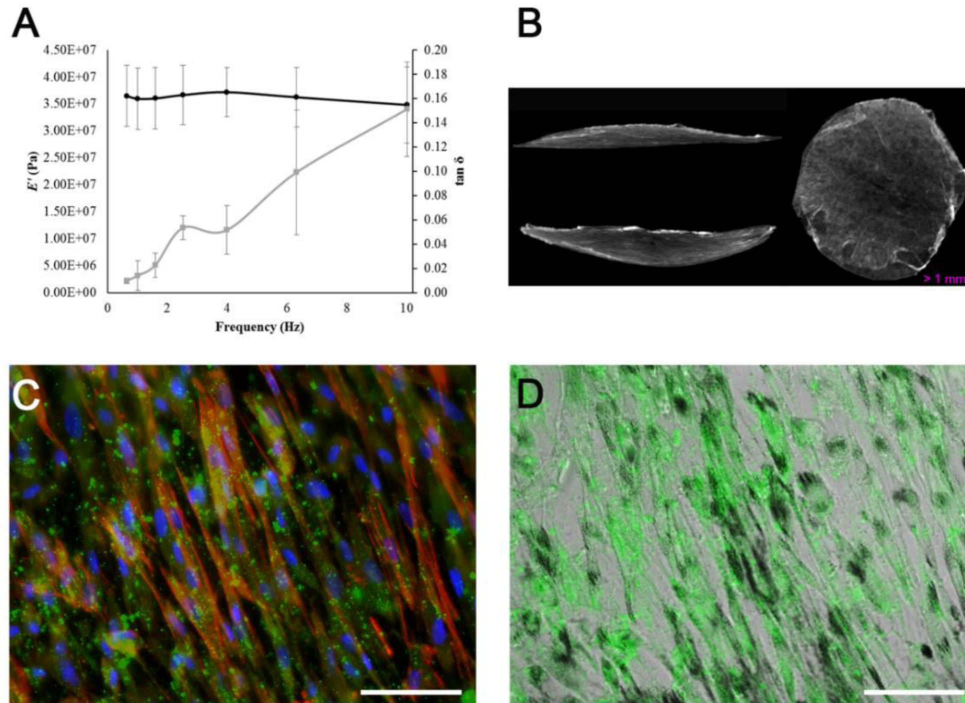


Figure 7-3. Stability and cohesiveness of magCSs. Biomechanical properties of magCSs assessed by DMA for storage modulus (E´) and loss factor (tan δ) (A), at 37°C in αMEM medium. (B) MicroCT images of magCSs. (C) Microscopy of vinculin immunolocation (green) in magCSs, counterstained with phalloidin (red) and DAPI (blue), and (D) Phase contrast microscopy evidencing vinculin (green) and MNPs (black); scale bar represents 100μm.

7.4.2. Intracellular Assessment of Magnetic Nanoparticles

Due to their nano scaled dimensions, MNPs were detected to some extent inside the cells (Figure 7-4 A1, A2). Moreover, the different MNPs concentrations studied after 4 or 16h of incubation did not negatively influence cells metabolic activity (Figure 7-4 A3), in some cases even increasing the absorbance levels ($p<0.001$). Cellular viability also increased with incubation time, showing that MNPs were efficiently internalized without affecting hASCs viability. The iron levels released into the culture medium after hASCs incubation with the MNPs were measured by ICP spectrometry (Figure 7-4 A4). The percentage of iron uptaken by cells was higher in dynamic conditions after 4h with values around 60-70% ($p<0.001$). This correlates with other studies showing that MNPs uptake to some extent, is necessary to obtain magCSs [7]. Actually, the magCSs construction is a two-step procedure consisting firstly of magnetic labelling, and posteriorly cells culture under magnetic force.

These results suggest MNPs internalization seems to occur after a short time period of magnetic stimulation. However, and unlike lower MNPs concentrations, after a 16h incubation with 400μg/mL the percentage of iron uptake is around 70%. Thus, according to cellular viability and iron uptake results together with the ability of MNPs to mechanically reinforce cell sheets, the concentration of 400μg/mL was selected for the magCSs construction. The fact that about 99.8% of the TNMD+ cell subpopulation positive for CD90 marker were also positive for PE channel (Figure 7-4 B1), which was associated to the red fluorescent label of MNPs, suggests the co-localization of MNPs with cells. The cytoplasmic localization of MNPs was also observed after staining the sorted CD90+ TNMD+ cells with Prussian blue for iron detection (Figure 7-4 B2). Since intracellular iron oxide MNPs have been reported to undergo lysosomal metabolism [44] that is also associated to mechanisms of nanoparticle degradation [45, 46], we carried out immunolocation of LAMP-1. After a 16h incubation, MNPs were positively co-localized with LAMP-1 marker (green), indicating their presence within the lysosomal compartment (Figure 7-4 B3) which was confirmed by the overlap of red and green channels (orange). These results are in agreement with several studies showing that MNPs are taken up by cells and undergo the endocytosis pathway (early endosome, late endosome, lysosome) [47-50].

Moreover, ultrastructural Transmission Electron Microscopy (TEM) examinations (Figure 7-4 B4) confirmed the intracellular fate of the MNPs which were mainly localized in phagocytic vacuoles (PV). Other works also reported chitosan MNPs within membranous vesicles upon 2h of incubation [51]. Altogether, the findings of MNPs internalization, metabolic activity and distribution within cells show that MNPs are unlikely to accumulate intracellularly, thus avoiding toxicity. Indeed, internalized MNPs seem to follow the typical endocytic mechanisms and are also likely to be short-termed recycled and utilized by cells via endogenous iron metabolism [52, 53] or cleared through regular excretion routes [54].

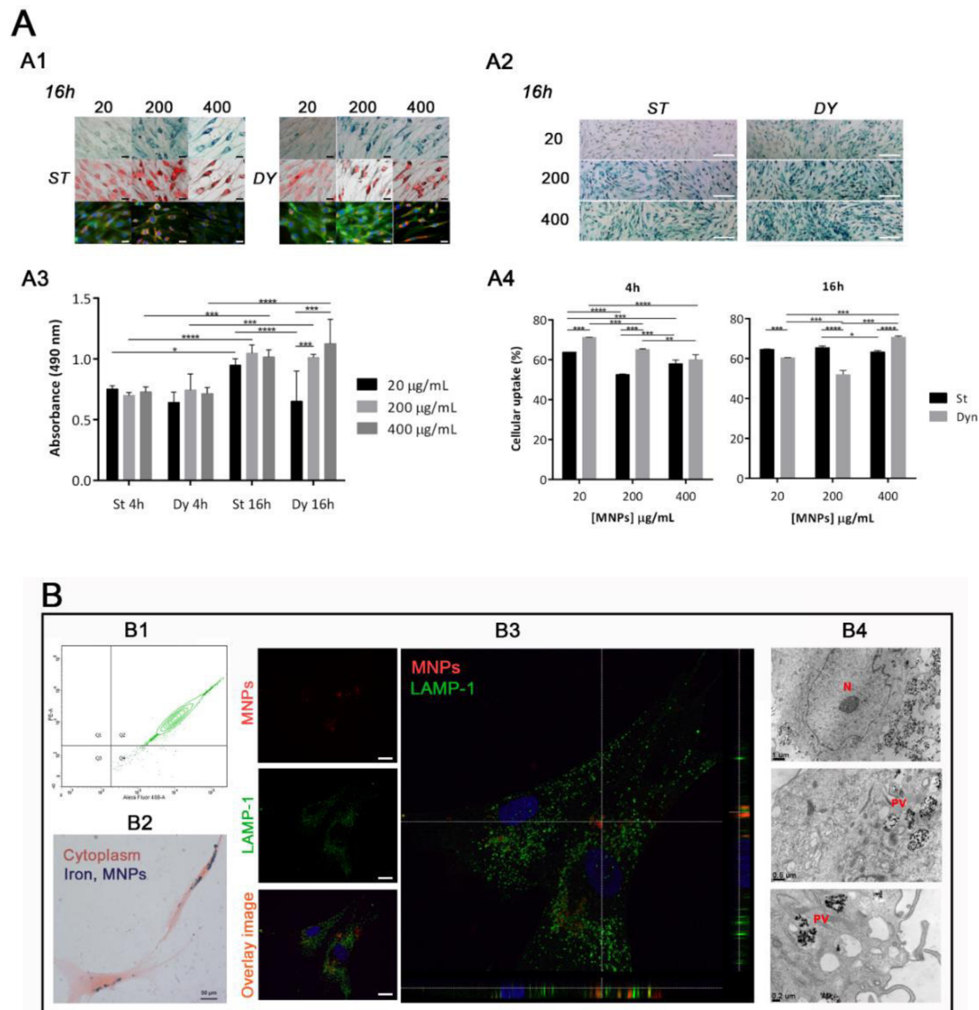


Figure 7-4 Internalization assessment (A) and intracellular location of MNPs within TNMD+ hASCs (B). (A1) Fluorescence microscopy images of TNMD+ hASCs cultured with red fluorescent MNPs at 20, 200 or 400µg/mL under static (St) or dynamically magnetic stimulated conditions using a low frequency oscillating magnetic field (Dy) for 16h; cell cytoskeleton is counterstained with phalloidin (green), while cell nuclei stain with DAPI (blue); Prussian blue staining identifies the MNPs iron core (blue); Scale bar represents 25µm. (A2) Prussian blue staining identifies the MNPs iron core (blue); Scale bar represents 100µm. (A3) Metabolic activity by MTS assay and (A4) ICP analysis for cellular uptake efficiency of MNPs after 4 or 16h of stimulation. (B1) Sorting of TNMD+ hASCs double positive for CD90 FITC and red fluorescent MNPs (PE-A channel). (B2) Prussian blue staining identifies the MNPs iron core (blue) in the TNMD+ hASCs cytoplasm (pink). (B3) Confocal micrographs of cells cultured with MNPs (red) incubated with the LAMP-1 (green), a lysosome specific antibody, and cell nuclei counterstained with DAPI (blue); Scale bar represents 25µm; The larger fluorescent image represents an orthogonal projection of z-stacks marked with LAMP-1, MNPs and nuclei; The orange color confirms the co-localization of MNPs in lysosomes

and results from green and red overlay. (B4) TEM micrographs of MNPs in phagocytic vacuoles (PV) of TNMD+ hASCs. Symbol *, **, *** and **** denotes study groups with statistical significant difference: $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ respectively.

ROS Production and Cell Viability

The influence of internalized MNPs in the production of reactive oxygen species (ROS) was also investigated using oxidized DCFDA and flow cytometry (Figure 7-5). ROS are generated by cells as a consequence of their natural aerobic metabolism [55], playing a role in normal cell signal transduction and cell cycling. However, in stressfull situations ROS expression is significantly increased and can cause several deleterious events.

When TNMD+ hASCs were stained with DCFDA, which is oxidized by ROS into 2', 7'-dichlorofluorescein (DCF), an increment in the number of cells with fluorescence is observed (Figure 7-5 A). However, this percentage and the fluorescence intensity is considerably lower than the levels reached by the positive control TBHP. The fact that MNPs are chitosan-coated is an additional advantage since polymer coatings were reported to protect against pro-oxidant agents, suppressing oxidative damage [56].

A viability assay was also performed with 7-AAD viability staining solution to assess possible cytotoxicity caused by increased ROS generation. MNPs do not particularly influence ROS formation nor cytotoxic effects on TNMD+ hASCs (Figure 7-5 B). Moreover, at physiological concentrations ROS plays several roles in cellular and tissue homeostasis. For instance, nanoparticles-mediated ROS can influence the activation of the mitogen-activated protein kinase (MAPK) pathways [35, 57], which can be important prompting cell differentiation. On the other hand, Fe_3O_4 MNPs were reported to possess intrinsic peroxidase-like activity [58]. In this sense, after the internalization of MNPs and transfer to lysosomes, the degradation and consequent release of free iron (Fe) into cytoplasm can occur [44, 59]. Consequently, Fe^{2+}/Fe^{3+} ions in solution may catalyze the breakdown of hydrogen peroxide, which can positively affect cell growth [60].

Despite the increment of about ~20% in 7-AAD in the presence of MNPs, the internalization of iron oxide MNPs, their non-toxic behavior to cells and applicability for tissue engineering approaches is generally accepted. Several MNPs have been FDA approved for biomedical applications [61] and have

been reported not to evidence a cytotoxic behavior [62]. Moreover, it is unlikely that detrimental cytotoxic accumulation of these particles might occur in an *in vivo* scenario upon implantation of a magCSs patch as iron-containing MNPs are biocompatible in a concentration dependent manner and can be metabolized into its basic elements [53], recycled or excreted upon biological needs [54].

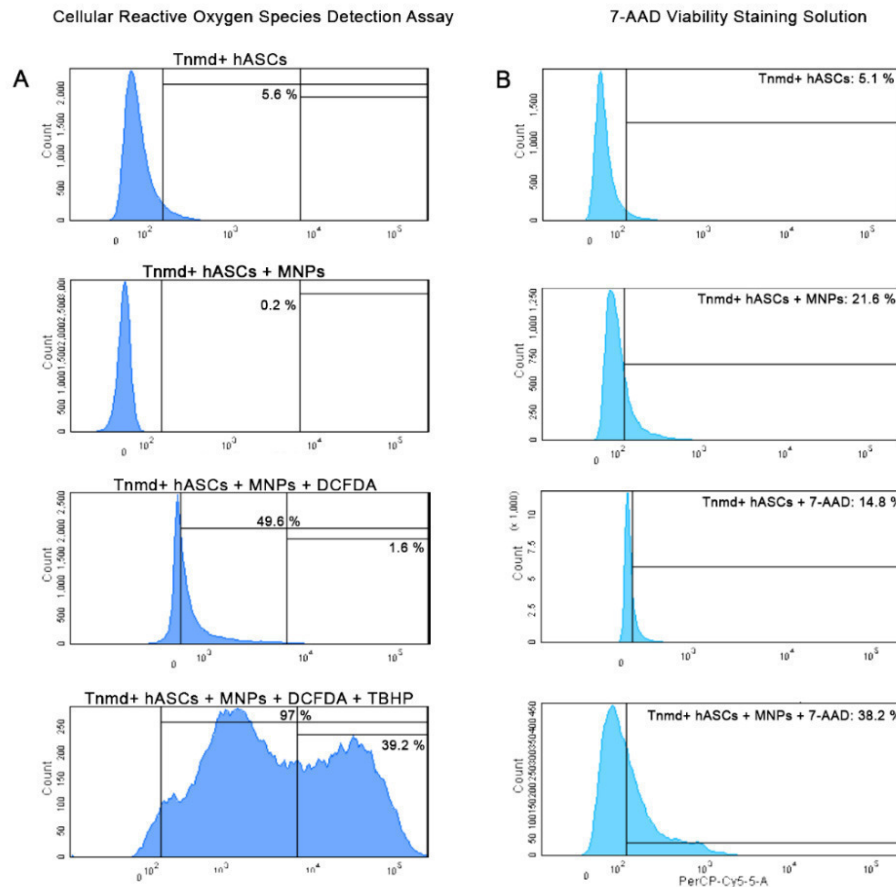


Figure 7-5. Cellular reactive oxygen species and viability assays of TNMD+ hASCs cultured with red fluorescent MNPs for 16h, by flow cytometry analysis. (A) TNMD+ hASCs were stained with DCFDA which is oxidized by ROS into 2', 7'-dichlorofluorescein (DCF), a highly fluorescent compound (FL1 channel); TBHP mimics ROS activity oxidizing DCFDA to fluorescent DCF, being considered the positive control of ROS measurement. (B) TNMD+ hASCs were stained with 7-AAD viability solution after 16h of incubation; cells without MNPs served as experimental control.

7.4.3. Assessment of Tenogenic Potential and ECM production by Magnetic Cell Sheets

Cell Viability and Proliferation of Tnmd+ hASCs in magCSs

Besides TNMD+ hASCs response to the internalization of MNPs, it is also relevant to understand how TNMD+ hASCs behave within a much more complex system that is a magCSs construct. Thus, metabolic activity and proliferation assays (Figure 7-6) were examined up to 7 days in culture. In general, a slight decrease in the metabolic activity of TNMD+ hASCs with the time in culture, suggests that the high cellular confluence associated to the magCSs construction influences metabolic activity. However, TNMD+ hASCs still exhibit high values of metabolic activity showing they are viable when assembled and cultured into magCSs. Interestingly, the metabolic activity tends to be higher in magnetic stimulated conditions at all timepoints, being significantly increased at day 3 in comparison to magnet conditions ($p<0.05$).

Similarly to metabolic activity outcomes and regardless of the high dsDNA values, cell proliferation decreases with the time in culture. Although TNMD+ hASCs assembled in magCSs are metabolically active by 7 days, their dsDNA content decreased in time ($p<0.05$) which may relate to the overgrown confluence of the cells and spatial restrains within the construct that confines cellular proliferation. These results are in accordance to previous studies that reported the incorporation of MNPs caused no prejudice on cellular viability and proliferation nor affected differentiation of adipose derived stem cells [48, 63-65].

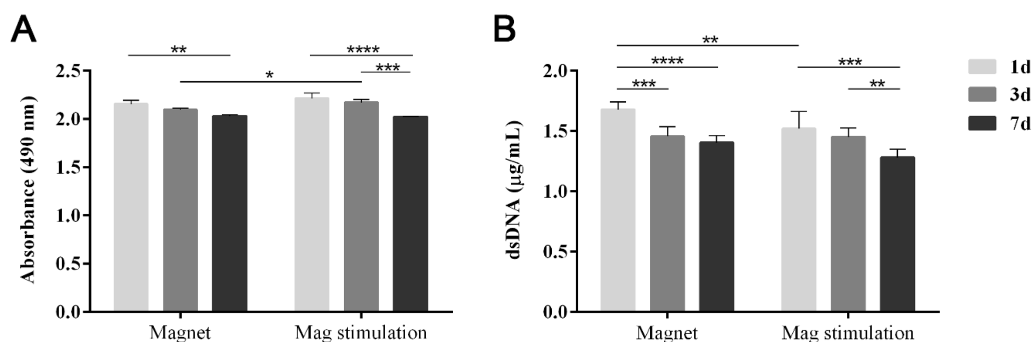


Figure 7-6. TNMD+ hASCs viability analysis (A) and proliferation (B) assembled in magCSs cultured for up to 7 days in αMEM medium under a magnet or magnetic stimulation conditions. Symbol *, **, *** and **** denotes study groups with statistical significant difference: $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ respectively.

Real Time RT-PCR

To understand the influence of magnetic sheets in the tenogenic commitment of TNMD+ cells, the expression of tendon related genes was investigated (Figure 7-7).

Magnetic actuated conditions seem to influence the expression of tendon related genes with a significant up-regulation of *COL3A1* and *TNC* after 3 days ($p<0.05$). These findings confirm the benefit of magnetic actuation to stimulate cell-based magnetic constructs prior to implantation and are consistent with reports about the upregulation of osteogenic, chondrogenic and tenogenic genes upon magnetic stimulation [66, 67].

However, after 7 days in culture, the tendency is just the opposite, where magnetic stimulation conditions reveal a lower genetic expression for the same genes. This is especially clear for *COL3A1*, *SCXA* and *TNC* ($p<0.05$). In the case of *TNMD*, the gene expression tends to increase from 3 to 7 days of culture. Despite the fact that the values found in magnetic stimulated groups tend to be lower, *TNMD* expression is maintained in time and between conditions. Moreover, *TNMD* and *SCX* expression follow a similar trend, which is consistent with the fact that *SCX* has been suggested to positively regulate the expression of *TNMD* [25, 68]. The conversion of mechanical signals into biochemical signaling from the cellular membrane to the nucleus, is mediated by a multitude of signaling cascades, some of them oriented towards cell differentiation such as TGFβ and MAPK pathways. Magnetic-mechano actuation has been described to elicit stem cells differentiation and has been complemented with MNPs to allow additional levels of control over specific mechanosensors and mechanotransduction, and therefore cell fate [69]. The lower expression of tendon genes can be explained by the fact that exposing the magCSs to an external magnetic field may be a tunable time dependent parameter, which may trigger different responses on cells.

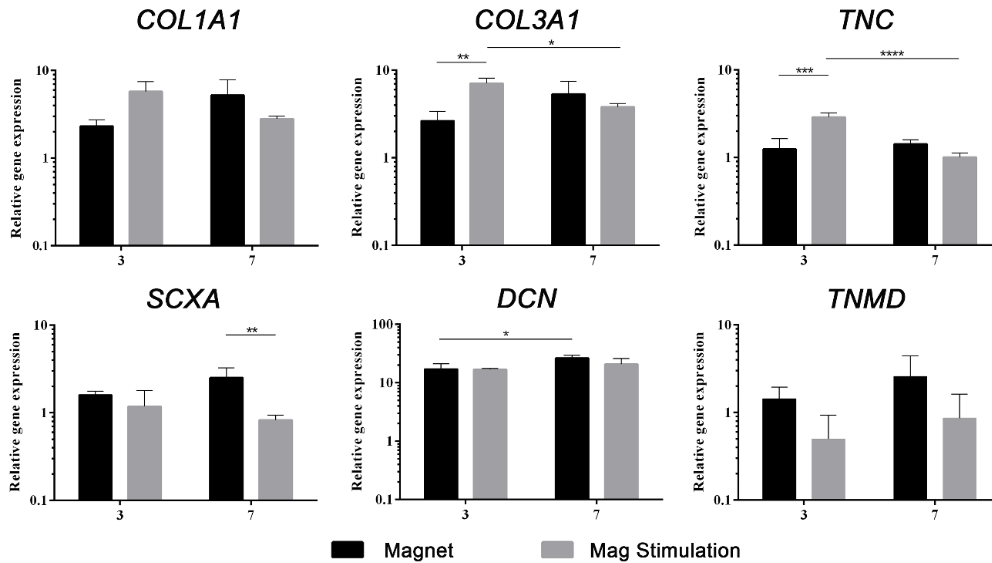


Figure 7-7. Real time RT-PCR analysis of collagen type I (*COL1A1*), collagen type III (*COL3A1*), tenascin C (*TNC*), scleraxis (*SCXA*), decorin (*DCN*) and tenomodulin (*TNMD*) gene expression of magCSs. Symbol *, **, and **** denotes study groups with statistical significant difference: P<0.05, P<0.01, and P<0.0001 respectively.

Collagen and Non-collagenous Proteins

The relevance of cell sheet technologies relies in the recreation of a scaffold-free environment with bioinstructive cues to guide and stimulate cells upon implantation. Thus, understanding matrix composition and organization, and how cells interact with it, is key to understand the function, homeostasis, and repair of tendons [70]. Herein, the use of prone stem cells committed to the tenogenic lineage is an advantageous factor envisioning the synthesis of a tendon-like ECM. Of the tendon dry weight, 60% is collagen type I arranged in tensile-resistant fibers [71], but other types are available, namely collagen types III (0-10%), IV (~2%), V, and VI. The non-collagenous matrix is primarily made up of glycoproteins which include the proteoglycans such as decorin and tenascin C [72]. The results of collagen and non-collagenous proteins produced by TNMD+ cells in magCSs was quantitatively translated into collagenous and non-collagenous ECM after normalization with total protein (Figure 7-8). MagCSs cultured for 7 days under magnetic stimulation produce significantly (p<0.05) more collagen than under magnet condition (Figure 7-8 A), suggesting that the actuation of an external magnetic stimulation promotes the production of a collagen rich ECM (p<0.05). The interactions between collagen types I and III are essential for normal fibrillogenesis and regulation in tendon [73]. Moreover, the interactions of

collagen fibrils with small leucine-rich proteoglycans such as decorin have been implicated as important regulators of collagen fibrillogenesis [73, 74]. Overall, the production of ECM proteins by TNMD+ hASCs benefits from the actuation of an external magnetic field, as observed by immunocytochemistry and the respective semi-quantitative analysis (Figure 7-8 D), which confirmed the increased deposition of tendon related ECM proteins upon magnetic stimulation ($p<0.05$).

These results, together with the outcomes from histological stains support the tenogenic-like ECM produced in developed magCSs that can be an added value to CSs functionality aiming at tendon tissue engineering and regenerative medicine strategies. Although the precise mechanism of recreating the structural of the tendon-like matrix was not deeply investigated in this work, we hypothesize that there are several elements to be accounted for, namely the TNMD+ cell subpopulation, the presence of MNPs and the actuation of an external magnetic field. The fact that cells formed a cohesive cell sheet can also influence the production of an ECM as this structure favors cell communication and signaling between cells.

In a previous study by our group, TNMD+ cells were considered to be of significance susceptibility to commit towards the tenogenic lineage [17] whereas the utilization of MNPs and magnetic actuation have been described to elicit cell responses and signaling [75-77], especially in mechano-responsive tissues. Therefore, we hypothesized that all the elements may play a role in the process of creating a successful magnetic tendon patch and synergistically act, enhancing the production of a tendon-like ECM.

Collectively, the quantitative and qualitative data of tendon related markers support the tenogenic potential of the developed magCSs with a relevant deposition of a tendon-like ECM. However, forthcoming work is needed to overcome limitations of this work. First, a more comprehensive study on the gene transcriptional responses of magCSs to the protein level should futurely be considered. Second, the current study was an *in vitro* study, thus whether this magnetic cell patch would indeed ameliorate tendon healing and regeneration ability is unknown. The obtained results should, therefore, be validated *in vivo* in order to address the clinical significance of magCSs patch for rotator cuff healing, and the implications of such *in vitro* encouraging results.

In summary, this work provided promising magnetic cell sheets with a tenogenic prone stem cell population demonstrating good mechanoelastic properties and capable of magnetic responsiveness. Tendon cells seeded in aligned structures were observed to maintain *in vitro* phenotype and promote

aligned ECM deposition [78, 79]. However, whether surface topography can be correlated into a clinically functional response *in vivo* is uncertain. English *et al* [80] showed that imprinted substrates didn't induce ECM orientation parallel to the substrate orientation nor neotissue formation in a rat patellar tendon model. Conversely, in our approach, this restraint doesn't apply as we show a cohesive tendon-like rich matrix with high biomechanical strength produced by suitable stem cells, bypassing the need for artificial carriers and their shortfalls. Thereby, magCSs as cells that create their own tissue-specific ECM are futurely envisioned to effectively interact with the host milieu, directing a coordinated functioning towards tendon repair.

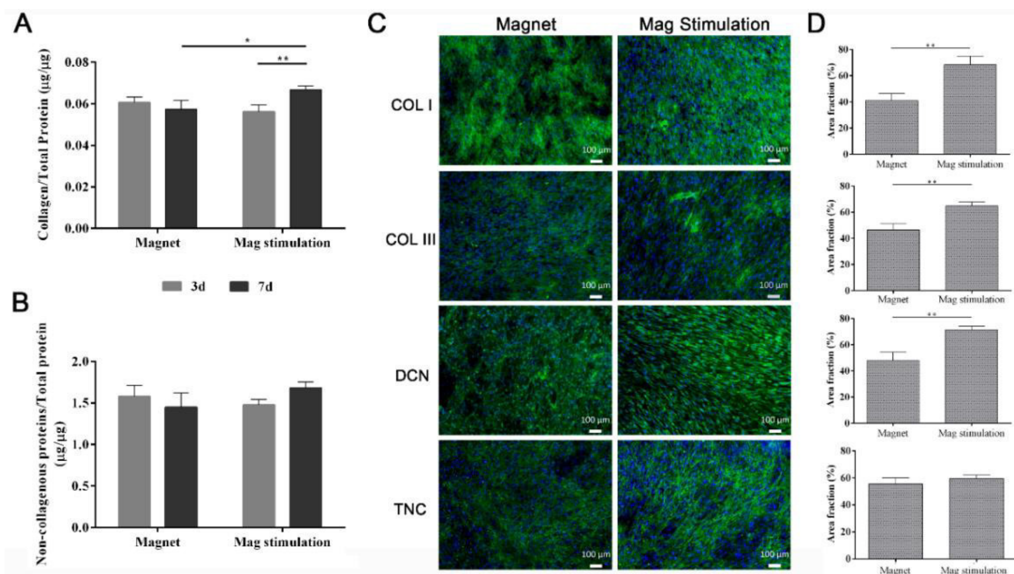


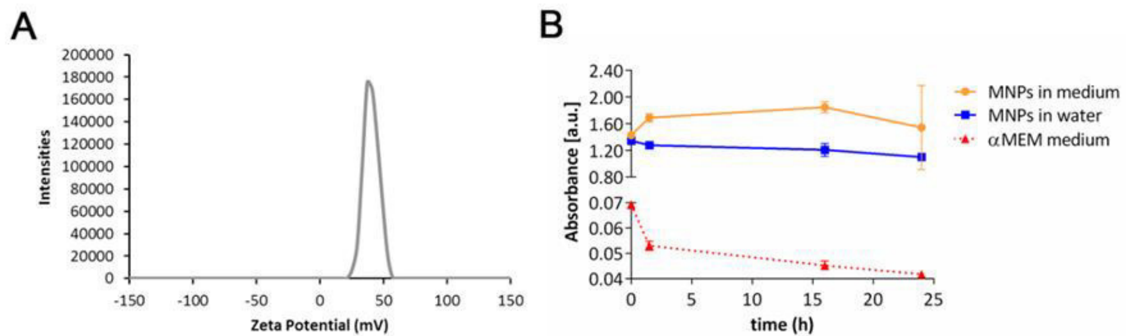
Figure 7-8. Quantification of collagen (A) and non-collagenous proteins (B) by Sirius Red/Fast Green Collagen staining kit. (C) Immunocytochemistry of Collagen type I, Collagen type III, Decorin and Tenascin C proteins deposited by TNMD+ cells in magCSs under a magnet or magnetic stimulation, and (D) Semi-quantification of immunocytochemistry by area fraction. DAPI (blue) stains cell nuclei and tendon related ECM proteins are stained in green. Symbol *, ** denotes study groups with statistical significant difference: $P<0.05$ and $P<0.01$, respectively.

7.5. Conclusions

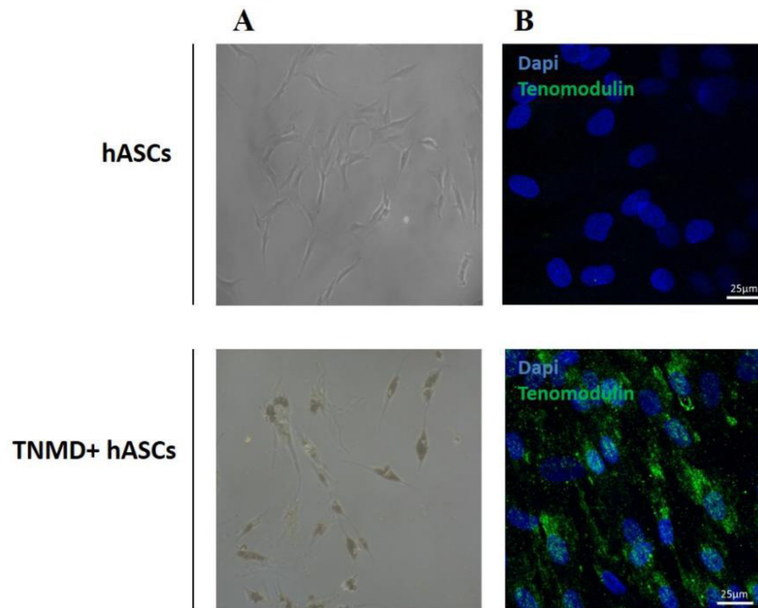
ECM rich patches can be successfully developed with TNMD+ cells as building blocks for magnetic cell sheets (magCSs) technologies. Being a living construct, magnetic scaffold-free constructs integrate a new platform of biocompatible and bioinstructive patches offering an ECM enriched structure to support

and guide the cells upon implantation with mechanical reinforcement provided by MNPs and with remote modulatory actuation. Furthermore, cell sheet engineering for tendon defect augmentation could allow multilayered cellular architectures within their own microenvironment and establish paracrine signaling with nearby tissues and/or interfaces to patch tendon defects. Overall, magnetically actuated stem cells labelled with MNPs may modulate the local environment through autocrine/paracrine and/or cell homing mechanisms. Developing bottom-up bio-instructive patches for tendon tears augmentation is a promising strategy for surrounding host tissue integration, causing minimal inflammation while guiding the cells toward tenogenic phenotype using less invasive procedures and stimulating endogenous regenerative mechanisms.

7.6. Supplementary material



Supplementary Figure 7-1. Iron oxide nanoparticles characterization: (A) Zeta-potential distribution of chitosan iron oxide MNPs (04-00-152, Micromod), (B) Turbidimetry assay by UV/visible spectroscopy at 450nm of a MNPs suspension in basic culture medium (αMEM) monitored over a period of 24 hours, namely 0, 1.5, 16 and 24 h. αMEM medium relates to the MNPs-free control solution of the assay and values are presented as mean ± standard deviation (SD).



Supplementary Figure 7-2. Microscopy analysis of hASCs and TNMD+ hASCs subpopulation 3 days after IMS sorting. A) Microscopic observation using a phase contrast microscope; Magnification 10x. B) Immunofluorescence of TNMD marker (green) under confocal microscopy observation.

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Chapter 8

Exploring the Potential of Starch/Polycaprolactone Aligned Magnetic Responsive Scaffolds for Tendon Regeneration

This chapter is based on the following publication:

Gonçalves AI, Rodrigues MT, Carvalho PP, Banobre-Lopez M, Paz E, Freitas P, Gomes ME. Exploring the Potential of Starch/Polycaprolactone Aligned Magnetic Responsive Scaffolds for Tendon Regeneration, Advanced Healthcare Materials 2016; 5:213-222. doi: 10.1002/adhm.201500623

8. EXPLORING THE POTENTIAL OF STARCH/POLYCAPROLACTONE ALIGNED MAGNETIC RESPONSIVE SCAFFOLDS FOR TENDON REGENERATION

8.1. Abstract

The application of magnetic nanoparticles (MNPs) in tissue engineering (TE) approaches opens several new research possibilities in this field, enabling a new generation of multifunctional constructs for tissue regeneration.

This study describes the development of sophisticated magnetic polymer scaffolds with aligned structural features aimed at applications in tendon tissue engineering (TTE). Tissue engineering magnetic scaffolds were prepared by incorporating iron oxide MNPs into a 3D structure of aligned SPCL (starch and polycaprolactone) fibers fabricated by rapid prototyping (RP) technology. The 3D architecture, composition and magnetic properties were characterized. Furthermore, the effect of an externally applied magnetic field was investigated on the tenogenic differentiation of adipose stem cells (ASCs) cultured onto the developed magnetic scaffolds, demonstrating that ASCs undergo tenogenic differentiation synthesizing a Tenascin C and Collagen type I rich matrix under magneto-stimulation conditions. Finally, the developed magnetic scaffolds were implanted in an ectopic rat model, evidencing good biocompatibility and integration within the surrounding tissues. Together, these results suggest that the effect of the magnetic aligned scaffolds structure combined with magnetic stimulation has a significant potential to impact the field of tendon tissue engineering towards the development of more efficient regeneration therapies.

8.2. Introduction

Scaffold guiding regeneration is very important in tendon tissue engineering (TTE), as scaffold architectures have a primordial role in directing cells fate [1] towards a desired tenogenic identity, but also because tendon hierarchical structure of aligned fibers is responsible for their unique mechanical properties and function [2]. In addition, the importance of combining structural features with appropriate biochemical and mechanical stimulus in order to achieve the tenogenic differentiation in stem cells based tissue engineering approaches has been recognized [3-5].

Magnetic nanoparticles (MNPs) have already demonstrated a great potential in various biomedical

applications including for tagging, tracking and activation of stem cells into desirable cell responses as migration, proliferation or differentiation [6, 7], offering effective means of monitoring and stimulating *in vitro* and *in vivo* behavior [8, 9], but such features have been barely explored in combinatory tissue engineering strategies. MNPs can be directly associated with stem cells [10, 11], to be subsequently laden in a 3D construct or integrated into the scaffold structure providing basis for obtaining multifunctional magnetic responsive construct [12-17] with the capacity to be remotely stimulated by the actuation of an external magnetic field. The incorporation of the MNPs influences stem cells response, through magnetic driven stimulation, while allowing monitoring their behavior along time in culture [9]. The concept of fabricating a magnetically active scaffold as a tissue-like substitute with improved functionality is quite innovative and may become the basis for a new generation of tissue engineered constructs. However, and despite the advantageous physical stimulation of external magnetic forces in biological functionality, iron based nanoparticles [18] are a matter of debate regarding their biocompatibility and non-cytotoxicity for utilization in regenerative medicine [8]. Nevertheless, iron oxide particles have been reported not to affect viability [19, 20], proliferation [21] and differentiation [22, 23] capacity of stem cells.

In the present study we fabricated a biomimetic scaffold architecture inspired on the natural tendinous fibrous structure, focusing on aligned arrangements of SPCL fibers incorporating magnetic ferric oxide (magnetite) nanoparticles. Moreover, we investigated the importance of magnetic stimulus in combination with the aligned structure of the magnetic scaffolds in driving human adipose stem cells (hASCs) towards tendon-like cells, enhancing the functionality of magnetic constructs, and assessing the safety and biocompatibility of the magnetic scaffolds in subcutaneous defects in rats. Overall, results evidenced that the application of a magnetic field enhanced the biological performance of hASCs, in terms of cell proliferation and differentiation, and demonstrated low inflammatory response in an ectopic rat model.

8.3. Materials and Methods

8.3.1. Magnetic Scaffolds Development and Characterization

Aligned fibrous magnetic scaffolds were obtained from a biodegradable blend of SPCL (30/70 % wt,

Novamont, Italy) by rapid prototyping (RP) through the incorporation of commercially available iron oxide particles (Fe3O4, magnetite; size 250 nm) (45-00-252, Micromod, Germany) in SPCL powder (0.018:1 ratio, w/w) with a Bioplotter® (Envisiontec GmbH B10, Germany). The polymer-to-MNPs weight ratio used is lower than ones found in literature [51, 52] for the inclusion of nanoparticles in PCL matrix.

For this purpose, SPCL and iron oxide MNPs mixture was placed in a stainless steel syringe and processed at 120 °C through a heated cartridge unit, then plotted through a 22 gauge needle by air pressure control. A metal piston plunger with a teflon seal was used to apply pressure to the melted magnetic polymer. The design parameters of the scaffold were loaded on the bioplotter CAD software (PrimCam) and 2 layered scaffolds were plotted along the 0° direction.

Samples fabricated with SPCL (without iron oxide MNPs) were used as experimental controls of this experiment and designed as SPCL scaffolds. All samples were cut into 5×5 mm scaffolds and sterilized using ethylene oxide.

Iron oxide particles of 250 nm in size (45-00-252, Micromod, Germany) were characterized in terms of stability using a Zetasizer Nano ZS (Malvern Instruments, United Kingdom) in water dispersant.

The structure and morphological characterization of the scaffolds was analyzed by high-resolution micro-CT with a Skyscan 1072 scanner (Skyscan, Kontich, Belgium) and scanning electron microscopy (SEM, NanoSEM, FEI Nova 200, USA). The elemental characterization was performed with an energy dispersive spectrometer (EDS, Pegasus X4M) coupled to the SEM.

The X-ray source was set at 65 kV of energy and 154 μA of current. Scans of magnetic and control scaffolds were obtained with a resolution of pixel size set at 7.53 μm, and an exposure time of 1.7s was used. Representative data sets of 215 projections were acquired with a rotation range of 180° and a rotation step of 0.45°. Data sets from the scaffolds acquisition were reconstructed using NRecon (version 1.6.6.0, SkyScan) software. DataViewer (version 1.4.4, Skyscan) and CT Vox (version 2.3.0, Skyscan) software were used to build 3D virtual models of the scaffolds from acquired data sets.

Prior to SEM observation, the magnetic scaffolds were coated with gold–palladium by ion sputtering and graphite coating was used for energy dispersive spectroscopy (EDS) analysis to confirm the presence of Fe element.

Magnetization (M) measurements of the magnetic scaffolds were performed in a superconducting quantum interference device (SQUID-VSM) magnetometer from Quantum Design at several temperatures

between 300 K and 77 K and under an applied magnetic field (B) up to 5 T. According to the manufacture information, the particles have a density of 5.35 g/cm3, with a magnetization of 46 emu/gFe at B = 0.1 T and a saturation magnetization > 71 emu/gFe for B > 1 T.

8.3.2. Cytotoxicity assessment

The cytotoxicity of the magnetic scaffolds was assessed by a Minimum Essential Medium (MEM) extract test, according to ISO/EN 109935-5 guidelines, in order to verify the potential cytotoxicity of leachables from the polymeric/magnetic materials. A cell line of mouse lung fibroblasts (L929), acquired from European Collection of Cell Cultures (ECACC), was exposed to an extract for up to 7 days. The cells were grown as monolayers in basic medium, composed of Minimum Essential Medium (MEM) alpha Medium (α-MEM) (Invitrogen) supplemented with 10 % fetal bovine serum (FBS, Alfagene) and 1 % of antibiotic-antimycotic (A/A) solution (Alfagene). Trypsin (Alfagene) was used to detach the L929 cells from the culture flasks. Afterwards, cells were seeded into 96-well plates (Falcon) at a density of 10.000 cells per cm2 in order to reach 80 % confluent monolayer after 24 h of incubation at 37 °C in a humidified atmosphere with 5 % CO_2. The magSPCL scaffolds were extracted in complete culture medium (the same used for cell culture) for 24 h at 37 °C and agitation (60 rpm). A latex rubber extract was used as positive control medium of cellular death. The extracts were filtered (filter pore size: 0.22 μm) and placed in contact with the monolayer of the L929 cells. For this purpose, the culture medium from the well plates with the L929 cells monolayers was replaced for the same amount of extraction fluid. Cells grown in the presence of standard culture medium (basic α-MEM) were included as negative controls. Cells metabolic activity was evaluated after 24, 48, 72 h and 7 days of incubation by MTS assay. This assay is based on the bioreduction of the substrate (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (Cell Titer 96® Aqueous Solution Cell Proliferation Assay, Promega) into a brown formazan product. Briefly, after 1, 2, 3 and 7 days of culture, the extraction fluid was removed and a mixture containing serum-free culture medium without phenol red and MTS reagent was added to each well and incubated for further 3 h at 37 °C and 5 % CO_2 atmosphere. Supernatants were then transferred to new 96-well plates and the optical density (OD) was read at 490 nm in a microplate reader (Synergy HT, Bio-TeK Instruments).

8.3.3. Cell Isolation and Expansion

Human adipose-derived stem cells (hASCs) were obtained from lipoaspirate samples from the abdominal region, under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee.

Human ASCs were isolated from tissue samples and cultured as described before [53, 54], and have been previously characterized by RT-PCR for CD44, STRO-1, CD105 and CD90 markers [53]. Briefly, the tissue was rinsed in phosphate buffered saline (PBS, Sigma-Aldrich) containing 10 % of an A/A solution. The fat solution was immersed in a 0.05 % collagenase type II (Sigma/C6885) solution for 45 minutes at 37 °C under mild agitation. The digested tissue was centrifuged at 304 g for 10 minutes at 4 °C, after which the supernatant was eliminated. Lysis buffer was used to dissolve the pellet followed by a centrifugation at 304 g for 5 minutes. Cells were expanded in basic medium composed of α-MEM supplemented with 10 % FBS, and 1 % A/A solution.

8.3.4. Culture of hASCs on SPCL or magSPCL Scaffolds Under Magnetic Stimulating Conditions

hASCs were seeded at a density of 6.5 × 104 cells per scaffold and cultured in α-MEM medium for 7 days. Experiments were conducted under static and magnetic stimulating (oscillation frequency of 2 Hz and 0.2 mm of displacement) conditions in a magnefect nano device (nanoTherics Ltd, Keele, UK) composed by an oscillating magnet array system (0.35 T per magnet per scaffold). The magnetic stimulation induced by the oscillation frequency was constant and maintained without interruption for 7 days. Cells seeded on magnetic (magSPCL) and bare SPCL scaffolds (SPCL) were also cultured in static non-stimulating conditions as an experimental control.

Constructs were further investigated for cell metabolic activity and iron release by inductively coupled plasma atomic emission spectrometry (ICP). Tenogenic differentiation was evaluated on constructs by the quantification of collagen and non-collagenous proteins and immunolocation of tenogenous ECM related proteins as Collagen I and Tenascin C.

hASCs Metabolic Activity

Cell metabolic activity of hASCs was evaluated by MTS assay (Cell Titer 96® Aqueous Solution Cell Proliferation Assay, Promega). Briefly, after 7 days of culture, constructs were transferred to another multi–well plate, rinsed in PBS and a mixture of serum-free culture medium without phenol red and MTS solution (5:1 ratio) was added to each construct. The plate was incubated for 3 h at 37 °C and 5 % CO_2 atmosphere. Then, 100 μl of each sample was transferred to a new 96-well plate and the absorbance read at 490 nm (Synergy HT, Bio-TeK Instruments). Triplicates of each sample were made and a blank reading was performed. Iron Release Quantification: The iron (Fe) release from the magnetic scaffolds cultured for 3 and 7 days was evaluated by ICP (JY2000-2, Jobin Yvon, Horiba, Japan) analysis. Culture medium samples were collected after 3 and 7 days of culture, filtered with a 0.22 μm filter (VWR) and frozen at -80°C until performing ICP analysis.

Due to the presence of iron in α-MEM powder constitution, a sample of α-MEM medium was used as blank solution. An iron (Fe, 1000 μg/mL) standard solution (13830, Specpure®) was used to prepare the standard concentration solutions.

Immunofluorescence

Constructs were rinsed in PBS and fixed in formalin (INOPAT) prior to the detection of collagen type I (Rabbit polyclonal Anti-Collagen I, ab292, Abcam) and Tenascin C (Mouse monoclonal Anti-Tenascin C antibody [BC-24], ab6393, Abcam) deposition was assessed. After cell permeabilization with 0.025 % Triton-X100 (Sigma/X100)/PBS solution, the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector). Then, cells were incubated overnight with the primary antibodies above mentioned, diluted in antibody diluent with background reducing components from Dako (S3022, Dako) at 4 °C. The dilution ratio used was 1:500, 1:3000 for Collagen I and Tenascin C antibodies, respectively. Afterwards, samples were rinsed in 0.025 % Triton-X100 (Sigma/X100)/PBS solution, following incubation for 1 h at room temperature (RT) with the respective fluorescent secondary antibody (donkey anti-rabbit Alexa Fluor 488/A21206 or rabbit anti-mouse Alexa Fluor 488/A11059, Invitrogen; dilution 1:200), considering the host species of the primary antibodies. After the incubation with secondary antibodies, samples were rinsed in 0.025 % Triton-X100 (Sigma/X100)/PBS solution and stained with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μl, D9564, Sigma) for 10 minutes.

Negative controls assessed for immunofluorescence detection were incubated in Dako diluent in the absence of the primary antibody. The samples were left overnight in PBS and then observed at laser scanning confocal microscopy (Olympus FluoViewTMFV1000, Germany). A representative area of each sample was selected and two sequential pictures were acquired as individual slices and then built up from the series of stacked images with the software Olympus FluoView 1000 (FV viewer v.3.1.).

Quantification of Collagen and Non-collagenous Proteins

The amount of collagen and non-collagenous proteins expressed by hASCs cultured on the scaffolds was determined using a semi-quantitative assay, namely Sirius Red/Fast Green Collagen Staining Kit (9046, Chondrex). Cells were fixed in 95 % ethanol/5 % glacial acetic acid for 10 minutes at RT. Then, the dye solution was added and incubated for 30 minutes in order to completely immerse the fixed cells. A dye extraction buffer was then mixed and the OD values read in a spectrophotometer (Synergy HT, Biotek Instruments) at 540 nm and 605 nm.

8.3.5. MagScaffolds Subcutaneous Implantation

The *in vivo* biocompatibility of the magnetic scaffolds was evaluated after subcutaneous implantation in rat. Six adult Wistar male rats (16-week-old Wistar male rat with an average weight between 400 and 475 g) were used in this experiment. The animal study was performed according to the national guidelines and conducted in accordance with Portuguese legislation (Portaria n°1005/92) and international standards on animal welfare as defined by the European Communities Council Directive (86/609/EEC). Surgeries were performed under general anesthesia with an intraperitoneal (IP) administration of a combination of medetomidine (Dormitor - 0.5 mg/kg) with ketamine (Imalgene - 75 mg/kg). Four subcutaneous defects were created on the dorsum of each rat, and grouped under the following experimental groups: (i) empty pocket (negative experimental control), (ii) pocket filled with SPCL scaffolds (non-magnetic), and (iii) pocket filled with magnetic scaffolds (magSPCL). SPCL and magSPCL materials were prepared as described above. All groups underwent the same surgical procedure, and during surgery, each animal was fully anesthetized. Briefly, the hair was shaved and the site of implantation was aseptically prepared with a povidone-iodine solution. Four full thickness skin longitudinal incisions (about 1 cm) containing the subcutis and the panniculus carnosus (skin and

smooth muscle) were performed in the dorsum of each animal (anterior and posterior incisions). Cranial and lateral oriented subcutaneous pockets were created by blunt dissection, on the incision site. The scaffolds were inserted into these pockets, one scaffold per pocket and a total of 4 scaffolds per animal, and the incisions were carefully closed using surgical staples. After surgery, animals were monitored daily to confirm full recovery from surgery and to check for signs of infection until the end of the experiment at 7 days post implantation. One administration of Carprofen (Rimadyl, 2.5–5 mg/kg) was subcutaneously administered to each animal at the end of the surgery to minimize any post-surgical pain or distress. After recovery, animals were fed ad libitum and allowed unrestricted cage activity in individual cages. None of the animals used showed clinical symptoms of pain or distress during the experiment. After 7 days of implantation, animals were anesthetized with isofluorane and euthanized with an intraperitoneal injection with an overdose of pentobarbital sodium (Eutasil from Ceva Saúde Animal). Afterwards, pockets were retrieved for further characterization and evaluation.

Collection of Explants

After 7 days of implantation, explants were retrieved from a total of 4 explants per animal. Explants were fixed in a formalin solution until histological processing and evaluation.

Histological Characterization: Explants containing the scaffold and surrounding tissues were transversally sectioned in two halves before being subjected to standard histological tissue processing followed by paraffin embedding. Sections with 4 μm thickness were obtained using a microtome (HM355S, Microm, Thermo scientific) and stained with hematoxylin and eosin (H&E, 05–12011/L, 05-M10003, Bio-optica) in an automatic stainer equipment (HMS740, Microm, Thermo scientific), accordingly to a standard H&E protocol, and mounted into histology slides. Stained sections were observed under a transmitted and reflected light microscope with apotome 2 (Axio Imager Z1m, Zeiss, Germany), and images were acquired with an MRc5 camera and the AxioVision V.4.8 software (Zeiss).

Explants embedded in paraffin were visualized under a stereo microscope (Schott KL 200, Stemi 1000, Zeiss), and images were captured by a digital camera (Power-Shot G11, Canon).

8.3.6. Statistics

All quantitative results are expressed as the mean ± standard deviation. Two-Way ANOVA followed by

Bonferroni's Multiple Comparison test was assessed to determine whether differences between sample groups were significant. Differences were considered significant when the P value was < 0.05.

8.4. Results and Discussion

8.4.1. Magnetic Scaffolds Development and Characterization

Scanning Electronic Microscopy (SEM) and Micro-CT Analysis

Starch and polycaprolactone (SPCL, 30:70% w/w blend) has been widely studied for TE applications with successful outcomes[24-26] and was therefore selected as starting material for the production of the magnetic scaffolds (magSPCL). magSPCL scaffolds were obtained by the incorporation of iron oxide MNPs in the SPCL, subsequently extruded into aligned fibers through a rapid prototyping (RP) process with a 0° pattern.

The morphology of the scaffolds was analyzed by SEM (Figure 8-1), showing the magnetic scaffold microstructure with small brilliant spheres observable at the surface of the scaffold, corresponding to iron oxide MNPs aggregates. Also, the energy dispersive spectroscopy (EDS) analysis detected the presence of Carbon (C), Oxygen (O) and Iron (Fe) elements on the surface of the scaffold and the elemental mapping confirmed the presence of a uniform and homogeneous distribution of Fe in the fiber network surface, meaning that iron oxide MNPs (the Fe source) are also homogenously spread out all over the surface.

Micro-CT images (Figure 8-1) show the parallel alignment of the fibrous pattern of the magSPCL scaffolds. Despite the fact that iron oxide MNPs are nano-scaled and the micro-CT resolution is at a micrometer range, it is possible to detect MNPs due to the high density of MNPs iron core in comparison to the SPCL blend, and due to the overlapping slides used to reconstruct the 3D model of the scaffold, which further confirms the effective incorporation of iron oxide MNPs into the bulk material.

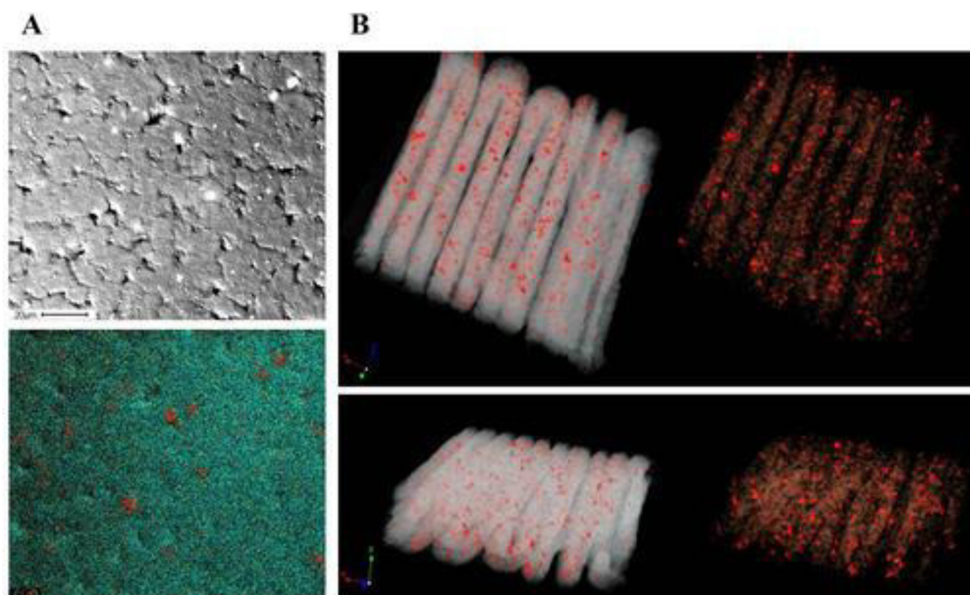

Figure 8-1. Representative images of the developed magnetic scaffolds. (A) SEM micrograph of the magSPCL scaffolds surface (upper) showing the presence of iron oxide MNPs (Fe, red) by EDS mapping (lower); (B) 3D reconstructed model by micro-CT analysis reveals the highly parallel and aligned microstructure of magSPCL. The polymeric matrix is represented in grey (SPCL) and iron oxide MNPs are observed in red (Fe).

Zeta-potential Measurements of MNPs and Magnetization Analysis of magSPCL Scaffolds

The determined average zeta potential (ζ -potential) of the water soluble iron oxide nanoparticles used was – 11.7 ± 0.14 mV (Figure 8-2), indicating particle stability being the repulsive charge between the particles enough to prevent agglomeration. In order to determine the room temperature magnetic properties of the developed magSPCL scaffolds, the magnetization curve as a function of the applied magnetic field (hysteresis loop), M vs. B, was measured at 300 K between B= -5 T and B= 5 T (Figure 8-2). The observed absence of remanence and coercive forces at zero magnetic field confirms the superparamagnetic behavior of the MNPs-doped SPCL scaffolds. On the other hand, a saturation magnetization (Ms) value of 1.22 emu/g was in good agreement with that found in previous reported studies aimed at TE [12, 13, 15-17].

Afterwards, and envisioning, for example, the possibility of cryopreserving cell-seeded scaffolds as means to obtain off-the-shelf products for TE, successive hysteresis loops were performed in zero-field-cooling conditions from room temperature at several temperatures between 300 K and 77 K. A complete collapse of all the magnetic curves was verified (Figure 8-2), so no differences in the magnetic behavior

of the material were observed after several cooling/heating cycles, as compared to the behavior at room temperature. Hence, it is conceivable that cell-magSPCL scaffolds aimed at ready-to-use applications can be preserved through a cryopreservation process and still maintain their intrinsic magnetic properties upon thawing, if to be used in later studies or to provide off-the-shelf tissue-like substitutes ready to use upon immediate clinical needs, as suggested in previous studies for bare SPCL scaffolds [27].

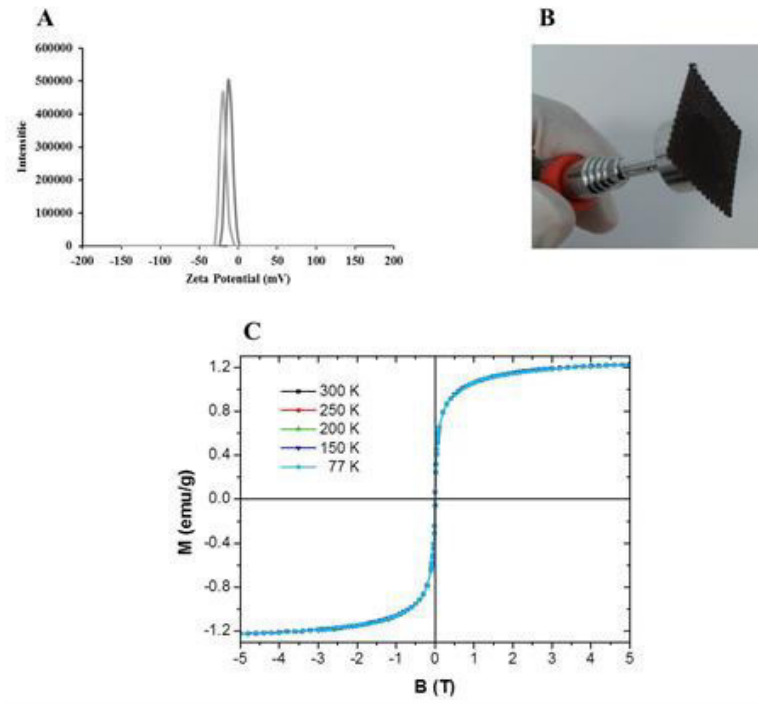

Figure 8-2. Iron oxide nanoparticles and scaffolds characterization: (A) zeta-potential distribution of iron oxide MNPs; (B) magnetic-responsive magSPCL scaffold under the magnetic field generated by a magnet; (C) Hysteresis loops of magSPCL scaffolds at different temperatures between 300 K and 77 K under an applied magnetic field up to B= 5 T.

In vitro Biocompatibility Assessment

The *in vitro* cytotoxicity of the magnetic extracts was assessed as a first screening of the scaffolds biocompatibility. In this work, the cytotoxicity of the developed magSPCL scaffolds was assessed by a MTS assay, after culturing L929 cells for 24, 48, 72 h and 7 days with the extracts of the materials. Results obtained from the MTS assay (Figure 8-3) revealed that the metabolic activity of L929 cells after being in contact with the scaffold extracts increased with time of culture, clearly suggesting that the iron oxide MNPs present on the polymer matrix of the scaffolds does not affect the viability of the cells.

Different studies report the use of iron based particles for cell labeling not affecting cell viability or functionality [23, 28] and from several MNPs comparatively tested, iron oxide MNPs were shown to be one of the safest, with no measurable effect on cells on concentrations lower than 200 μg/mL [19]. Furthermore, an investigation on the effect of increasing concentration of iron oxide nanoparticles (6, 12.5, 25, 50, 100 μg/mL) on human mesenchymal stem cells (hMSCs) concluded that no decrease in cell viability was observed for the studied concentrations [29]. The compositional analysis of the collected cultured medium samples was performed in order to evaluate the possible release of iron (Fe) element from the magnetic scaffolds, by inductively coupled plasma atomic emission spectrometry (ICP). The values of iron concentration were normalized in relation to a blank solution which was the α-MEM medium (supplemented with 10 % FBS and 1 % A/A) that presented an iron concentration of 219 ± 0.01 μg/L. Overall, the release of iron from magnetic scaffolds into the culture medium was initially higher (day 3, $p<0.05$) under the influence of an extern magnetic field, as compared to static culture. This may be explained by the vibrational and magnetic forces generated by the magnetic device which in turn may promote particles release from the surface of the fibers. Despite this early increment in iron release under magnetic stimulation, values obtained from day 3 and day 7 are negligible when compared to the α-MEM medium control. This may also explain the non-cytotoxicity of the magSPCL scaffolds extracts with cell viability levels similar to bare SPCL scaffolds, reported above.

Moreover, the effect of magnetic stimulation on the cellular metabolic activity of human adipose derived stem cells (hASCs) seeded onto magSPCL scaffolds was assessed by MTS assay (Figure 8-3). Thus, an oscillating magnetic field was applied to influence the magnetic responsiveness in terms of viability and proliferation of hASCs.

Human ASCs metabolic activity was found higher in magSPCL constructs than in non-magnetic (SPCL), cultured either in static and dynamic conditions. Thus, hASCs viability seems not to be negatively affected by the presence of MNPs and may even enhance cells metabolic activity. This also suggests that the iron oxide MNPs concentration used to fabricate the magnetic scaffolds and the iron released from the scaffolds does not induce an adverse effect on cultured stem cells, as it was already suggested by the results obtained in the cytotoxicity assay. In fact, previous published studies reported that the incorporation of MNPs into PLGA scaffolds was the key factor in increasing cell proliferation [12] without resorting to magnetic field appliance. Enhanced cell proliferation was also observed on magnetic

scaffolds composed of hydroxyapatite due to the incorporation of MNPs [15]. When the magnetic stimulus is applied (Figure 8-3), a significant increment in cell viability levels was observed both in SPCL and magSPCL constructs. The stimulus provided by the magnetic device promotes the proliferation of cells even in the SPCL scaffolds without iron oxide MNPs, suggesting that the cellular behavior of hASCs is also favored by indirect magnetic forces. These results are also in agreement with a previously reported study, in which electromagnetic field responsiveness on tendon cells has been investigated, demonstrating a positive influence in the proliferation and in the expression of tendon specific markers [30]. In short, cells sense the magnetic stimulus whether cultured on SPCL or on SPCL doped with iron oxide MNPs (magSPCL). However, magSPCL constructs exhibit significant higher absorbance values in comparison to SPCL constructs, under magnetic stimulation. This fact highlights the synergistic effect of combining iron oxide MNPs with magnetic actuation, at the cellular level, stimulating cell mechanisms.

Thereby, and accordingly to the cell metabolic activity assay, hASCs maintain their viability when cultured onto SPCL and magSPCL scaffolds after 7 days of culture.

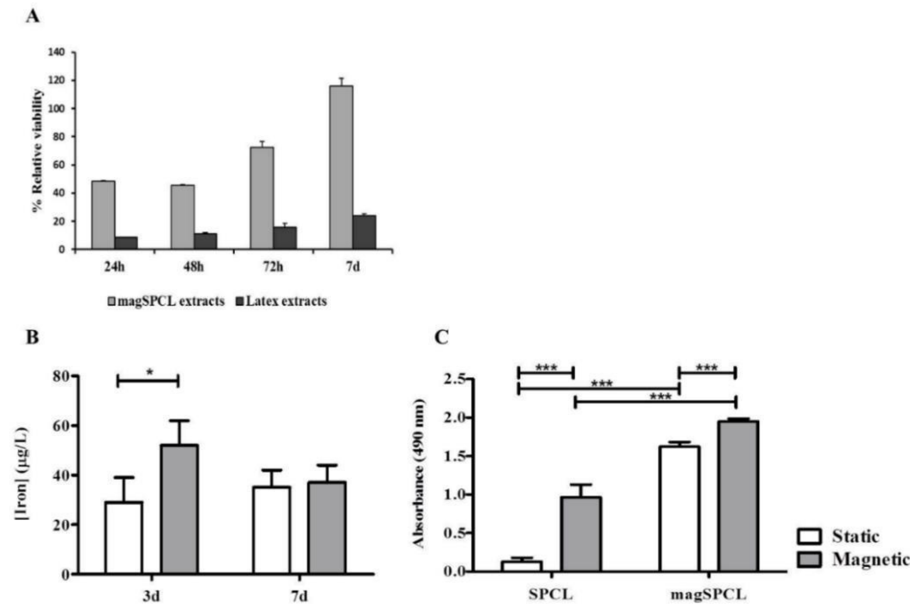

Figure 8-3. (A) Relative viability of L929 cells in contact with magSPCL scaffold leachables after 24, 48, 72h and 7 days, data obtained by MTS assay, and (B) ICP analysis for iron quantification released to the culture medium. The culture medium collected was sampled from hASCs-magSPCL culture medium after 3 and 7 days of culture; MTS assay results obtained upon culture under static or magnetic conditions for 7 days of (C) hASCs-magSPCL constructs. SPCL scaffolds without the incorporation of iron oxide MNPs were used as experimental

controls. Values represent the mean ± SD. Symbols (*) and (***) denote study groups with statistical significant difference $P < 0.05$ and $P < 0.001$, respectively.

8.4.2. The Role of Magnetic Stimulus in the Tenogenic Differentiation

Synthesis of a Tendon-like ECM

The immunolocalization of Collagen type I, the predominant fibril-forming collagen in tendons [31], and Tenascin C, a glycoprotein that function to enhance mechanical stability allowing tendons to return to their pre-stretched lengths after physiological loading [32], was assessed as a tool to characterize the tendon-like matrix synthesized by hASCs cultured on magSPCL scaffolds. hASCs were selected as candidates for tenogenic differentiation because they present multipotential capacity, high proliferation and renewal rates, besides their wide availability and safe harvesting procedures. Furthermore, other studies have demonstrated the potential of hASCs in tendon different TE strategies [33-37]. However, apart from biochemical, mechanical or topographical stimuli, the employment of magnetic actuation on ASCs responses towards tendon regeneration still remains relatively unexplored. Concerning the effect of magnetic stimulus in stem cells differentiation, it was recently reported that the exposure to electromagnetic fields accelerates osteogenic differentiation of ASCs *in vitro* [38]. In accordance, some works reported that magnetic composites induce a higher proliferation rate, faster differentiation and ECM secretion of pre-osteoblast cell line under exposure to a magnetic field *in vitro* [15, 16], and also *in vivo* [39, 40].

In the present study, the immunofluorescence analysis revealed that hASCs developed a Collagen type I rich matrix after 7 days of culture (Figure 8-4), that seems to be more abundant under magnetic stimulation, especially in magnetic scaffolds (magSPCL). Other published studies suggested that the application of magnetic force conjugated with MNPs activates mechanosensitive ion channels [22, 41] which in turn convert mechanical signals into biochemical signals.

The present study does not include cell internalization of iron oxide MNPs but their integration in the fiber network of the scaffold. Hence, we can only infer about iron oxide MNPs nanoscale interaction with the cultured hASCs as part of the scaffold structure. In fact, similar studies with seeded cells onto magnetic scaffolds reported the positive influence of MNPs within the biomaterials on ECM production and osteogenic differentiation [13, 42], specially augmented when an external magnetic field is applied

[15, 16, 39]. This information suggests that both iron oxide MNPs and the provided magnetic stimulus might contribute to the production of protein molecules, specifically collagen networks. The modulation of ECM synthesis and remodeling through mechanotransduction occurs by the activation of mechano-sensitive receptors at the cell surface such as ion channels, integrins, cadherins, that are able to trigger intracellular signaling mechanisms in response to mechanical changes in the extracellular microenvironment [43]. Furthermore, tenogenic differentiation might also be achieved by activating other signalling pathways such as the mitogen-activated protein kinase (MAPK) pathway, RhoA/ROCK (small G-protein) and focal adhesion kinase (FAK) signaling events [44-46]. Thus, magnetic actuation on cell-scaffold constructs may induce local deformations on the surface of the scaffold which is sensed by the adhered cells resulting in magneto-mechanical stimulation [47] that will likely influence a series of intracellular processes, namely reorganisation of the actin cytoskeleton, transcriptional factors activation and expression of specific genes, ultimatly promoting tenogenic differentiation. Under magnetic stimulation, the alignment of collagen fibers and the cytoskeletal alignment of hASCs is more evident on magSPCL scaffolds. Tendon functional capacity derives from a unique ECM composed primarily of type I Collagen arranged in a highly organized structure. A collagen network that resembles the collagenous extracellular matrix of these connective tissues was detected.

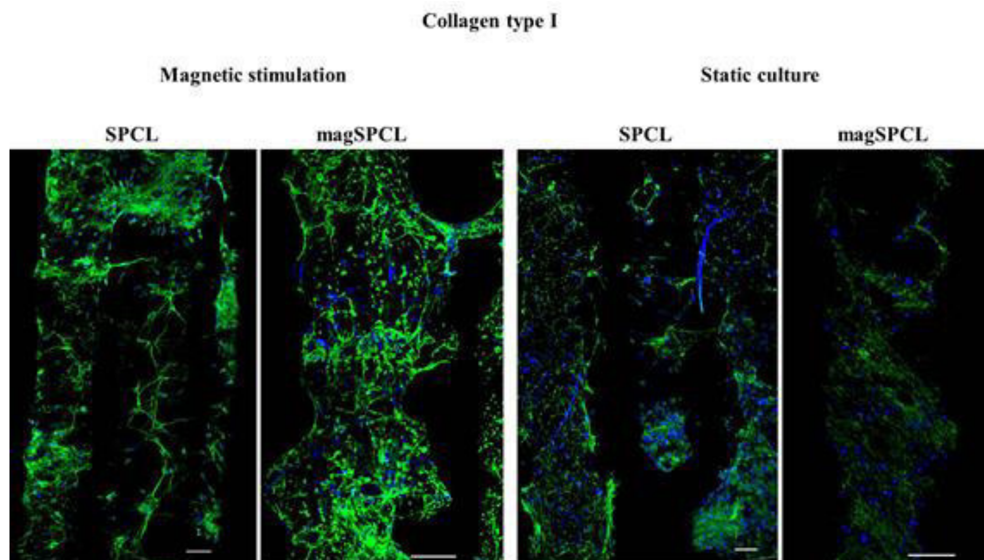

Figure 8-4. Collagen I immunolocation in hASCs-mag/bare SPCL constructs, cultured for 7 days under magnetic stimulation or static culture. Photomicroimages are representative of 1 to 2 fibers of the aligned scaffolds. DAPI (blue) stains cell nucleus and collagen type I (green) represents the major tendon ECM protein.

Bare SPCL scaffolds (without iron oxide MNPs) were used as experimental controls (SPCL). Scale bar represents 100 μm.

The fluorescent signal of Tenascin C was detected as early as 7 days of culture. Moreover, in both types of scaffolds (SPCL and magSPCL), the Tenascin C production is stronger in constructs cultured under magnetic stimulated conditions. Apart from being highly organized, tendons are mechanically responsive tissues [32] meaning that their biological response and further tenogenic ECM formation may be altered in response to mechanical loading. ECM protein Tenascin C is known to be a tendon marker up-regulated in response to mechanical stress [48]. Congruent with these findings, our results showed that the degree of Tenascin C deposition (Figure 8-5) tends to increase when a magnetic stimulus is applied to constructs. Since external mechanical stimulation regulates the expression of Tenascin C [48, 49], the mechano-magnetic stimulation provided by the magnetic bioreactor may likely influence biological processes including the synthesis of tendon ECM proteins.

Herein, magSPCL scaffolds incorporating iron oxide nanoparticles in the SPCL polymeric blend allows magSPCL scaffolds to reach high magnetization values under the application of an external magnetic field, which may explain a higher deposition of Tenascin C in magSPCL constructs. As mentioned previously, it is plausible that the external magnetic forces exerted on the cellular membranes may induce the activation of mechano-sensing pathways that will lead to a series of molecular responses resulting in protein synthesis, as a mean of cellular adjustment to the changes in the microenvironment. In agreement, both magnetic scaffolds and external magnetic fields have been reported to enhance cellular osteogenic responses [15, 16], suggesting that the activation of magnetic elements in cell niches may have a stimulatory role in the differentiation process of stem cells.

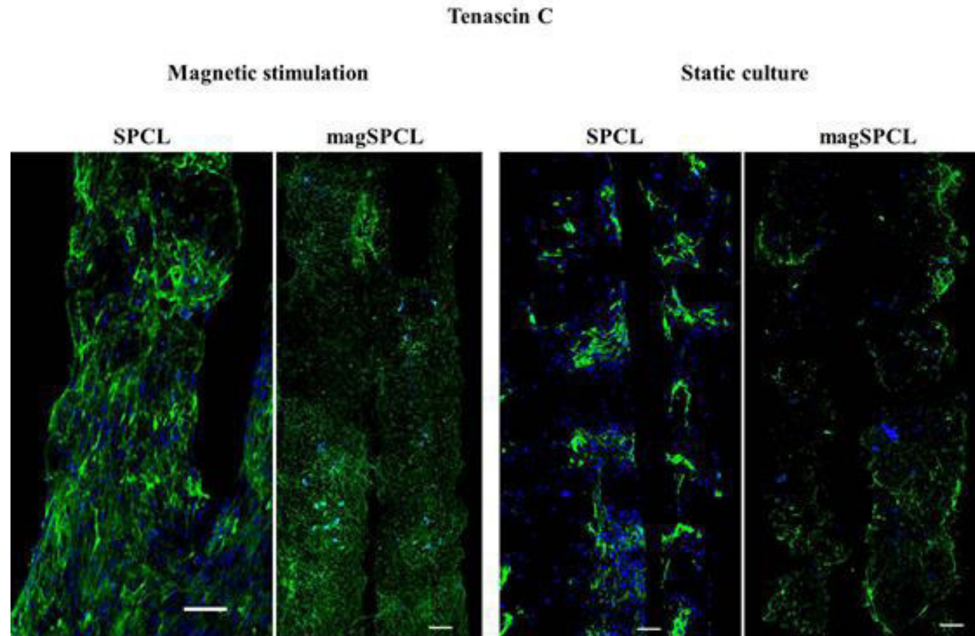

Figure 8-5. Tenascin C immunolocation in hASCs constructs, cultured for 7 days under magnetic stimulation or static culture. Photomicroimages are representative of 1 to 2 fibers of the aligned scaffolds. DAPI (blue) stains cell nucleus and tenascin C (green) represents a tendon ECM protein. SPCL scaffolds without the incorporation of iron oxide MNPs were used as experimental controls (SPCL). Scale bar represents 100 μm.

Quantification of Collagen and Non-collagenous Proteins

The amount of collagen and non-collagenous proteins (Figure 8-6) produced by hASCs cultured onto SPCL under magnetic stimulus was found to be higher in both static and dynamic conditions than for hASCs-magSPCL constructs. Collagen and non-collagenous values are enhanced under magnetic stimulation, although this difference was not found statistically significant ($p>0.05$). In summary, results suggest that magnetic actuation tends to accelerate the production of collagen and non-collagenous proteins by hASCs.

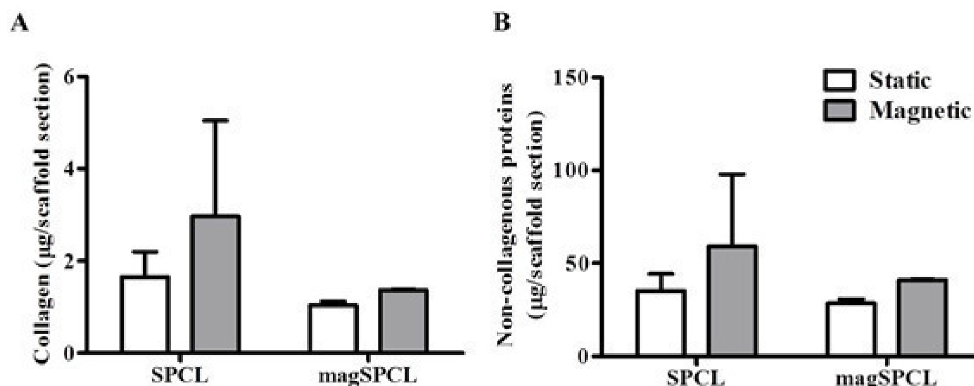

Figure 8-6. Quantification of collagen (A) and non-collagen proteins (B) secreted by hASCs cultured on magnetic scaffolds (magSPCL) under static or magnetic stimulation for 7 days, by Sirius Red/Fast Green Collagen staining kit. Bare SPCL scaffolds (without iron oxide MNPs) were used as experimental controls (SPCL). Values represent the mean ± SD.

8.4.3. *In vivo* Subcutaneous Implants

Cell-free magnetic scaffolds (magSPCL) were implanted in a rat subcutaneous model to assess tissue response. The macroscopic observation of magnetic scaffolds explants after 7 days of implantation showed tissue infiltration in between scaffold fibers. Upon 7 days of implant it was observed the maintenance of the scaffold integrity and structure, without visible indications of degradation as expected for these materials upon such a short period of implantation [50]. No edema or necrosis was observed in any of the explants retrieved. Also, no exacerbated inflammatory response was observed at the implantation site (Figure 8-7) for all studied conditions, with apparent absence of inflammatory cells. Nevertheless, a thin fibrotic capsule was observed in the presence of magSPCL and SPCL scaffolds. The magnetic particles, which were completely integrated within the SPCL fibers of the magnetic scaffolds, did not disperse into the nearby tissues nor elicited a detrimental host tissue response.

Overall, it was also observed a good integration of both types of scaffold with the surrounding tissues, revealing promising scaffold-host interactions.

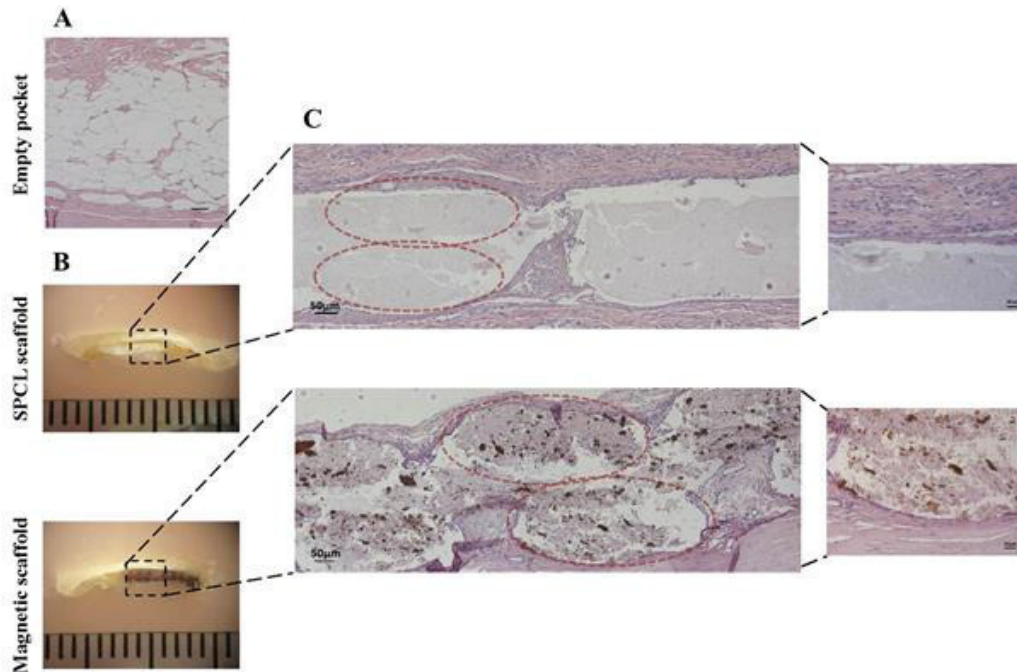

Figure 8-7. Micrographs of histological sections stained with H&E of tissue retrieved from empty pockets (A) and from SPCL scaffolds and magnetic scaffolds (C). Photographs of explants surrounded by nearby tissue, containing the SPCL and the magnetic SPCL scaffolds (B).

8.5. Conclusion

The present study focused on the development and characterization of magnetic scaffolds with potential for tendon tissue engineering (TTE).

Magnetic aligned SPCL scaffolds were successfully prototyped through a feasible and simple technique. Furthermore, *in vitro* cell studies with hASCs suggested the potential of these scaffolds for TTE approaches as iron oxide MNPs input on the scaffold did not negatively affect cell viability or differentiation. The stimulation provided together with the architectural fiber alignment achieved by RP, played a determinant role in promoting hASCs differentiation. The various functionalities that can be achieved in such systems may provide new tools to overcome some of the most common limitations associated with regenerative medicine approaches, enabling the combination of therapeutic and diagnostic tools. The developed scaffolds also showed to be cytocompatible in an ectopic rat model, highlighting its potential for TERM strategies.

8.6. References

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Chapter 9

Triggering the activation of Activin A type II receptor in human adipose stem cells towards tenogenic commitment using mechanomagnetic stimulation

This chapter is based on the following publication:

Gonçalves AI, Rotherham M, Markides H, Rodrigues MT, Reis RL, Gomes ME, Haj, AE. Triggering the activation of Activin A type II receptor in human adipose stem cells towards tenogenic commitment using mechanomagnetic stimulation, *submitted*

9. TRIGGERING THE ACTIVATION OF ACTIVIN A TYPE II RECEPTOR IN HUMAN ADIPOSE STEM CELLS TOWARDS TENOGENIC COMMITMENT USING MECHANOMAGNETIC STIMULATION

9.1. Abstract

Tendon injuries are a considerable problem worldwide and increase with ageing. Available treatments fail to fully restore the tendon's functionality and stem cell therapies hold potential to stimulate tendon regeneration and homeostasis. Tendon tissue structure and function is maintained in response to the mechanical environment with multiple mechano-receptors present on the tendon cell membrane. Activins are members of the mechano-responsive TGF-β superfamily which participate in regulation of several downstream biological processes. Mechanosensitive membrane receptors such as activin can be activated in different types of stem cells via magnetic nanoparticles (MNPs) through remote magnetic actuation resulting in cell differentiation.

In this work, we target the Activin receptor type IIA (ActRIIA) in adipose derived stromal stem cells, using anti-ActRIIA functionized MNPs, externally activated through a vertical oscillating magnetic bioreactor. Upon activation of this receptor, the phosphorylation of Smad2/3 is induced allowing translocation of the complex to the nucleus, which activates the transcription of tendon related genes driving tenogenic differentiation. Our study demonstrates the potential remote activation of MNPs tagged hASCs to trigger the Activin receptor leading to tenogenic cellular responses. These results may provide insights toward tendon regeneration therapies.

9.2. Introduction

During tendon development, mechanical forces are transmitted to tendon cells through mechanosensitive receptors available in cell membranes [1, 2]. These changes initiate intracellular transduction cascades through the activation and the stimulation of transmembrane receptors such as serine/threonine kinase receptors, inducing structural changes in the cytoskeleton and promoting regulated transcriptional responses. However, the mechano-sensing mechanisms that regulate homeostasis and that are involved in mature tendon repair are not well established, hampering the development of successful cell based therapies toward tendon regeneration.

Signaling cascades are the main routes of communication between the membrane and intracellular regulatory targets. Among them, TGFβ/Smad2/3 was reported as one of the most relevant pathway involved in tenogenic differentiation [3, 4]. The transforming growth factor-β (TGF-β) superfamily comprises of the TGFβs, activins, NODAL, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and anti-Müllerian hormone (AMH) [5]. Both TGF-β and mechanical stimulation were found to activate the Smad2 and Smad3 molecules, downstream mediators of TGF-β type I (activin receptor-like kinases, ALKs) and type II receptors, suggesting stress-activated TGF-β signaling drives tenogenesis [6, 7]. ActivinβA has been suggested to activate SMADs pathway [8, 9] and to be involved in the regulatory pathway of tenomodulin [10], a type II transmembrane glycoprotein suggested as tendon specific marker [11, 12].

Since tendons are notably sensitive to mechanical forces, the use of magnetic mechano-activation might constitute an effective approach to commit stem cells towards the tenogenic lineage through mechanosensing signaling cascades such as TGF-β/Smad2/3 pathway. The technology of remote activation of mechanotransduction via magnetic nanoparticles (MNPs) has been successfully employed using the MICA Bioreactor (MICA Biosystems Ltd) to deliver the magnetic field to MNPs-tagged cells in both 2D and 3D environments [13, 14]. MNPs-tagged cells renders magnetic responsiveness to the engineered systems with potential to be remotely controlled and tuned by the actuation of an external magnetic field stimulating cells *in vitro* and upon implantation [15]. Additionally, the magnetic field was shown to impact biological processes [16, 17] and to render a positive outcome in tissue healing [18, 19]. Thus, in this study we propose to investigate a magnetically actuated TE approach using an externally applied oscillating magnetic field over stem cells tenogenic phenotype commitment (Figure 9-1). Human adipose-derived stem cells (hASCs) were labelled with MNPs previously functionalized with anti-Activin receptor type IIA antibody. The magnetic actuation is expected to directly activate the mechanosensitive membrane receptor via functionalized MNPs, stimulating hASCs towards controlled cellular responses, more specifically regulating the transcription of tenogenic associated genes and driving differentiation. In this way, we can compare specific MNPs tagging with non specific effects of an oscilating magnetic field at the cell level.

Previous works using biofunctionalised MNPs targeted to the mechano-responsive ion channel TREK1, Wnt Frizzled or PDGFRα and β receptors, have shown the promotion of an osteogenic, or smooth

muscle cell phenotype, respectively, in hMSCs *in vitro* and *in vivo* [13, 14, 20, 21]. This raised the hypothesis that this approach can be extended to other tissues of the musculoskeletal system, as tendons for instance, and of what cell surface receptors are likely to be mechano-magnetically targeted affecting cell signalling pathways upon stimulation.

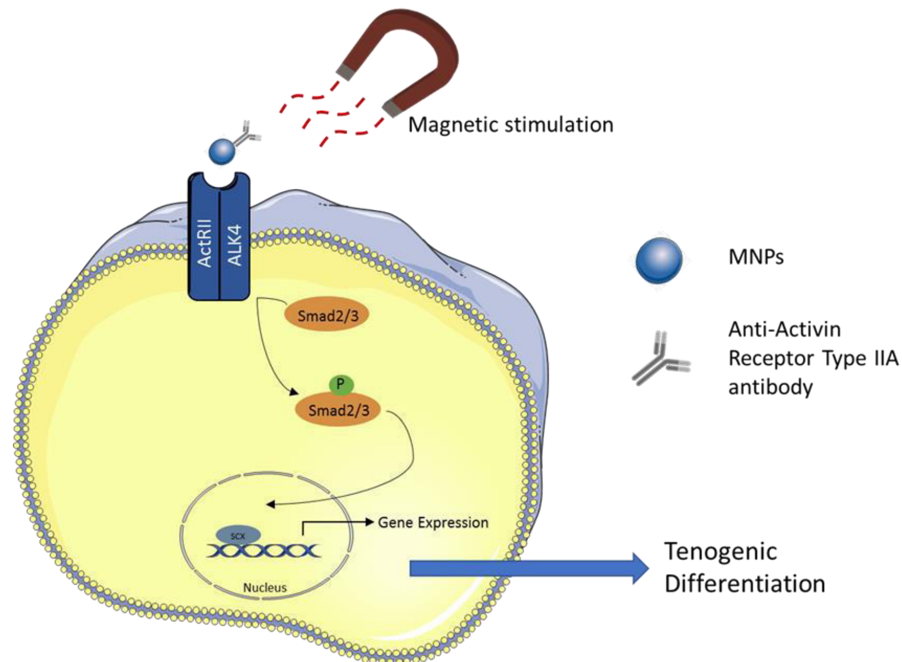


Figure 9-1. Schematic representation of cellular Activin A receptor targeting with MNPs functionalized with Anti-Activin type IIA antibody (MNPs-ActRIIA) for tenogenic differentiation.

9.3. Materials and Methods

9.3.1. Magnetic nanoparticles (MNPs) conjugation with antibody

Carboxyl functionalized magnetic nanoparticles (nanomag®-D, 09-02-252, Micromod) were covalently coated with Anti-Activin Receptor type IIA (ActRIIA) antibody (ab135634) herein termed as „MNPs-ActRIIA“, or with Anti-Rabbit-IgG Fc antibody (ab97196), „MNPs-IgG“, by carbodiimide activation as described previously [14]. Briefly, particles were activated using EDAC (03449, Sigma) and NHS (130672, Sigma) dissolved in 0.5M MES buffer pH6.3 (Sigma) for 1h at room temperature under continuous mixing. The particle suspension was washed and re-suspended in 0.1M MES buffer containing 60μg of anti-rabbit secondary antibody (ab97196). The particle suspension was continuously

mixed overnight at 4°C and then washed and re-suspended in 0.1mL MES buffer containing 10μg of Anti-Activin Receptor type IIA antibody. Particle suspensions were mixed for 3h at room temperature and then blocked with 25mM Glycine (Sigma) for 30 minutes before final washing and re-suspension in distilled water. Functionalized nanoparticles were then analyzed for surface charge and size using a Zetasizer 3000 HSa (Malvern Instruments). Particles were diluted in dH20 and measurements performed at 25°C. The size and surface charge of antibody-coated nanoparticles was compared to IgG-coated nanoparticles.

9.3.2. Cell Isolation and Expansion

Human adipose-derived stem cells (hASCs) were obtained from lipoaspirate samples from the abdominal region and isolated from surplus tissue samples, under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee.

Human ASCs were isolated from tissue samples and cultured as described before [22, 23], and have been previously characterized by RT-PCR for CD44, STRO-1, CD105 and CD90 markers [22]. Briefly, the tissue was rinsed in phosphate buffered saline (PBS, Sigma-Aldrich) containing 10% of an A/A solution. The fat solution was immersed in a 0.05% collagenase type II (Sigma/C6885) solution for 45min at 37°C under mild agitation. The digested tissue was centrifuged at 800g for 10min at 4°C, after which the supernatant was eliminated. Cells were expanded in basic medium composed of αMEM (BE12-169F, Lonza) supplemented with 10% FBS (10270, Gibco) and 1% A/A solution (DE17-603E, Lonza).

9.3.3. Phosphorylation assays

hASCs were seeded at a density of 100,000 cells/well in tissue-culture 6-well plates (Falcon) and incubated with carboxyl functionalised MNPs previously coated with anti-ActRIIA antibody or with anti-Rabbit-IgG antibody. After a 30min labelling period with conjugated MNPs, hASCs were incubated/stimulated for 2, 10, or 30min in basal αMEM medium under magnetic stimulated conditions (magnetic field of 25–120mT from an array of permanent magnets (NdFeB) situated beneath the culture

plates at a frequency of 1Hz) with a vertical oscillating magnetic bioreactor (MICA Biosystems Ltd) and non-stimulated cells kept in identical conditions in incubator. hASCs cultured without MNPs in αMEM medium supplemented with Recombinant Human/Mouse/Rat Activin A Protein (338-AC-010, R&D systems) or TGF-β3 at 10 and 20 ng/mL [24] were considered positive controls of ActRIIA activation. The dose of ligands was chosen based on literature and according to the phospho-Smad2/Smad3 ELISA kit. The MNPs conjugated only with secondary IgG antibody constitute the negative control of the ActIIRA activation. After each incubation period, cells were analysed by enzyme-linked immunosorbent assay (ELISA) that recognizes endogenous levels of phospho-Smad2 (Ser465/467) and phospho-Smad3 (Ser423/425) proteins (PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA, 12001, Cell Signaling Technology), following the manufacturer's instructions.

9.3.4. hASCs culture

hASCs were seeded at 10,000 cells/well in 24-well plates and cultured in the followiing conditions for up to 14 days: i) αMEM medium in static conditions („ST"), ii) αMEM medium in magnetically stimulated conditions („MICA"), iii) αMEM medium supplemented with Activin A (20ng/mL) in ST, iv) αMEM medium supplemented with Activin A (20ng/mL) in MICA, v) αMEM medium in the presence of MNPs-ActRIIA in ST, or vi) αMEM medium in the presence of MNPs-ActRIIA in MICA. Medium was changed every 3 days. Tenogenic commitment of hASCs was evaluated based on the deposition of tendon ECM related proteins and on real time RT-PCR analysis for tendon-related markers as described in detail below.

Magnetically stimulated groups were placed in a commercially available vertical oscillating magnetic bioreactor (MICA Biosystems Ltd), providing a magnetic field of 25–120mT from an array of permanent magnets (NdFeB) situated beneath the culture plates at a frequency of 1Hz, provided every other day for 1h sessions. Non-stimulated groups were kept in identical conditions but without magnetic field.

9.3.5. Dextran Immunolabelling

Fixed cells labeled with MNPs-ActRIIA or MNPs-IgG undergoing dextran immunolabelling were permeabilized with 0.025% Triton-X100 (Sigma/X100)/PBS solution, blocked with 2% BSA (A9418, Sigma) in PBS (21-040-CVR, Corning) for 1h at room temperature and incubated overnight with mouse

monoclonal anti-Dextran antibody (60026FI.1, Stem Cell technologies) in 0.1% BSA in PBS at 4°C. Cells were rinsed in PBS, following incubation for 1h at room temperature with anti-mouse IgG FITC antibody (F2012, Sigma). Samples were stained with 4,6-Diamidino-2-phenyindole dilactate (DAPI, 40009, VWR) for 10min and Phalloidin Tetramethylrhodamine B isothiocyanate (P1951, Sigma) for 20min. The images were acquired under a Fluorescence Inverted Microscope (Axio Observer, Zeiss).

9.3.6. mRNA Extraction and Quantitative Polymerase Chain Reaction

Total RNA was extracted using TRI reagent (T9424, Sigma) according to the manufacturer's instructions. Briefly, 800µL of TRI reagent was added to each sample and stored at -80oC. After defrosting, samples were incubated with 160µL of chloroform (Sigma) for 15min and 12,000g centrifuged for 15min at 4oC. The aqueous fraction was collected and an equal part of isopropanol (Sigma Aldrich) was added. After 10min, samples were centrifuged at 12,000g for 10min at 4°C. RNA pellet was washed with 800µL of 70% ethanol and subsequently centrifuged at 7,500g for 5min at 4°C. Air-dried RNA samples were resuspended in 15µl of RNase/DNase free water (Gibco). RNA quantity and purity were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The cDNA synthesis was performed with the qScript cDNA Synthesis kit (Quanta Biosciences) and using the Mastercycler Realplex (Eppendorf) using an initial amount of total RNA of 1µg in a total volume of 20µL. The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences) following the manufacturer's protocol, in a Real-Time Mastercycler Realplex thermocycler (Eppendorf). The primers were pre-designed with Primer 3 software (Table 9-1) and synthesized by MWG Biotech. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene. The 2-ΔΔCt method was selected to evaluate the relative expression level for each target gene [25]. All values were first normalized against GAPDH values, and then to the hASCs cells collected at day 0.

Table 9-1. Primers used for quantitative RT-PCR analysis.

Gene	Sequence	NCBI reference

Human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F- GGGAGCCAAAAGGGTCATCA R- GCATGGACTGTGGTCATGAGT	NM_001256799.1
Collagen, Type I, alpha 1 (*COL1A1*)	F- CCCCAGCCACAAAGAGTCTAC R- TTGGTGGGATGTCTTCGTCT	NM_000088.3
Collagen, Type III, alpha 1 (*COL3A1*)	F- CCTGAAGCTGATGGGGTCAA R- CAGTGTGTTTCGTGCAACCAT	NM_000090.3
Tenascin C (*TNC*)	F- ACTGCCAAGTTCACAACAGACC R- CCCACAATGACTTCCTTGACTG	NM_002160.3
Scleraxis (*SCXA*)	F- AGAACACCCAGCCCAAACAGAT R- TCGCGGTCCTTGCTCAACTTT	NM_001080514.2
Decorin (*DCN*)	F- CTAGTCACAGAGCAGCACCTAC R- CCAGGGAACCTTTTAATCCGGGAA	NM_001920.4
Tenomodulin (*TNMD*)	F- CCGCGTCTGTGAACCTTTAC R- CACCCACCAGTTACAAGGCA	NM_022144.2

9.3.7. Quantification of Collagen and Non-collagenous Proteins

The amount of collagen and non-collagenous proteins expressed by hASCs cultured in 2D was determined using a semi-quantitative assay, namely Sirius Red/Fast Green Collagen Staining Kit (9046, Chondrex). After cells fixation, the dye solution was added and incubated for 30min in order to completely immerse the samples. A dye extraction buffer was then mixed and the OD values read in a spectrophotometer (Synergy HT, Biotek Instruments) at 540 nm and 605 nm.

9.3.8. Immunofluorescence

Cells were rinsed in PBS and fixed in formalin (INOPAT) prior to the detection of Tenomodulin (sc-49324) and Fibromodulin (ab81443) deposition. After cell permeabilization with 0.025% Triton-X100 (Sigma/X100)/PBS solution, the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector). Then, cells were incubated overnight with anti-tenomodulin and anti-

fibromodulin antibodies, diluted in antibody diluent with background reducing components from Dako (S3022, Dako) at 4°C. Samples were then rinsed in 0.025% Triton-X100 (Sigma/X100)/PBS solution followed by incubation for 1h at room temperature with the respective Alexa fluor 488 (Alfagene) considering the host species of the primary antibodies. Samples were stained with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μl, D9564, Sigma) for 10min and with Phalloidin–Tetramethylrhodamine B isothiocyanate (P1951, Sigma) for 20min. Samples were observed with a Fluorescence Inverted Microscope (Axio Observer, Zeiss).

9.3.9. Statistics

Quantitative results are expressed as the mean ± standard deviation. Two-Way ANOVA followed by Bonferroni's Multiple Comparison test was assessed to determine whether differences between sample groups were significant, unless specified. Differences were considered significant when the P value was < 0.05.

9.4. Results and Discussion

9.4.1. Assessment of Smad2/Smad3 phosphorylation in hASCs triggered by TGF-β-like ligands

Signal transduction molecules of the Smad family are components of a critical intracellular pathway that transmit signals mediated by transmembrane serine/threonine kinase type II receptors into the nucleus. Upon binding of TGF-β family ligands, such as Activins or TGF-β3, receptors leading to the recruitment, phosphorylation and activation of type I Activin receptor (ALK4), which provides a binding site for the downstream substrates, initiating the intracellular signaling through activation of Smad proteins [26].

Herein, TGF-β-like ligands, Activin A and TGF-β3 known for their functions in tendon development [10, 27], were investigated as inducers of serine/threonine receptor's activation and consequent phosphorylation of Smad2/3. Using two different ligand doses (10 and 20ng/mL) and at varying temporal exposures, Smad2/3 signaling and subsequent gene expression has been assessed. Moreover,

Activin A and TGF-β3 ligand-receptor binding will serve as a positive experimental control of the activation of ActRIIA receptor mediated by MNPs-antibody complexes.

The activation of the ligand-receptor complex is described as a relatively fast step in the TGF-β signal transduction, occurring within 2min after ligand stimulation, and the signal is relayed to the activation of Smad proteins, which reach at maximal levels in up to 30-60 minutes [28].

Considering this, we investigated the levels of pSmad2/3 following 2, 10, 30 and 60 min of treatment. The detection of pSmad2/3 proteins was rapid (at 10 min) and significantly more intense in Activin A supplemented medium at 20ng/mL than in medium supplemented with TGF-β3 ligand (Figure 9-2), suggesting a more effective phosphorylation of Smad proteins. Therefore, Activin A at this concentration was selected as the positive control for ActRIIA activation in the subsequent experiments.

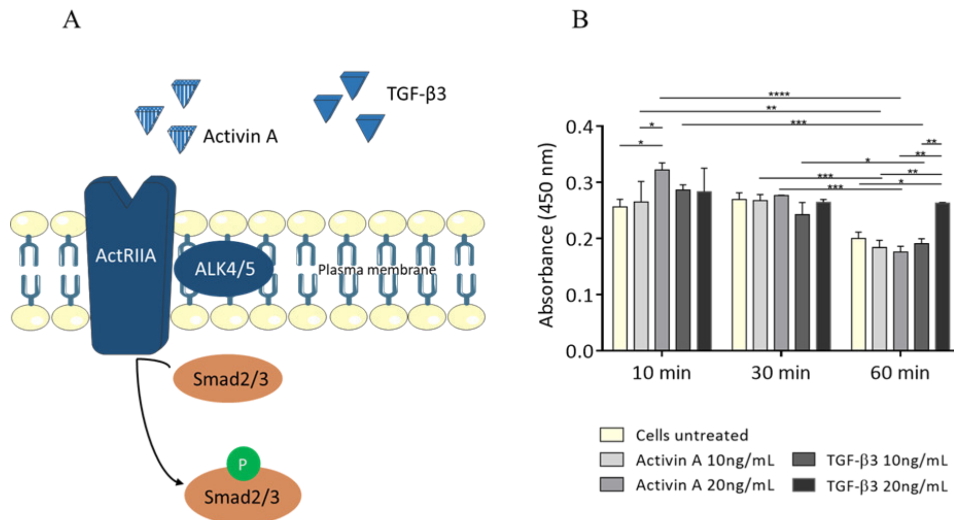


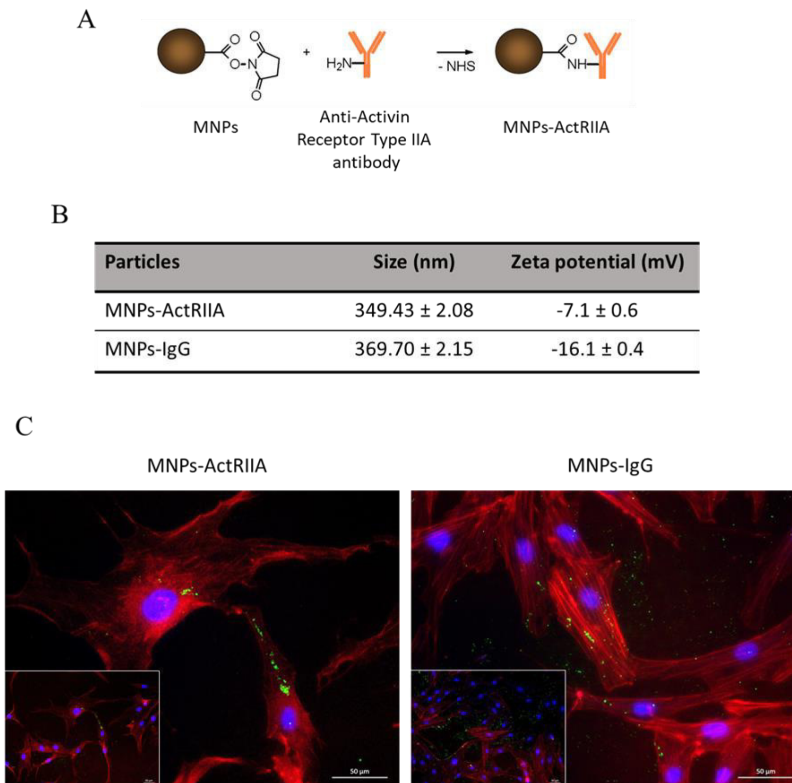
Figure 9-2. Phosphorylation assessment of Smad2/Smad3 in hASCs. A) Schematic of the experimental design for the phosphorylation assay. Treatment of hASCs with Activin A and TGF-β3 ligands for up to 60min and subsequent phospho-Smad2/Smad3 (pSmad2/3) detection; B) Assessment of pSmad2/3 by ELISA kit assay, in hASCs cultured in αMEM medium (cells untreated) or cultured in αMEM medium suplemented with Activin A or TGF-β3 at 10 and 20ng/mL for 10, 30 and 60min. Values represent the mean ± SD. Symbol *, **, *** and **** denotes study groups with statistical significant difference P<0.05, P<0.01, P<0.001 and P<0.0001 respectively.

9.4.2. Development and characterization of functionalized MNPs complexes

After antibody functionalization by carbodiimide activation (Figure 9-3A), the size and surface charge of MNPs-ActRIIA and MNPs-IgG were characterized. The particle size increased after IgG coating of MNPs

in comparison to MNPs coated with anti-ActRIIA (MNPs-ActRIIA). Conversely, the surface charge increased in MNPs-ActRIIA (Figure 9-3B), suggesting improved electrostatic interactions with the negatively charged cell membrane.

In order to co-localize functionalized MNPs within hASCs, MNPs-ActRIIA or MNPs-IgG labelled cells were visualized by fluorescence microscopy after immuno-labelling the MNPs dextran shell with an anti-dextran antibody (Figure 9-3C), suggesting that 30min after labelling, there is a clear association of MNPs-ActRIIA and MNPs-IgG with cells.



Particles	Size (nm)	Zeta potential (mV)
MNPs-ActRIIA	349.43 ± 2.08	-7.1 ± 0.6
MNPs-IgG	369.70 ± 2.15	-16.1 ± 0.4

Figure 9-3. Development and characterization of functionalized MNPs complexes. A) Schematic representation of MNPs functionalization with anti-activin receptor type IIA antibody by carbodiimide activation; B) Size and zeta potential analysis of MNPs functionalized with anti-activin receptor type IIA antibody (MNPs-ActRIIA) or with IgG secondary antibody (MNPs-IgG); C) Dextran (green) immunofluorescence images of hASCs labelled with MNPs-ActRIIA or MNPs-IgG. Cell nuclei are shown by DAPI (blue) and the cytoskeleton by phalloidin (red). Insets represent lower magnification images.

Triggering Smad2/Smad3 phosphorylation in hASCs by MNPs complexes

Furthermore, the capability of functionalized MNPs with anti-activin receptor type IIA antibody (MNPs-ActRIIA) to directly target and remotely activate the Activin receptor via magneto-mechanical stimulation was investigated using MICA bioreactor (Figure 9-4A). The activin receptor activation was indirectly assessed through detection of downstream phosphorylation of Smad2/3 using an ELISA kit assay that recognizes endogenous levels of these proteins. The detection of phospho-Smad2/3 proteins seems to be more intense after 10min in hASCs labeled with MNPs-ActRIIA under magnetic stimulation, which highlights a more efficient ActRIIA activation. Magneto-mechanical stimulated groups showed significantly higher phosphorylation levels in relation to non-stimulated and to Activin A control groups after 2 and 10min, decreasing after 30min (Figure 9-4A) in MNPs-ActRIIA ST and MNPs-ActRIIA MICA conditions.

Results also show a significant elevation of absorbance values in comparison to the negative control group, MNPs-IgG, or to the positive control group, Activin (Figure 9-4B). These outcomes suggest that ActRIIA is a mechanosensitive receptor that can be remotely activated using Ab coated MNPs, whose action is favoured by the actuation of an external magnetic field provided by MICA bioreactor.

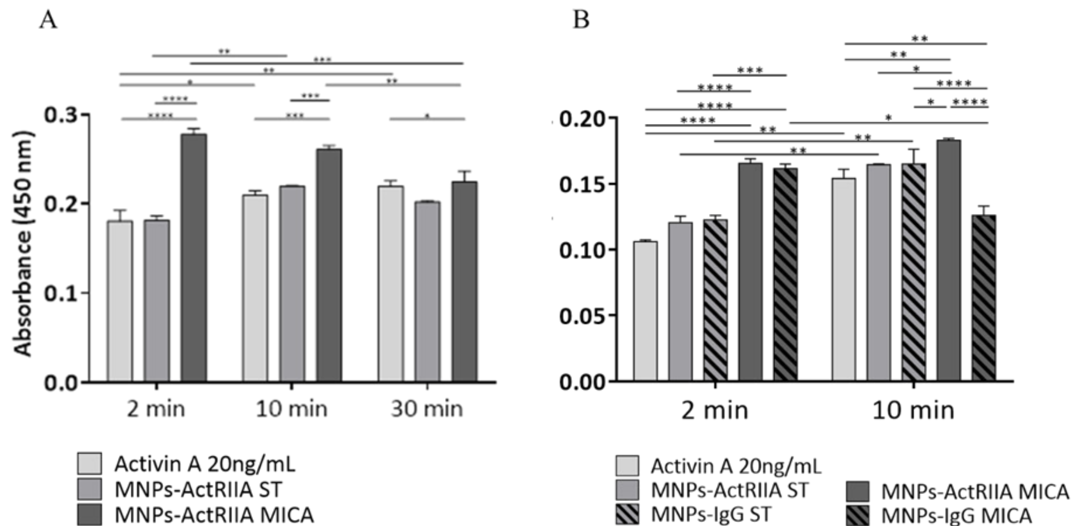


Figure 9-4. A) Phospho-Smad2/Smad3 assay of hASCs cultured in Activin A (positive control) and with MNPs-ActRIIA under static (ST) or under actuation of a vertically oscillating magnetic field (MICA) for 2, 10, and 30min; B) Phospho-Smad2/Smad3 ELISA assay of hASCs cultured with MNPs-ActRIIA or MNPs-IgG under static (ST) or under actuation of a vertically oscillating magnetic field (MICA) for up to 10min; Values represent the mean ± SD. Symbol *, **, *** and **** denotes study groups with statistical significant difference $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ respectively.

9.4.3. Triggering tenogenesis in hASCs using MNPs-ActRIIA complex

Tenoblasts maturation to tenocytes is characterized by the formation of enriched extracellular matrix and controlled by key tenogenic transcription factors, such as SCX [29]. The TGF-β signaling pathway regulates a diverse group of cellular behaviors such as proliferation, differentiation, and growth arrest [30] and has also been suggested as the most active pathway in tendon cells during mouse limb development [31]. In this study, real time RT-PCR analysis, the production assessment of collagen and non-collagenous proteins and the deposition of Tenomodulin (TNMD) and Fibromodulin (FMOD) tendon-related proteins were conducted up to 14 days upon ActRIIA activation under magnetic field stimulation provided by MICA bioreactor.

Gene expression analysis

Havis *et al* reported that blocking the TGF-β pathway with SB43 inhibitor, significantly downregulated *SCX* and *COL1a1*, among other gene expression [31] concluding that TGF-β is required via the Smad2/3 intracellular pathway for expression of tendon-related markers in mouse forelimbs [31].

The expression of tendon associated genes in hASCs was found to be higher in ASCs bound with MNPs-ActRIIA with and without the magnetic stimulation after 7 days (Figure 9-5). Overall, magnetic actuation of MNPs coated with ActRIIA bound to the receptor enhance the expression of all tendon related genes studied. PCR results evidence higher gene expression values in MNPs-ActRIIA after 7 days under magnetic stimulation. Targeted mechano-activation of the ActRIIA using magnetic actuation of MNPs attached to the membrane receptor resulted in enhanced upregulation of tenogenic genes, *TNC*, *TNMD*, *SCXA*, *DCN*, and *COL3A1* but not *COL1A1*. Interestingly, MNPs-ActRIIA binding alone enhanced *TNC* expression but not other tenogenic genes, as for example *TNC* gene expression is significantly higher in MNPs-ActRIIA ST ($P<0.05$) in comparison to Activin A ST and to αMEM ST ($P<0.01$).

We can infer that MNPs-ActRIIA and magnetic actuation synergistically influenced Smad2/3 mediators. This agrees with other studies using alternate mechanical activation methods where phosphorylation assays, and *SCX* and *TNMD* transcriptional activity was up regulated in response to strain [12, 32, 33].

Fluctuations in gene expression from 7 to 14 days (Supplementary Figure 9-1) may be related with ligand depletion from the environment causing cells to lose the stimulation and shut down the

phosphorylation of Smad2, which can occur within a few hours post stimulation [34, 35]. Dose and time of TGF-β-like ligands stimulation have effects on Smad signaling and short-term ligand pulse stimulation results in transient pSmad2, whereas serial pulses or continuous stimulation results in sustained pSmad2 [34]. Moreover, there is a time-delay for reaching the maximum pSmad2/3 phosphorylation, which we determined to be 10min after treatment with MNPs-ActRIIA or with Activin A at 20ng/mL, and nuclear accumulation and subsequent regulation of transcriptional responses. Considering these two assertions and the results of gene expression we can infer that as the complex MNPs-ActRIIA was provided to hASCs once at the beggining of culture, the transcriptional responses observed were time-dependent and transient mostly affecting gene expression in early culture periods.

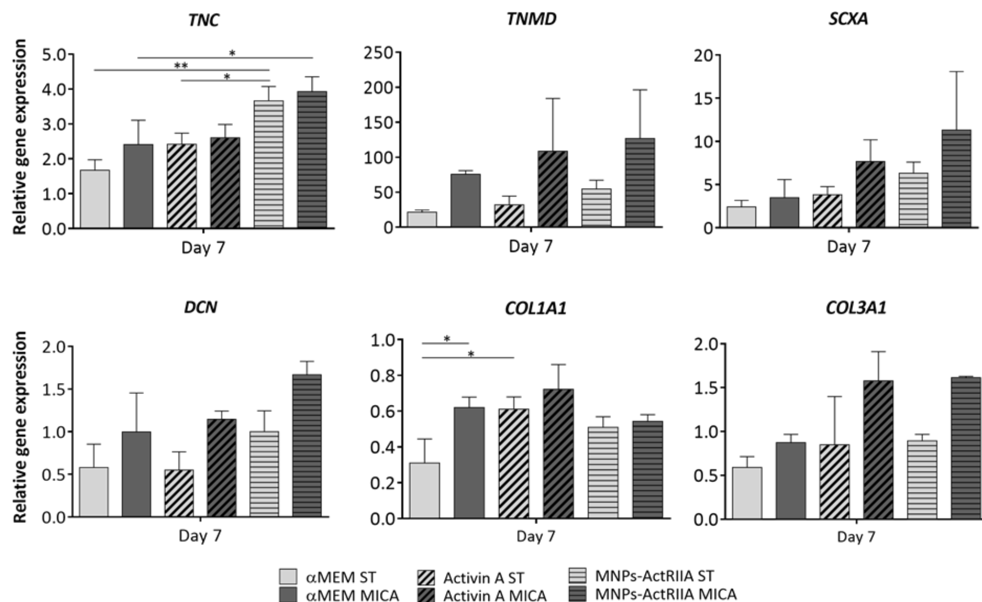


Figure 9-5. Real time RT-PCR analysis of tenascin C (TNC), tenomodulin (TNMD), scleraxis (SCXA), decorin (DCN), collagen type I (COL1A1) and collagen type III (COL3A1) gene expression of hASCs cultured for 7 days in αMEM medium, αMEM supplemented with Activin A (20ng/mL) or labeled with MNPs-ActRIIA in αMEM medium, under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). Values represent the mean ± SD. Symbol * and ** denotes study groups with statistical significant difference $P<0.05$ and $P<0.01$, $P<0.001$, by one-way ANOVA.

Extracellular matrix formation assessment

Of the tendon dry weight, 60% is collagen type I arranged in tensile-resistant fibers [36] and other types such as collagen types III (0-10%), IV (~2%), V, and VI. The non-collagenous matrix is primarily made up of glycoproteins which include the proteoglycans such as decorin (DCN), tenascin C (TNC), fibromudulin (FMOD) and tenomodulin (TNMD) [37]. ECM formation was assessed by production of collagen and non-collagenous proteins as depicted in Figure 9-6.

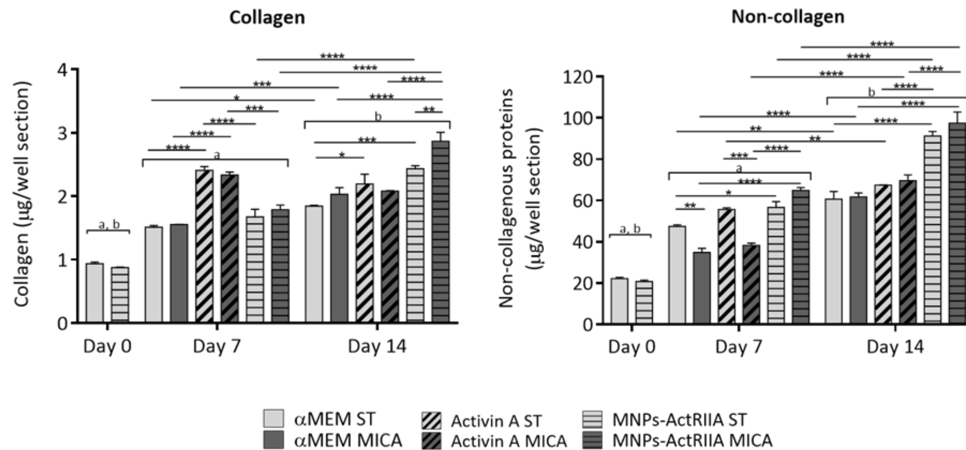


Figure 9-6. Quantification of collagen and non-collagenous proteins by Sirius Red/Fast Green Collagen staining kit in hASCs labeled with MNPs-ActRIIA in αMEM medium, under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). hASCs cultured for up to 14 days in αMEM medium and in αMEM supplemented with Activin A (20ng/mL) were considered the negative and positive experimental controls, respectively. Values represent the mean ± SD. Symbol *, **, *** and **** denotes study groups with statistical significant difference P<0.05, P<0.01, P<0.001 and P<0.0001 respectively; a and b letters represent statistically different groups.

Collagen and non-collagenous proteins production by hASCs significantly increased with time in culture from day 0 to 7 days and from 0 to 14 days, respectively (statistical significant groups: a and b). Interestingly, the collagen values obtained in MNPs-ActRIIA MICA condition were significantly higher than in static conditions after 14 days. These values were also higher than αMEM or Activin A controls. The same trend was observed in non-collagenous protein production.

The synthesis of such complex matrix supports the activation of activin receptor by MNPs-ActRIIA complex under magnetic actuation in a more efficient way than using the Activin ligand to trigger hASCs response.

Tendon specific markers assessement

Considering the role of TNMD as specific tendon marker and Fmod as a crucial proteoglycan component of tendons that regulate physiological ECM assembly, we investigated the deposition of these markers by hASCs tagged MNPs-ActRIIA.

Fmod is highly expressed in the tendon and identified as a critical component of the tendon stem cell niche [38], immunodetection of TNMD and Fmod is presented in Figure 9-7A and B, respectively.

Tendon-specific transcription factors such as SCX [3] are essential and involved in mechanoresponsive tenogenesis through regulation of its downstream ECM proteins such as the tendon marker TNMD [33, 39] and proteoglycans such as FMOD.

Tenomodulin and Fibromodulin proteins deposition was increased by hASCs previously labeled with MNPs-ActRIIA in comparison with hASCs cultured in basal αMEM or in αMEM supplemented with Activin A (negative and positive controls, respectively). Also, the detection of these proteins after 7 and 14 days of culture was higher when the oscillating magnetic field, provided by MICA bioreactor, was applied.

Tenomodulin has been sugested to be expressed upon Activin II receptor stimulation by myostatin, triggering the Smad2/3 signaling cascade and increasing the expression of SCX that ultimately results in the TNMD gene expression [10]. Thus, upon MICA stimulation, MNPs-ActRIIA complexes are likely to act over Activin II receptor, activating the signaling cascade and ultimately inducing the transcription of TNMD and its translation into protein. Moreover, FMOD suggested to play a role in the formation of extracellular matrix and regulate TGF-β levels [40]. Also, mutations in the small leucine-rich proteoglycan gene FMOD result in irregularities in the diameter of collagen fibers in the tendons [41].

Overall, these results together with Sirius Red/Fast Green Collagen staining assay and RT-PCR confirms our hypothesis of magnetically actuated MNPs-tagged hASCs towards a tenogenic commitment of hASCs.

Moreover, it was clear in the present work that the activation of Activin receptor and subsequent induction of Smad2/3 signaling, elicited hASCs tenogenic response. However, it is known that several

variables outside the target cell determine the extent of stimulation by a TGF-β cytokines [42]. Thus, future studies will have to take into account other extracellular regulators's impact in Smad signaling dynamics, such as ligand exposure, and also investigate the effect of antagonistic ligands.

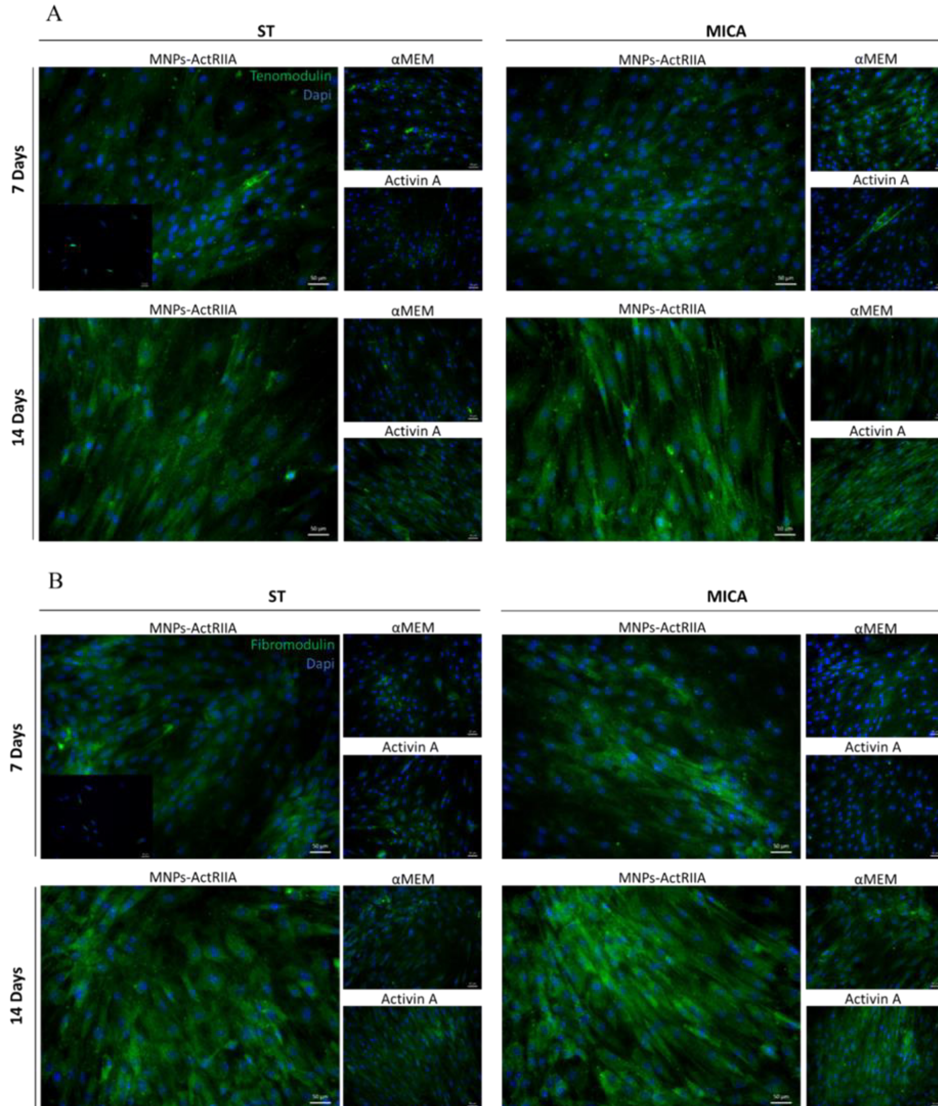
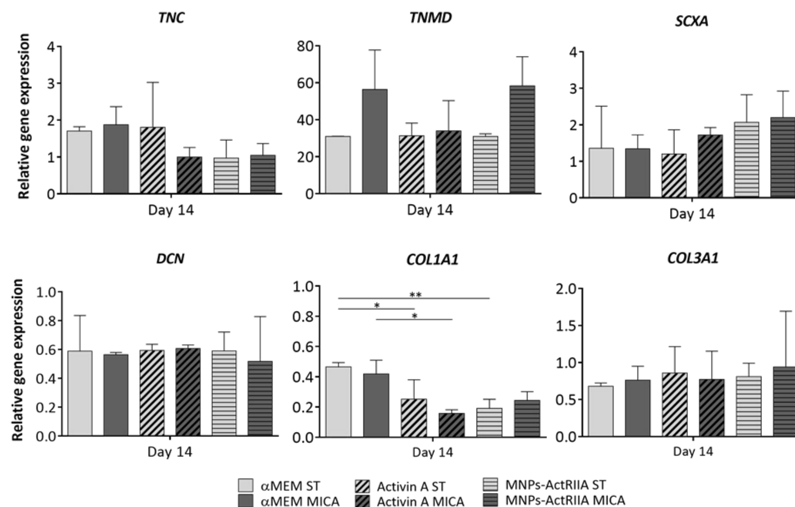


Figure 9-7. Immunocytochemistry of A) Tenomodulin and of B) Fibromodulin proteins (green) deposited by hASCs labeled with MNPs-ActRIIA in αMEM medium, under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). hASCs cultured for up to 14 days in αMEM medium and in αMEM supplemented with Activin A (20ng/mL) were considered the negative and positive experimental controls, respectively. DAPI (blue) stains cell nuclei. Insets represent hASCs labeled with MNPs-ActRIIA in αMEM medium at day 0.

9.5. Conclusions

Smad2/3 signaling pathways are associated with tendon development and transcriptional responses. To date no one has attempted to locally target and activate Activin receptors upon mechano-magnetic stimulation, in order to modulate Smad2/3 signaling. Our work has shown that the remote activation of MNPs tagging mechanosensitive receptors of hASCs may have potential for controlling stem cell differentiation resulting in successful cell therapies for tendon regeneration. The TGF-β ligand, Activin A, has been demonstrated to be a putative mechano-sensitive candidate for magnetic MNPs tagging which can be utilised in tendon lineage comitment via ActRIIA/Smad2/3 signaling cascade, as shown by phosphorylation of Smad2/3 proteins in MNPs-ActRIIA tagged hASCs. The mechanism behind signal activation through MNPs-ActRIIA and the effects on hASCs fate and differentiation requires further investigation. Also the remote activation of other mechanosensitive receptors for the modulation of TGFβ/Smad2/3 pathways using MNPs technology should be investigated in tendon tissue engineering field. The development of this technology raises the possibility of remotely controlling TGF-β/Smad2/3 signaling and consequently the control of stem cell behaviour. Overall, the remote activation of MNPs tagged hASCs may have potential for controlling stem cell differentiation following cell therapy and modulation of signaling pathways involved in tendon formation.

9.6. Supplementary material



Supplementary Figure 9-1. Real time RT-PCR analysis of tenascin C (TNC), tenomodulin (TNMD), scleraxis (SCXA), decorin (DCN), collagen type I (COL1A1) and collagen type III (COL3A1) gene expression of hASCs

cultured for 14 days in αMEM medium, αMEM supplemented with Activin A (20ng/mL) or labeled with MNPs-ActRIIA in αMEM medium, under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). Values represent the mean ± SD. Symbol * and ** denotes study groups with statistical significant difference $P<0.05$ and $P<0.01$, $P<0.001$, by one-way ANOVA.

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Section IV

GENERAL CONCLUSIONS

Chapter 10

Concluding Remarks

10. CONCLUDING REMARKS

The ideal treatment of tendon injuries is yet to be encountered since the current approaches are merely conservative (e.g., reduced activity and immobilization) or based on surgical repair (e.g., suturing techniques and/or replacement with tendon grafts), which are not successful enough to consistently restore the original properties of a functional tendon. Therefore, feasible and clinically efficient strategies to fully restore tendon function are still an unmet need in the regeneration of tendon tissues. Thus, the ideal biomimetic approach aiming at successful tendon tissue engineering (TTE) resolutions is an increasingly pursued objective by researchers in this field. Seeking this issue, and taking advantage of the emergent and sophisticated nanotechnology techniques, we were able to successfully propose different TTE strategies, while providing new insights on the development and characterization of cellular sources and magnetic tools. Moreover, the scientific and technological knowledge obtained might be widen in future studies exploiting the fundamental mechanisms behind the observed results and underlying the tenogenic phenotype achievement, namely in the follow up of the several works carried out under the scope of the present thesis.

The systems developed in this thesis comprising cellular sources together with bioactive factors, the developed scaffolds and the cell sheets with magnetic responsiveness, are distinct approaches yet complementary and a starting point and motivation for further research and refinement toward tissue engineering (TE) and regenerative medicine strategies aiming to improve tendon regeneration.

An important development in tendon field would consider breakthroughs on the specific role of the bioactive molecules in tendon biology. This could be of particular interest in the standardization of cell culture media, for controlling the *in vitro* tenogenic differentiation and providing insights in tenogenesis during tendon development and maturation.

In this thesis, we questioned the need of an adequate stem cell source or a subpopulation of stem cells more prone for tenogenic differentiation, together with finding an adequate tenogenic differentiation cocktail, are of beyond requirements in the TTE field. So, the work developed in Chapters 5 and 6 was centered on the understanding of i) different stem cell sources, ii) bioactive agents, iii) stem cell subpopulations, for use in TTE strategies.

Tissue regeneration in general has been relying in the use of stem cells, available at high amounts and from different reachable tissues, as building blocks of tissue substitutes and/or as manufacturers

of bioactive molecules *in situ*. Thus, although more studies are needed to infer about the capacity of autologous stem cells to treat tendon injuries, reports in literature suggest that autologous strategies encompassing the use of tendon resident cells are still challenging. This lead us to find alternative cell sources with evidenced ability to promote tissue repair, as human adipose stem cells (hASCs) and human amniotic stem cells (hAFSCs), or subsets of hASCs with improved capability towards tenogenic differentiation. The results obtained showed that hASCs and hAFSCs have a distinct response in the presence of different GFs under evaluation (EGF, bFGF, PDGF-BB and TGF-β1) which is indicative that the origin of these cells may have an effect on the process of differentiation, although showing potential to be biochemically induced towards tenogenic commitment. Nevertheless, EGF and bFGF influenced the expression of tendon related genes in hAFSCs, while EGF and PDGF-BB stimulated the genetic expression in hASCs.

Moreover, we pursued the hypothesis that in a crude population of hASCs could reside a subpopulation susceptible to commit towards the tenogenic lineage. In fact, the use of a Tenomodulin positive subpopulation of hASCs, among others under study in Chapter 6, promoted tenogenic lineage commitment and concomitantly contributed with new perspectives for the stem cell field and toward translational therapies.

Furthermore, in Chapters 7, 8 and 9 of this thesis, we unleashed innovative magnetically assisted approaches, namely the v) construction of magnetic cells sheets (magCSs), vi) biofabrication of rapid prototyped magnetic scaffolds (magSPCL), and vii) targeting of a mechanosensitive cell receptor by means of magnetic actuation. In native tendon tissue, cells are embedded within the extracellular matrix, therefore, using extracellular-rich cell sheets seems an obvious choice for the development of a bioinstructive tendon patch. In this sense, in Chapter 7, we used magnetic force-based tissue engineering for the development of magnetically responsive cell sheets with a Tenomodulin positive subpopulation of hASCs, which revealed good mechanoelastic properties and a rich collagenous matrix. In clinical mindsets, the use of tendon substitutes specifically tailored to meet the native structure and biological milieus of the different tendon tissues, from different anatomical regions, is a complex demand. This cell-sheet perspective, reinforces the importance of stem cell players in recreating the native milieu of the tissue to be explored as tendon healing promoters, in this case towards the repair of tendon rotator cuff. Furthermore, and on a 3D scaffolding design perspective, magnetic polymer scaffolds with aligned

structural features were also fabricated by rapid prototyping technique, inspired on the natural tendinous fibrous structure and focusing on aligned arrangements of SPCL fibers incorporating magnetic nanoparticles. The work developed in Chapter 8 focused on the *in vitro* tenogenic potential of such system laden with hASCs, as well as on their *in vivo* biocompatibility evaluation in an ectopic rat model. Our *in vitro* findings suggest a synergistic effect of combining iron oxide MNPs with magnetic actuation, at the cellular level, stimulating cell mechanisms toward tenogenic differentiation. With regard to the *in vivo* study, the developed scaffolds showed to be cytocompatible and to be well integrated with the surrounding tissue.

The functionalities of the developed systems could also be pursued and complement/enrich innovative applications in the field. In fact, the research herein carried out relied on the development of unique cell-laden magnetically responsive systems which have the capacity to be remotely modulated *in vivo* by the application of external magnetic stimuli. Thus, in order to validate the findings of Chapter 7 and 8, future *in vivo* assessment of functionality and tendon healing of the developed magnetic cell sheets and magnetic scaffolds, or a combination of both, for tendon repair are due. Indeed, taking into consideration the dissimilar structural integrity requirements of different tendons, TTE pursuits optimized constructs with suitable cells, which could benefit from the assembling of cell sheets with fiber scaffolds in engineering transplantable tendon substitutes optimized from a mechanical, biological and nutrient transport point of view.

Moreover, the manipulation and control of cells and sub-cellular structures through magnetic nanoparticle-based actuation offers novel advantages such as the impact in cell performance and also modulation of the inflammation events. In fact, magnetic systems such as the magnetic cell sheets or the magnetic scaffolds offered the possibility of remote stimulation in the studies carried out, and can further be applied for the combination of therapeutic and diagnostic tools, such as magnetotherapy and MRI analysis, respectively.

Firstly, an ectopic model should be considered to assess the biocompatibility of developed magCSs, including degradation/integration rate, cell migration and recruitment, and screen the eventual biodistribution of MNPs that could be released with time resultant from the degradation/remodeling of the magCSs in the integration process with surrounding tissues. Then, the functionality and potential for stimulating neo-tendon formation and tendon healing should be considered in tenotopic rotator cuff

models where implanted magCSs could work as potential remotely actuated tendon patches. In fact, forthcoming works should also focus on the relevance of studying magnetic forces *in vivo* and on the application of external magnetic fields across the implanted bioengineered substitutes (magCSs or magSPCL scaffolds), which can additionally be monitored by MRI providing valuable information on their specific role in the regeneration of tendon defects. Moreover, pre-clinical studies are also expected to enable further understanding on the influence of the inflammatory process in the tendon repair mechanisms and how inflammation can eventually be modulated through the application of an external magnetic field.

Moreover, there is a growing interest to decipher the molecular mechanisms in the modulation of signaling cascades underlying tendon differentiation and healing/repair. Therefore, we also investigated the targeting of a Activin cell-receptor type IIA by tagging functionalized magnetic nanoparticles with anti-ActRIIA antibody and externally activated through a vertical oscillating magnetic bioreactor. This study demonstrated the possibility to trigger the downstream Smad2/3 pathway leading to tenogenic cellular responses. The withdrawn conclusions of this work contributed to the understanding of the effective pathway regulators of transcript and protein levels in the acquaintance of a tenogenic phenotype. However, targeting for other specific cellular mechanoreceptors of the widely and easily obtained human adipose derived stem cells would certainly contribute to unveil the cellular signaling pathways that trigger tenogenic differentiation.

The best comprehension of these issues and the establishment of cell based therapies combined with remote magnetic actuation conditions is of fundamental interest and will definitely contribute to improved translational strategies in the development of refined tendon regeneration responses. This constitutes an added value in the enhancement of tissue response that can significantly broaden the therapeutic window of current tendon TE systems and eventually be transposed to the regeneration of other tissues of the musculoskeletal system.

In summary, under the scope of this thesis it was possible to remark that the selection of a source of stem cells predisposed to tenogenic commitment, also combined with biochemical/bioactive molecules and remote magnetically assisted stimulation showed to be valuable tools in providing insights towards tendon regeneration therapies.