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Mesenchymal stem cells in degenerative joint diseases

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Summary

Abstract

In the setting of cell therapy, mesenchymal stem cells (MSCs) are the most investigated and used cell type. Recent evidences about their perivascular localization and ability to release a multitude of growth and trophic factors in reponse to an injury strengthen the concept that they can be actually used as a drug in many clinical conditions. Hence the alternative explanation of the acronyms MSC as “medicinal signaling cells”. Indeed, in addition to their multi-potency and consequent ability to replace directly tissue-specific cells, MSCs can contribute to tissue healing and homeostasis restoration via paracrine action, through an intense crosstalking with the tissue resident cells.

The purpose of this PhD project has been to investigate the MSCs therapeutic properties for the treatment of degenerative joint disorders, focusing on different approaches and different ways to exploit their wide potential.

The primary goal was the development of an *in vivo* model of tissue degeneration. A reproducible pre-clinical model of rat Achilles tendinopathy was developed to deeply investigate the progression of this condition, as well as to study the possible role of MSCs in tendon healing. The results of this study suggested that the tendinopathy induction by a chemical agent such as collagenase enzyme, represents a reliable model in rats that resembles the human disease. The findings of this study permitted to observe the progression of the tendinopathy during time, with its proper distinct stages, thus offering the opportunity to accurately develop future novel MSCs-based therapies.

An extensive *in vitro* characterization of adipose, bone marrow- and tendon-derived stem cells was performed too with the aim to identify the best MSCs source for each specific clinical purpose. In particular, to face the lack of consensus within the scientific community about the tenogenic potential of MSCs, a biochemical tenogenic induction protocol specifically was developed and implemented during this PhD path. It was able to give efficient results using all the three different MSCs sources, confirming that MSCs are a valid cell replacement tool in tissue engineering approaches.

Two studies, still in progress, were planned to identify the best way to exploit the paracrine activity of MSCs. In particular, one study was aimed to observe the anti-inflammatory and immunomodulatory properties of adipose stem cells secretome that is the pool of molecules released by these cells in responce to specific stimuli. The preliminary results showed that the secretome was able to decrease the proliferation of pre-activated peripheral blood mononuclear cells (PBMCs) and to increase the levels of catabolic factors (such as metalloproteinases 1-MMP1) in chondrocytes previously treated with a pro-inflammatory stimulus.

The second study has focused on the evaluation of MSCs as a therapeutic target rather as a therapeutic agent. This rationale underneath this concerns the hypothesis that each tissue is provided of a stem/progenitor cell subpopulation that in physiological condition takes part to tissue healing and homeostasis. For those tissues characterized by a poor self healing ability, the external stimulation of this progenitor subpopulation could represents a good strategy to improve this process. In this view, basing on the previous findings obtained from the rat tendinopathy study, the effects of the biophysical stimulation with pulsed electro-magnetic fields on this condition were evaluated, focusing on the resident MSCs and progenitor cells activation. Although still in progress, this study has shown that the best therapeutic answer is obtained applying biophysical stimulations during the proliferative and the remodeling phase of the pathology rather than during the inflammatory one. Further evaluations will be carried on to identify a stimulation protocol able to maximize the therapeutic effect.

Since the success of the cell therapy approach strongly resides on the efficiency of cell delivery, one of the main aim of this PhD project was the development and optimization of a cell-delivery system intended as a medical device for one-step local MSCs applications. In particular, the idea was to develop a concrete one-step surgical tool for degenerative joint disorders, such as osteoarthritis and tendinopathy that was able to satisfy the safety requirements, as well as to respect the strict surgical time allotted to such kind of procedure in the clinical practice. A silk-coated sodium-alginate microcarrier system (FAMs, fibroin-coated alginate microcarriers) was first produced and then characterized in term of physical and chemical properties. After a first seeding protocol that allowed to obtain a firm cell adhesion in about 2 hours while maintaining an optimal cell viability and multi-differentiation potential, additional *in vitro* studies using the DoE (Design of Experiment) has lead to the identification of proper parameter conditions able to maximize the cell seeding efficiency, in a more homogeneous way.

Moreover, the biocompatibility of FAMs and their performances as delivery system were tested *in vivo* in a rat model in collaboration with the Andalusian Center for Nanomedicine & Biotechnology BIONAND of Malaga (Spain). The results confirmed the FAMs biocompatibility, as shown by the lack of any adverse inflammatory reactions, as well as satisfactory performances as cell carriers, as demonstrated by the persistent cell viability at the target site.

All the studies carried on during this doctoral path has permitted to increase the knowledge about the multiple properties that make MSCs an effective tool in regenerative medicine. In particular, the data collected in these years as well as the results reported in literature show that MSCs can be really considered as drugs, mainly thanks to their paracrine ability based on the release of a plethora of different molecules with different effects. The ability of MSCs to respond adaptively to the different microenvironment stimuli make them to be

a potential step forward in comparison with traditional drugs for the treatment of many disorders, both as therapeutic agents and targets.

Introduction

The “cells as a drug” paradigm: the transformation of the mesenchymal stem cell concept

The concept of cell therapy arose during the 16th century from the theory of the Swiss physician Philippus Aureolus Paracelsus who in the *Der Grossen Wundartzney* stated that “the heart heals the heart, lung heals the lung, spleen heals the spleen; like cures like" *(Der Grossen Wundartzney, Great Surgery Book, PA Paracelsus, 1536).* From this hypothesis, the idea of repairing an organ by the use of living components and tissue has been progressively consolidated over the centuries.

Nowadays, cell therapy is recognized as the transplantation of human or animal cells to replace and repair a tissue damage. In specific, the Regulation (EC) No 1394/2007 defines a somatic cell therapy medicinal product as “a biological medicinal product which has the following characteristics: (a) it contains or consists of cells or tissues that have been subjected to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor; (b) it is presented as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues”.

Currently, cell therapy is considered an alternative therapeutic approach, belonging to the regenerative medicine field and it is progressively evolving in a potential future tool for the treatment of pathologies that are not adequately treated using the current approaches. A typical example of a cell therapy application, with or without the combination with suitable scaffolds, is the treatment of pathologies in which a tissue is severely damaged, where the main goal of cell therapy is to restore its physiological and functional properties. During the last years, cell therapy has been applied to several fields, including hormonal dysfunctions (diabetes and growth hormone deficiency), neurodegenerative diseases, cardiovascular lesions, peripheral vascular ischemia, cornea lesions, musculoskeletal injuries and skin patholol gies *(Sánchez A et al, 2012).*

In the context of cell therapy, mesenchymal stem cells (MSCs) are the most investigated and used cell type and play a crucial role in the majority of the application protocols.

After the first discovery of a MSCs population in bone marrow in the 1970s *(Friedenstein AJ et al, 1966),* in the following decades their presence was assessed in almost each vascularized tissues, not necessarily of mesodermic origin. These findings, together with difficulties to describe a univocal immunopheonotype of these cells, led to many questions about their real nature, leading to a very fast overturning process in regard of their nature

and its great potential. Hence, at present MSCs represent one the most debated issue of this field.

The recent discoveries demonstrate that MSCs are associated to a perivascular district and that can derive from perivascular cells, named as “pericytes”, located in the proximity of blood vessels *(Crisan M et al, 2008; Caplan AI, 2016; Chen WC et al, 2015)*. This theory has completely changed the awareness about the origin and function of MSCs, giving them an even higher importance.

Pericytes belong to the so called “perivascular niche” *(Chen WC et al, 2012)* where they live in a quiescent condition until external signals, such the local surrounding plethora of molecules that occur after an injury, activate their mesenchymal stem profile to produce and release anti-inflammatory and immunomodulatory bioactive molecules, initiating the tissue restoration process *(Kean TJ et al, 2013; Singer NG and Caplan AI, 2011; Caplan AI 2015; Caplan AI and Dennis JE, 2006; Oh M et al, 2015)*. When an injury occurs, the response of body consists of three phases that represent the stages of a normal wound healing: inflammation, proliferation and remodeling. Inflammation, often seen as a negative event, actually is an essential component of tissue healing, where many of the traditional pro-inflammatory molecules, play a critical role in supporting tissue regeneration. On the other side, persistence of inflammation, as in chronic wounds, causes a progression of tissue damage and inhibits healing process. In this context, maintenance or re-establishment of a correct balance of cytokines and molecules with immunomodulatory potential is among the best way to obtain the restoration of tissue homeostasis. In a normal process of wound healing, when inflammation starts to decrease, tissue resident cells migrate to the site of injury and start to proliferate. At last, remodeling phase occurs and implicates a slowing down process of extracellular matrix (ECM) production in order to obtain a balance between synthesis and degradation of tissue exerted by metalloproteinases (MPPs).

In such a complex chemical and cellular response of human body to a tissue injury, MSCs act as one of the main participant, especially for their peculiar localization.

Indeed, the MSCs presence in each vascularized tissues, their immunomodulatory and homing potentials, as well as their chemotactic properties similar to other immune cells that respond to injury and sites of inflammation *(Caplan AI and Dennis JE, 2006; Caplan AI and Correa D, 2011; Bernardo ME et al, 2013)* are the keys of MSCs role in the normal tissue turnover and in the physiologic reparative process that normally occurs after a damage.

Despite the first and more traditional interest for MSCs was focalized on their direct participation to tissue regeneration through their multi-differentiation potential and recognized by the multitude of existing researches *(Caplan AI 1991; Caplan AI 1994; Jackson L et al, 2007; Gimble JM et al, 2008; Lee AY et al, 2015)*, today their effective in *vivo* function and the therapeutic effects are thought to be related to a paracrine and trophic action *(Caplan AI 2015, Caplan AI 2016).*

In fact, when a vessel is damaged as normally occurs during an injury, this event lead to the release of pericytes that from a quiescent (resting) phase pass to an activated phase, finally acquiring a MSCs phenotype. Activated MSCs release a cascade of bioactive molecules to counteract the overaggressive immune response *(Kean TJ et al, 2013)* and trophic factors to (re)-establish a regenerative microenvironment, inhibiting ischemic events, apoptosis and scar formations, promoting angiogenesis and stimulating proliferation of tissue specific progenitor cells *(Caplan AI and Correa D, 2011) (Figure 1)*. In particular, the specific immunomodulatory response depends on the maintenance of MSCs in the site of injury and their contact with the local environment. Due to these recent findings, Arnold Caplan and his colleagues from Case University, Cleveland, USA, after many years spent studying MSCs, recently defined MSCs as a "drugstore", since their ability to release a wide range of growth factors and cytokines in the surrounding microenvironment actually mimic the effect of a very powerful drug administered locally *(Caplan AI and Correa D, 2011).* Therefore, the term "Stem" has gradually lost its importance and become more and more insufficient to describe their real potential *(de Girolamo L et al, 2013)*. For this reason, the acronyms MSCs, initially "mesenchymal stem cells", has progressively changed during time, first to "mesenchymal stromal cells" to highlight their stromal origin *(Horwitz EM et al, 2005),* and then to "medicinal signalling cells", to underline their paracrine activity able to create an intense crosstalk with resident cells *(Caplan AI 2010).*

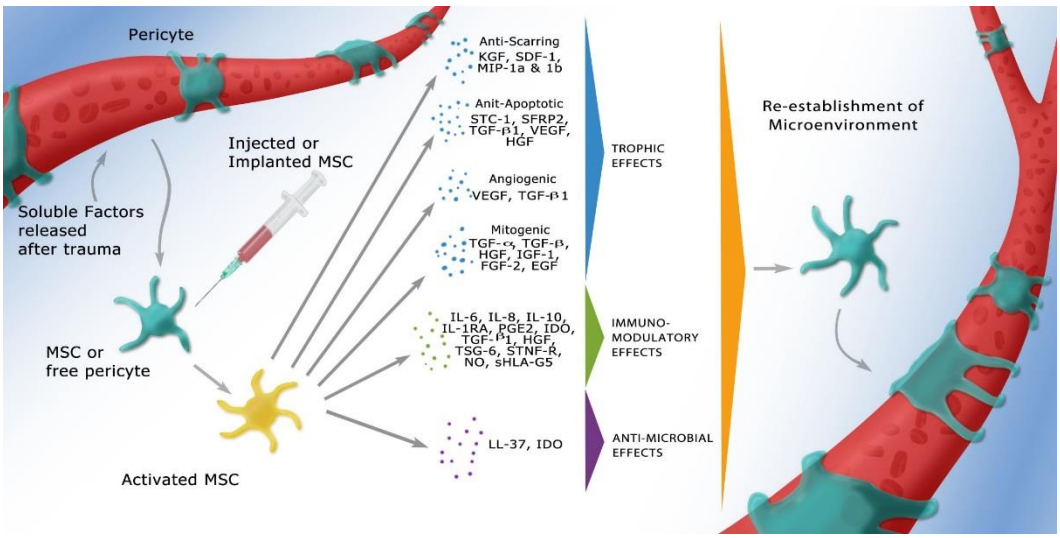

Figure 1: "Murphy MB, Moncivais K, Caplan AI. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. Exp Mol Med. 2013 Nov 15;45:e54"

MSCs as a therapeutic agent

Despite the natural ability of a tissue to heal spontaneously through different mechanisms, including the one just mentioned, sometimes an impairment can occur during one or more of the three phases of the healing process. For example, in case of a severe damage, the tissue is not able to completely regenerate itself. Similarly, the poor vascularization of certain tissue such as tendon, cartilage and ligament leads to an insufficient supply of nutrients, oxygen and especially of reparative cells, including pericytes/MSCs. In other cases, the prolonged inflammation, peculiar of chronic disorders, may arise from an inappropriate balance of the immunomodulatory mediators, thus leading to an overproduction of MPPs and catalytic species. The resolution key of these conditions has not been clarified so far, and innovative approaches might be considered to re-establish the balance between pro- and anti-inflammatory factors.

In this view, the necessity to identify new treatments for these kinds of conditions, especially for those that exert a deep impact in the social life of affected people, had led to focus more deeply on MSCs. Many aspects have been identified and carefully investigated, starting from the MSCs multi-differentiation and clonogenic ability, up to the more recent findings about their immunomodulatory activity and their great sensitivity to the local microenvironment.

Thanks to the huge amount of in *vitro* and pre-clinical data collected over the last years *(De Bari C et al, 2001; ZuK PA et al, 2001; Bi Y et al, 2007; de Girolamo L et al, 2013; Kobolak J et al, 2016; Yun JW et al, 2016)*, nowadays the use of MSCs have been tested in clinical trials to treat several conditions including but not limited to cardiovascular diseases, blood diseases, neurological disorders, graft versus host disease (GVHD) following bone marrow transplantation, autoimmune diseases, liver diseases, skin injuries, orthopedic injuries and disease, generally with good results. Up to today, 560 clinical trials on MSCs have been carried on most of them are still ongoing (*www.clinicaltrials.gov*).

Among them, many are in the orthopedic field, where the MSCs potential seem to be particularly promising for the treatment of conditions such as chondral lesion *(Deng Z et al, 2016),* osteoarthritis *(de Girolamo L et al, 2016)*, tendinopathy *(Gaspar D et al, 2014),* intervertebral disc disease *(Richardson SM et al, 2015)* and bone non-union/delayed fractures *(Stanovici J et al, 2016).*

For several decades, bone marrow has represented the primary source of MSCs (BMSC, bone marrow stem cells), in particular in orthopaedic applications due to the homology between donor and recipient site. However, it must be considered that in bone marrow aspirate only 0.01% - 0.0001% of nucleated cells are identified as BMSCs and that this subpopulation is strongly affected by ageing, often showing a progressive considerable

decrease of both BMSC number and functionality at advanced ages *(Chamberlain G et al, 2007; Caplan AI 2007).*

Driven by the need to identify alternative MSCs sources to bone marrow and aware of the currently accepted thesis that MSCs are strictly correlated with the presence of blood vessels, adipose tissue, that is provided of a consistent vasculature, has been progressively recognized as a plentiful source of these cells that can be easily collected in abundance with a lower invasiveness for the patient and fewer age-related restrictions. Thanks to these properties, adipose-derived stem cells (ASCs) currently represent together with BMSCs the most used MSCs in a variety of clinical applications. Subcutaneous adipose tissue is rich in mature adipocytes (67.6%), but it is also composed of blood vessels, leukocytes, fibroblasts, macrophages and pre-adipocytes, identified as Stromal Vascular Fraction (SVF*) (Pettersson P et al, 1984; Tsuji W et al, 2014; Peinado JR et al, 2012; Lin CS et al, 2010).* The SVF composition has been deeply studied and has revealed the presence of heterogeneous cell subpopulations: blood derived cells (CD45+), endothelial (progenitor) cells (CD31+, CD34+, CD45-, CD90+, CD105low CD146+), adipose derived stem cells (CD31-, CD34+, CD45-, CD90+, CD105-, CD146-) and pericytes (CD31-, CD34-, CD45-, CD90+, CD105-, CD146+) *(Yoshimura K et al, 2006).*

Cell populations with similar features of BMSCs and ASCs have been identified in several tissues and organs such as skin, muscle, tendon, bone, brain, liver, kidneys, lungs, spleen pancreas thymus, synovial membrane, and umbilical cord *(Meirelles Lda S et al, 2009).* However, with the exception of fetal annexes that can be an alternative smart source for allogeneic cell therapy *(Dziadosz M et al, 2016; Tsuda H et al, 2014)*, the other tissues do not represent feasible sources for MSCs collection, especially in view of a clinical translatability.

Even if the past ten years have seen thousands of studies aimed to improve the knowledge about the mechanisms of MSCs function during the reparative process of injured organs and tissues, the way how MSCs effectively act in the body and the way how we can exploit their potential in treating some conditions still represents one of the most confusing and controversial issue in cell biology.

The use of MSCs as a therapeutic agent and the modalities of action such as the way of release/activation of MSCs are deeply influenced by the specific final therapeutic target. In all the MSCs applications, the first focus is their engraftment at the target site that can be long lasting or just temporary.

In the context of applications in which MSCs are requested in specific locations, such as in focal musculoskeletal injury like chondral defect or fracture site, their administration by the combination with a vehicle or a carrier is preferable *(Kurtz A 2008; Eggenhofer E et al, 2012*). In this regard, in the 1993 arose the concept of tissue engineering that is an interdisciplinary field that combine the principles of engineering with those of biological sciences, in order

to obtain biological substitutes able to restore, maintain and improve the tissue properties *(Langer R and Vacanti JP, 1993).*

In tissue engineering, the traditional idea about the usefulness of MSCs related to their multi-differentiation potential still represents the key concept. Together with a suitable cell source, a decisive role is fulfilled by bioactive, biodegradable and biocompatible tridimensional scaffolds that aim to replicate the principal features of tissue specific extracellular matrix *(Anderson HJ et al, 2016).* During the last decades the identification of the best scaffold for a given applications has involved a very large number of researchers and many studies are still ongoing *(Howard D et al, 2008; Dhaliwal A et al, 2016; Zhou X et al, 2016)*.

Today the most common scaffolds are generally made of natural polymers such as collagen and alginate or synthetic such as polyglycolic acid (PGA), polylactic acid (PLA), and polyethylene glycol (PEG) and have to be chosen accordingly to the target tissue to replace *(Koh and Atala, 2004; Murphy and Atala, 2013).* The importance of scaffold is not only related to their ability to temporarily provide a structural support while cells attempt to rebuild tissue matrix, but also to provide an anchorage site to cells and therefore to support their attachment and proliferation, avoiding apoptotic events *(Dalby MJ et al, 2014).* However, despite the positive outcomes often reached using tissue engineering approaches, these applications necessarily require an extensive cell manipulation, thus falling within the competences of ATMPs regulations (Advanced Therapy Medicinal Products) that make the concrete application of MSCs on a large scale very hard.

To at least partially overcome such limitations, alternative ways to use minimally manipulated MSCs have been developed. For example, the standard procedure to obtain ASCs from adipose tissue is based on an enzymatic digestion of the tissue by collagenase enzyme to separate the SVF from the other predominant cell component *(Peinado JR et al, 2012*) and on the purification of ASCs via plastic adherence *(Zuk PA et al, 2001).* To avoid all these passages, the use of whole fresh SVF have been proposed *(Faustini M et al 2010; Michalek J et al, 2015; Rodriguez JP et al, 2012).* To further support this approach, it has been proposed that the implantation of the whole SVF could be more beneficial than purified ASCs. Indeed, the SVF transplantation can be considered as the transplantation of the complete microenvironment of the stem cell niche and this can represents a more powerful and a safer therapeutic agent in comparison to the use of only the in *vitro* selected ASCs *(Bajek A et al, 2016),* since it has been demonstrated that the interaction of stem cells with other cellular components of their niche is critical for self-renewal and the maintenance of an active stem cell pool and for the determination of their differentiation fate *(Caplan AI 2015; Oh M et al, 2015).* So, in this view the therapeutic approach aimed to maintain the stem cell niche has claimed to be important not only to minimize the cell manipulation but also to achieve better results.

The more recent findings about the anti-inflammatory immunomodulatory effect resulting from paracrine activity of MSCs support the hypothesis that MSCs can also be used in wider sites of disorder, such as osteoarthritis, that involve the whole joint, or tendinopathy, that usually affect a large portion of tendon. In these cases, the key role of MSCs is not their stable engraftment at the lesion site and direct participation to re-build the target portion of tissue, but rather their ability to reach the site and start an intense crosstalk with resident cells through the paracrine mechanism previously reported, in the attempt to restore a physiological tissue homeostasis. In the clinical setting of osteoarthritis, intraarticular injections of cell concentrates offer great advantages by allowing cells within joint space to target the injured tissues through interaction with recipient cells and surfaces eventually leading to positive outcomes. In addition, as already mentioned, such approach is minimally invasive, is cost-effective, and has a better patient compliance *(Murphy MB et al, 2013).*
Pioneering studies have explored the systemic administration of MSCs, mainly by intravenous injection in a peripheral vein, using the ability of MSCs to spontaneously migrate into the site of inflammation and to reach sites such as cartilage, lung and liver *(Wei X et al, 2013; De Becker A and Riet IV, 2016; Sakaida I et al, 2004; Zhu XY et al, 2013)* and intra-arterial administration of MSCs, that is characterized by a better permanence and by an increased accumulation of cells into the target site but a high rate of invasiveness *(Mohamadnejad M et al, 2007).* However, further studies are required to assess the safety of these approaches.

MSCs as a therapeutic target

Not all pericytes are potential MSCs and MSCs/pericytes present in perivascular district of each vascularized tissue do not strictly correspond to tissue specific progenitor cells present in each tissue and that are responsible of maintenance of tissue homeostasis. Indeed, tissue specific progenitor cells are defined as a potentially heterogeneous population of functionally undifferentiated cells, capable of homing to an appropriate growth environment, proliferation, production of a large number of differentiated progeny, self-renewing or self-maintaining their population and regenerating the functional tissue after injury *(Loeffler M and Roeder I; 2002).*
Therefore, with specific reference to mesodermal tissues it is not correct to identify a unique class of MSCs but it could be more suitable to consider the general concept of MSCs as a heterogeneous cell type, with different developmental origin and with peculiar tissue-specific progenitor features *(Sacchetti B et al, 2016).*
In this view, the possibility to stimulate resident MSCs in order to improve physiological tissue healing appears a promising approach, although very few studies are reported in literature on this topic up to date. However, this idea is supported by several findings

collected in pathological conditions demonstrating that MSCs, exposed to damage associated molecular pattern (DAMP) or pathogen associated molecular pattern (PAMP), could react by reverting the production of pro-inflammatory to anti-inflammatory molecules *(Hengartner NE et al, 2015; Li D et al, 2016)* and by inducing the switch from pro-inflammatory M1 macrophages maturation to the anti-inflammatory M2 phenotype *(Qi Y et al, 2014).* These observations open the field to the hypothesis that an enhancement of MSCs potential would benefit the healing of many conditions, including those affecting musculoskeletal tissues, representing a less-invasive and cost-effective therapeutic strategy. Their plasticity and ability to response to external stimuli, both physical and chemical, make MSCs a very good target for different treatments intended to improve tissue regeneration.

In particular, the recent advancements in the knowledge about the mechanism of biophysical stimulations, such as shock waves and pulsed electromagnetic fields, support the hypothesis that they would be effective in the stimulation of endogenous mesenchymal progenitor cells activity.

However, since recent findings have confirmed that MSCs are able to actively interact with the components of the innate immune system and throughout these connections they can exert a role of both anti-inflammatory and pro-inflammatory *(Bernardo ME et al, 2013; Keating A 2012; Le Blanc K and Mougiakakos D, 2012; Prockop DJ and Oh JY, 2012)*, further studies are needed to clarify the best stimulation approach or combination of stimulations to find the perfect balance for each given condition to treat.

Rationale of MSCs-based approach for degenerative musculoskeletal pathologies

Degenerative musculoskeletal pathologies are characterized by the presence of two main components, inflammation and tissue degeneration. The timing and mechanism onsets of both phases are still not completely understood. Therefore, despite the high incidence on worldwide populations of degenerative musculoskeletal disorders, such as osteoarthritis (OA) and tendinopathy, the current conservative pharmacological treatments are not able to guarantee a complete restoration of tissue homeostasis, since they do not overcome the proper causes of the pathology but they are more specifely aimed to symptoms relief. Hence, in the majority of the cases the spontaneous reparative tissue is mainly constituted by scarring tissue lacking of the peculiar physiological native tissue properties.

Osteoarthritis (OA) is a pathology involving the whole joint and it affects differet tissues such as cartilage, synovium and subchondral bone at later stages. The etiology of this pathology can be attributed to many different factors, such as age, body mass index (BMI), gender, mechanical loading, excessive physical activity, and nutrition *(Yucesoy B et al,*

2015). This pathology can be classified as primary or secondary, due to the variable nature of these factors. OA derived from genetic predisposition and biochemical profile are considered as primary whereas the one originated from traumatic events, chronic inflammatory conditions and metabolic disorders *(Kapoor M et al, 2011*) is named as secondary OA. In both cases, the syntomps are pain, joint stiffness and loss of function.
The principal events characterizing the progression of OA are the presence of whole joint inflammation and consequent cartilage degeneration. Indeed, the chronic inflammatory condition increases the production of Matrix Metalloproteinases (MMPs), with the subsequent reduction of cartilage extracellular matrix (ECM) *(Goldring MB and Marcu KB, 2009; Mort JS et al, 2016; Troeberg L and Nagase H, 2012).* The re-activation of chondrocytes metabolic activity can lead to a not balanced homeostasis with the presence of cellular hypertrophy and calcification process *(Dreier R, 2010).* The presence of an inflammatory microenviroment entails the activation of immune cells at the synovia with the consequent production of further inflammatory agents, such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) *(Bondeson J et al, 2006; Ishii H et al, 2002).* These mediators induce the production of secondary inflammatory mediators such as cyclooxygenases (COX-1 and COX-2), prostaglandins (PGE2) and reactive oxygen species (ROS) that could increase the presence of apoptotic events in chondrocytes *(Reed KN et al, 2014; Sokolove J and Lepus CM, 2013; Wang P et al, 2013; Intekhab-Alam NY et al, 2013).*
Current therapies comprise conservative and surgical approaches. Pharmacological administration of non-steroidal anti-inflammatory drugs (NSAID) is the most popular conservative treatment, but they are not effective in the reduction of tissue degeneration causing also several side effects after prolonged administrations *(Lanas A et al, 2011; Mandegaran R et al, 2013)*. For the most severe OA grade, joint arthroplasty is the only effective radical solution. Less invasive procedures aimed at least to reduce the degeneration of early OA have been proposed too, with no completely comprehensive outcomes though.
Similarly, the typical degenerative process that accompanies the progress of tendinopathy is caused by both endogenous (BMI, abnormal weight bearing pattern or joint laxity) and exogenous factors (trauma and overuse). They may cause a primary tissue damage that could be not efficiently overwhelmed by resident cells, thus causing the production of weaker and low quality scar tissue. In general the term “tendinopathy” represents a specific clinical condition characterized by tissue degeneration and inflitration of immune cells and inflammatory mediators *(Rees JD et al, 2014),* whose severity ranges from transient pain and inflammation to chronic conditions leading to tears or, in the most severe cases, to total tendon ruptures. Tendons are generally characterized by a poor self healing capacity, resulting from a scarce blood supply and low cellularity. The first events that occur after a trauma is the recruitment of immune cells and fibroblasts *(Lin TW et al, 2004)* resulting

from the secretion of cytokines (IL-1β) and the subsequent neovascularization process promoted by vascular endothelial growth factor (VEGF). This condition leads to the development of a fibrous tissue *(Evans CH, 1999),* with altered mechanical and physiological properties. The replacement of this fibrous tissue with the physiological one represents the last stage of tendon healing and consists in the substitution of collagen type III with collagen type I *(Docheva D et al, 2015*). However, repetitive traumas or patient own characteristics can obstacle this process *(Lin TW et al, 2004; Voleti PB et al, 2012; Yang G et al, 2013)* and impede the normal tissue regeneration in favor of the establishment of a chronic disease.
The administration of NSAIDs are most used therapeutic strategy, especially for pain relief, but their efficiency to overcome the causes of the pathology is not adeguate *(Wang JC and Shapiro MS, 1997; Vogel HG, 1977; Carlstedt CA et al, 1987; Forslund C et al, 2003).*
Similarly, since the prolonged administration of corticosteroids provoked a wide range of side effects, other therapeutic strategies should be preffered *(Speed CA, 2001).*
Further conservative approaches have been developed, comprising eccentric exercise, cryotherapy and biophysical stimulations. Even if all of the mentioned above treatments are able to ameliorate the patient's clinical condition, in several cases the complete rupture of the tissue cannot be avoided and in these cases the surgical intervention becomes mandatory.
It is therefore clear how also in this setting the possibility to use a conservative treatment able to restore tissue homeostasis and prevent the pathological degeneration would be of great impact on the social and economic burden represented by tendon disorders *(Docheva D, 2015).*

In both OA and tendinopathy scenario, the opportunity to set up an innovative conservative treatment, directly aimed to contrast the tissue degeneration in early OA, opens the field to the use of MSCs as a therapeutic agent and to exploit their great potential. In particular, the dynamic response performed by MSCs during tissue healing MSCs could provide an excellent solution to scar formation, by secreting another spectrum of molecules with antiscarring properties and then stimualating the tissue regeneration process *(Caplan and Dennis, 2006).*

Regulatory aspects of cell therapy

As mentioned above, the great interest of the scientific community on MSCs must be connected to their potential to open new perspectives in the treatment of chronic diseases in favor of the establishment of a regenerative mechanism. If a cell is considered as a drug, this drug automatically represents the most complex existing biopharmaceuticals *(Feyen DA et al, 2016).* In fact, a cell is a much more complex system than a protein or a nucleic

acid and the critical point is that a cell is a dynamic system with multi-dimensional therapeutic potential able to actively interact with the surrounding microenvironment but also to be deeply influenced by it. Hence, the great challenge is that the therapeutic activity of cell after transplantation into a tissue can be completely different from the one observed in *vitro* studies.

The use of stem cells as therapeutic drugs could exert an enormous social impact as they could be used in diseases such as Parkinson's disease, multiple sclerosis, heart disease, liver disease, spinal cord damage, cancer and others. However, the lacking understanding of the complexity of stem cell products and the still evolving acknowledgment about the basic mechanism of their in *vivo* activity, reveal a difficult challenge that limit the concrete application of these products, especially in the treatment of those pathologies for which the pathophysiology is still not completely determined *(Nagpal A et al, 2016).*

In the last years, the lack of a well-framed regulatory framework regarding this field had led to a slowing down in the development of regenerative medicine products, accompanied by continuous demands for further feedbacks *(Johnson PC et al, 2014).*

Currently, both the United States (US) and European Union (EU) are working in order to delimitate the regulatory guidelines in this field. Up to today, it is still difficult to have the complete control of the safety, the efficacy and the quality of cell-based therapy products, thus leading to an unavoidable slowing down for what concerns their commercial use.

In Europe, the passage from the basic science toward the development of cell-therapy product is governed by several regulations. First of all the *European Directive 2001/83/EC* and the *Directive 2009/120/EC amending Directive 2001/83/EC* furnished a definition of somatic cell therapy products that must be considered as an Advanced Therapy Medicinal Product (ATMP) with a peculiar regulatory framework.

The European regulatory recognizes that the pharmacology and the toxicological assessment of cell-based products must unavoidably have different pathways in the regard of traditional drugs. The definition of ATMPs, that include the cell-therapy products, has been provided in the *Regulation (EC) No 1394/2007* and has been immediately applied in all Member States of the European Parliament and of the council, strongly binding the applications in this context. Under these regulatory directives, cell populations aimed for cell therapy approaches must be considered ATMPs, when they are subjected to substantial manipulation and/or when they are destined for non-homologous use, thus meaning that the administration of minimally manipulated cells is not possible if they are not normally present in the target site or if they have to perform a function that is not the one they normally have. Despite the numerous efforts to better define the regulatory framework of the cell-based therapy and ATMPs, up to today the knowledge in this context is still weak.

Also in the United States all cell-based therapies and their relative products are firmly defined by severe regulations imposed by the Food and Drugs Administration (FDA) *(CFR-*

Code of Federal Regulations Title 21, Part 1271). Within this set of regulations, human cells, tissues, or cellular or tissue-based products (HCT/Ps) are defined as an article "containing or consisting of human cells or tissues that are intended for implantation, transplantation, infusion, or transfer into a human recipient" *(Code Federal Regulations Title 21, 1271.3).*
Also in this case, the developmental process of cell-therapy products are strictly related to the rate of cell manipulation and the homologous or non-homologous use of the product. Indeed, the remarkable manipulation and the extensive handling of tissues and cells which this therapeutic approach is based on, still cause several worries and hesitations in the scientific community and in FDA administrators too. For this reason, also in this regulatory framework considerable efforts will be needed to validate the process in order to permit a tangible future in the clinical practice.
Therefore, waiting for new regulatory pathways that evolve with new technologies and that facilitate the generation of new regenerative therapies that can help the patients and maintain their safety, it will be important going forward with the researches, contributing to increase the universal knowledge about these innovative approaches in the regenerative medicine field. An evident existing link between scientific research and regulatory system implies a continuous exchange of informations and the need for a deep collaboration between the two systems, for a better achievement in term of future commercialization of these products.

Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use (Consolided version: 16/11/2012).

European Commission, Regulation (EC) No 1394/2007 of the European Parliament and of the council of 13 November 2007 on advanced therapy medicinal products and amending directive 2001/83/EC and regulation (EC) no 726/2004. Off. J. Eur. Union. L 324: 2007, p. 121–137

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Food and Drug Administration, CFR-Code of Federal Regulations Title 21, Part 1271: Human Cells, Tissues, and Cellular and Tissue-Based Products, US Food & Drug Administration, 2014

Aim of the research

The above-mentioned scenario attempts to give an overview of the current state of knowledge regarding MSCs. Facing with the enormous potential of MSCs and the likewise impressive necessity of a deeper understanding, this PhD path has been directed to achieve a deeper awareness of MSCs potential and of their possible therapeutic uses in skeletal disorders.

In this context, the main purpose was to evaluate the specific features of MSCs strengthening the concept that a cell can be actually seen and used as a drug, with an additonal peculiar dynamic activity. All these researches were aimed to further consolidate the clinical large-scale employment of MSCs for the treatment of those pathologies that up to today are treated with not very satisfactory results.

In this view, this study was addressed to investigate one of the most representative degenerative pathology in the orthopaedic field that up to today still represents a challenging issue: the tendinopathy. Indeed, despite the high incidence of this disorder among population, the mechanisms and the development of tendinopathy are still not completely understood, thus implying the need of further investigations for the subsequent obtainement of innovative and more effective treatments. To fill this gap, an *in vivo* animal model was developed to finely understand the progression of the pathology and to better exploit the possible ways of intervention of MSCs in the treatment of it (**Aim 1**).

Hence, multiple therapeutic options based on the use of MSCs that could be relevant in the treatment of tendinopathy were evaluated (**Aim 2**)**,** firstly studying their potential in a tissue engineering view considering their direct participation to tissue regeneration (i.e. differentiation into tissue-specific cells). In this view, a wide in *vitro* characterization focused on the multidifferentiation capacity of MSCs isolated from different sources, including tendons, was performed. In addition, two preliminary studies aimed to do the groundwork for alternative therapeutic approaches were carried on: one focused on the potential of MSCs paracrine activity to recreate a regenerative microenvironment, the other investigated the intrinsic tissue regenerative capacity to improve the healing process stimulating the resident MSCs and tissue-specific progenitor cells.

Finally, a broad study regarding the delivery of MSCs at the target site was performed. In particular, the study aimed to develop a suitable cell delivery system for the local administration of MSCs in degenerative musculoskeletal diseases, allowing the maintenance of their peculiar features and properties (**Aim 3**)**.**

Chapter 1

Development of an *in vivo* model of tissue degeneration: the tendinopathy

Aim 1

A deeper awareness about the possible therapeutic role exerted by MSCs in the treatment of tissue degeneration can be better reached if the cause of the degenerative process and the ways of pathology progression are known. Due to its poor healing activity, caused by the weak vascularization and the poor cellularity, tendon represent a good model to investigate the dual therapeutic role of MSCs, either the endogenous stimulation of the tendon resident ones or their local delivery at the lesion site. However, since the literature lacked of comprehensive findings about the different phases of tendinopathy and timing of tendon healing, we developed a reproducible pre-clinical model of Achilles tendinopathy in rat (Chapter 1, Manuscript 1, Perucca Orfei C et al, 2016, published on Plos One), which allowed to increase our knowledge in term of disease progression and tissue spontaneous healing (inflammation, proliferation and remodeling).

The rationale of this study relied on the possibility to predict the possible best timing and way to use MSCs-based therapies and to set up the following research steps aimed to verify these observations.

Manuscript

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RESEARCH ARTICLE

Dose-Related and Time-Dependent Development of Collagenase-Induced Tendinopathy in Rats

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Dose-related and Time-dependent Development of Collagenase-induced Tendinopathy in Rats

A Model of Tendinopathy Progression

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ABSTRACT

Tendinopathy is a big burden in clinics and it represents 45% of musculoskeletal lesions. Despite the relevant social impact, both pathogenesis and development of the tendinopathy are still under-investigated, thus limiting the therapeutic advancement in this field. The purpose of this study was to evaluate the dose-dependent and time-related tissue-level changes occurring in a collagenase-induced tendinopathy in rat Achilles tendons, in order to establish a standardized model for future pre-clinical studies. With this purpose, 40 Sprague Dawley rats were randomly divided into two groups, treated by injecting collagenase type I within the Achilles tendon at 1 mg/mL (low dose) or 3 mg/mL (high dose). Tendon explants were histologically evaluated at 3, 7, 15, 30 and 45 days. Our results revealed that both the collagenase doses induced a disorganization of collagen fibers and increased the number of rounded resident cells. In particular, the high dose treatment determined a greater neovascularization and fatty degeneration with respect to the lower dose. These changes

were found to be time-dependent and to resemble the features of human tendinopathy. Indeed, in our series, the acute phase occurred from day 3 to day 15, and then progressed towards the proliferative phase from day 30 to day 45 displaying a degenerative appearance associated with a very precocious and mild remodeling process. The model represents a good balance between similarity with histological features of human tendinopathy and feasibility, in terms of tendon size to create lesions and costs when compared to other animal models. Moreover, this model could contribute to improve the knowledge in this field, and it could be useful to properly design further pre-clinical studies to test innovative treatments for tendinopathy.

INTRODUCTION

Tendinopathy is a big burden in clinics and it represents 45% of musculoskeletal lesions [1]. In particular, athletes and middle-aged people are frequently affected by tendinopathy of Achilles, patellar and supraspinatus tendons. The severity of tendon injuries ranges from transient pain and inflammation to chronic conditions leading to tears or total tendon ruptures [1, 2]. The poor healing capability of damaged tendons is related to their scarce blood supply and the compromised metabolic activity of resident cells [3] that determine an impaired tissue homeostasis [4]. The histopathological appearance of injured tendons shows collagen degeneration and fiber disorganization, increased vascularization and increased presence of resident cells, tissue metaplasia, and occasionally formation of fatty and bony deposits [5, 6]. However, despite its clinical significance, the pathogenesis and development of the tendinopathy are still under-investigated, thus limiting the therapeutic progress in this field. In fact, the current conservative treatments are still mainly symptomatic, whereas surgical approaches have a poor success rate and require a long recovery time [7].

In this contest, a better knowledge of tendinopathy progression throughout its phases could be reached through the development of an efficient in vivo model focused on the choice of the most effective dose of collagenase at an exact temporal window to induce the acute phase of the disease. Despite an ideal animal model able to reproduce all aspects of human tendinopathy has not been identified so far, rat represents the most popular species to model the Achilles tendinopathy, thanks to its suitable size for surgical approaches and tissue retrieval, and its easy handling. Moreover, the rat model of Achilles tendinopathy has been extensively used in preclinical research, because of the similar conditions [8, 9] and the genetic homology to humans [10]. The most common techniques to develop rodent models of tendinopathy are based on mechanical overuse or chemical factors, such as collagenase, corticosteroids, cytokines (TGF-β1) and substance P [8, 9]. However, the mechanical overuse model is not completely accepted due to its scarce reproducibility and to the role of inflammation that does not equate to tendinopathy [8, 11]. Among the chemical-induced tendinopathy models, it has been shown that collagenase type I can provoke collagen fiber disruption and changes in biochemical and biomechanical features of the tendon, better resembling the main histopathological characteristics and functional impairments of human tendinopathy [8, 12, 13]. Thus, this injection model can represent a valid approach to induce and study the development of this pathology [14]. However, despite collagenase seems to be the most interesting agent to generate a consistent and reproducible model of tendinopathy, to date, standardized

protocols have not been defined yet. Indeed, there is no agreement on which is the concentration, volume and site of injection, and time of occurrence of collagenase-induced lesions [13, 15-20].
The purpose of this study was to evaluate the cellular and tissue-level changes occurring in a collagenase-induced Achilles tendinopathy in rats at different time points by using two collagenase concentrations. In particular, we want to accurately investigate the development of the disease throughout its phases, in order to establish a standardized model for future pre-clinical studies that resembles as closely as possible the human pathology.

MATERIALS AND METHODS

Ethics Statement

The Mario Negri Institute for Pharmacological Research (IRFMN) Animal Care and Use Committee (IACUC) approved the study (Permit N. 06/2014-PR). The IRFMN adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2008 – Reg. N° 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01). The animals were regularly checked by a certified veterinarian responsible for health monitoring, animal welfare supervision, experimental protocols and procedure revision. All surgeries were performed under general anesthesia, and all efforts were made to minimize suffering.

Study design

Forty 12-weeks-old male Sprague Dawley rats (Rattus norvegicus) (Envigo, Huntingdon, UK) (mean body weight 347 ± 9 g) were used in this study. In the absence of suitable data to perform the power analysis, the sample size was calculated according to the Mead's resource equation (E=N-T, 10<E<20). Thus, considering two treated limbs per animal for a total of 16 treated tendons per time-point, the sample size was calculated as follows: E=(16-1)-(4-1)=12. The rats were randomly divided into two treated groups that were injected within the Achilles tendon with collagenase type I (collagenase from Clostridium histolyticum, Worthington Lakewood, NJ, USA, 185 IU/mg): 1) collagenase 1 mg/mL defined as low dose (LD); 2) collagenase 3 mg/mL defined as high dose (HD). In each group, the contralateral tendon was treated either with phosphate buffered saline (PBS) and served as control (CTRL), or left untreated and served the sham control (S-CTRL). For each time point, 4 tendons were injected with LD, 4 tendons with HD, 4 tendons received PBS and 4 tendons were left untreated (n=4). Tendon explants were analyzed at 3, 7, 15, 30 and 45 days.

In vivo procedures

The rats were anesthetized via inhalation of isoflurane (3%; Merial, Duluth, Georgia, USA) and maintained with an intraperitoneal injection of ketamine hydrochloride (80mg/kg; Imalgene, Merial, Milan, Italy) and medetomidine hydrochloride (1mg/kg; Domitor, Pfizer, New York City, NY, USA). All animals also received a preoperative intramuscular single injection of cefazolin (30mg/kg; Cefamezin, Teva, Petah Tikva, Israel). Using aseptic technique, after shaving and disinfection, a longitudinal 0.5 cm skin incision was performed through a medial approach to expose by blunt dissection the central region of the Achilles tendon. Then, all animals were injected with LD or HD of type I collagenase dissolved in 30 µL of PBS by means of a 30G needle into the central portion of the tendon, and with PBS into the contralateral tendon as controls (Fig 1). Finally, the skin was sutured by separated stitches with Prolene 4/0 (Johnson & Johnson, New Brunswick, NJ, USA). Atipamezole (1mg/kg; Antisedan, Pfizer, New York City, NY, USA) was administered subcutaneously to recover the animals from general anesthesia. After 3, 7, 15, 30 and 45 days, the rats were euthanized by CO2 inhalation to harvest the Achilles tendons for the histological investigations.

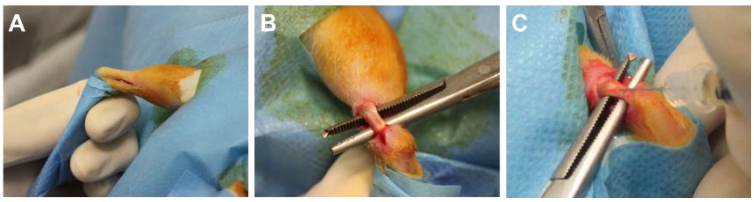


Figure 1. *Surgical approach. A) Longitudinal incision of the skin in the medial portion of the hind limb. B) Achilles tendon exposure by blunt dissection. C) Injection of collagenase type I within the Achilles tendon structure.*

Histological analysis

Tendon specimens were fixed in 10% formalin for 24h. Then, they were dehydrated in alcohol scale before embedding in paraffin and cutting into 5 µm longitudinal sections. The slides were stained with haematoxylin and eosin (H&E) to evaluate the tendon morphology of collagenase-treated groups compared to tendons treated with PBS (CTRL) or untreated native tendons (S-CTRL). Photomicrographs of the tissue were captured through an Olympus IX71 light microscope and an Olympus XC10 camera (Japan). Four sections of each sample were randomly selected and evaluated by two blinded observers to assess the tendon morphology according to a modified semi-quantitative grading score from 0 to 3 proposed by others [21, 22] (see S1 Table). The score analyzed the fiber structure and arrangement, resident cell density and appearance, infiltration of inflammatory cells, neovascularization and fatty deposits. According to this grading system, a perfectly normal tendon obtained score 0, whereas a score of 18 was assigned to maximally abnormal tendons.

Statistical analysis

Comparisons between groups and time points were analyzed by the Mann-Whitney U test (GraphPad Prism v5.00 Software, La Jolla, CA, USA). All data are expressed as means ± standard deviation (SD). Values of $p<0.05$ were considered statistically significant. The interrater reliability of the examiners' scores for each technique was calculated with intraclass correlation coefficient (ICC): ICC = 1, perfect reliability; ICC > 0.75, excellent reliability [23].

RESULTS

Histopathological findings

The S-CTRL tendons showed a uniform appearance of compact, well-aligned collagen fibers with a normal presence of spindle-shaped tenocytes disposed parallel to the fiber pattern, and, as expected, no degenerative events were observed during the whole study period (Fig 2).
Differently, at day 3, the CTRL group exhibited a loss of fiber organization that also appeared partially fragmented along the route of the needle injection (Fig 3A). However, a complete recovery of the tendon structure was spontaneously achieved starting from day 7 (Fig 3D) and maintained until the last time point (day 45; Fig 3A).
The LD-treated tendons showed a mild degeneration throughout the time points. At day 3, the LD-treated tendons showed an abnormal presence of fatty deposits associated with the loss of fiber organization (Fig 3B, black arrows) disseminated with an increased number of slightly rounded resident cells (Fig 3B, white arrow). At later time points, the presence of a high number of rounded cells was the most distinctive feature of degeneration in these samples, by the way decreasing progressively until day 45 (Fig 3E, H, K, N).
Three days after the collagenase injection, the HD-treated tendons exhibited a moderate to marked collagen matrix disorganization with a great increase of cell density consisting mostly in rounded cells (Fig 3C). Moreover, a marked neoangiogenesis associated with the presence of several inflammatory cells was found (Fig 3C, white arrow). At days 7 and 15, the HD-treated tendons showed a complete fiber disorganization, in which the pattern was no more identifiable and the increased resident cells showed a rounded morphology (Fig 3F, I). Particularly on day 7, a marked increase in vascularity was detected related to the presence of several inflammatory cells (Fig 3F, asterisk). Furthermore, at day 15, a substantial presence of lipid vacuoles was found (Fig 3I, black arrow). Thirty days after the HD collagenase injection, the collagen fibers appeared disseminated of rounded and proliferative cells towards a new connective tissue deposed within the disrupted collagen fibers (Fig 3L). From day 30 to day 45, the collagen structure was resized and reshaped in a parallel organization, and a decreased cell number was detected at 45 days (Fig 3O).
In general, comparing the two treated groups, the HD collagenase induced a progressive degeneration of the tendon tissue, with a peak around day 15, while the injection of LD collagenase exerted its effect earlier (day 3) with no further increase of the pathological aspects during the following time points. Moreover, the LD treatment produced a mild degeneration with respect to the HD, particularly in terms of angiogenesis, fatty infiltration and fiber disorganization. Indeed, even

if both treatments led to a spontaneous regeneration of the tissue at day 45, the effect of the treatment was still evident in the HD group, while the LD-treated tendons appeared similar to the CTRL (Fig 3N, O).

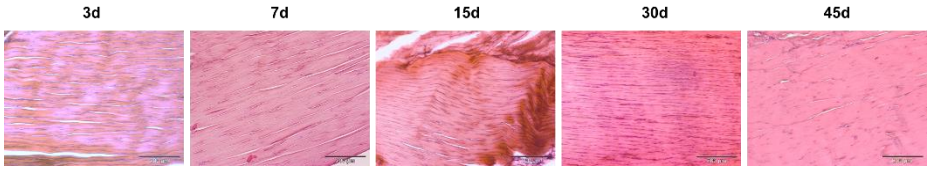


Figure 2. *Histologic appearance of S-CTRL tendons at different time points. Representative micrographs of the histopathological analysis; H&E staining. Scale bars 200 µm (10X).*

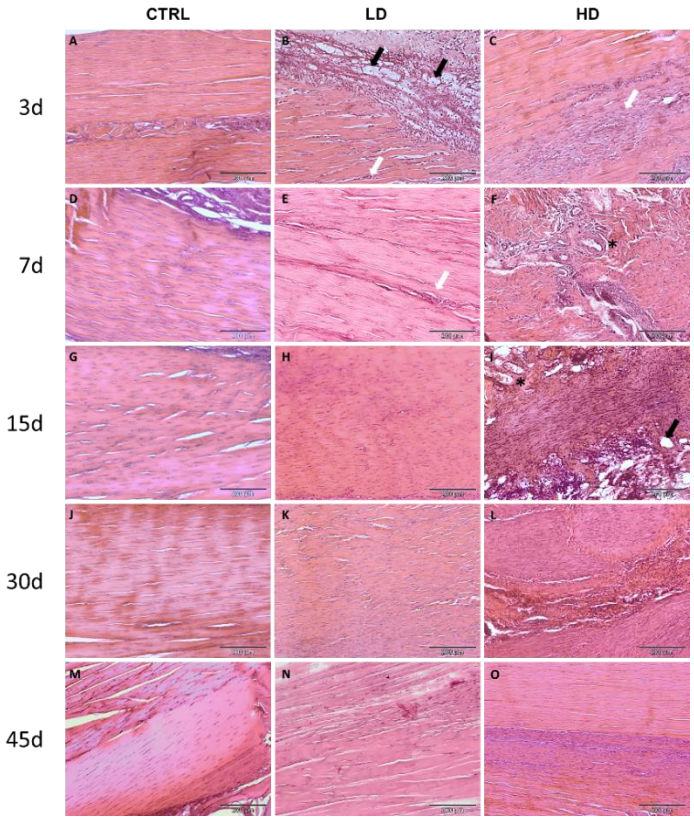


Figure 3. *Histological appearance of CTRL, LD and HD-treated tendons at different time points. Representative micrographs of the histopathological analysis, H&E staining. Black arrows indicate fatty deposits; White Arrows: representative zone with high cellularity; *: vessels. Scale bars 200 µm (10X).*

Histopathological score analysis

The interrater reliability of the scoring analysis performed by two blinded examiners was good (ICC 0.73). The total and specific histopathological scores are presented in the histograms in Fig 4A.
The injection of collagenase induced deep changes in the histological appearance of the treated tendons resulting in a significantly worse total score of the HD and LD groups compared to the CTRL group at all time-points (with the exception of LD at day 15; Fig 4A). The HD group reached the worst total score at day 15 (16.5±2.1), while the LD one reached a maximum of 9.7±0.4 at day 3. Remarkably, the total score in the HD group decrease significantly between day 3 and 45 ($p<0.05$). Both concentrations of collagenase produced a visible damage on fiber arrangement, though significant differences were just observed at day 15 in the HD group respect to CTRL ($p<0.05$) and to LD ($p<0.05$). Analyzing the temporal changes in terms of disorganization of collagen fibers, we found a progressive score worsening at least up to 15 days (1.75±0.4 at day 3, 3±0.0 at day 15, $p<0.05$), but then a subsequent spontaneous regeneration was observed (1.17±0.3 at day 45, $p<0.05$) (Fig 4B).
At day 3, cell density was increased by all the treatments with a significant increase between CTRL and HD ($p<0.05$) or LD groups ($p<0.05$) (Fig 4C). The cell density of the HD group was higher at all time-points respect to CTRL and LD treatments, with a significant increase respect to LD at day 15 ($p<0.05$). Cell morphology was also affected by the treatments. In fact, a higher number of rounded cells was observed in the LD and HD groups with respect to CTRL at all time-points (Fig 4D). In particular, at day 7, both LD and HD group showed a higher score with respect to CTRL ($p<0.05$). Moreover, the HD group showed significant increases also at day 15 and 45 compared to CTRL ($p<0.05$). Nevertheless, in the HD group, a progressive decrease of rounded cells was observed at day 45 with respect to day 3, 7 and 15 ($p<0.05$).
The infiltration of inflammatory cells was observed in all samples at day 3. By day 7, they almost disappeared in CTRL group, while they were persistent in LD and HD ones. In particular, in the LD group, a significant increase was observed with respect to CTRL at day 7 and day 30 ($p<0.05$), whereas in the HD group it was found only at day 15 ($p<0.05$) (Fig 4E).
The presence of new vessels was evident in all collagenase-treated tendons (Fig 4F), specifically, their number significantly increased in both LD and HD groups with respect to CTRL up to 15 days ($p<0.05$). In particular, HD groups showed a greater presence of vessels compared to LD group at day 15 ($p<0.05$). A similar behavior was observed in terms of fatty deposits (Fig 4G). In fact, at day 3, both doses of collagenase caused greater fatty deposits with respect to CTRL ($p<0.05$). Their increase was found at day 7 in the LD group and at day 15 in the HD group with respect to CTRL. The HD group showed higher levels of fatty deposits at all time-points, in particular, a significant difference was observed with respect to LD at day 15 ($p<0.05$). Overall, the LD-treated tendons showed a lower degenerative score with respect to the HD group. Moreover, the pathological changes in tendons treated with LD collagenase did not significantly vary during the experimental observation with any differences observed between time points neither for the total score nor for the specific features.

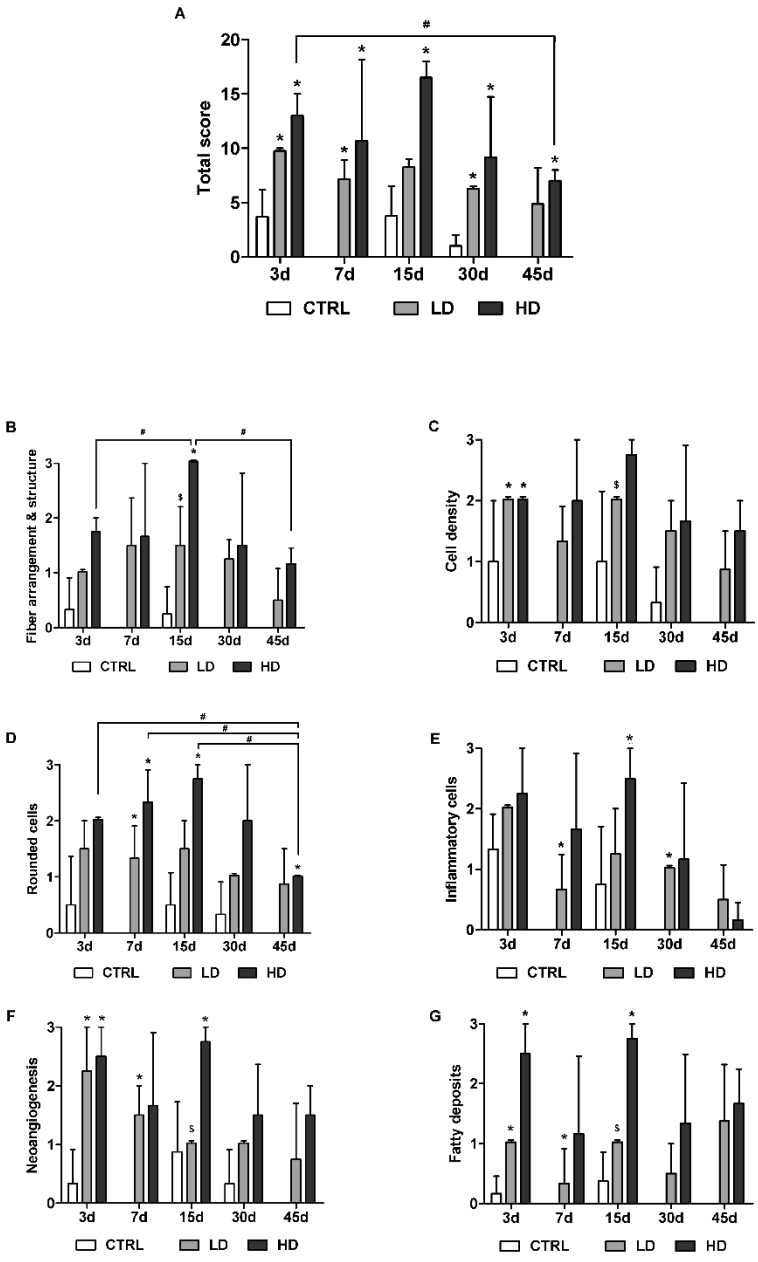


Figure 4. *Histological scores. Total (A) and specific scores for fiber arrangement (B), cell density (C) and morphology (D), presence of inflammatory cells (E), neoangiogenesis (F) and fatty deposition (G). Data are reported as mean±SE. *p<0.05 respect CTRL; $p<0.05respect to HD; #p<0.05 respect to different time point; n= 4.*

DISCUSSION

Tendinopathy is an umbrella term that refers to tendon injury with unknown etiology [24]. More precisely, tendinopathy has been defined as tendinitis when a non-rupture tendon injury is associated with a very precocious inflammatory process [25]. This process brings to mechanical-related chronic lesions, commonly known as tendinosis [25]. The lack of knowledge about the physiopathology of tendinopathy leads to misleading opinions in the presence of a host inflammatory response and development phases of this disorder. In this context, the current failure to offer a complete clinical picture of such a multiple etiology disease [14, 26] increases the need of a valid animal model able to mimic the histological features of tendinopathy in humans and to establish a standardized tool suitable for future preclinical studies.

Collagenase is considered an effective method to induce the Achilles tendinopathy in preclinical models, as widely described in the literature [14, 15, 17-21, 27-30]. However, the use of different rodent strains, different protocols in terms of amount and type of collagenase administration and follow-up time points determined a poor reproducibility of the model [13].

Aiming at standardizing a rat model of collagenase-induced tendinopathy, we carefully compared the degenerative potential of two different concentrations of collagenase type I. In particular, we provided a complete time course evaluation of collagenase-induced Achilles tendinopathy in rats, focusing on the tendon histopathology at different time points (3, 7, 15, 30 and 45 days), in order to define the most effective dose of collagenase at an exact temporal window able to generate histological evidences of tendon lesions.

The choice of the collagenase doses included in this work was based on our previous in vitro studies, in which the dose inducing the collagen fiber disruption was validated in tendon explants [31, 32].

Our results revealed that both LD- and HD-treated tendons displayed a disorganization of the collagen fibers and increased the number of rounded resident cells, suggesting that a single intra-tendinous injection was sufficient and effective to induce a prompt and severe impairment of Achilles tendon integrity, above all in tendons treated with HD of collagenase type I. Our findings were consistent with other studies performed in large animals, in which the severity of the pathology was related to the amount of the injected collagenase [33]. The morphological changes - especially occurring at day 15 - resembled the histological appearance of tendinopathy in humans. In fact, human Achilles tendinopathy exhibits disorganized and smaller collagen fibers, loss of their parallel orientation, and an increased amount of rounded-shaped tenocytes [6].

Overall, in this model, we demonstrated that the acute phase occurred from day 3 to day 15 and evolved towards a proliferative phase from day 15 to day 45 displaying a degenerative appearance associated to a very precocious and mild remodeling process, according to what observed by previous in vivo and human studies [6, 34]. Moreover, 25% of cases treated with both LD and HD collagenase showed the presence of chondrocyte-like cells disseminated within the damaged tendon fibers (see S1 Figure). This finding was consistent with other studies describing both the presence of chondrocytes and the up-regulation of chondrogenic genes in rat patellar and Achilles tendons after four weeks of collagenase injection [14, 27]. Similarly, chondrocyte markers were also expressed in human clinical samples of patellar [35], supraspinatus [36] and Achilles tendons [6]. The impairment of the tendon feature in favor of a fibrocartilaginous one assumes a pathological

significance. Indded, the progressive lack of elasticity and tensile strength makes tendon more subjected to ruptures, even if no molecular mechanisms and pathways occurring in human Achilles tendinopathy have been investigated yet [37]. This condition was strengthened by some in vitro studies that demonstrated the capability of tendon stem/progenitor cells to effectively transdifferentiate towards the chondrogenic lineage [38-40].

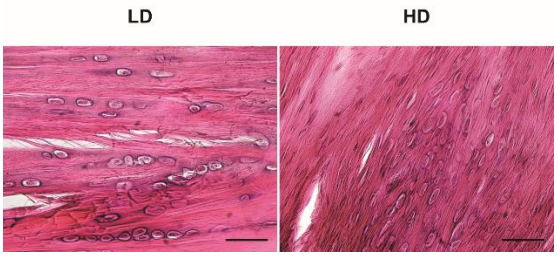
The presence of fatty deposits was observed in tendons treated with HD collagenase, while they were rarely found in the LD-treated ones. The fat infiltration is retrieved in case of poor tendon repair. Moreover, fatty deposits were found in chronic tendinopathy in humans and in large animals [35, 41]. Our findings showed increased neo-angiogenesis in collagenase treated samples, more markedly visible in the HD-treated tendons than in the LD ones, above all at the earlier stages of the disease. The presence of new vessels was mainly restricted to the peritenon and it was combined with an initial increase of the cell number from day 3 to day 15, followed by a decrease from day 15 to day 45. The neovascularization and the increased amount of cells could be correlated to an inflammatory reaction. Our data were supported by several studies that demonstrated an inflammatory reaction in humans and animal models, both in the early overload response and in the established tendinopathy [42]. However, the role of inflammation in tendon healing is still a greatly debated topic. Inflammation is highly beneficial to the tissue repair thanks to the release of cytokines and growth factors that together promote neoangiogenesis and the recruitment of resident and progenitor cells, and macrophages [43]. Nevertheless, how the inflammatory reaction can influence the progression of the pathology and how it can possibly contribute to the healing process are still unanswered questions. So, since its role in tendinopathy is uncertain, the presence (or absence) of an inflammatory response in our model would not have represented a crucial parameter to be considered in comparison with the human disease, thus, it was not deeply investigated. Despite our study was based only on histological evaluations, it was able to resemble the most important tendinopathy features in terms of tissue damages. These outcomes need to be examined in depth through quantitative analyses to assess the correlation of the histological findings with biochemical analyses, such as glycosaminoglycan, collagen and DNA contents, as well as with biomechanical parameters.

Overall, the results of this study suggested that the HD collagenase-induced tendinopathy is a reliable model in rats, resembling the human disease. In particular, our results demonstrated that collagenase type I efficiently induced three distinct stages of the disease over time, thus offering the opportunity to accurately investigate the pathological progression in a well-controlled establishment of this complex injury. More importantly, this model could be used to test novel therapies during the three-stage tendon disorder to achieve the most effective results in patients.

Supporting Information

	0	1	2	3
Fiber structure and arrangement	Normal: continuous, parallel collagen fibers	Slightly abnormal: partially disorganized and fragmented fibers	Abnormal: moderately disorganized, fragmented, crossed and wavy fibers	Markedly abnormal: total disorganized and non-identifiable fiber pattern
Cell density	Normal	Slightly increased	Moderately increased	Markedly increased
Cell appearance	Spindle-shape cells	Slightly rounded cells	Moderately rounded cells	Markedly rounded cells
Inflammatory cell infiltration	<10%	10-20%	20-30%	>30%
Neovascularization	Normal presence of vascular bundles	Slight increase of vascular bundles	Moderate increase of vascular bundles	Marked increase of vascular bundles
Fatty deposits	Absence of lipid vacuoles	Slight increase of lipid vacuoles	Moderate increase of lipid vacuoles	Marked increase of lipid vacuoles

S1 Table. Grading system for the tendon histological evaluation



S1 Figure. *Chondrocyte-like cells in LD and HD treated tendon at day 45. Representative micrographs of the histopathological analysis; H&E staining. Scale bars 100 µm (20X).*

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Chapter 2

Therapeutic alternatives based on MSCs: agents or targets?

Aim 2

The multiple roles of MSCs showed by the most recent literature opened the way to multifactorial studies that have to take into consideration several interconnected aspects of MSCs biology.
In the first part of this chapter, we observed possible uses of MSCs as a therapeutic agent for the treatment of musculoskeletal diseases, considering them from a tissue engineering point of view, highlighting their multidifferentiation potential and their potentiality as a direct replacement tool.
Although BMSCs and ASCs are still considered as gold standards in regenerative medicine, we decided to focus also on alternative sources of MSCs to find out the best candidate for each specific clinical application, especially for the treatment of tendinopathy and other tendon-related pathologies.
Basing on the identification of a progenitor cell population in tendon tissue, defined as tendon stem/progenitor cells (TSPCs) *(Bi Y et al, 2007)*, we compared this cell type with ASCs, assessing their stemness features and multi-differentiation potential (Chapter 2, manuscript 2, Stanco D et al 2015, published on RegenMed).
Then, we focused on the selection of tendon progenitors within whole tendon cells, testing different culture conditions and isolation procedures, such as the clonal selection. The best cell culture condition was selected to allow the prevalence of the progenitor components over the other terminally differentiated cells (Chapter 2, manuscript 3, Viganò M et al 2016, submitted JEO).
Still focusing on tendon degenerative disorders and aware of the necessity to further increase the knowledge about tendon regeneration, we also conducted an in-depth evaluation of the tenogenic differentiation of MSCs isolated from bone marrow, adipose tissue and tendons. Basing on the premise that after an injury progenitor cells involved in tendon regeneration can undergo to an undesired chondro-osteogenic events *(Harris MT et al, 2004)*, we hypothesized that an in *vitro* pre-differentiation of MSCs could be useful for the maintaining of the tenogenic phenotype. The great challenge of this work was the identification of the best biochemical stimulation to obtain the tenogenic differentiation of MSCs, since there is still a lack of consensus within the scientific community (Chapter 2, manuscript 4, Perucca Orfei C et al 2016, paper in preparation).

Another aspect of MSCs is their paracrine activity that is their ability to secrete and release a plethora of different molecules with different actions, including immunomodulatory, thopic, anti-scarring and anti-inflammatory, which can even lead to consider not to use the whole cells but just their products. This ability makes MSCs a potential candidate not exclusively as a therapeutic agent but also a therapeutic target, just for their ability to

influence the surrounding microenvironment and to stimulate the reparative process, by the activation of the tissue resident MSCs and progenitor cells. In this view, we performed two studies aimed to exploit and investigate deeper the therapeutic potential of MSCs with this recent awareness: one is based on the immunomodulatory and anti-inflammatory properties of cell-free conditioned medium obtained from ASCs cultures in an *in vitro* model of inflammation (Chapter 2, research in progress, Viganò et al 2016). The second, basing on the hypothesis that biophysical stimulation can also influence the immunomodulatory and trophic activity of resident MSCs, we stimulated these tendon plastic cells *in situ* using pulsed electromagnetic fields. This approach avoids cell harvesting and transplantation and thus could represent a valid and less invasive treatment (Chapter 2, research in progress, Perucca Orfei C et al 2016).

Manuscripts

* Stanco Deborah, Viganò Marco, **Perucca Orfei Carlotta**, Di Giancamillo Alessia, Peretti Giuseppe, Lanfranchi Luciano, de Girolamo Laura. *Multidifferentiation potential of human mesenchymal stem cells from adipose tissue and hamstring tendons for musculoskeletal cell-based therapy.* Regen Med. 2015;10(6):729-43.

* Vigano Marco*, **Perucca Orfei Carlotta** *, Colombini Alessandra, Stanco Deborah, Randelli Pietro, Sansone Valerio, de Girolamo Laura. *Different culture conditions affect the growth of human tendon stem/progenitor cells (TSPCs) within a mixed tendon cells (TCs) population.* Submitted to Journal of Experimental Ortopaedics

* **Perucca Orfei Carlotta***, Viganò Marco*, Santos-Ruiz Leonor, Pearson John R., Colombini Alessandra, Girolamo de Laura*. Development of an effective strategy for the tenogenic differentiation of human Adipose-, Bone Marrow-and Tendon-derived Mesenchymal Stem Cells.* Paper in preparation

Ongoing research projects

* Viganò Marco, **Perucca Orfei Carlotta**, Colombini Alessandra, de Luca Paola, Chlapanidas Theodora, Laura de Girolamo, Torre Maria Luisa*. Cell-free approaches in Regenerative Medicine: paracrine mediators of ASCs possess immunomodulatory and chondro-protective abilities.* Research in progress

* **Perucca Orfei Carlotta**§, Lovati Arianna B.§, Viganò Marco, Bottagisio Marta, Di Giancamillo Alessia, Setti Stefania, de Girolamo Laura. *Biophysical stimulations with pulsed electromagnetic fields (PEMFs) for the treatment of Achilles tendinopathy in a rat model*. Research in progress

Research Article

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Regenerative Medicine

Multidifferentiation potential of human mesenchymal stem cells from adipose tissue and hamstring tendons for musculoskeletal cell-based therapy

Aim: Adipose-derived stem cells (ASCs) have been deeply characterized for their usefulness in musculoskeletal tissue regeneration; recently, other mesenchymal stem cell (MSC) sources have also been proposed. This study compares for the first time human tendon stem/progenitor cells isolated from hamstring tendons with human ASCs. **Materials & Methods:** Human TSPCs and ASCs were isolated from hamstring tendon portions and adipose tissue of healthy donors undergoing ACL reconstruction or liposuction, respectively (n = 7). Clonogenic ability, immunophenotype and multi-differentiation potential were assessed and compared. **Results:** Both populations showed similar proliferation and clonogenic ability and expressed embryonic stem cell genes and MSC surface markers. Tendon stem/progenitor cells showed lower adipogenic and osteogenic ability, but after the chondrogenic differentiation, they produced more abundant glycosaminoglycans and expressed higher levels of aggrecan with regards to ASCs. The tenogenic induction with BMP-12 upregulated *SCX* and *DCN* gene expression in both populations. **Conclusion:** Our results demonstrate that waste hamstring tendon fragments could represent a convenient MSC source for musculoskeletal regenerative medicine.

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Multidifferentiation potential of human mesenchymal stem cells from adipose tissue and hamstring tendons for musculoskeletal cell-based therapy

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Materials and Methods: Human TSPCs and ASCs were isolated from hamstring tendon portions and adipose tissue of healthy donors undergoing ACL reconstruction or liposuction, respectively (n=7). Clonogenic ability, immunophenotype and multidifferentiation potential were assessed and compared.

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Conclusion: Our results demonstrate that waste hamstring tendon fragments could represent a convenient MSC source for musculoskeletal regenerative medicine.

INTRODUCTION

Although bone marrow is still the most common source of MSCs, in the last years researchers, driven by a constant quest for the "most convenient" source, have demonstrated that these cells can be identified in several alternative sites. In particular MSCs have been found in tissues that are discarded after surgical interventions, including adipose tissue, periodontal ligaments and deciduous teeth. Many studies have been performed to compare the features of MSCs from different origins, with the final aim to identify the possible best MSC tissue source for a given clinical situation. The choice of the most appropriate cell type is crucial, since, although mesenchymal stem cells deriving from different tissues share common properties, it is known that they are influenced by the surrounding microenvironment and tissue specific characteristics that can ultimately may influence the final treatment outcome. Moreover, age, site, gender and pathological conditions have been shown to affect the number, the proliferation potential and differentiation capacity of MSCs [1-4]. Recently, it has been shown that also discarded tendon fragments derived from knee ligament reconstruction [5] or tendon cuff repair [6] contains a MSC population, named tendon stem/progenitor cells (TSCPs) [7]; this observation has allowed remarkable advancements in understanding the physiopathology of this tissue, as well as the possibility to use this cell source as a potential tool for tendon regenerative treatments.
In this study for the first time the in vitro immunophenotype, embryonic stem cell (ESC) marker expression, clonogenic and proliferation ability and multilineage differentiation potential, including the tenogenic one, of TSPCs and adipose-derived stem cells (ASCs) were compared. Indeed, ASCs have been already well characterized and demonstrated to be potentially useful in musculoskeletal cell-based therapy, but to date very few in vitro studies have investigated their in vitro tenogenic potential [8-10]. Thus the main aim of this study is to provide further knowledge about the potential use of TSPCs in musculoskeletal tissue regeneration strategies, where our hypothesis is that it could be more advantageous to use this cell source for tendon regenerative purpose in comparison to ASCs.

MATERIALS AND METHODS

TSPCs and ASCs isolation and culture

All the procedures were carried out at Galeazzi Orthopeadic Institute (Milano, Italy) with the Institutional Review Board approval (M-SPER-014.ver7 for use of surgical waste). All the donors gave their written consent to the use of surgical waste material for research purpose. TSPCs were isolated from discarded fragments of semitendinosus and gracilis tendons collected from 7 donors (mean age 33 ± 14 years) who underwent anterior cruciate ligament (ACL) reconstruction; ASCs were isolated from raw lipoaspirates of 7 donors (mean age 47 ± 14 years) who underwent aesthetic liposuction (Table 1). The samples of tendon tissue were minced into small pieces (0.5-0.8 cm), placed in 100 mm Petri dish and covered with control medium (Table 2, CTRL medium). During the

first 10 days in culture, tendon cells migrated from tissue and started to proliferate [11, 12]; about 3 weeks later they reached 80-90% of confluence.

ASCs were isolated as previously described [2]. Briefly, adipose tissue was washed with phosphate-buffered saline (PBS) and digested with 0.075% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C for 30 min. The stromal vascular fraction (SVF) was centrifuged (1200 g, 10 min) and then filtered through a 100 µm nylon cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); the collected SVF cells were plated in CTRL medium at 104 cells/cm2 of density and in about 7 days they reached the 80-90% of confluence. TSPCs and ASCs were maintained in culture changing medium every 3 days.

TABLE 1
Characteristic of patients and cellular yield at passage 1

	TSPCs	**ASCs**
Number of patients	N=7 (2 F, 5 M)	N=7 (6 F, 1 M)
Age (years)	33 ± 14	47 ± 14
Grams of tissue for each donor	1.7 ± 0.9 ***	21.5 ± 13.3
Number of cells at P1/grams of tissue	2.2 ± 1.8 X 10^5 *	0.7 ± 0.5 X 10^5

* p<0.05, *** p<0.001 *vs* ASCs

TABLE 2
Composition of differentiation media

Medium	Main components	Serum	Supplements
Control (CTRL)	HG-DMEM 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine	10%FBS	/
Adipogenic (ADIPO) -induction	CTRL	10%FBS	1µM dexamethasone, 10 µg/mL insulin, 500 µM IBMX (3-isobutyl-1-methyl-xanthine), 200 µM indomethacin
Adipogenic (ADIPO) -maintenance	CTRL	10%FBS	10 µg/mL insulin
Oteogenic (OSTEO)	CTRL	10%FBS	10 nM dexamethasone, 10 mM glicerol-2-phosphate, 150 µM L-ascorbic acid-2-phosphate, 10nM cholecalciferol
Chondrogenic (CHONDRO)	CTRL	1%FBS	1 mM sodium pyruvate, 1% ITS+1 (1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, 0.5 µg/ml sodium selenite, 50 mg/ml bovine serum albumin and 470 µg/ml linoleic acid), 0.1 µM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate 10 ng/ml TGF-β1
Tenogenic (TENO)	CTRL	1%FBS	50 ng/ml BMP-12

TGF-β1 : Transforming growth factor β1; BMP-12: bone morphogenetic protein 12

Cell doubling time

For TSPCs and ASCs cell-doubling time (DT) was calculated from passages 2 to 4. The cells were plated at a density of 3 x 103 cells/cm2 in CTRL medium. Fresh medium was supplied every 3 days and at 80–90 % confluence, the cells were splitted cells using trypsin/EDTA (0.5 % trypsin/0.2 % EDTA; Sigma-Aldrich). DT was calculated according to the following formula: DT = CT / ln (Nf-Ni) / ln2, where CT is the cell culture time (hours), Nf the final number of cells, and Ni the initial number of cells [13].

Fibroblast-Colony Forming Unit Assay (CFU-F)

Clonogenic ability of ASCs and TSPCs from passage 2 to passage 4 was evaluated by colony forming unit assay as previously described [2]. Briefly, cells were plated in six-well plates at low density by limiting dilution (starting dilution: 100 cells/cm^2, ending dilution: 13 cells/cm^2) and cultured in control medium supplemented with 20% FBS for 14 days. Then the cells were fixed with 4% paraformaldehyde solution, and counted after 2.3% Crystal Violet staining (Sigma-Aldrich) for 10 minutes at room temperature. The frequency of CFU-F was established by scoring the individual colonies composed of at least 30 cells and expressed as a percentage relative to the number of the initial seeded cells.

Cell metabolic activity

For both TSPCs and ASCs $1.5x10^4$ cells at passage 4 were seeded in 96-well plates and cell metabolic activity assay was performed at 1, 3 , 7 and 14 days. Briefly, a final concentration of 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added to the culture medium and incubated for 4 hours at 37°C. After medium removal, 100% DMSO (dimethylsulfoxide) was added to each well to solubilize the formazan precipitate. The absorbance of this solution was read at 570 nm (VictorX3, Perkin Elmer microplate, Waltham, MA, USA).

FACS analysis

TSPCs and ASCs at passage 4 were detached and washed twice in cold FACS Buffer (PBS w/o Ca/Mg^{2+}, 2% FBS, 0.1% NaN_3). 2.5 x 10^5 cells were incubated with anti-human primary monoclonal antibodies fluorescein isothiocyanate (FITC)-conjugated CD90, CD13, CD31, CD44, CD45, CD166; biotinylated (BIOT)-conjugated CD29, CD34, CD54, CD71, CD105 (all Ancell Corporation, Bayport, MN, USA) and phycoerythrin (PE) conjugated CD73 (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation, Streptavidin-PE and FITC conjugated goat anti-mouse Ab (Ancell Corporation) were used as secondary antibodies for cells stained with biotinilated antibodies. Background fluorescence was established by negative controls and data on 10000 cell fluorescence events were acquired by flow cytometry using a FACSCalibur flow cytometer and analyzed by CellQuest software (BD Bioscences, San Jose, NJ, USA).

Adipogenic differentiation

Both cell populations at passage 4 were seeded at 10^4 cells/cm^2 and then induced to differentiate toward adipogenic lineage for 21 days using a repeated pulsed protocol, consisting in 3 days of adipogenic induction medium followed by 3 days in adipogenic maintenance medium (Table 2, ADIPO medium). To quantify the lipid vacuoles, cells were rinsed and fixed in 10% neutral buffered formalin for 1 hour, stained with Oil Red O (Sigma-Aldirch) for 15 min, then unstained with 100% isopropanol. Absorbance was read at 490 nm.

Osteogenic differentiation

Cells at passage 4, seeded at 104 cells/cm2 were differentiated into osteogenic lineage by culturing in osteogenic medium (Table II, OSTEO medium). Cells were assessed for alkaline phosphatase (ALP) activity and extracellular calcified matrix deposition [2]. Briefly, after 14 days of differentiation, cells were lysed with 0.1% Triton X-100 (Sigma-Aldrich) and the enzymatic ALP activity was determined by incubating cellular lysates at 37°C with 1 mM p-nitrophenylphosphate (Sigma-Aldrich) in alkaline buffer (100mM diethanolamine and 0.5 mM MgCl2, pH 10.5). The absorbance was read at 405 nm (Victor X3, Perkin Elmer microplate) and the ALP activity was normalized on the total protein content determined (BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA).

The extracellular calcified matrix deposition was evaluate after 21 days of differentiation. Briefly, the cells were stained with 40 mM Alizarin Red S (pH 4.1, Fluka) for 15 min. The dye was extract with 10% cetylpyridinium chloride monohydrate (CPC, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) and the absorbance read at 550 nm.

Chondrogenic differentiation

At passage 4, 5.0 x 105 TSPCs and ASCs were centrifuged (250 g, 5 min) to obtain cell pellets. Pellets were cultured in chondrogenic (CHONDRO) medium (Table II) for 21 days. For histological analysis, pellets were fixed for 24 hours in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 µm. Sections were stained with haematoxylin-eosin (Sigma-Aldrich) and safranin O to evaluate extracellular matrix and glycosaminoglycans (GAGs) deposition [14]. GAGs were also quantified digesting pellets (16 h, 60°C) in 500 µl of PBE buffer (100 mM Na2HPO4, 10 mM NaEDTA, pH 6.8) containing 1.75 mg/ml L-cystein (Sigma-Aldrich) and 14.2 U/ml papain (Worthington). Samples were incubated with 16 mg/l dimethylmethylene blue (Sigma-Aldrich) and absorbance was read at 500 nm (Perkin Elmer Victor X3 microplate reader). The same samples were used for DNA quantification by CyQUANT Kit (Life Technologies). The amount of GAGs produced for each sample was normalized on DNA content and expressed as µg of GAGs per µg of DNA.

Tenogenic differentiation

Both TSPCs and ASCs populations were seeded at passage 4 and at cell density of 10^4 cells/cm^2. After 7 and 14 days of tenogenic differentiation in inductive medium (Tab II) the expression of tendon-related genes was assessed by RT-PCR.

Gene expression

The total RNA was purified from the cell lysates using the RNeasy Mini kit (Qiagen, Duesseldorf, Germany) and reverse-transcripted to cDNA (5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C) using iScriptcDNA Synthesis Kit (Bio-Rad Laboratories, Benicia, CA, USA). Ten ng of cDNA were incubated with a PCR mix (50 °C for 2', 95 °C for 10', 40 cycles at 95 °C for 15 seconds, and 60 °C for 1') containing TaqMan Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies, Grand Island, NY, USA) for the following genes: leptin (LEP, Hs00174877_m1), runt-related transcription factor 2 (RUNX2, Hs00231692_m1), collagen type I alpha 1 (COL1A1, Hs01076777_m1), collagen type III alpha 1 (COL3A1, Hs00943809_m1), SRY-box 9 (SOX9, Hs00165814_m1), aggrecan (ACAN, Hs00153936_m1), scleraxis (SCX, Hs03054634_g1), decorin (DCN, Hs00370385_m1), octamer-binding transcription factor 4 (POU5F1, Hs04260367_gh) and kruppel-like factor 4 (KLF4, Hs00358836_m1). Reaction was performed with Applied Biosystems StepOnePlus (Life Technologies). The fold change in the expression was normalized on the expression of the housekeeping GAPDH gene (glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Normal distribution of values was assessed by the Kolmogorov-Smirnov normality test. Statistical analysis was performed using Student's t-test for data with a normal distribution and Wilcoxon test for data with a non-normal distribution (GraphPad Prism v5.00; GraphPad Software, San Diego, CA, USA). Level of significance was set at $p< 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Correlation between age and each parameter was assessed by Pearson's correlation test.

RESULTS

Undifferentiated TSPCs and ASCs show similar stem cells features

TSPCs and ASCs were harvested from 7 different donors each (Table 1). The mean donors' age of the two cell populations was different, but the large variability within each group make this difference not significant (n.s). The regression analysis performed to assess possible correlation between age and all the parameters evaluated revealed no significant correlation (data not shown).
Although the weight of the starting tendon fragments was significantly lower with respect to lipoaspirates ($p<0.001$), the number of cells at passage 1 normalized per grams of tissue was 2.2 ± 1.8 X 10^5 and 0.7 ± 0.5 X 10^5, respectively for TSPCs and ASCs ($p<0.05$). During passages in culture,

TSPCs showed a typical fibroblastic-like morphology and proliferated with a rate similar to the ASCs one (Figure 1a, b).

Indeed, at passage 2 the average doubling time (DT) was 87.0 ± 7.6 hours for TSPCs and 70.8 ± 23.6 hours for ASCs (n.s); then TSPCs and ASCs proliferation rate progressively decreases until reaching at passage 4 an average DT of 176.7 ± 108.1 hours and 190.3 ± 65.8 hours, respectively (n.s.). The clonogenic ability was also similar between the two cell populations along all the passages analyzed; in particular the highest number of colonies was observed at passage 4 for TSPCs (4.4 ± 1.9%) and at passage 3 for ASCs (3.5 ± 1.9%) (Figure 1c). In order to evaluate the in vitro cell viability of TSPCs and ASCs, considered an important parameter concerning primary cell survivorship after isolation and during culture, we observed their metabolic activity using MTT assay. No significant differences were also observed in term of cell metabolic activity at passage 4 between TSPCs and ASCs (Figure 1d).

The expression of stemness-specific transcription factors KLF4 and POU5F1 of TSPCs was also comparable to that observed for ASCs (n.s.) (Figure 1d).

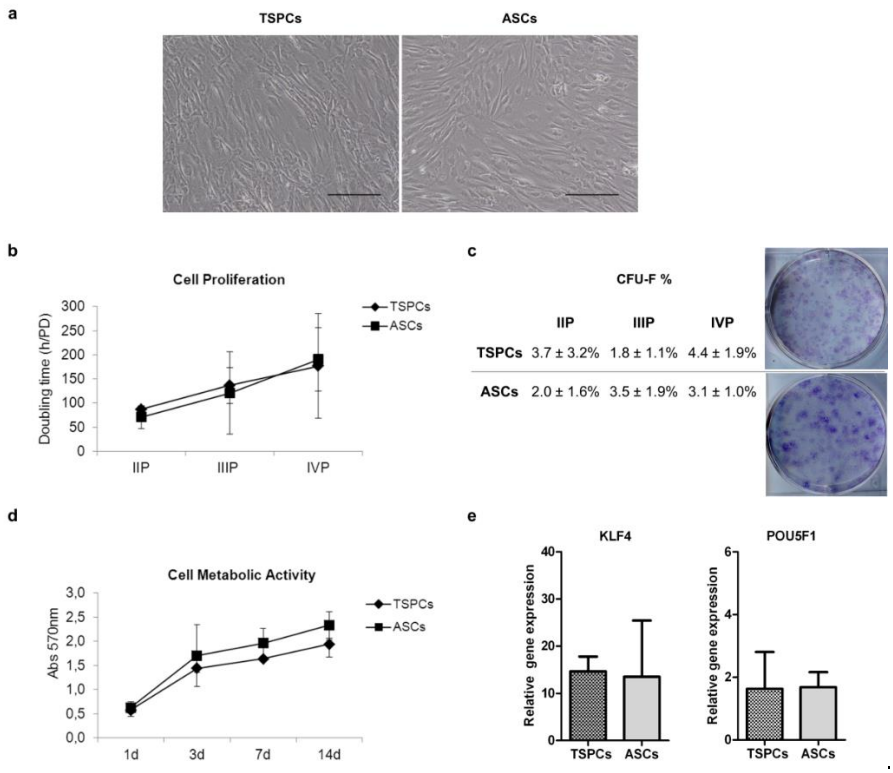

Figure 1: *Features of TSPCs and ASCs. (a) Cell in culture at passage 4 (optical microscopy, 10 X, scale bar 200 µm). (b) Proliferation ability during passages in culture, expressed as mean of doubling times (DT). (c) Percentage of clonogenic cells from passage 2 to passage 4 (scored colonies were normalized on number of seeded cells) and representative pictures of stained CFU-F for both cell population at P4. (d) Cell viability of TSPCs and ASCs at passage 4 at several time points (1, 3, 7, 14 days of culture). (e) Evaluation of KLF4 and POU5F1*

gene expression determined by quantitative real-time PCR in both cell populations. Data were normalized on the expression of the housekeeping GAPDH gene.

The cell size and granularity of both populations were also similar (Figure 2). Moreover, TSPCs were found to possess the characteristic mesenchymal immunophenotypic profile, without any significant difference with regards to ASCs. Indeed they both were highly positive for CD13, CD73, CD90 , CD29, CD44, CD105, CD166 and CD54, and both cell populations did not express hematopoietic markers such as CD31, CD34, CD45 and CD71 (Figure 2).

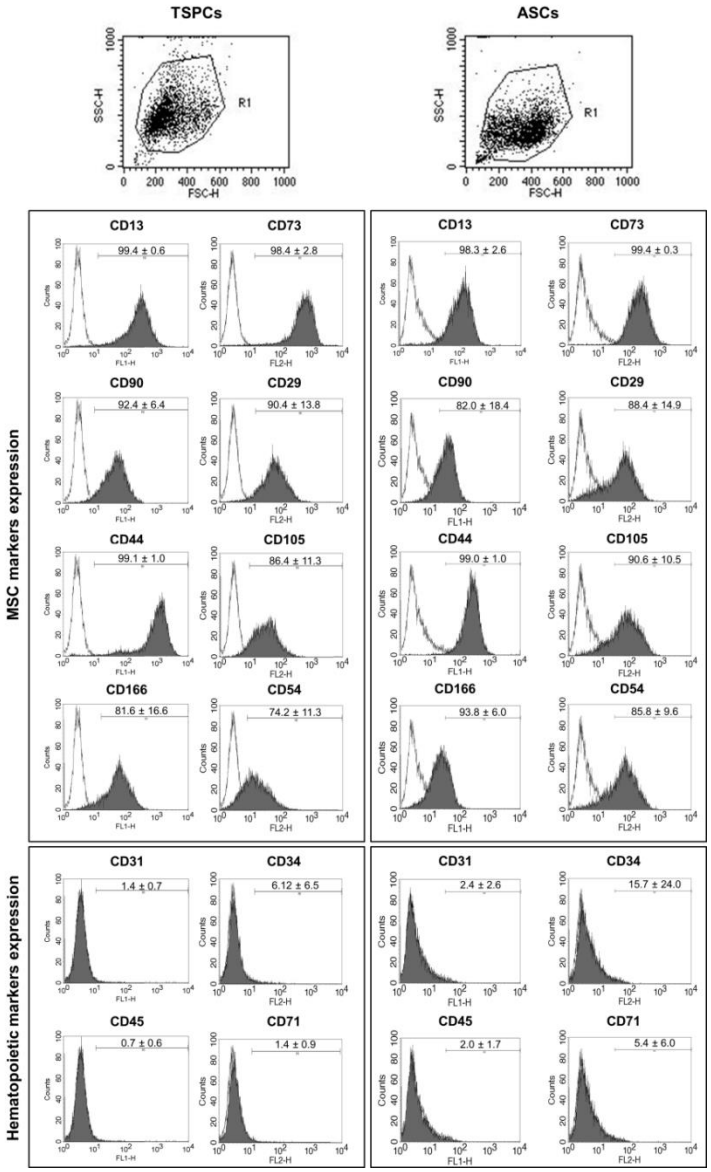

Figure 2: *MSCs surface markers pattern expression. TSPCs and ASCs distribution based on forward scatter (FSC) and side scatter (SSC) and the representative expression of the typical MSCs surface markers and hematopoietic*

markers for both population at passage 4 (markers are represented as grey histograms and isotype control antibodies are represented as white histograms).

TSPCs have a lower adipogenic potential than ASCs

TSPCs cultured for 14 days in adipogenic medium just showed a slight intracellular lipid vacuoles increase respect to cells maintained in non-inductive medium (+40.3%, n.s.) (Figure 3a,b). The lipid vacuoles content observed in TSPCs was significantly lower than that observed in ASCs ($p<0.05$) that, when cultured in adipogenic medium, showed a significant increase in comparison to CTRL ASCs (+136.3%, $p<0.05$). However, both differentiated cell populations expressed significantly higher levels of *leptin* in comparison to their respective CTRL cells ($p<0.05$) and without any difference between them (Figure 3c).

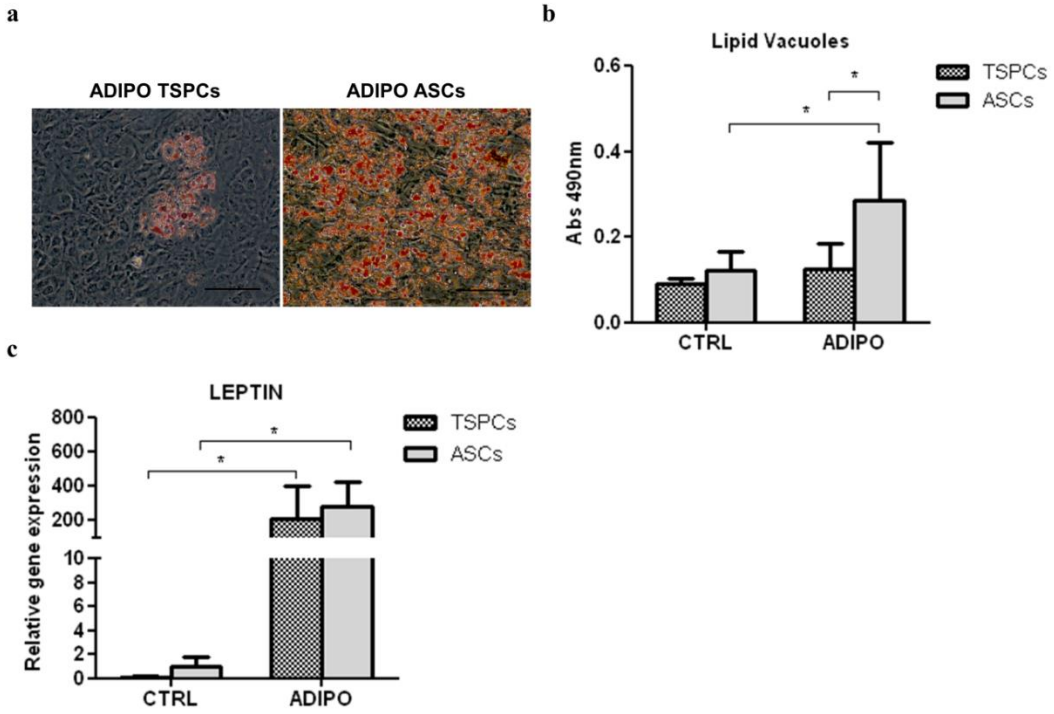

Figure 3: *Adipogenic differentiation. (a) Micrographs of TSPCs and ASCs differentiated towards the adipogenic lineage after Oil Red O staining (scale bars 200 µm). (b) Quantification of lipid vacuoles in undifferentiated (CTRL) and adipogenic-differentiated (ADIPO) cells. (c) Gene expression of LEPTIN normalized to GAPDH. Level of significance * p<0.05.*

TSPC osteogenic potential is lower than ASCs one

After 14 days of culture in osteogenic medium, both TSPCs and ASCs showed significant increases in term of ALP activity of 173% and 177%, respectively ($p<0.05$) in comparison to CTRL cells (Figure 4a). Significant up-regulation of *RUNX2* expression was observed in both osteo-differentiated TSPCs (+35%, $p<0.05$) and ASCs (+113%, $p<0.05$) in comparison with the respective CTRL cells. Comparing the two cell types, significantly higher mRNA levels of *RUNX2* were observed in differentiated ASCs

with respect to TSPCs (+136% ASCs vs TSPCs, p<0.01) (Figure 4b). After 21 days of culture in osteogenic medium, the calcified matrix deposition, as revealed by alizarin red S staining and extraction, was significantly higher in comparison to CTRL cells for both TSPCs and ASCs (+46% and +410%, respectively, both p<0.05) (Figure 4c,d). However, the amount of calcified matrix produced by differentiated TSPCs was very similar to undifferentiated ASCs, that after the osteogenic induction produced significantly higher amount of calcified matrix with respect to TSPCs (+ 411%, p<0.001). All these data suggest a lower capacity of TSPCs to differentiate toward osteogenic lineage.

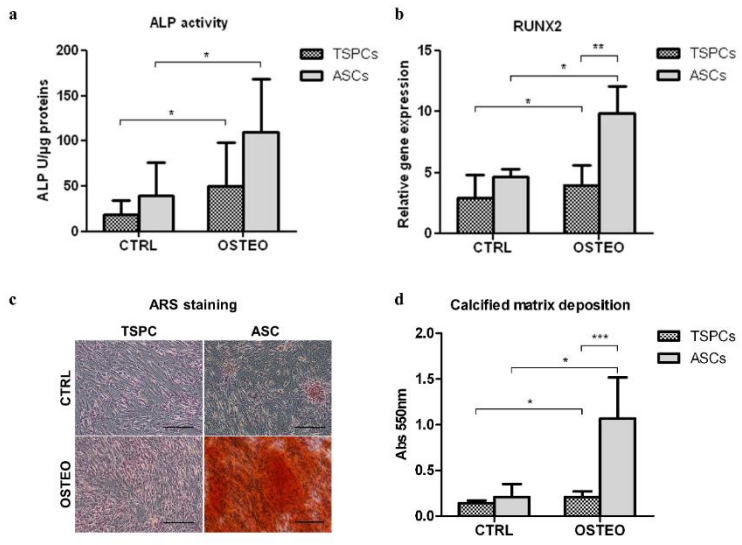

Figure 4: *Osteogenic differentiation. (a) ALP activity determined at 14 days of culture in undifferentiated (CTRL) and osteo-differentiated (OSTEO) cells. (b) Gene expression of RUNX2 normalized to GAPDH at 14 days of culture. (c) Micrographs of CTRL and OSTEO cells stained by AR-S (scale bars 200 µm). (d) Quantification of calcified matrix deposition by AR-S staining and extraction. Levels of significance * p<.05, ** p<0.01, *** p<0.001.*

TSPC chondrogenic potential is higher than ASCs

Both cell populations were cultured in pellet in chondrogenic medium for 21 days. As revealed by the histological evaluation, the pellets cultured in non-inductive medium were smaller and less organized in comparison to the ones cultured in chondrogenic medium for both types of cells (Figure 5a). Moreover, only the chondro-differentiated pellets of TSPCs showed a marked extracellular matrix production which was intensively stained by safranin-o dye. Chondro-induced TSPCs also showed a significant increase in term of glycosaminoglycan deposition respect to undifferentiated cells (+83%; p<0.01); however, undifferentiated ASCs showed significantly higher levels of GAGs in comparison to undifferentiated TSPCs (+96%; p<0.05). Both *SOX9* and *ACAN* were strongly up-regulated in chondro-induced pellets (Figure 5c,d) (+251% and +44% for *SOX9,* +1279% and +145% for *ACAN,* for TSPCs and ASCs, respectively) in comparison with CTRL pellets. Moreover, both undifferentiated and differentiated TSPCs showed higher ACAN expression with respect to ASCs (+1208% and +7256% increases for undifferentiated and differentiated TSPCs vs the respective ASCs). However all these differences, due to the wide inter-donor variability, were not significant.

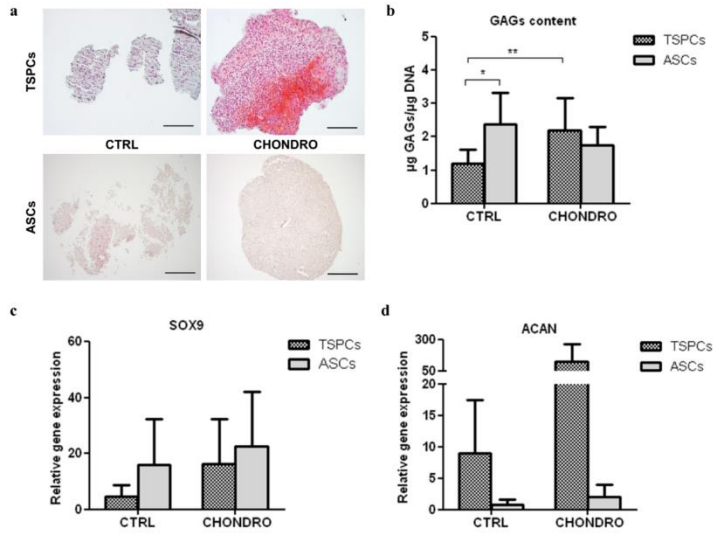

Figure 5: *Chondrogenic differentiation. (a) Histological evaluation using haematoxylin-eosin and safranin staining in undifferentiated (CTRL) and chondro-differentiated (CHONDRO) pellets of both TSPCs and ASCs (scale bars, 200 µm). (b) DNA and GAGs content in TSPCs and ASCs pellets. (c, d) Gene expression of SOX9 and ACAN normalized to GAPDH. Level of significance * p<.05, ** p<0.01.*

BMP-12 increases scleraxis and decorin expression in both TSPCs and ASCs

The BMP-12 dosage used in this study (50 ng/ml) was chosen accordingly to previously experiments carried on ASCs (data not shown).

The gene expression of specific tendon markers was evaluated after 7 and 14 days in undifferentiated and differentiated cells in both cell populations. In undifferentiated cells, SCX was more expressed in TSPCs than in ASCs (+ 461%, $p<0.05$ and +344% at 7 and 14 days, respectively) (Fig.6a, left column). The tenogenic medium was able to induce a further increase in the expression of this transcription factor in both populations; indeed, differentiated TSPCs and ASCs showed higher levels of SCX in comparison with undifferentiated cells both at 7 days (+86% and +89%, respectively) and at 14 days of culture (+50% and +24%, respectively), although these differences were not statistically significant (Fig.6a right column). For what concern the extracellular matrix components, DCN, COL1A1 and COL1A3 gene expression was evaluated. After 7 days of culture in undifferentiated medium TSPCs showed higher levels of DCN in comparison with ASCs (+93%, ns); however, at 14 days of culture this difference was not present anymore as ASCs DCN expression increased with time in culture (Fig.6b left column). Tenogenic medium induced a significant gene up-regulation of this marker in both population already at 7 days of differentiation respect to undifferentiated cells (+50%, $p<0.05$ and +164%, $p<0.01$, respectively). However, after 14 days of differentiation the DCN expression in ASC significantly decreased with respect to the previous time point, whereas TSPCs maintained similar expression level (Fig. 6b right column). Unexpectedly, undifferentiated ASCs showed very higher level of COL1A1 and COL3A1 expression respect to TSPCs (+1296%, $p<0.01$ and% + 1279%, $p<0.01$, respectively) (Fig. 6c, d left column). Moreover, both cell populations did not show any further increase after tenogenic induction respect to undifferentiated cells (Figure 6c, d right column).

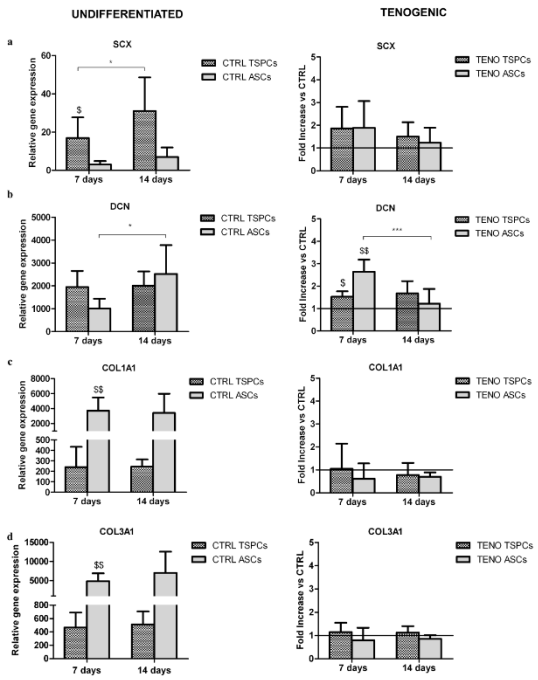

Figure 6: *Tenogenic differentiation. (a-e) Gene expression of scleraxis (SCX), decorin (DCN) and type I and III collagen (COL1A1 and COL1A3) normalized to GAPDH in undifferentiated (CTRL, left panel) and teno-differentiated (TENO, right panel) TSPCs and ASCs at 7 and 14 days of culture. Data for the tenogenic differentiated cells are expressed as average fold increase ± SD respect to undifferentiated ones (fixed line set at 1). Levels of significance * p<0.05, ** p<0.01; ASCs vs TSPCs.*

DISCUSSION

To the best of our knowledge this is the first study comparing stem cell properties and multi-lineage differentiation potential of human TSPCs and ASCs. The main finding of our study is that TSPCs and ASCs show a similar cell proliferation, viability and clonogenic ability, as well as stem cell markers expression. On the other hand, although both MSC populations show multi-differentiation potential, some significant differences were observed partially related to their tissue origin, supporting the idea that a given MSC tissue source could be more appropriate for a given clinical situation. Little is known about the tendon biology also for the scarce knowledge of the cell population resident in tendon tissue. Just recently it has been demonstrated that tendon contains stem/progenitor cells that can provide a new tool to study tendon physiology, pathology and possible innovative tendon therapies based on their properties [7]. Indeed, TSPCs are supposed to play a primary role in maintaining the tissue homeostasis and in promoting the repair after injury and they could be addressed as a new potential therapeutic target. In particular, TSPCs could be the main effector of the in vitro biophysical stimuli such as pulsed electromagnetic field and extracorporeal shock waves that have been proposed as promising alternatives for the treatment of tendinopathies [15-17]. Another perspective might involve the use of TSPCs as cell source for cell-based therapy approaches to improve the regeneration of tendon or of other tissues of mesodermal origin such as bone and cartilage. Currently, adipose tissue is considered one of the smartest and most convenient source for MSC isolation due to its wide availability as surgical waste material [18] and for this reason in the last years several cell/tissue banks have focused their activity on its preservation and of the related MSC population. Similarly, as already demonstrated and confirmed by this study, it is possible to efficiently isolate TSPCs from surgical waste tendon fragments, including hamstring tendons that are used in

ligament reconstruction. Considering that each year worldwide about 200.000 anterior ligament reconstruction procedures are performed [19], it could be noteworthy to ameliorate the knowledge of the potential of these cells to possibly exploit them for future allogeneic applications, also thanks to their low immunogenicity [20]. In this study we have compared the in vitro behavior and the stemness features and multidifferentiation potential of human TSPCs with those of ASCs. The number of cells is crucial for some regenerative medicine applications; in our study the cellular yield of TSPCs was higher with respect to ASCs. Since above all at the first passages in culture the tendon cell population is composed not only by TSPCs but also by terminally differentiated tenocytes, the yield could have been influenced by this factor. However, tenocytes lose their phenotype in vitro with time and passages in culture and, although specific markers have yet to be found, they differ from TSPCs in term of morphology, proliferation potential and expression of stem cells markers such as OCT4 (POU5F1) [20-24]. For all these reasons, the experiments were performed starting from cells at passage 4 (about after 5 weeks of culture), when it was observed that the cell population possessed features peculiar of progenitor cells, including the expression of transcription factor essential for self-renewal maintenance and pluripotency in embryonic stem cells such as POU5F1 and KLF4 [25], and, in agreement with the minimal criteria for defining multipotent mesenchymal stromal cells [26], the expression of specific mesenchymal stem cell markers in standard culture conditions, without any difference with ASCs. Clonogenic assay for fibroblast-like colonies revealed a very similar frequency of cells able to form colony in TSPCs to that observed in ASCs. All TSPC and ASC populations did not show differences in cell metabolic activity suggesting similar cell viability when they are cultured in vitro. Moreover, we also observed that TSPCs and ASCs possessed a comparable doubling time of around three days at passage two. These results are consistent with some previous ones, indicating that human and mouse TSPCs proliferated faster than BMSCs [7, 27], which have been demonstrated to possess a lower proliferation ability than ASCs [28, 291], thus explaining the comparable behavior observed between ASCs and TSPCs. The differences in term of multi-differentiation potential of MSCs from different sources have been deeply investigated: previous studies reported higher multi-differentiation potential of mouse and rat TSPCs respect to BMSCs [7, 27]; others found that human ASCs were able to more efficiently differentiate into the adipogenic lineage, but not into the chondrogenic one in comparison to BMSCs [28-30]. In our study also TSPCs and ASCs presented some differences in term of multi-differentiation potential. In particular, although adipo- and osteo-differentiated TSPCs showed significant increases in the related tissue specific marker expression respect to undifferentiated cells, they possess lower adipogenic and osteogenic potential with regard to ASCs The more pronounced adipogenic potential of ASCs could be attributed to their pre-commitment, whereas the more marked osteogenic potential is also indicated by the very high type I collagen expression that we observed in undifferentiated ASCs, as also already reported in previous studies [31, 32]. Quite unexpectedly, TSPCs seemed to be more susceptible to chondrogenic medium in comparison to ASCs, above all in term of GAG production, although they was already high in undifferentiated ASCs. The more dramatic difference observed between TSPCs and ASCs that underwent the chondrogenic differentiation protocol was the aggrecan gene expression; aggrecan is a proteoglycan, present in cartilage as well as in compressed tendon regions. So, the very high expression of this marker already in undifferentiated TSPCs could be partially related to their tissue origin and consequently to their pre-commitment. Anyway, as

articular cartilage has only a poor capacity for self-repair [33], it could be important to further investigate this aspect as hamstrings can be easily harvested during knee surgery, causing minimal additional morbidity to patients which possibly require autologous regenerative treatment for knee concomitant chondral defects. Collectively, all these data confirm that MSCs from different sources can respond differently to stimuli, posing the question whether optimal conditions of differentiation should be properly adapted to the specific MSC type. To achieve a more efficient in vitro differentiation protocol, it could be useful to further investigate the cell niche factors, as it is widely accepted that several different environmental factors contribute to the overall control of stem cell activity. As little is known about the maturation of tendon and its related tendon cell niche factors, consequently few information are available to develop an efficient tenogenic differentiation medium. In previous in vitro studies on ESCs, ASCs, and BMSCs, the tenogenic induction has been attempted using a variety of growth factors, including bone morphogenic proteins (BMP-2, -5, and BMP-12, -13, -14, also known as GDF-7, -6, and -5, respectively), insulin-like growth factor-1 (IGF-1), TGF-β1, TGF-β3 [8-10, 34-37]. The influence of BMP-12 on tenogenic differentiation was evaluated in several types of MSCs [37, 38], but only few studies were conducted on ASCs [8, 10], and to our knowledge none of them on human cells. Here, for the first time, we evaluated the effect of BMP-12 on the tenogenic induction of human ASCs and TSPCs. Regarding tendon related markers expression, our results demonstrated that scleraxis, transcription factor involved in tendon development, and decorin, proteoglycan that stabilize and align collagen type I and III fibrils [39, 15, 24], were more expressed in undifferentiated TSPCs compared to undifferentiated ASCs, also in this case probably due to their pre-commitment. However, BMP-12 was able to induce in both populations a strong up-regulation of scleraxis expression, as well as a significant increase of decorin one. On the other hand, tenogenic induction did not affect neither collagen type I nor collagen type III expression in both TSPCs and ASCs, probably suggesting that BMP-12 alone is not able to induce a complete up-regulation of tendon extracellular matrix marker. Our data on ASC tenogenic differentiation confirm what observed by Shen who demonstrated that BMP-12 is capable of inducing tenogenic differentiation in canine and mouse ASCs, but our hypothesis that it could be more advantageous to use TSPCs for tendon regenerative applications in comparison to ASCs was not confirmed, at least with the protocol here used [8]. Other tenogenic factors, such as GDF-5/BMP-14, should been tested on human ASCs and TSPCs to assess if it is able to induce a more evident tenogenic induction, although previous studies reported that GDF-7/BMP-12 induced the tenogenic differentiation of canine ASCs more efficiently in comparison to GDF-5/BMP-14 [8]. Moreover, the identification of more specific targets could allow to develop new factors able to induce cells toward tendon differentiations more efficiently. Since tendon is a mechanoresponsive tissue, it can be speculated that, together with biochemical stimuli, appropriate mechanical loads would be helpful to improve the tenogenic differentiation of progenitor cells, in particular of TSPCs, as the modulation of physical stress response is part of their physiological role in tendon tissue; indeed external physical stimuli seem to enhance or accelerate the differentiation into tenocyte-like cells [40]. For this reason, further studies are needed to clarify the cell response to mechanical stimuli and also how they are able to influence the fate of MSCs. Another criticism in this research field is that the markers usually used to assess the tenogenic differentiation are not strictly specific for tendon, as they can be also found in other tissues and cells. For this reason the evaluation can be sometimes misleading and

thus, to obtain more reliable results, the identification of more specific tendon markers may provide some better insight into the in vitro tenogenesis. One of the limitations of the study is the lack of a donor-matched comparison that could have partially reduced the high interdonor variability that often represent a critical point when dealing with primary cells. However, in this case the use of donor-matched cells would have involved ethical issues, since part of the collected tissues should have been intentionally harvested, whereas in our study all the samples used to isolate cells were surgical waste. Nevertheless, the possibility to obtain data form a donor-matched comparison study could be considered in future studies to better characterize MSCs from different sources. Another limitation of this study is the use of the differentiation protocols that were previously developed and adapted for ASCs, and that could have disadvantaged the TSPC differentiation ability. Moreover, the assessment of just tendon specific gene expression without the related protein expression and the testing of a single growth factor without any mechanical stimulation to induce the tenogenic differentation represent other limitations of this study. Further comparative studies about MSC immunogenicity could be useful to better identify the best cell type for allogeneic use.

CONCLUSIONS

Taken together, our results demonstrated that TSPCs do not significantly differ from ASCs in term of clonogenic ability, proliferation and immunophenotypic profile. In comparison to ASCs, TSPCs are less prone to differentiate into adipogenic and osteogenic lineage using our protocols, but are more able to differentiate into chondrogenic- and tenogenic-like cells, potentially opening the possibility to use them as a new cell type to be used for some regenerative medicine applications in the musculoskeletal field.

FUTURE PERSPECTIVES

Preclinical studies have shown the possibility to exploit the properties of ASCs and BMSCs to manage tendon disorders. Our findings, together with others concerning TSPCs, allow to speculate that tendon could represent in the future an appealing and sustainable cell source to isolate MSC for musculoskeletal regenerative purposes. Moreover, thanks to the low immunogenicity of MSCs, it could be possible to preserve TSPCs isolated from surgical waste material for allogeneic use, as currently done for other MSC types, including umbilical cord- and adipose tissue-derived ones.

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Manuscript 3

Different culture conditions affect the growth of human tendon stem/progenitor cells (TSPCs) within a mixed tendon cells (TCs) population

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ABSTRACT

Background: Tendon resident cells (TCs) are a mixed population made of terminally differentiated tenocytes and tendon stem/progenitor cells (TSPCs). Since the enrichment of progenitors proportion could enhance the effectiveness of treatments based on these cell populations, the interest on the effect of culture conditions on the TSPCs is growing.
In this study the clonal selection and the culture in presence or absence of basic fibroblast growth factor (bFGF) were used to assess their influences on the stemness properties and phenotype specific features of tendon cells.
Results: The clonal selection allowed to isolate cells with a higher multi-differentiation potential, but at the same time a lower proliferation rate in comparison to the whole population. Moreover, the clones express a higher amounts of stemness marker *OCT4* and tendon specific transcription factor Scleraxis (*SCX*) mRNA, but a lower level of decorin (*DCN*). On the other hand, the number of cells obtained by clonal selection was extremely low and most of the clones were unable to reach a higher number of passages in cultures.
The presence of bFGF influences TCs morphology, enhance their proliferation rate and reduce their clonogenic ability. Interestingly, the expression of CD54, typical marker of mesenchymal stem cells, is reduced in presence of bFGF at early passages. Nevertheless, bFGF does not affect the chondrogenic and osteogenic potential of TCs and the expression of tendon specific markers, while it was able to downregulate the *OCT4* expression.
Conclusion: This study showed that clonal selection enhance progenitors content in TCs populations, but the extremely low number of cells produced with this method could represent an insurmountable obstacle to its application in clinical approaches. We observed that the addition of bFGF to the culture medium promotes the maintenance of a higher number of differentiated cells, reducing the proportion of progenitors within the whole population. Overall our findings demonstrated the importance of modulating different culture protocols to obtain the most suitable tendon cells for each given clinical application.

INTRODUCTION

Tendon cells (TCs) represent 5% of tendon tissue weight and are the main responsible for the maintenance of tissue homeostasis. The majority of these cells (90-95%) consists in a mixed population made of terminally differentiated tenocytes, representing the predominant cell type,

and tendon stem/progenitor cells (TSPCs), which show several features of stem cells (Bi Y et al 2007; Lui PP et al 2011); the remaining 5-10% is represented by chondrocytes, synoviocytes and vascular endothelial cells (Kannus P et al, 2000). The possibility to use TCs, and specifically TSPCs, in regenerative medicine approaches is currently under investigation, with promising preliminary results expecially deriving from pre-clinical models. Their application ranges from injective treatment into pathological tendons (Ni M et al 2012; Chen L et al 2012) to the use of cell-seeded scaffolds as a form of surgical augmentation (Cao Y et al 2002; Chen JM et al 2007; Stoll C et al 2011; Chen J et al 2011). To date, due to the absence of specific cell markers suitable for the sorting of tendinous subpopulations, culturing tendon cells at low density represents the most used method to isolate specific progenitor subpopulations. Although their real effectiveness is still to be clearly demonstrated, several studies have reported satisfactory results using both low density culture and single colony harvesting to isolate TSPCs from a mixed tendon cell population in rat and rabbit (Lui PP et al 2011, Zhang P et al 2010, Bi Y et al 2007, Rui YF et al 2010). Nevertheless, how the culture conditions can influence the TCs population features and its enrichment in progenitor content is still debated, at least for what concern human-derived cells. Different growth factors have been also used in order to enhance or reduce the proportion of either terminally differentiated tenocytes or tendon stem/progenitor cells within TCs culture. TGF-β, IGF-1, BMPs and PDGF were found to be able to induce tenocyte proliferation and, at the same time, to enhance the expression of tendon markers such as type I collagen (Gaspar D et al 2014). In addition, IGF-1 could enhance the stem properties of TSPCs in culture (Hollyday C et al 2013), whereas basic fibroblast growth factor (bFGF) is known to enhance proliferation of fibroblasts (Yun YR et al 2010) and to maintain tendon marker expression in cultured tenocytes as well as to enhance their proliferation in combination with PDGF or IGF-1 (Caliari SR et al 2013; Qiu Y et al 2013; Costa MA et al 2006).
In this study we have compared different sorting and culture conditions of human TCs, assessing their possible influences on the stemness properties and phenotype specific features of tendon cells. In particular, we focused on the use of bFGF and clonal isolation, with the aim to investigate if these approaches will allow to increase the number of tendon progenitors having a translational potential for regenerative medicine applications.

MATERIALS & METHODS

Tendon cells (TCs) isolation and culture

All of the procedures were carried out at Galeazzi Orthopedic Institute (Italy) with Institutional Review Board approval (M-SPER-014.ver7 for the use of surgical waste). The donors gave their written informed consent to the participation to the study. Gracilis and semitendinous tendons were harvested from leftover tissue that would otherwise be discarded of 8 donors (6 males, 2 females; mean age 31.1±10.9 years) who underwent anterior cruciate ligament reconstruction. Tendon cells were isolated from fragments by enzymatic digestion (24 hours, 37°C,) with 0.3% w/v type I collagenase (Worthington Biochemical Corp., NJ, USA) in high glucose DMEM (HG-DMEM, Life Technologies, Carlsbad, CA, USA) (Rui Y et al 2010; de Girolamo L et al 2013). After the digestion, the samples were filtered through a 100 µm cell strainer (Becton, Dickinson and Co., NJ, USA) and centrifuged (300 g, 5 minutes). The cells were counted and plated at a density of $5x10^3$ cells/cm^2 in

complete medium (CM) consisting of HG-DMEM supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM L-glutamine (all from Life Technologies), and maintained in incubator at 37°C in a humidified atmosphere with 5% CO_2. Once they reached 80%–90% of confluence, the cells were detached with 0.5% trypsin/0.2% EDTA (Sigma-Aldrich) and plated at a density of $3x10^3$ cells/cm^2 and cultured in CM in absence (TCs-) or in presence (TCs+) of 5 ng/mL bFGF (Peprotech, NJ, USA). The cells were used for the experiments until passage 4 (P4). A clonal selection was also performed on all the 8 cell populations included in this study. Cells at P1 were plated at low density, 50 cells/cm^2 (Rui Y et al 2010), and cultured in CM without bFGF. After 3 weeks of culture, the colonies were detached by 0.5% trypsin/0.2% EDTA using Pyrex® cloning cylinder (Corning, NY, USA). Clones were further expanded at normal density to P4 and the multi-differentiation potential and the gene expression of each colony were investigated. Cells cultured at normal density ($5x10^3$ cells/cm^2) were used as controls.

Morphological evaluation

TCs+ and TCs- were daily observed at the optical microscope and their morphology at P4 was evaluated: cells were fixed in 4% paraformaldehyde solution, nuclei were stained with DAPI (1 µg/ml, Life Technologies) and F-actin filaments were stained with Phalloidin (6.6 ⍰M, Life Technologies). The samples were then imaged through a fluorescence microscope (Olympus IX71).

Doubling time evaluation

The doubling time (DT) of both TCs+ and TCs- was recorded from P2 to P4 and calculated according to the following formula: DT = CT/ln(Nf-Ni)/ln2, where CT is the cell culture time (hours), Nf is the final number of cells and Ni is the initial number of cells (Staszkiewicz J et al 2008).

Clonogenic ability assay

A colony-forming unit-fibroblast (CFU-F) assay was performed at P2 and P4. TCs + and TCs - were plated in 6 well plate at different seeding density (1 cell/cm^2; 3 cells/cm^2; 6 cells/cm^2; 12 cells/cm^2; 24 cells/cm^2; 48 cells/cm^2) and cultured in CM with 20% of FBS (Lopa S et al 2014). After 14 days, cells were fixed with 4% paraformaldehyde, stained with 2.3% Crystal Violet staining (Sigma-Aldrich) for 10 min at room temperature and then counted. CFU-F frequency was established by scoring the individual colonies composed of at least 30 cells and expressed as a percentage relative to the number of seeded cells.

Flow cytometry

The immunophenotype of TCs+ and TCs- at P2 and P4 was evaluated by Fluorescence-Activated Cell Sorting (FACS) analysis. Cells were washed twice in cold FACS buffer (phosphate-buffered saline without Ca/Mg^{2+}, 2% fetal bovine serum and 0.1% NaN_3). For each sample, 2.5×10^5 cells were incubated with the following anti-human primary monoclonal antibodies: fluorescein isothiocyanate-conjugated CD90, CD13 and CD45 (Ancell Corp., MN, USA); biotinylated-conjugated CD34 and CD54 (Ancell Corp.); and phycoerythrin-conjugated CD73 (Miltenyi Biotec, Germany).

Streptavidin–phycoerythrin and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies (Ancell Corp.) were used to reveal the expression. Background fluorescence was set up by negative controls and data (10,000 cell fluorescence events) were acquired using a FACSCalibur™ flow cytometer (BD Bioscences, NJ, USA) and analyzed by CellQuest™ software (BD Bioscences).

RNA extraction and Real Time PCR

Gene expression of TCs+, TCs- and clones at P2 and P4 was evaluated by real time PCR (StepOne Plus, Life Technologies). Total RNA was extracted by PureLink® RNA Mini Kit (Life Technologies) and reverse transcripted to cDNA (5 min at 25°C, 30 min at 42°C and 5 min at 85°C) using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA). Twenty ng of cDNA were used as template and were incubated with a PCR mix (50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min) containing TaqMan® Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies) for the following genes: *KLF4* (Hs00358836_m1), *OCT4* (Hs04260367_gh), *MKX* (Hs00543190_m1), *DCN* (Hs00370385_m1) and *SCX* (Hs03054634_g1). Reactions were performed with Applied Biosystems® StepOnePlus™ (Life Technologies). The fold change in expression was normalized against the expression of the housekeeping gene *GAPDH* (Hs99999905_m1). Two replicates were analyzed for each experimental group. Data were expressed according to the ddCt method.

Multi-differentiative potential

Adipogenic potential

TCs+, TCs- and clones at P4 were seeded in 24-well plates at 10^4 cells/cm^2 and differentiated for 21 days in a pulsed adipogenic medium (de Girolamo L et al 2009) 3 days of induction in CM supplemented with 1 µM dexamethasone, 10 µg/mL insulin, 500 µM 3-isobutyl-1-methylxanthine and 200 µM indomethacin (all from Sigma-Aldrich), followed by 3 days of maintenance in CM supplemented with 10 µg/mL insulin. The cells were fixed in 10% neutral buffered formalin for 1 h and stained with Oil Red O (Sigma-Aldrich) for 15 min to evaluate lipid vacuoles formation. Oil Red O was unstained with 100% isopropanol and then quantified by absorbance at 490 nm (Perkin Elmer Victor X3 microplate reader; Perkin Elmer, Waltham, MA, USA).

Osteogenic potential

TCs+, TCs- and clones at P4 were seeded at 10^4 cells/cm^2 and differentiated for 21 days in osteogenic medium consisting of CM supplemented with 10 nM dexamethasone, 10 mM glycerol-2-phosphate, 150 µM L-ascorbic acid-2-phosphate and 10 nM cholecalciferol (all from Sigma- Aldrich) (de Girolamo L et al 2009). The extracellular calcified matrix deposition was measured using Alizarin Red-S staining. Cells were fixed with ice-cold 70% ethanol for 1 h and stained with 40 mM Alizarin Red S (pH 4.1; Fluka-Sigma Aldrich, MO, USA) for 15 min. The dye was extracted with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) and the absorbance was read at 550 nm (Perkin Elmer Victor X3 microplate reader).

Chondrogenic potential

TCs+, TCs- and clones at P4 were seeded at 10^4 cells/cm^2 and cultured for 21 days in chondrogenic medium consisting of HG-DMEM supplemented with 1% of FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, 1 mM sodium pyruvate (all from Sigma-Aldrich), 1% ITS+1 (1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, 0.5 µg/ mL sodium selenite, 50 mg/mL bovine serum albumin and 470 µg/mL linoleic acid, Sigma-Aldrich), 0.1 mM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate, 10 ng/ml TGF-β1 (Peprotech) (Barbero A et al 2004). The cells were fixed with 10% neutral buffered formalin solution, rinsed with distilled water, and stained with Alcian Blue solution (pH 2.5) for 30 minutes (Sigma-Aldrich) to evaluate glycosaminoglycan deposition. The dye was extracted with guanidine hydrochloride (6M) and the absorbance read at 650nm (Perkin Elmer Victor X3 microplate reader) (Ruzzini L et al 2014).

Statistical analysis

Data are expressed as means ± standard deviations. GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used to perform all the analysis. To assess for adjustment of series of values to normal distribution, the Kolmogorov-Smirnov test was applied. Student's t test was applied to compare values between groups when data were normally distributed, otherwise Mann-Whitney's test was performed. p values <0.05 were considered as statistically significant. (*p < 0.05).

RESULTS

Clones obtained by TCs seeded at low density show a higher multi-differentiation potential but a lower proliferation rate in comparison to the whole population

Among the 8 TC populations seeded at low density (50 cell/cm^2), only three gave rise to clones and allowed to proceed with the further experiments.

From each of these, a range of 3-11 clones were isolated, with $6.3\pm4.9x10^3$ cells per clone at P2. At P3 the number of cells increased to $4.8\pm4.2x10^4$, even if only the 72±27% of clones survived. At P4, this proportion decreased to 21±29%, with a mean cell count of $1.1\pm0.6x10^4$.

The osteo-differentiated clones showed an increase of +36% in matrix deposition with respect to whole population, but this difference was not statistically significant. Similarly, an increase in their chondrogenic ability was observed in chondro-differentiated clones with respect to whole population, but the high variance among clones prevent the identification of statistical significances (+102%, n.s.). Indeed, each clone demonstrated extremely different features in terms of differentiation potential, ranging from great to none ability to produce calcified matrix or glycosaminoglycans.

Clones obtained by TCs seeded at low density possess a higher expression of OCT4 and SCX but a lower level of DCN

Just one population over the 8 tested allowed a suitable cell harvesting for mRNA extraction. The clones from this population showed a quite variable relative expression in all analyzed markers, with no particular correlation with their differentiation ability. Interestingly, the mean relative expression of *OCT4* and *SCX* in the clones resulted 6-fold higher than the whole population, while a 10-fold decrease was observed for what concern *DCN*. Moreover, a positive correlation was found between the expression of *OCT4* and *SCX* among clones of the same population (Pearson's R^2=0.89, $p<0.05$), while a negative correlation was observed comparing *SCX* and *KLF4* (Pearson's R^2=-0.91, $p<0.05$).

The presence of bFGF influences TCs morphology, proliferation rate and clonogenic ability

TCs showed a spindle shaped morphology when cultured without bFGF, while a more fibroblast-like morphology was observed in cultures with bFGF (Fig. 1A). As expected, the proliferation rate was higher in presence of bFGF, with a doubling time of 70±26 and 62±16 hours at P3 and P4, respectively (-18% and 31% vs TCs-, $p<0.05$) (Fig. 1B). On the other hand, TCs- showed a higher clonogenic ability both at P2 and P4 (3.2%±2.3% and 4.2%±1.6%, respectively) in comparison with TCs+ (0%±0.0%and 1.6%±1.1%) (Fig. 1C, D).

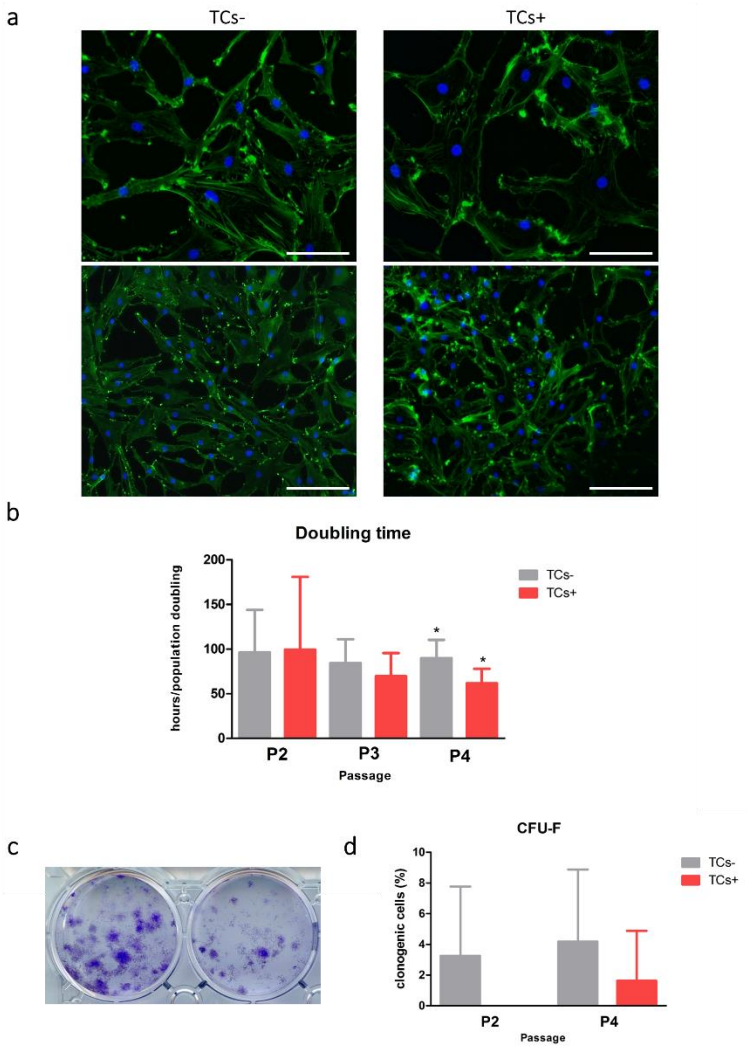


Figure 1. *Morphology and clonogenic potential. (a) Cells in culture at P4 and after DAPI/phalloidin staining (scale bars 50 µm for upper panel and 100 µm for lower panel). (b) Proliferation ability during passages in culture expressed as mean of doubling times. Levels of significance: *p<0.05 (c) Representative micrographs of CFU-F at P4. (d) Percentage of clonogenic cells at P2 and P4 (n=4).*

Surface marker expression of CD54 is reduced in presence of bFGF at early passages

Cell surface marker analysis was performed during expansion (P2 and P4). The presence of bFGF did not influence the percentage of cells positive for the stemness-related markers CD13, CD73, CD90, as well as the percentage of cells negative for CD34, CD45 expression, which are known to be hematopoietic cell markers. The only difference between TC+ and TC- was observed in CD54 expression, which was significantly higher expressed in TCs- at both P2 and P4 in comparison with TCs+ ($p<0.5$). For both the cell types, an increasing trend of CD54 expression from P2 to P4 was observed. The complete data concerning cell surface marker expression are shown in Table 1.

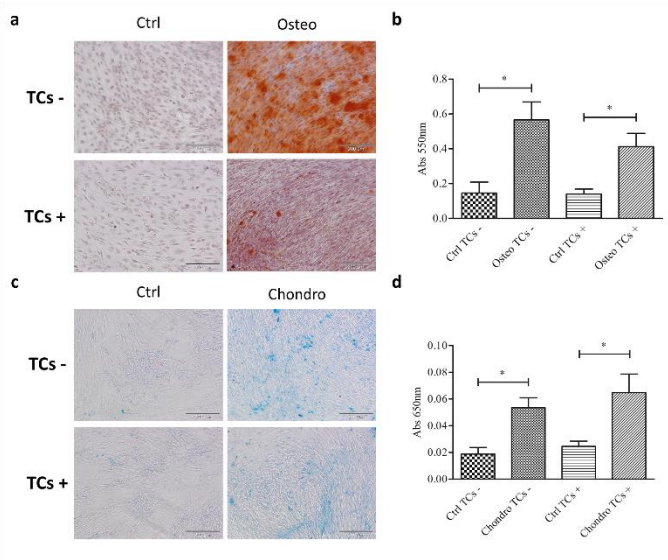
	Surface Markers	CD13	CD34	CD45	CD54	CD73	CD90
TCs -	P2	99.7%±0.1% +++	3.7%±1.9% -	2.6%±1.6% -	85.7%±6.5%* ++	99.8%±0.1% +++	99.1%±0.7% +++
	P4	99.9%±0.1% +++	1.7%±0.7% -	0.8%±0.7% -	92.1%±3.0%* +++	99.9%±0.1% +++	96.8%±0.5% +++
TCs +	P2	99.7%±0.1% +++	2.9%±3.2% -	0.8%±0.7% -	46.6%±29.2% +	99.7%±0.4% +++	99.0%±0.5% +++
	P4	99.7%±0.4% +++	2.7%±1.5% -	0.4%±0.3% -	82.5%±9.6% ++	99.8%±0.4% +++	98.6%±0.2% +++

bFGF does not affect the chondrogenic and osteogenic potential of TCs

Both TCs+ and TCs- cultured for 14 days in adipogenic medium showed no appreciable intracellular lipid vacuole production in comparison with cells maintained in non-inductive medium (data not shown).

Both in presence or absence of bFGF, TCs were able to differentiate towards the osteogenic lineage. Indeed, after 14 days of culture in osteogenic medium the deposition of calcified matrix was significantly higher in differentiated cells with regards to controls (3.9 fold in TCs- and 3.0 fold in TCs+ $p<0.05$, Fig. 2A,B), with no differences between culture conditions. At the same manner, the chondrogenic potential measured after 21 days of culture in chondro-inductive condition, was similar between the two populations with significantly higher amount of glycosaminoglycans in comparison with the respective controls (2.9 fold in TCs- and 2.6 fold in TCs+, $p<0.05$, Fig. 2C, D).

Figure 2. *Multi-differentiative potential. (a) Osteogenic differentiated TCs- and TCs+ in culture and after AR-S (scale bars 200 µm). (b) Quantification of calcified matrix by AR-S staining and extraction in undifferentiated (Ctrl) and osteogenic-differentiated (Osteo) cells (n=6). (c) Chondrogenic differentiation of TCs- and TCs+ in culture and after Alcian Blue staining (scale bars 500 µm). (d) Quantification of glycosaminoglycans deposition by Alcian Blue staining and extraction in undifferentiated (Ctrl) and chondrogenic-differentiated (Chondro) cells (n=4). Levels of significance: * p<0.05.*



bFGF decreases the level of the stemness marker OCT4, but exerts no influence on the tendon markers expression

A significant decrease of 1.36 fold ($p < 0.05$) in the expression of *OCT4*, was observed in TCs+ in comparison with TCs- cultured for 4 passages. Moreover, the cells cultured in presence of bFGF showed a clear decrease in *OCT4* expression from P2 to P4, even if this difference was not statistically significant. On the contrary, *KLF4* expression was stable in both the cell culture conditions and during passages (Fig. 3).

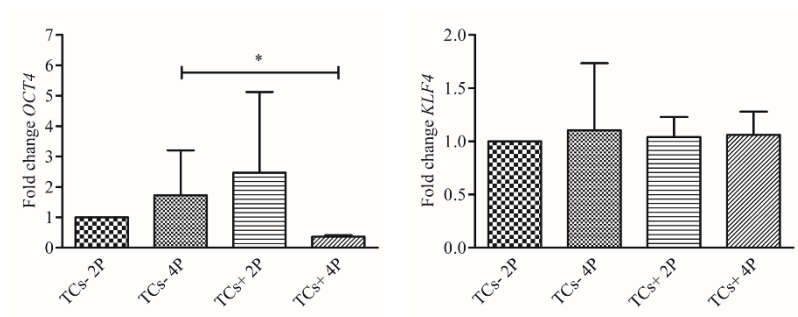


Figure 3. *Gene expression of stemness markers. OCT4 and KLF4 genes expression in TCs- and TCs+ at P2 and P4 normalized to GAPDH (n=6). Levels of significance: * p<0.05.*

The expression of *MKW*, *DCN* and *SCX* was measured at P2 and P4. Slight and no statistically significant differences were observed between TCs- and TCs+. In particular the TCs- expression of *DCN* increased with time in culture, and the presence of bFGF further up-regulated its expression (Fig. 4).

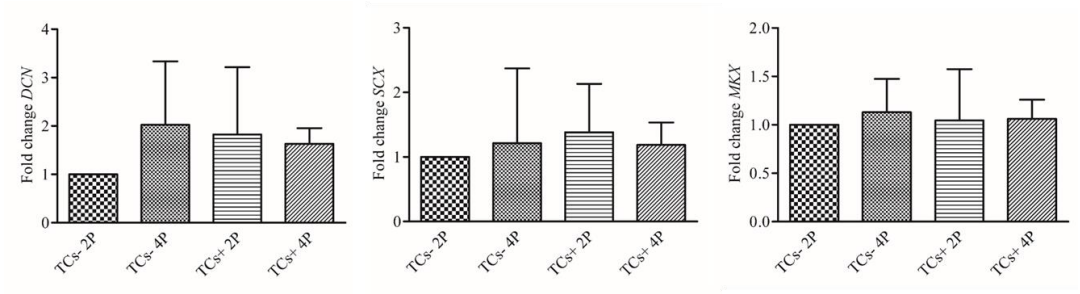


***Figure 4**. Gene expression of tendon markers. MKX, DCN and SCX genes expression in TCs- and TCs+ at P2 and P4 normalized to GAPDH (n=6).*

DISCUSSION

Adult mesenchymal stem cells represent a tool for clinical applications in regenerative medicine (Ménard C et al 2013). Indeed, the application of MSCs to tendon disorder is still under investigated, mainly due to the lack of knowledge about their tenogenic potential. Therefore, since the description of tendon stem/progenitor cells in 2007 by Bi and colleagues (Bi Y et al 2007), the possible use of autologous tendon cells in tendon regenerative medicine approaches is subject of a growing interest (Ho JO et al 2014). Nevertheless, the presence of different subpopulations among the tendon resident cells represents a largely unexplored field of investigation. The lack of a specific terminology and the difficulties in purifying, expanding and maintaining the different cellular subsets are the main obstacles in this field (Docheva D et al 2015). In view of future applications of regenerative approaches for the treatment of tendon disorders, it would be crucial to define the most suitable culture conditions to isolate the different sub-populations within tendon cells, and to improve their ability in promoting tendon healing and regeneration. For this purpose, different culture conditions have been proposed to enrich *in vitro* cultures with one population or the other, as the application of specific growth factors or patterned substrates, such as tendon derived matrix (ZhangJ et al 2011) to simulate the features of the native microenvironment of tenocytes (Gaspar 2014).

In our study we investigated the presence of Tendon Stem/Progenitor Cells (TSPCs) within the tendon resident cells (TCs) isolated from human gracilis and semitendinosus tendons, and we assessed the effects of bFGF and clonal selection as possible strategies to modulate their predominance in culture.

Almost 25% of the TC populations analyzed gave rise to few clones when cultured in clonal selection conditions. The clones showed a higher mean ability to differentiate toward osteogenic and chondrogenic lineages, as revealed by the production of calcified matrix and glycosaminoglycans, respectively, in comparison with the corresponding whole population, with high differences in the performance of each clone though. Interestingly, the stemness marker *OCT4*, as well as the early tenogenic marker *SCX*, were up-regulated in clones with regards to the whole population, and the expression of these two genes was positively correlated. On the contrary, the late tenogenic marker *DCN* was less expressed in clones in comparison with whole population. Moreover, the expression of the stemness marker *KLF4* resulted slightly higher in clones with respect to the cells cultured at

normal density, in particular in presence of bFGF. Nevertheless, the *KLF4* pattern of expression could vary between different conditions, such as stages of cell differentiation, and this could explain the negative correlation between this marker and *SCX* (Zhang P et al 2010).
As expected, all these data showed that the selected clones exhibited a more undifferentiated phenotype in comparison with whole TC population. Despite these encouraging data, the low number of cells that can be isolated by clonal selection make this approach hardly applicable to cell-based therapy.
b-FGF is often used to maintain the cell multipotency in many cells types, and, specifically in tendon cell populations, to enhance the tendon lineage differentiation (Tsutsumi S et al 2001; Hankemeier S et al 2005; Tokunaga T et al 2015). However, its role in promoting the predominance of tendon progenitor cells within the whole tendon cell population have not been investigated so far.
This growth factor, known to enhance cell proliferation in many cell types (Ornitz DM et al 2015), exerted the same effect on TCs. The evaluation of the immunophenotype of TCs treated or not with bFGF, showed no difference in term of CD13, CD73, CD90, CD34 and CD45 expression. On the contrary, starting from early passages till passage 4, the expression of the adhesion molecule CD54 (Intercellular Adhesion Molecule-1, ICAM-1) was higher in TCs cultured in absence of bFGF in comparison with TCs+. CD54 has been characterized as one of the peculiar mesenchymal stem cell markers (Calloni Ret al 2013) and it is important in inhibiting the osteogenic differentiation of mesenchymal stem cells (Xu FF et al 2014). The higher expression of CD54 in TCs- in comparison with TCs+ and the increase from passage 2 to passage 4 of its expression in both the populations could indicate a progressive enrichment of progenitors in tendon cells during culture, particularly when maintained in absence of bFGF.
Accordingly, for what concern stemness features, TCs- showed a higher clonogenic ability and OCT4 expression in comparison with TCs+. Since these gene is known to have a role in sustaining self-renewal capacity of adult stem cells (Niwa H et al 2007), the higher expression in TCs- even at the latest passage (P4) in comparison with TCs+, suggest that the lack of bFGF allowed for the maintenance of a more undifferentiated cell phenotype. The assessment of the multilineage differentiation of TCs in term of adipo, chondro- and osteogenic potential confirmed the findings of a previous study (Stanco D et al 2015). Indeed, our study showed that both TC- and TCs+ had no appreciable adipogenic potential, but they were able to deposit a consistent amount of calcified matrix and glycosaminoglycans in comparison with control cells, with no appreciable differences between them. Similarly, the expression of tendon markers such as *MKW*, *DCN* and *SCX* was not influenced by the presence of bFGF.

CONCLUSION

In conclusion, our study highlights the importance of modulating different culture protocols to obtain the most suitable tendon cells for each given clinical application. In particular, the addition of bFGF to the culture media allowed for the maintenance of a higher number of differentiated cells, without causing the loss of the tenogenic phenotype. On the other hand, in order to obtain a greater amount of tendon progenitor cells the use of bFGF is not suggested.

Taken together our results showed how much the different cell populations within the tendon tissue are sensitive to the biochemical environment. Further *in vitro* as well as pre-clinical studies are needed to better correlate the use of different stimuli to the cell responsiveness. Moreover, since tendons are known to be greatly affected to mechanical stress and forces, it would be interesting to identify a possible synergistic effect of biochemical with physical factors.

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Manuscript 4

Development of an effective strategy for the tenogenic differentiation of human Adipose-, Bone Marrow-and Tendon-derived Mesenchymal Stem Cells

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ABSTRACT

Even though the adipogenic, chondrogenic and osteogenic differentiation potential of Mesenchymal stem cells (MSCs) have been deeply studied, the in *vitro* tenogenic differentiation of MSCs has been not well established yet. Then, the purpose of the this work is to evaluate multiple biochemical stimulations to establish the most effective treatment to promote the tenogenic differentiation of MSCs, performing also a comparison of the tenogenic potential of Bone Marrow derived Stem Cells (BMSCs), Adipose derived Stem Cells (ASCs) and Tendon stem progenitor cells (TSPCs) and select the best performing cell source. Five different combinations of growth factors and supplements (including BMP-12, bFGF, TGF-β3, IGF-1, CTGF, AA) were used as tenogenic inductive treatments. The single addition of Bone Morphogenetic Protein 12 (BMP-12) was considered as positive control. First we selected the most effective growth factors combinations by the evaluation of the expression of two tendon markers, scleraxis (SCX) and decorin (DCN), in immunofluorescence assays. The quantification of these markers was then assessed by an immunofluorescence-based high-throughput system, Operetta® (Perkin Elmer). The gene expression of a wider range of tenogenic markers such as SCX, DCN, Mohawk homeobox protein (MKX), tenomodulin (TNMD), tenascin (TN-C) and type I collagen (COL1A1) was also evaluated. In all the cell types, BMP-12 alone increased the production of DCN, MKX and TNMD whereas the presence of Transforming Growth factors β3 (TGF β3) enhanced the production of SCX and COL1A1. In addition, we also observed that TGFβ3 actively inhibits the production of DCN and TNMD. TSPCs showed the highest levels of SCX and DCN expression with a lower donor-associated variability if compared to the other cell sources. Both BMSCs and ASCs showed to be good responders to tenogenic induction, but ASCs showed a higher predisposition to acquire the typical tenogenic phenotype. Basing on the large amount of the results obtained in this study, we can furtherly support the hypothesis that a more satisfactory tenogenic differentiation of MSCs is obtained using multiple factors rather than a single one, and that ASCs could be the best non tissue-specific cell source for cell-based tendon regeneration applications.

INTRODUCTION

Tendon disorders are a complex class of pathologies affecting a wide percentage of the population worldwide. Current conservative treatments and pharmacological therapy with non-steroidal anti-inflammatory drugs (NSAIDs) are efficient in pain relief but fail to restore tissue homeostasis, so that in several cases the progression of the pathology eventually lead to surgical intervention. On the

other side, surgical interventions are very invasive and the recovery from them is a long process and re-occurrence of the pathology is frequent (Maffulli N et al, 2006). Therefore, in the last decades the search for alternative treatment has increased and among the different possibilities Mesenchymal Stem cells (MSCs) seem to represent a valuable tool to improve the clinical outcome in case of tendon pathology. In fact, MSCs can promote the formation of a regenerative microenvironment thanks to their ability to act on resident progenitor cells and to differentiate themselves into tenocyte-like cells (Lee JY et al, 2011; Lui PP et al, 2014; Manning CN et al, 2015; Chen HS et al, 2015). However, the direct use of MSCs in the target site in in *vivo* models of tendon disorders could lead to the formation of ectopic bone or cartilage tissue (Lui PP et al, 2012; Harris MT et al, 2004). This side effect was already experienced in other MSCs application and many literature reports suggested that a priming of cells before treatment would reduce this issue. For example, the pre-differentiation of ASCs towards the osteogenic lineage resulted a successful strategy in bone repair application (Yoon E et al, 2007). Then, the previous possibility to induce MSCs toward the tenogenic lineage before using, may ameliorate the outcome avoiding an impaired tissue regeneration and remodeling. Nevertheless, differently from adipogenic, chondrogenic and osteogenic differentiation, the proper in *vitro* tenogenic differentiation protocol of MSCs is still unclear.
MSCs show dissimilar phenotypic aptitudes, depending on their specific tissue of origin. Therefore, the identification of the most suitable source of MSCs for this application is crucial to obtain an efficient and stable differentiation toward the tenogenic lineage.
In this context, the aim of the present work is to establish an effective tenogenic treatment, able to induce the expression of a stable tenogenic phenotype in MSCs, by the assessment of the tenogenic potential of Bone Marrow Derived Stem Cell (BMSCs), Adipose derived Stem Cells (ASCs) and Tendon Stem Progenitor Cells (TSPCs) in presence of different biochemical stimuli, with the final purpose of defining the optimal protocol and cell source for regenerative medicine applications of MSCs in tendon disorders.

MATERIALS AND METHODS

MSCs isolation and culture

Waste surgical samples were collected at our Institute as under written consent of the patients (M-SPER-014.ver7 for the use of surgical waste). In particular, hBMSCs were isolated from the femoral canal of five donors who underwent to hip replacement. Bone marrow aspirates were washed in phosphate-buffered saline (PBS) and centrifuged at 623 g for 10 minutes. The fraction of mononuclear cells was suspended in complete medium (CM) composed of Dulbecco's Modified Eagle Medium High Glucose (SIGMA Aldrich), 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM L-glutamine (Life Technologies) and plated in culture flasks at a density of $5x10^3$ cells/cm^2.
hASCs were isolated as previously described (de Girolamo L et al, 2013) from adipose tissue of five donors who underwent abdominoplasty. The adipose tissue was minced and then washed with PBS. The samples were digested digestion in 0.075% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C for 30 min. The obtained stromal vascular fraction (SVF)

was centrifuged (1200 g, 10 min) and then filtered through a 100 µm nylon cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); 10^4 cells/cm^2 were plated in CM.
hTSPCs were isolated from fragments of semitendinosus and gracilis tendons collected from five donors who underwent anterior cruciate ligament (ACL) reconstruction with hamstring. After 16 hours of enzymatic digestion with collagenase type I at 0.3% w/v (Worthington Biochemical Corporation, Lakewood, NJ, USA) (Rui YF et al, 2010; de Girolamo L et al, 2013), the samples were filtered through a 100 µm cell strainer (Becton, Dickinson and Co., NJ, USA) and centrifuged (300 g, 5 minutes). hTSPC were plated at a density of $5x10^3$ cells/cm^2 in CM and maintained in incubator at 37°C in a humidified atmosphere with 5% CO_2. All the populations were cultured until passage 4, when they were detached and seeded for the following experiments.

Tenogenic induction growth factors-mediated

At passage 4, hBMSCs, hASCs and hTSPCs were seeded at seeding density of 3000 cells/cm^2 and treated with different combinations of growth factors (Table 1) for three days. Briefly, all cell types were maintained in a medium composed of HG-DMEM, 1% FBS, 1% PSG, Ascorbic Acid 25µg/ml, b-FGF 5ng/ml supplemented with one or more of the following growth factors: bone morphogenetic protein 12 (BMP-12), Connective Tissue Growth Factor (CTGF), Transforming Growth Factor β3 (TGF β3) and Insulin Growth Factor 1 (IGF-1). After three days of culture, media were changed and the cells maintained in a maintenance medium composed of DMEM, 1%FBS, 1%PSG, AA 25µg/ml, b-FGF 5ng/ml until day 10. For each population, cells maintained in CM were considered as CTRL group.

	Medium	**Growth factor**
BMP-12 medium	DMEM, 1%FBS, 1%PSG, AA 25µg/ml, b-FGF 5ng/ml	BMP12 (50 ng/ml)
MIX 1		BMP12 50ng/ml
		CTGF 100 ng/ml
		IGF-1 50ng/ml
		TGF β3 20ng/ml
MIX 2		CTGF 100 ng/ml
		IGF-1 50ng/ml
		TGF β3 20ng/ml
MIX 3		CTGF 100 ng/ml
		IGF-1 50ng/ml

	BMP12 50ng/ml
MIX 4	CTGF 100 ng/ml
	BMP12 50ng/ml
	TGF β3 20ng/ml
MIX 5	IGF-1 50ng/ml
	TGF β3 20ng/ml
	BMP12 50ng/ml

Table 1: *the different inductive tenogenic media tested in these studies. The single addition of BMP-12 was considered as CTRL group*

Immunofluorescence analysis

Immunofluorescent staining for scleraxis (SCX, rabbit anti-human, 0.5 µg/ml) and decorin (DCN, mouse anti-human, 0.5µg/ml) was assessed in all samples. Cells were fixed with frozen methanol 100% for 5 minutes at room temperature and then washed with ice cold PBS. Samples were then treated with a blocking solution containing 1% of bovine serum albumin (BSA, Sigma Aldrich). Then, the diluted primary antibodies (for SCX, rabbit anti-human, 0.5µg/ml and DCN, mouse anti-human, 0.5µg/ml; Abcam,) were added to the samples and maintained overnight at 4°C. Then, cells were rinsed twice with PBS + 0.1% Tween 20 and then incubated with the diluted secondary antibody (Goat Anti-Rabbit IgG H&L, Alexa Fluor® 488, 2µg/ml; Rat monoclonal (SB74g) Anti-Mouse IgG2b gamma chain, Alexa Fluor® 647, 2µg/ml; Abcam) 1 hour at room temperature. Finally, the cells were washed and incubated with 0.1 µg/mL DAPI (DNA stain) for 1 min.
For an efficient quantification of these markers and for the qualitative evaluations of morphological modifications at different time points, an immunofluorescence-based high-throughput system, Operetta® (Perkin Elmer) was used at both time points.
Immunofluorescence images were captured by Operetta® (Perkin Elmer,MA, USA), and analyzed by Harmony Software. Basic Harmony software functions were used to evaluate mean fluorescence in all different channel (GFP, Far Red, DAPI), cell count (DAPI), cell dimension (phase contrast). Moreover, a protocol for identification of Decorin positive filament area in all fields was developed.

RNA extraction and gene expression analysis

A real time PCR (StepOne Plus, Life Technologies) was performed at day 3 and 10. Total RNA was extracted by PureLink® RNA Mini Kit (Life Technologies) and reverse transcripted to cDNA (5 min at 25°C, 30 min at 42°C and 5 min at 85°C) using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories,

CA, USA). Twenty ng of cDNA were used as template and were incubated with a PCR mix (50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min) containing TaqMan® Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies) for the following genes: SCX (Hs03054634_g1), DCN (Hs00370385_m1), COL1A1 (Hs01076777_m1), MKX (Hs00543190_m1), TN-C (Hs01115665_m1) and TNMD (Hs00943212_m1). Reactions were performed with Applied Biosystems® StepOnePlus™ (Life Technologies). The fold change in expression was normalized against the expression of the previously validated housekeeping gene YWHAZ (Hs03044281_g1). Two replicates were analyzed for each experimental group. Data were expressed according to the dCt/ddCt method.

Statistical analysis

Statistical analysis was performed by GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as the mean ± SD. Normal distribution of values was assayed by Kolmogorov–Smirnov normality test, while one-way analysis of variance (ANOVA) for repeated measures, with Bonferroni's correction, was used to compare data over time. *p values <0.05 were considered statistically significant.*

RESULTS

Screening study of Scleraxis and Decorin Expression

The expression of the early marker SCX and the late marker DCN was assessed by high throughput immunofluorescence experiments. Different conditions were compared to explore the effect of BMP-12, TGFβ3, CTGF, Ascorbic Acid, bFGF and IGF-I. In all the analyzed cell types, the expression of DCN was enhanced by BMP-12 alone and suppressed by TGFβ3 (Figure 1).

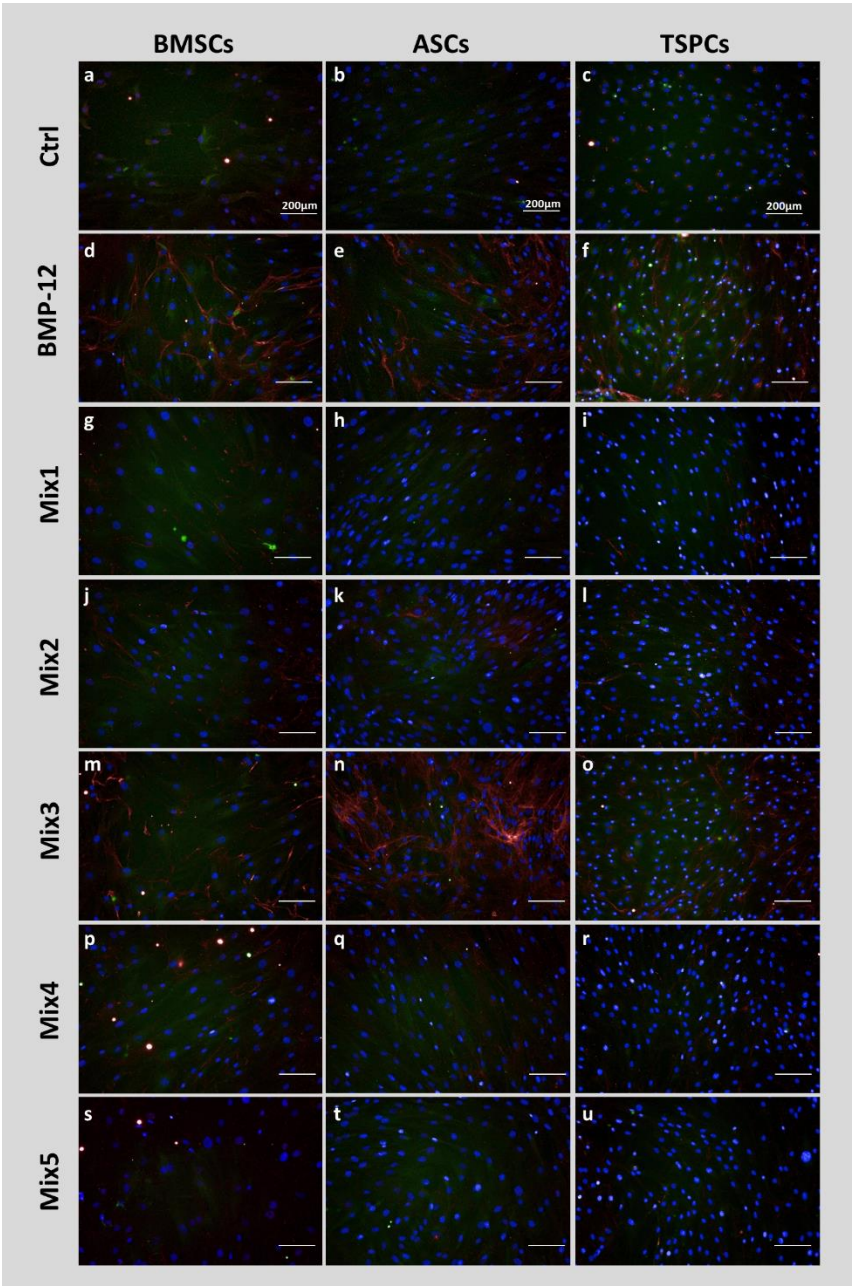

***Figure 1**: Representative micrographs of BMSCs (a-d-g-j-m-p-s), ASCs (b-e-h-k-n-q-t) and TSPCs (c-f-i-l-o-r-u) after three days of culture with different tenogenic media (Mix1, Mix2, Mix4, Mix4, Mix5). Un-treated (Ctrl, a-b-c) and BMP-12 induced (BMP-12, d-e-f) cells for each cell types were considered as negative and positive control respectively. Magnification 10X (200µm). Immunofluorescent staining for SCX (rabbit anti-human, 0.5µg/ml; secondary antibody Goat Anti-Rabbit IgG H&L, Alexa Fluor® 488, 2µg/ml) and DCN (mouse anti-human, 0.5µg/ml; secondary antibody Rat monoclonal (SB74g) Anti-Mouse IgG2b gamma chain, Alexa Fluor® 647, 2µg/ml). DCN -- > RED SCX -- > GREEN*

Indeed, all the samples treated with media containing TGFβ3 (Mix1, Mix2, Mix4 and Mix5) showed no expression of this marker after 3 days of tenogenic induction. In this setting, the expression of scleraxis did not resulted consistent within the experiments, with extremely slight differences between signal and background. At higher magnification it was possible to observe the presence of nuclear dots in BMP-12 treated ASCs, in accordance with the supposed subcellular localization of SCX (Figure S1). The lack of signal observed in Mix2 and Mix4 leaded to the exclusion of these media from further analysis.

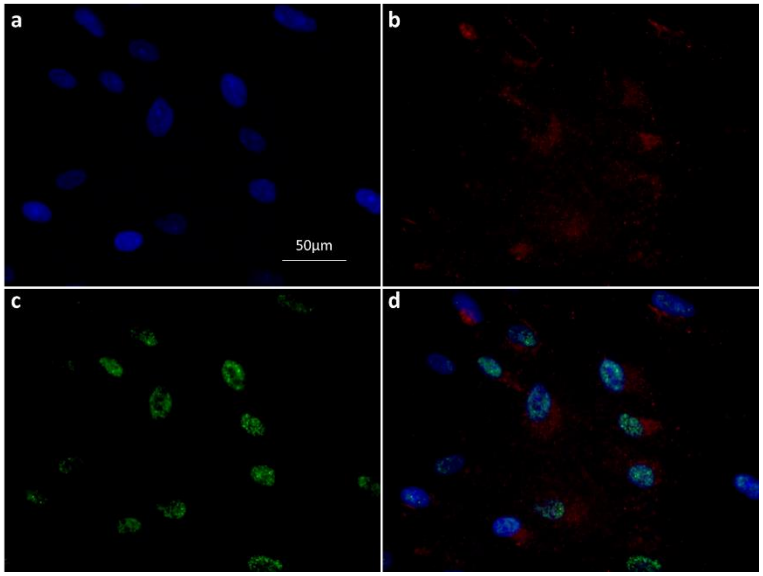

Figure S1: *Representative micrograph of ASCs treated with TGF ß3 (Mix1).* ***a****-DAPI;* ***b****-DCN;* ***c****-SCX;* ***d****-MERGED. Magnification 40X (50µm*)

Role of bFGF and Ascorbic Acid in the induction and maintenance of DCN expression

During the induction phase (days 0-3), the presence of bFGF and AA slightly enhances the expression of DCN in media supplemented with others growth factors (BMP-12, Mix1, Mix3, and Mix5). Nevertheless, a great difference was observed in the expression of DCN when cells were maintained in culture to day 10 in DMEM+1%FBS or with the same medium with bFGF and AA. Indeed, in presence of these factors, the expression of DCN increased during the time in culture, while it was lost in non-supplemented medium (Figure 2). This observation was also confirmed in all the different cell types.

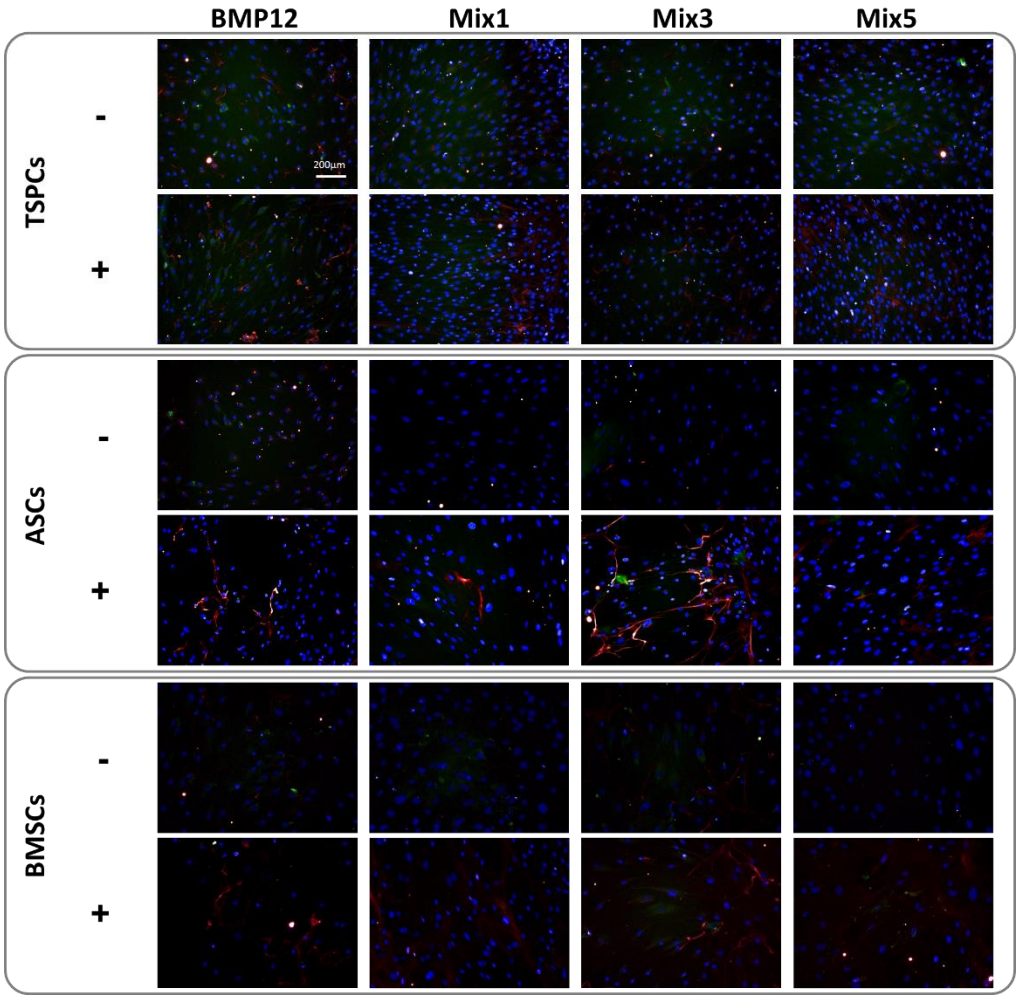

Figure 2: *Representative micrographs of differentiated TSPCs, ASCs and BMSCs without (-) and with (+) AA and b-FGF at 10 days. Magnification 10X (200µm).*

TGFβ3 induced the highest expression of SCX and COL1A1

The gene expression analysis of tendon specific markers revealed a clear role for TGFβ3 in the induction of Scleraxis and Collagen Type I, after 3 days of tenogenic induction. Indeed, while BMP-12 alone was able to upregulate slightly these markers, media containing TGFβ3 (Mix1 and Mix5) enhance their expression, in particular in TSPCs and ASCs. The inhibitory effect of TGFβ3 on DCN expression was confirmed by the gene expression analyses. The expression of the early tenogenic marker MKX was also enhanced by TGFβ3, but only in TSPCs cells, while all other media and cell types demonstrated lack of induction of this transcription factor (Figure 3).

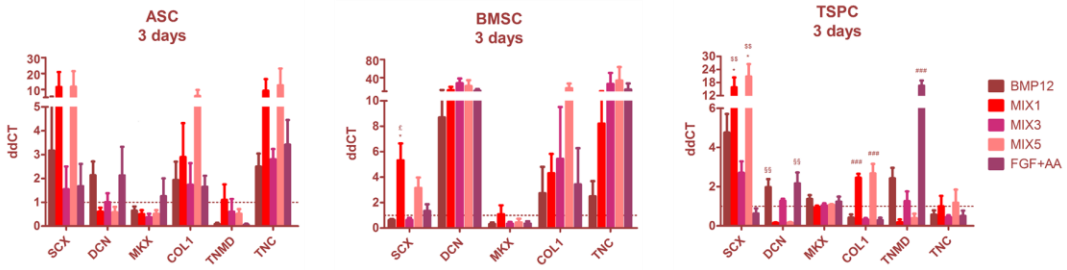

Figure 3: *Gene expression analysis of SCX, DCN, MKX, COL1, TNMD, TN-C. Data are expressed as ddCT; vs CTRL: * p<0.05; vs BMP12: £ p<0.05; vs all: ### p<0.001; vs MIX1 and MIX5: § p<0.01; vs FGF+AA: §§ p<0.01*

At 10 days, and in specific one week after the shift from induction to maintenance in bFGF+AA supplemented medium with 1%FBS, the expression of SCX and COL1A1 was found to be still upregulated in cells treated with TGFβ3 containing media (Mix1 and Mix5). Interestingly, the production of DCN resulted increased in the same media, especially for TSPCs, in contrast with what observed after 3 days of induction. In TSPCs the expression of MKX is significantly increased.

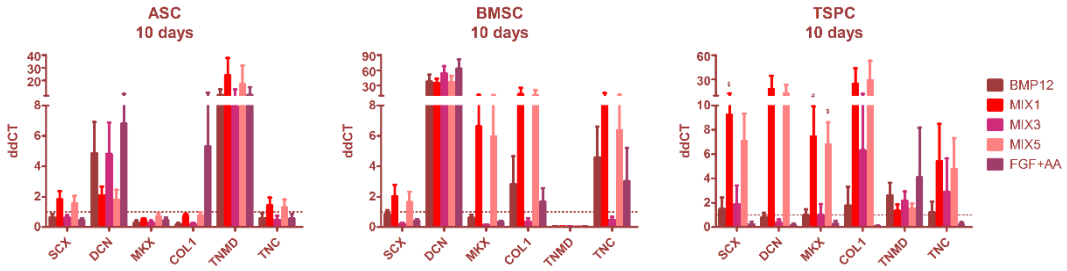

Figure 4: *Gene expression analysis of SCX, DCN, MKX, COL1, TNMD, TN-C. Data are expressed as ddCT; vs all: # p<0.05; vs FGF+AA: § p<0.05; §§ p<0.01*

TSPCs is the most suitable cell type for tendon differentiation and ASCs performed better than BMSCs

In terms of tendon markers expression and morphology, TSPCs appear as the best performing cell type in all of our experiments. Indeed, basal expression of tendon specific transcription factors (MKX and SCX) were higher with respect to ASCs and BMSCs. Moreover, BMP-12 alone was able to exert a prompt effect on these cells, while other growth factors were required by MSCs. Nevertheless, ASCs were able to express large amount of COL1A1 and DCN, even compared to TSCPs, and for what concern the expression of SCX, MKX and TNMD they provided higher level of expression with respect to BMSCs (Figure 5).

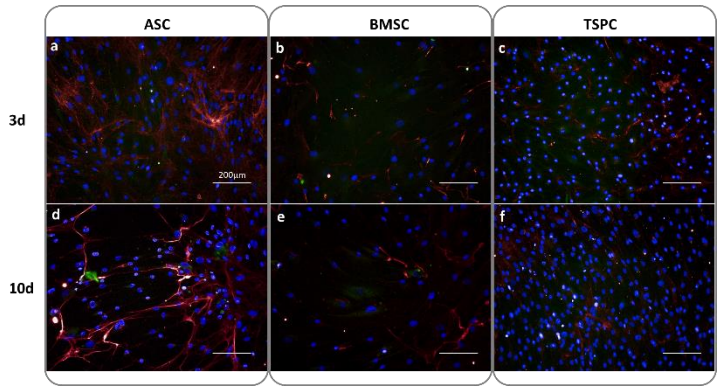

Figure 5: *Representative micrographs of ASCs (a-d), BMSCs (b-e) and TSPCs (c-f) treated with Mix3, at 3 and 10 days. Magnification 10X (200µm)*

DISCUSSION

The main finding of our work is that a proper in *vitro* tendon differentiation protocol should take in account different stages, from cell commitment to tenocyte maturation. Our proposed tenogenic differentiation protocol comprises a first phase of 3 days induction with BMP-12, TGFβ3, bFGF, Ascorbic Acid and IGF-I in presence of 1%FBS, followed by a second phase of at least 7 days where cell should be maintained in 1% FBS, ascorbic acid and bFGF, at concentration indicated in Materials and Methods section.

In the first phases the increase in tendon specific transcription factor is enhanced by TGFβ3 and BMP-12, while a second phase characterized by matrix deposition and fibres maturation then follows. This second phase appears to be induced by ascorbic acid under the guidance of previous inductive signals. Our observation are in accordance with the physiological tenogenic differentiation cascade (Dex S et al, 2016).

Transforming Growth Factor β superfamily (TGF β) has been extensively investigated in several literature reports and up to today together with TGF β, Bone Morphogenetic Proteins (BMPs) and Growth Differentiation Factors (GDFs) represent the most promising molecular signals driving the tenogenic differentiation of MSCs (Havis E et al, 2014; Park A et al, 2010; Lee YJ et al, 2011; Shen H et al, 2013; Stanco D et al, 2015; Barsby T et al, 2014).

Other growth factors such as Connective Tissue Growth Factor (CTGF), Insulin Growth Factor 1 (IGF-1), basic Fibroblast Growth Factor (b-FGF), that exert a more incisive role in the tendon healing rather than in tendon development, have been investigated and their involvement in cell differentiation, proliferation, chemotaxis and ECM synthesis have been reported in literature (Ackermann P et al, 2013; Muller SA et al, 2015; Lu YF et al, 2016). It has been demonstrated that b-FGF is associated with the maturation of tenocytes (Chan J et al, 1998) and that it is able to stimulate collagen type I and Type III production (Chan BP et al, 2000), whereas CTGF increases the expression of collagen type I and tenascin-C in MSCs (Lee CH et al, 2010). IGF-1 is particularly involved in the remodeling stages of the healing process and it is able to increase the levels of mRNA for collagen, tenomodulin and scleraxis (Herchenhan A et al, 2014). It is also currently known that the addition of ascorbic acid promotes the synthesis of collagen, the maintenance of the ECM in tendon and

ligament tissues, and therefore it facilitates the healing process of the tissue (Lui PP et al, 2016; Rehmann MS et al, 2016).
It is evident that the stimulation with TGFβ3 induced the expression of specific transcription factors in tendon derived progenitors, in particular of SCX that appears to be in the cascade right after tendon progenitor stage and before tenoblast phase, as well as it happens for the expression of collagen (Brent AE et al, 2003; Havis E et al 2014). After this phase, the tendon differentiation process should move forward up to the proper tenocyte, that assumes a longer morphology and express later tendon markers, such as TNMD and DCX (Dex S et al, 2016), an event that follows the treatment by ascorbic acid and bFGF, as also showed by our findings.
Since SCX represent the most recognized marker for tendon lineage commitment (Schweitzer R et al, 2001; Shukunami C et al, 2006), our first goal was to identify the best treatment to induce its expression. In fact, SCX is a bHLH transcription factor expressed in tendon progenitor cells in the mouse embryo (Schweitzer R et al, 2001), and its ectopic expression could drive human BMSCs to express markers of tendon progenitors (Alberton P et al, 2012).
Our results clearly show that this was obtained only when cells were cultured in presence of TGFβ3. The influence of TGFβ in tendon development was described in 2009 by Pryce and colleagues (Pryce BA et al, 2009) and the potential of this mediator in SCX induction was then verified in equine embryonic stem cells (Barsby T et al, 2013). Nevertheless, it is also known that TGFβ superfamily induce a wide number of target genes, and it is clearly involved in bone formation (Wu M et al, 2016; Labour MN et al, 2016; Ripamonti U et al; 2016). Thus, its solely application to MSCs would not allow for a precise tendon lineage induction. In our experiments, TGFβ was applied in combination with other growth factors, in particular BMP-12, that is described in literature as the main trigger of tendon differentiation. Indeed, BMP-12 is able to induce tendon specific markers in ectopic rat tissue, with a relatively low expression of bone and cartilage markers (Berasi SP et al, 2011), and many papers confirm its central role in tenogenic induction in both in *vitro* and in *vivo* scenario (Heisterbach PE et al, 2012; Lou J et al, 2001; Majewski M et al, 2008; Lee JY et al, 2011). Nevertheless, it was described as not sufficient for the induction of a proper tendon-like phenotype (Stanco D et al, 2015), but its use in combination with TGFβ3 could represent a strong and specific tenogenic inductive signal. In our experiments, BMP-12 alone showed a prompt increase of DCN expression, that could led to a reduced tendon repair ability and matrix deposition (Hildebrand A et al, 1994; Danielson KG et al, 1997). Thus, the possibility to avoid these events by the addition of TGFβ3 supports the use of both the growth factors in the first phases of tenogenic induction.
Moreover, the physiological expression of TGFβ was reported in adult tendon after injury (Chan KM et al, 2008, Fenwick SA 2001), as well as SCX but it is also correlated with hypertrophic scar tissue formation and fibrosis (Lian et al, 2016) suggesting caution in its possible clinical application. However, among the different isoforms, TGFβ3 possesses anti-scarring properties and has proven to be able to contrast the TGFβ1 and TGFβ2 action of inflammatory cells activation and abnormal connective tissue formation (Durani P et al, 2008).
Beside SCX induction, we observed that until its removal from culture medium, TGFβ3 inhibits the production of DCN mRNA and protein. DCN is a small leucine rich proteoglycan (SLRP) protein that possess both structural and signaling physiological functions, as it is involved in Collagen type I fibrils formation and mechanical properties (Reese SP et al, 2013; Kalamajski S et al, 1998; Orgel JP et al,

2009; Pogany G et al, 1992;), but it is also active in angiogenesis modulation (Järveläinen H et al, 2015). In particular, it has been suggested that this action depends on the ability of DCN to neutralize TGFβ action by direct binding (Yamaguchi Y et al, 2016; Border WA et al, 1992; Hildebrand A et al, 1994; Schönherr E et al, 1998). Despite the bi-unique inhibitory interaction between TGFβ and DCN, the real effects and cause of this process are still unclear and further studies are needed to clarify this mechanism. For what concern the structural role of DCN, its ability in binding collagen fibers could prevent the deposition of abnormal extracellular matrix resulting in an inhibitory effect on the possible development of fibrotic tissue (Hildebrand A et al, 1994). Then, the ability to induce DCN in tendon differentiated cells represents an encouraging aspect for the achievement of a physiological matrix production. BMP-12 showed to play a role in the induction of markers of tendon matrix maturation, but extremely higher results were obtained with ascorbic acid at both 3 and 10 days of differentiation protocols. Indeed, ascorbic acid was described as able to induce extracellular matrix deposition in a number of different approaches (Rehmann MS et al, 2016; Chen X et al, 2009), and its ability to induce Collagen type I (Takeuchi Y et al, 1993; Hering TM et al, 1994) is clearly coupled with the increased expression of DCN as resulting from our experiments. Nevertheless, ascorbic acid is not able to induce SCX, indicating that it would not be sufficient for tenogenic differentiation, but the ability in reducing collagen type II and osteocalcin expression (Ni M et al, 2013) make ascorbic acid an optimal effector of matrix deposition and maturation phases during tendon differentiation, as well as of tendon phenotype maintenance. At least for what concern TSPCs, it also showed the ability to increase TNMD expression, confirming its important role in the late stages of tendon development.

Another marker of tendon matrix is TN-C. It was used in the first years of tendon differentiation era (Lee et al, 2005), before the discovery of SCX (Chiquet M et al, 1984; Kardon G et al, 1998; Huang AH et al, 2015), and even if it is not as specific as other components of the extracellular matrix (Makie EJ et al, 1996), this glycoprotein represents an early marker of matrix (Huang AH et al, 2015). Indeed, the TNC expression pattern in our experiments was similar to the SCX and COL1 ones, since it is upregulated in presence of TGFβ3, confirming that this stimulation is able to enhance an early response towards tenogenic lineage.

Differently, a very early tendon marker such as MKX that is a transcription factor to SCX (Otabe K et al, 2015; Nakamichi R et al, 2016), was just slightly induced by our treatments and only in TSPCs, while no induction was observed after treatment of ASCs and BMSCs. Since its effect on tenogenic induction appear to be stimulated through TGFβ pathway (Liu J et al, 2015), it is possible that our direct induction of SCX by TGFβ bypassed the MKX involvement, reaching a more differentiated stage without the action of this particular transcription factor.

CTGF is another important growth factor for the induction of tendon matrix and specific transcription factor production (Ni M et al, 2013). Nevertheless, according to our results it was not able to induce any further increase in the expression of SCX, COL1 and TNC when used in combination with TGFβ3, BMP-12 and IGF-I, at least for what concern the treatment of ASCs and TSPCs. In fact, it showed a slight enhancement of the expression these markers in BMSCs, suggesting a role for CTGF in this particular cell type, as already suggested by other authors (Lee CH et al, 2006; Sassoon AA et al, 2012).

The differences among the analyzed cell types underlined the different potential of each cell source for tendon regeneration approaches. In particular, as widely accepted, TSPCs represent the gold standard for this particular application (Bi Y et al, 2007, Lui PP et al, 2011, Stanco D et al, 2015). The basal expression of the main specific markers, such as SCX, MKX and TNC was indeed higher in this population with respect to adipose and bone marrow derived counterparts, and these gaps were increased by tenogenic induction. Nevertheless, the low availability of TSPCs (Lui PP et al, 2011) and the rapid de-differentiation that occurs in vitro (Tan Q et al, 2012) suggested that other sources of MSCs could be a better choice for the clinical translation of this approach. Indeed, both ASCs and BMSCs showed potential in tenogenic differentiation but according to our data ASCs are more prone to produce tendon like extracellular matrix, while BMSCs are more easily induced in early stage of tendon differentiation. Indeed, it is known that adipose tissue derived cells spontaneously produce COL1 and TNMD (Stanco D et al, 2015; Saiki A et al, 2009), and they were able to increase also SCX, DCN and TNC expression after stimulation with BMP-12 and TGFβ3. Nevertheless, the ability to respond to these kind of stimuli, was accompanied by concerns about the induction of cartilage markers such as COMP and aggrecan (Park A et al, 2010; Martinello T et al, 2012), and then the possible formation of ectopic cartilage. On the other hand, BMSCs showed a higher basal expression of SCX with respect to ASCs but at the same time, lower levels of all the other matrix markers. After stimulation with BMP-12, TGFβ3 and in particular CTGF, the increase in COL1 and TNC expression indicated a good response of this cell type to early induction of tendon differentiation, while the later phase of matrix production and maturation was not induced as demonstrated by the lack of DCN and TNMD overexpression after treatments. Most of the previous works support these observations, even if TNMD expression was reported after BMSCs tenogenic induction by different methods (Leung M et al, 2013; Schneider PRA et al, 2011; Luo Q et al, 2009). Different experimental conditions and TGFβ-isoform specific effects could explain these apparent inconsistencies, while confirming our findings for what concern SCX, COL1 and TNC expression. Moreover, some concerns are present for possible ectopic tissue formation after tenogenic induction of BMSCs, in particular muscle and bone (Hankemeier S et al, 2005; Pietschmann M et al, 2013; Bi Y et al, 2007; Harris MT et al, 2004), while cartilage transdifferentiation did not emerged as a possible side effect (Tan SL et al, 2012). Taken together, all of these informations suggest that ASCs would produce a more tendon-like extracellular matrix with respect to BMSCs, and could be preferred for tendon regenerative applications.

A limitation of the present study is represented by the lack of bone and cartilage specific markers assessment during differentiation. Nevertheless, the induction of SCX was described as alternatively with respect to SOX9 (Sugimoto Y et al, 2013), thus suggesting that cartilage differentiation should be prevented by treatments inducing this tendon specific marker. Another limitation is represented by the lack of an efficient antibody for immunofluorescence application to SCX, resulting in a high background signal. Different previous works relied on this method for SCX investigation, but they often showed its expression in the cytoplasm, while the localization of SCX should probably be nuclear. The absence of mechanical induction of tendon differentiation in our experiments could have also produced an underestimation of tenogenic potential of our protocol and cell types, since the mechanical stretch was already described as an efficient method to enhance tendon specific markers expression and tenocyte-like morphologic appearance (Li Y et al, 2015).

In the future, a wider investigation over the effect of IGF-I, as well as the application of our protocols to 3D culture and the use of mechanical and biophysical stimulation, will be taken in account to increase tenogenic inductive effectiveness and to advance with the knowledge in this field. In particular, Pulsed Electromagnetic Fields will be applied in the same experimental environment, since they already demonstrated SCX-inductive ability (de Girolamo L et al, 2013; de Girolamo L et al, 2015).

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Research in progress and future perspectives

Cell-free approaches in Regenerative Medicine: paracrine mediators of ASCs possess immunomodulatory and chondro-protective abilities

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The interest in the paracrine role of MSCs in different pathophysiological context is growing, and many literature reports were published in the last five years, investigating this aspect (Caplan et al, 2011; Caplan et al, 2016). In fact, the possibility to apply the immunomodulatory potential of MSCs in therapeutic approaches, avoiding the direct transplantation of allogeneic or autologous cells would represent a great advantage in terms of safety and cost effectiveness of the treatments. MSCs in culture are able to produce cytokines and growth factors that could promote tissue homeostasis, and the possibility to tailor the expression of this molecules has been already described as a near-future possible scenario, in particular thanks to the responsiveness of this cells to biophysical, biochemical, environmental and pharmacological stimuli (Baum C et al, 2003; Haider H et al, 2008; Schumann D et al, 2006; Baraniak PR et al, 2010;). Nevertheless, the investigation on this topic is still on-going and the definition of standardized protocols for each application must be validated before moving any step in the clinical practice. For example the choice of a particular cell source must acknowledge that significant differences were found among cells types.

The aim of our future researches is the evaluation of the immunomodulatory ability of ASCs conditioned medium, concentrated by filtration, in a model of inflammation, developed with phytohaemagglutinin (PHA)-treated peripheral blood mononuclear cells (PBMCs).

Peripheral blood was collected from healthy donors in presence of EDTA, and diluted with 1:1 PBS. Mononuclear cells were isolated by gradient centrifugation with Ficoll® Isopaque (GE Healthcare) for 10 minutes, and then PBMC were harvested with a micropipette. PBMCs were seeded at $5x10^4$ cell/well in a 96-well plate and incubated with different doses of MSCs conditioned medium concentrate were performed, in presence of PHA 10 µg/ml. PBMC activation was measured by proliferation assay (BrdU proliferation assay kit, Roche) and pro-inflammatory cytokine IL-6 production. When PBMCs were incubated with the product of $1.5x10^5$ MSCs per milliliter, the proliferation resulted inhibited by 52%, and further dilution of MSCs product resulted similarly active, up to $2.5x10^4$ cells/ml. While this results support the hypothesis that MSCs conditioned medium would contrast the action of inflammatory stimuli, its action in musculoskeletal degenerative disorders should involve a wider range of mediators, thus providing tissue-protective effectiveness and a contrast to the pathological catabolic microenvironment. A chondro-protective assay was then performed to investigate the ability of MSCs in this context.

An in *vitro* model of inflammation, involving IL-1β-treated human chondrocytes was established. Primary cultures of human articular chondrocytes were harvested starting from waste surgical material derived by hip arthroplasty. After isolation by collagen type II digestion, cells were cultured until passage 3 and then seeded in 6-well plate, at a density of $5x10^3$ cell/cm^2. When cultured in presence of IL-1β, increase in metalloproteinases (MMP-1, -3, -13) and a decrease in TGFβ were

found, suggesting the establishment of a catabolic response. When added to culture media, at a concentration equivalent of $1,5x10^5$ cell/ml, MSCs secretome was able to reduce levels of MMP-1, TNFα and the pro-angiogenic factor VEGF, whilst it induced higher levels of TIMPs and IL-1ra, suggesting its efficacy as a chondroprotective agent in this *in vitro* model. These data are just starting results of a larger study in progress, but represent the proof of concept for further advances in the identification of the mechanism of action of MSCs secretome in the field of musculoskeletal degenerative pathologies. In the future, after providing an optimization of the protocols, the application of the expertise acquired from previous works on biophysical stimulations would allow the tailoring of MSCs secretome for different applications. Nevertheless, the possibility to explore the differences in MSCs sources, starting from the work showed in the present thesis, represent a challenge but, at the same time, a further opportunity to enhance the efficacy of this product in orthopaedic disorders.

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Research in progress and future perspectives

Biophysical stimulations with pulsed electromagnetic fields (PEMFs) for the treatment of Achilles tendinopathy in a rat model

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Disorders affecting tendons still represent a big burden in orthopedics with particular reference to initial degenerative changes of collagen structures and acute traumatic lesions. Indeed, recent studies demonstrated the presence of inflammatory process in pathologic tendons (Sharma et al., 2006; Abate et al., 2009). Among conservative treatments, physical therapies (extracorporeal shockwaves and pulsed electromagnetic fields) have been investigated for their potential effectiveness in tendon regeneration (Rosso et al., 2015). In particular, the pulsed electromagnetic fields (PEMFs) have been extensively used in clinics for the treatment of delayed-union fractures and chronic wounds being simple, non-invasive, cost-effective and without side effects (Strauch et al., 2006; Akai et al., 2002). Despite the lack of evidences of positive effects on tendon healing after PEMF treatment (Uzunca et al., 2007; Dingemanse et al., 2014), a very recent clinical randomized study described the decrease of inflammation, swelling and pain in patients underwent to PEMFs treatment after rotator cuff repair at a short terms follow up (Osti et al., 2015). Only few old studies in animal models reported discordant results about the function of PEMFs on the healing process of flexor tendon tissue (Lee et al., 1997; Strauch et al., 2006; Robotti et al., 1999). Recently Tucker and colleagues (Tucker et al., 2016) suggested that PEMFs exposure had biomechanical and histological positive effects on early rat rotator cuff healing in rats. These observations from clinical and preclinical studies encouraged to have a deeper insight into the role of PEMFs to promote resident cell metabolism favoring tendon repair.

In the last decades, inductive low frequency PEMFs have been exploited for their interaction with cell membrane, stimulating intracellular pathways linked to cell proliferation, cytokine and growth factor release (de Girolamo et al., 2013; Rosso et al., 2015), thus modulating the tissue restoration, from an initial phase characterized by pain and swelling to blood vessel growth and tissue remodeling (Strauch et al., 2006). Moreover, our recent in vitro studies showed the increased proliferation of human tendon cells after PEMF treatment associated to an up-regulation of specific tenogenic genes (scleraxis, vascular endothelial growth factor and collagen type I) (de Girolamo et al., 2013; de Girolamo et al., 2015). More importantly, in these studies, the enhanced release of interleukins (IL-6, IL-10) and transforming growth factor-β by cells treated with PEMFs determined the activation of cell metabolism and proliferation.

Taken together, the lack of knowledge about PEMFs effectiveness on tendon disorders makes undoubtedly necessary further in *vivo* studies to evaluate the effects of PEMFs mechanical stimuli on resident multipotent cells and collagen pattern during the restoring process. In the present study, we employ a validated rat model of acute collagenase-induced tendinopathy (Perucca Orfei et al., 2016) to explore whether the low-intensity PEMFs exposure contributes to sustain the progression of tendon healing.

MATERIALS AND METHODS

Ethics Statement

The Mario Negri Institute for Pharmacological Research (IRFMN) Animal Care and Use Committee (IACUC) approved the study (Permit N. 06/2014-PR). The IRFMN adheres to the principles set out in the following laws,

regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate–UNI EN ISO 9001:2008 –Reg. N° 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01). The animals were regularly checked by a certified veterinarian responsible for health monitoring, animal welfare supervision, experimental protocols and procedure revision. All surgeries were performed under general anesthesia, and all efforts were made to minimize suffering.

Collagenase-induced acute tendinopathy in rats

Twelve 12-weeks-old male Sprague Dawley rats (Rattus norvegicus) (Envigo, Huntingdon, UK) (mean body weight 347 ± 9 g) were used in this study. According to our previous model, we determined an acute tendinopathy in rat Achilles tendons, by injecting 3 mg/mL collagenase type I (collagenase from Clostridium histolyticum, Worthington Lakewood, NJ, USA, 185 IU/mg) (Perucca Orfei et al., 2016). Briefly, under general anesthesia, the Achilles tendon was exposed through a medial skin incision, and injected with 30 µL type I collagenase dissolved in of PBS (COLL group). The controlateral tendon of each animal was used as negative control by injecting 30 µL PBS (CTRL group). After skin closure and anesthesiologic recovery, rats were free for weight bearing after surgery. For the following 15 days, rats were maintained in controlled conditions of temperature and lightning and food and water were provided ad libitum.

Treatment with pulsed electromagnetic field

After 15 days from surgery, rats were randomly assigned to a control group which did not received any additional treatment (PEMFs-) and a group treated with PEMFs (field intensity 1.5 ± 0.2 mT; 75 Hz) for 8 hour per day for 15 days (PEMFs+). To expose animals to PEMFs, a custom made coil was placed at the bottom of cages (Figure 1). The coil (40 x 18 cm) was connected to a PEMFs generator system (IGEA, Carpi, Italy) and electromagnetic field was evaluated as described in our previous studies (de Girolamo et al., 2013; de Girolamo et al., 2015). To perform the 8 hours length of exposure, the PEMFs generator system was connected to a timer, which automatically turned on and off the PEMFs emission. Thirty days after surgery, rats were sacrificed by CO_2 inhalation to harvest the Achilles tendons for the histological investigations.

Figure 1. *PEMF treatment setup. Pulsed electromagnetic field device (PEMF generator) connected to the coil fixed at the bottom of rat cages and controlled by a timer for 8h/day for 15 days.*

Histological analysis

Tendon specimens were explanted, fixed in 10% formalin for 24h and in 70% ethanol for 48h. Samples were dehydrated in alcoholic scale and embedded in paraffin. Then, they were sectioned in 5 µm slides and stained with haematoxylin and eosin (H&E) to evaluate the tendon morphology and the healing process of the tissue. Photomicrographs of the tissue were captured through an Olympus IX71 light microscope and an Olympus XC10 camera (Japan) and analyzed by two blinded observers, according to the histological score reported elsewhere (Perucca Orfei et al., 2016). Specifically, the following parameters were evaluated: cellular repopulation and distribution, collagen pattern, vascularization and inflammation.

PRELIMINARY RESULTS

The identification in our previous study (Perucca Orfei et al., 2016) of the different phases and times of tissue healing in the rat tendinopathy model allowed us to properly design a study aimed to evaluate the effect of biophysical stimulations (pulsed electro-magnetic fields) on Achilles tendinopathy.
The present study is still in progress, but the preliminary results obtained by the histological analysis with H&E staining show that a preferable therapeutic answer and an improvement in the restoration of the tissue homeostasis are better obtained applying the biophysical stimulations during the proliferative and the remodeling phase of the pathology rather that in the inflammatory one. The application of PEMFs promotes the re-establishment of the aligned conformation of the tendon fibers, decreasing also the neovascularization process and the formation of adipose depots. However, these evidences must be further confirmed.
The study will be continued with the final aim to determine a specific procedure to follow to maximize the therapeutic answer and to provide an effective therapeutic conservative treatment for Achilles tendinopathies.

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Chapter 3

Delivery of MSCs

Aim 3

The current findings about MSCs deeply encourage their clinical applications, especially for musculoskeletal disorders. However, although their enormous therapeutic potential has been already recognized, many issues still remain unresolved, including way and time of delivery, concentration and specific indications. This is mainly due to the fact that mesenchymal stem cells are the most complex existing drugs and we are not still able to exactly determine their real fate after their administration. In addition to this lack of knowledge, the concrete employment of MSCs in the clinical practice is further hampered by the regulatory authority that is still debating about this topic. The current very restrictive rules related to the use of MSCs imposed by the regulatory system are justified by the unavoidable need for a deeper knowledge about their in *vivo* activity, also considering that the cell is a high-sensitive responsive unit and it can be strongly affected by the external microenvironment. In this view, the use of minimally manipulated product, for example the whole stem cell niche that is their native microenvironment, is among the best way to obtain the safest approach and probably the most effective activity, partially escaping from the restrictive rules related to the use of ATMPs. In this context I worked at the development of a vehicle for MSCs delivery aimed to obtain a MSCs-based product for the conservative treatment of joint diseases, in particular for osteoarthritis and intervertebral disc degeneration. The challenge of this research project was to obtain a safe and effective system that allows a good maintenance of the physiological features of cells while increasing the effectiveness of their local delivery.

The study was conducted in three different phases: the first phase of the project was intended to the development of a biocompatible, biodegradable and cytocompatible system able to allow for MSCs adhesion and subsequent delivery (Chapter 3, manuscript 5, Chlapanidas T et al, patent application drafting).

Secondly, we tested the biodegradability and the biocompatibility of this device in a rat model, also evaluating the cell maintenance and viability at the target site (Chapter 3, manuscript 6, Perucca Orfei C et al, patent application drafting).

At last, using the Design of Experiment (DOE) method, we attempted to optimize the cell seeding protocol onto the carrier previously developed (Chapter 3, manuscript 7, Perucca Orfei C et al, patent application drafting). In particular The DOE method has been used to allow for the identification of the minimal experimental conditions and the evaluation of specific parameter modifications, with the aim to identify the cell seeding conditions able to give the best performing cell adhesion on the carrier.

Manuscripts (waiting to be submitted)

* Chlapanidas Theodora, de Girolamo Laura, Martella Elisa, **Perucca Orfei Carlotta**, Pierini Michela, Fumagalli Valentina, Pintacuda Valeria, Perteghella Sara, Marco Viganò, Torre Maria Luisa, Lucarelli Enrico. *Silk/alginate microcarriers for stem cell delivery: physicochemical-technological characterization and in vitro cytocompatibilty.*

* **Perucca Orfei Carlotta**, de Girolamo Laura, Perteghella Sara, Pintacuda Valeria, Fumagalli Valentina, Amat Daniel, Chlapanidas Theodora, Torre Maria Luisa, Lucarelli Enrico, Santos-Ruiz Leonor. *In vivo biocompatibility and efficiency of silk/alginate microcarriers as delivery system of adipose-derived stem cells*

* **Perucca Orfei Carlotta**, Talò Giuseppe, Chlapanidas Theodora, Marco Viganò, Perteghella Sara, Fabro Fontana Francesca, Torre Maria Luisa, de Girolamo Laura. *Optimization of cell adhesion rate on silk/fibroin microcarriers for one-step mesenchymal stromal cell delivery: Design of Experiment approach*.

Manuscript 5

Silk/alginate microcarriers for stem cell delivery: physicochemical-technological characterization and in vitro cytocompatibilty

Chlapanidas Theodora, de Girolamo Laura, Martella Elisa, Perucca Orfei Carlotta, Pierini Michela, Fumagalli Valentina, Pintacuda Valeria, Perteghella Sara, Marco Viganò, Torre Maria Luisa, Lucarelli Enrico

ABSTRACT

Background and objectives: Alginate is a natural polysaccharide employed only for cell encapsulation. The presence of negative charges and the deficiency of integrin domains inhibits cell adhesion on alginate surface. In this study a new fibroin-coated alginate microcarrier (FAM) was described.

M&M: An alginate microcarrier (AM) was coated with a fibroin solution to improve cell adhesion. The microcarriers were characterized for size distribution, coating homogeneity, and cytocompatibility, culturing human adipose stem cells (hASCs) on their surface.

Results: Microcarrier analysis indicated that the size is not influenced by the coating process (diameter mean of AM 464.34 ± 62.19 µm and FAM 421.94 ± 46 µm). Fibroin completely covered microcarrier's surface that appeared as a spherical rough structures. Cytocompatibility of FAM was evidenced by the surface colonization of hASCs after 3 days of culture and the cell proliferation up to 14 days. Metabolic assays revealed that cells were metabolically active and proliferate on the FAM surface for at least 7 days. hASCs maintained the multilineage differentiation potential when cultured on microcarriers surface.

Conclusions: Our results demonstrate that FAM support *in vitro* cell adhesion, proliferation maintaining cell multipotency and encourage FAM application as 3D injectable system for cell therapy

INTRODUCTION

In 1967, Van Wezel introduced for the first time the concept of microcarrier as method to produce large scale vaccines and to improve mammalian cell growth (van Wezel 1967). Nowadays in regenerative medicine applications microcarriers can be used as a fast and reliable method for ex vivo cell expansion, to meet the highly demanding cell dose for peculiar cell therapies, and to the following cell delivery to the target site after implantation (A. K.-L. Chen, Reuveny, and Oh 2013). A wide range of microcarriers, with different properties (degree of porosity, chemical composition and surface topography), and diameter comprised between 100 and 400 μm, are now commercially available (Malda and Frondoza 2006; Markvicheva and Grandfils 2004). Using microcarriers as a delivery system, cells are maintained for longer time into the lesion site (Quittet et al. 2015), giving them the possibility to secrete growth factors and actively participate to tissue matrix deposition promoting its regeneration (A. K.-L. Chen, Reuveny, and Oh 2013; Georgi, van Blitterswijk, and Karperien 2014). Many and different natural or synthetic biopolymers have been investigated for microcarrier formulation for regenerative medicine (M. Chen et al. 2011; Sun et al. 2010; Yang, Rossi, and Putnins 2007; Zhou et al. 2011). Commonly employed for cell and drug/growth factors encapsulation (Gaetani et al. 2008; Goh et al. 2013; Della Porta et al. 2014; Villani et al. 2008), alginate is a bedrock biomaterial for cell transplantation (Chang 1964)., due to its properties of fast sol-gel transition in contact with divalent cations, *in vivo* biocompatibility, permeability, and dissolution.(Gasperini, Mano, and Reis 2014). However, alginate surface is unsuitable for cell adhesion due to the presence of negative charges and its deficiency of integrin domains (Rowley, Madlambayan, and Mooney 1999; Steward, Liu, and Wagner 2011). To overcome this inconvenience, alginate can be conjugated with an arginine-glycine-asparagine (RGD) sequence to increase its cell adhesion properties (Schmidt, Jeong, and Kong 2011) or combined with natural proteins, such as silk fibroin. Due to its peculiar characteristics, such as versatility (Omenetto and Kaplan 2010; Vepari and Kaplan 2007), biodegradability and biocompatibility (Panilaitis et al. 2003; Yucel, Lovett, and Kaplan 2014), silk fibroin is widely used to develop scaffolds for different applications, mainly to be used in association with cells. Indeed, silk fibroin is able to promote cell adhesion, proliferation and differentiation particularly of mesenchymal stem/stromal cells (MSCs) (Altman et al. 2010; Chlapanidas et al. 2011, 2016; Hofmann et al. 2007; Mauney et al. 2007; Meinel et al. 2004; Uebersax, Merkle, and Meinel 2008; Wang et al. 2008).

MSCs have been widely studied for regenerative medicine application due to their immune privileged nature and multi-lineage differentiation ability (Manferdini et al. 2013; Mizuno, Tobita, and Uysal 2012), and currently they are employed in more than 500 clinical trials (www.clinicaltrials.gov). Their use ranges from immune (e.g. graft-versus-host-disease, Crohn's disease) to degenerative/post traumatic pathologies. In particular in orthopaedic field, cell-based therapies have been proposed to treat bone, cartilage, ligament, tendon and muscle injuries (de Girolamo et al. 2013; Marmotti et al. 2014; Maumus, Jorgensen, and Noel 2013; Sharma et al. 2014; Yang, Rossi, and Putnins 2007; Zhou et al. 2011). Among the different sources of MSCs, adipose tissue is one of the most convenient sources due to its availability and accessibility. Moreover, the yield of MSCs from adipose tissue (adipose-derived stem/stromal cells, ASCs) is higher to that of bone marrow MSCs (Guilak et al. 2006; De Ugarte et al. 2003), as well as their immunomodulatory properties (Peng et al. 2012; Puissant et al. 2005).

At the best of our knowledge, today alginate/fibroin composite microcarriers are not available for ASCs expansion and differentiation, as well as for implantation: in the present work, these biopolymers have been selected as the first choice materials for their safety and consolidate use for cell transplantation. Therefore, we developed and characterized new composite microcarriers, constituted by a core of alginate and a silk fibroin shell, evaluating in vitro cytocompatibility and suitability as delivery system for ASCs in regenerative medicine applications. This approach could open new insights toward the development of an injectable microcarrier-based system aimed to the local delivery of cells to the injury site, focusing on a potential use in joint-related pathologies, such as early osteoarthritis, and intervertebral disk.

MATERIALS AND METHODS

Preparation of microcarriers

The novel production process has been structured in four steps (Figure 1): preparation of silk fibroin solution (Figure 1A); preparation of alginate cores (alginate microcarriers, Figure 1B); coating of alginate cores with silk fibroin solution obtaining fibroin/alginate microcarriers (Figure 1C); silk fibroin conformational transition, obtaining stable composite devices (Figure 1D).

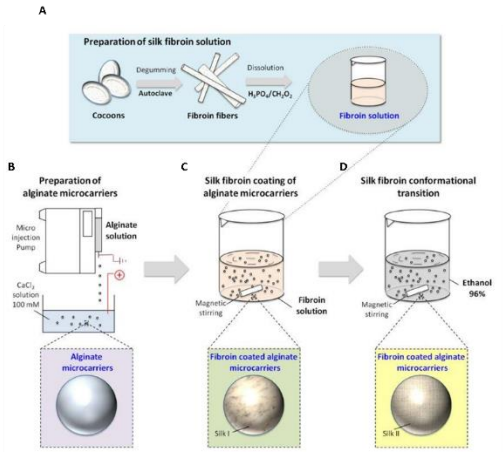


Figure 1. *Schematic representation of FAM production process: preparation of silk fibroin solution (A); preparation of alginate cores (B); coating of alginate cores with silk fibroin solution obtaining fibroin/alginate microcarriers (C); silk fibroin conformational transition, obtaining stable composite devices (D)*

Preparation of silk fibroin solution

Bombyx mori cocoons were degummed in autoclave (Systec V-65, Wettenberg, Germany) to separate fibroin fibers and sericin solution (Chlapanidas et al. 2013, 2014). Silk fibroin fibers were rinsed three times with distilled water at 60 °C, dried at room temperature (RT), cut in small pieces and solubilized in phosphoric acid/formic acid (80:20 v/v) (Sigma-Aldrich) under stirring at room temperature. Raw fibroin solution was dialyzed against distilled water using a modified polyethersulfone membrane (cut off 12 kDa, Visking, London, UK) at RT. The final concentration of silk fibroin aqueous solution was 1.5% w/v.

Preparation of alginate microcarriers

A solution of 1% w/v sodium alginate medium viscosity (Sigma-Aldrich, Milan, Italy), in distilled water was prepared. The solution was added dropwise by a syringe pump through a 0.17-mm diameter nozzle using a bead generator (Encapsulator VAR V1, Nisco Engineering AG, Zurich, Switzerland), with a differential charge of 7 kV, into a solution of distilled water containing calcium chloride (Sigma-Aldrich) 100 mM under magnetic stirring. When alginate droplets reached calcium chloride

solution, calcium ions diffused into the droplets, leading alginate gelation. Alginate microcarriers were then collected by filtration and washed twice with water.

Silk fibroin coating of alginate microcarriers

Alginate microcarriers (AMs) were shaken into fibroin solution (volume ratio alginate microcarriers: fibroin solution 1:2) under mild magnetic stirring for 5 minutes. Microcarriers were collected by filtration, and immersed in 96% (v/v) ethanol (Carlo Erba Reagents, Milan, Italy) for 15 minutes to induce silk conformational transition and β-sheet formation. The procedure was performed three times to assure the homogeneous and complete coating. Fibroin-coated alginate microcarriers (FAMs) were suspended in ethanol and stored at 4 °C until use.

Characterization of FAMs

Particle size

Granulometric analysis of microcarriers was performed with a laser light scattering analyzer (Mastersizer 2000, Malvern Instruments Ldt, Worcestershire, UK) equipped with Hydro SM wet sample dispersion unit; the refractive index was set at 1.359 for ethanol. Results are expressed as the mean value of the five replicates for each batch; the volume weighted mean D [4,3] was considered.

Fourier Transform Infrared Spectroscopy (FTIR)

In order to evaluate the fibroin molecular conformation, samples were analyzed before and after fibroin coating, on a Bruker Alpha-E IR Fourier Transform Spectrophotometer (Bruker, Milan, Italy), equipped with a MIRacle attenuated total reflection Diamond crystal cell in reflection mode, using a resolution of 4 cm-1. The spectra were collected in the middle IR (400-4000 cm-1).

Scanning Electron Microscopy (SEM) –Energy Dispersive X-ray (EDX) analysis

Samples were placed on aluminum stubs and coated with gold/palladium (60/40% w/w). The microcarrier morphology, before and after coating, was evaluated by a scanning electron microscope (SEM-FEG) Mira 3 (Tescan, Brno, Czech Republic), mode High Vacuum, 15 kV and secondary electrons (SEs) detector. EDX analysis was carried out with iXRF system and EDS-2004 system (IXRF Systems, Inc. Austin, TX, USA) for revelation.

Confocal Laser Scanning Microscopy (CLSM)

A confocal laser scanning microscopy analysis of fibroin coating was performed using a NikonTiE microscope equipped with a fully automated A1 confocal laser which incorporates the resonant scanner with a resonance frequency of 7.8 kHz allowing high-speed imaging (A1R, Nikon, Amsterdam, Netherlands). FAMs were washed three times with saline solution (NaCl 0.9%) and placed in 35 mm μ-dish glass bottom (high, Ibidi Gmbh, München, Germany) for imaging acquisition. In order to identify the correct laser source to detect fibroin fluorescence, a sequential excitation of

microcarriers was performed at four different wavelength (405, 488, 561, and 638 nm, respectively). The emission signal was separated by using a dichroic mirror (500 nm) and the spectral images were acquired in sequential bandwidths of six nm spanning the wavelength range of 500 to 692 nm to generate a lambda stack containing 32 images. The confocal pinhole was set to 4 Airy disk and a 10X Plan-Apochromatic Ph1 DL 0.25NA objective lens was used.

Cytocompatibility of FAMs, cell proliferation, and cell differentiation potential

Isolation and monolayer culture of human stromal cells (hASCs)

hASCs were isolated from subcutaneous abdominal fat of 3 informed female donors (37 ± 14 years) who underwent aesthetic liposuction. All of the procedures were carried out at Galeazzi Orthopedic Institute (Milan, Italy) with Institutional Review Board approval (M-SPER-014.ver7 for the use of surgical waste). Adipose tissues were washed with phosphate buffered saline solution and digested with 0.075% w/v type I collagenase (Worthington Biochemical Corporation, LakeWood, NJ, USA) at 37 °C for 30 minutes (de Girolamo et al. 2009). Then, the recovered cells were centrifuged at 1200 g for 10 minutes, filtered with a 100 µm cell strainer and seeded in monolayer culture in complete medium (CM), containing Dulbecco's Modified Eagle's Medium-HIGH GLUCOSE (DMEM-HIGH, Sigma-Aldrich), 10% fetal bovine serum (FBS, HyClone, EuroClone, Milan, Italy), 50 U/ml Penicillin, 50 mg/ml Streptomycin, 2 mM L-glutamine (Sigma-Aldrich) (Catalano et al. 2014; Faustini et al. 2010). hASCs were then expanded up to 70-80% of confluency. Then, cells were detached using TripLE™ Select Enzyme solution (Life Technologies, Carlsbad, CA, USA) for 3-5 minutes at 37 °C in 5% humidified CO_2 atmosphere. Cell pellet, obtained after centrifugation at 450 g for 3 minutes, was mixed with CM and cells were counted using nucleocounter device (Chemotemec, Allerod, Denmark). hASCs were maintained at 37 °C in humidified atmosphere with 5% CO_2, changing culture medium every 3 days; cells up to passage 5 were used for the following experiments.

Cell seeding on FAMs

FAMs were washed in saline solution (50% v/v), conditioned for 24h in complete medium, and then transferred in 2 mL sterile centrifuge tubes. Afterwards hASCs were added to the FAMs suspension in order to obtain a final seeding density of 5000 cells/cm^2 of microcarriers' surface area (Schop et al. 2010). The centrifuge tubes were tightly closed and stirred on an oscillating shaker (Rotamax 120, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) for 2h at 37 °C, 5% CO_2, at 70 rpm to permit the cell adhesion. After the seeding step, fresh medium was added in each tube.

Cell Viability and Adhesion

The presence of viable cells on FAMs was qualitatively observed with Live and Dead staining (Life Technologies) after 1, 3, 7, and 14 days from cell seeding. Briefly, an aliquot of microcarriers was incubated with 2.5µM of Calcein-AM and 10µM of Ethidium Homodimer-1 in saline solution for 10 minutes at 37°C and 5% CO_2. After two washes with saline solution, images were acquired using epifluoresence microscope (Nikon Eclipse TE2000-U inverted epifluorescence microcope) using filter

for Calcein-AM: ex. 465-495 nm, em. 515-555 nm; Ethidium Homodimer-1: ex. 510-560 nm, em. 590 nm).

Metabolic assays

Five batches of FAMs were tested with hASCs. Metabolic activity of hASCs seeded onto FAMs was measured by the MTT Assay immediately after the stirring period (2 hours) and at 1 and 7 days from the cell seeding. A final concentration of 0.5 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) solution was added into each well and after 4h of incubation at 37 °C and 5% CO_2 the medium was removed and formazan crystals solubilized in dimethyl sulfoxide (DMSO; Carlo Erba Reagents). The absorbance of the resulting solution was read at 570 nm (Victor X3, Perkin Elmer microplate, Waltham, MA, USA).
A further evaluation of the metabolic activity of adherent hASCs was performed by Alamar Blue Assay. In details, aliquots of cell-seeded FAMs were treated with 10% v/v Alamar Blue solution (Life Technologies) for 4h at 37°C. The fluorescence of the obtained solution was measured by Victor X3 (Ex/Em 560/590 nm).

Immunofluorescent staining for cytoskeletal actin

At each time point (3, 7, and 14 days of culture) hASCs seeded FAMs were fixed with 4% paraformaldehyde solution supplemented with 50 mM $CaCl_2$ solution for 45 minutes with agitation. Samples were washed in saline solution and permeabilized with 0.1% Triton-X (Sigma-Aldrich) in saline solution for 15 minutes. After three washes in saline solution, the cell seeded FAMs were incubated with TRITC-Phalloidin (Sigma-Aldrich #P1951, [1:500] in saline solution) for 40 minutes. The nuclei were stained with 5 µg/mL Hoechst 33342 (Life Technologies #H3570, [1:2000] in saline solution) for 10 minutes. After three washing in saline solution, confocal images were acquired using Nikon A1 confocal laser with a Plan Apo VC DIC N2 20X 0,75NA objective and z-stack setting of 2 µm/step. For TRITC-Phalloidin visualization a 561 nm laser and a band filter of 570/620 nm range was used.

Transmission Electron Microscopy (TEM)

After 7 and 14 days of culture, samples were fixed with glutaraldehyde 2.5% in cacodylate buffer 0.1 M for 4 h at 4 °C, then with osmium tetroxide 0.1% and finally with alcohols. The scaffold was included in Epon 812/Araldite resin overnight before being sectioned using Ultracut S Ultramicrotome. The thin sections were treated with Toluidine Blue staining (Sigma-Aldrich) and observed under an optical microscope, while the ultrathin sections were observed for transmission electron microscopy with a JEOL JEM 1200 EX instrument (Jeol, Tokyo, Japan).

Multilineage differentiation of hASCs

The osteogenic, chondrogenic and adipogenic potential of hASCs was evaluated both culturing them in monolayer and on FAMs.

Osteogenic differentiation was induced by seeding hASCs in 24-well culture plates at 5000 cells/cm^2; the same density was used to seed cells onto 100µL of microcarriers (50% v/v of suspension). After a first period of culture in DMEM-HG medium supplemented with 2% FBS , once cells were confluent, medium was supplemented with 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid, and 1 µM dexamethasone (Pierini et al. 2013)(all from Sigma-Aldrich) and cells cultured for 28 days changing medium twice a week. Cells cultured in basal medium were used as control. Samples were then stained with Alizarin Red S (Sigma-Aldrich) to detect the deposition of mineralized matrix as described previously (Stanford et al. 1995). To confirm mineralization, the OsteoImageTM (Lonza, EuroClone) assay was carried out according to the manufacturer's protocol. Briefly, cells were firstly washed with saline solution and then fixed in 70% ethanol for 20 minutes at RT. After fixation, samples were rinsed twice with wash buffer provided and stained with diluted staining solution, incubating in dark at RT for 30 minutes. Finally, samples were rinsed with wash buffer and images were captured using epifluoresence microscope.

For adipogenic differentiation, hASCs were seeded at the same density reported above. On the following day, the basal medium was replaced with adipogenic differentiation medium (adipogenic induction medium) containing DMEM-HIGH, 10% FBS, 1 µM dexamethasone, 0.2 mM indomethacin, 1.75 µM bovine insulin, and 0.5 mM isobutylmethylxanthine (all from Sigma-Aldrich). After 3 days medium was changed and supplemented with 1.75 µM bovine insulin (adipogenic manteinance medium), alternating the two medium for 21 days (Barbero et al. 2003). Cells cultured in basal medium were used as control. After 21 days from induction, Oil Red O and Nile Red staining were performed to visualize lipid droplets production. Cultures were washed, fixed with 4% paraformaldehyde in saline solution and stained with 0.3% Oil Red O (Sigma-Aldrich) in an isopropanol solution for 15 minutes with agitation. Finally, the cultures were washed two times with saline solution and images were captured to evidence the lipid droplets. Nile Red staining was done fixing the cultures in 4% paraformaldehyde solution supplemented with 50 mM CaCl2 for 10 minutes at RT, washed three times with saline solution, and finally stained with 5 µg/mL Nile Red for 20 minutes in dark and RT. Cells nuclei were stained with Hoechst as described above. After one wash in saline solution, samples were imaged with confocal with Nikon A1 confocal laser microscope with a Plan Apo VC DIC N2 20X 0,75NA objective and z-stack setting of 2.5 µm/step for a lambda stack containing 86 images for the control and 54 for the induced sample. For Nile Red visualization a 514 nm laser and a band filter of 570/620 nm range was used.

Chondrogenic differentiation was performed in pellet culture using a StemPro Chondrogenesis Differentiation Kit (Life Technologies), whereas DMEM-HIGH supplemented with 10% FBS was used as control. For pellet culture, 2.5 x 10^5 cells were mixed with 100 µL of a 50% (v/v) FAMs suspension in a 15 mL centrifuge tube and spun in a benchtop centrifuge at 450 g for 5 minutes. Pellet cultures without FAMs were used as controls. The samples were put at 37 °C and 5% CO_2 for a week in a humidified incubator; after that CM was changed in inducing or maintained medium for 21 days (Control and induced pellet, respectively), changing the medium twice a week. After differentiation, pellets were digested with buffered papain (Sigma-Aldrich) solution for 3 h at 65 °C, then spun at 10000 g for 10 minutes. The glycosaminoglycans (GAG) content was evaluated with use of a Blyscan Sulfated Glycosaminoglycan Assay kit (Biocolor, Carrickfergus, UK) according to the manufacturer's instructions. The total GAG content was assessed by reading the optical density at 656 nm in a

microplate reader (Synergy HT, BiotTek, Winooski, United States) and interpolating with use of a reference standard curve (Yoneno et al., 2005). In addition, a histochemical evaluation with Safranin O was performed. Pellets were fixed for 24 hours in 10% calcium formalin, dehydrated and then embedded in paraffin. Four-micron sections were stained with Safranin O to evaluate extracellular matrix deposition.

Statistical analysis

The effect of fibroin coating on microcarrier size distribution was assessed by one-way ANOVA, considering the coating process (Yes or No) as factor and the volume weighted mean D[4,3] as dependent variable.Results on hASCs metabolic activity were processed by multifactor ANOVA, considering the time of culture (after 2 hours, 1 and 7 days from cell seeding), the cell line (n = 2) and the microcarrier batch (n=5) as fixed factors, the optical density (for MTT assay) and the fluorescence intensity (for Alamar Blue assay) as independent variables. The differences between groups were analyzed with the post-hoc LSD's test for multiple comparisons. Unless differently specified, data are expressed as mean ± standard deviation. The statistical significance was fixed at $p \leq 0.05$.

RESULTS

The production process of FAMs has been developed and standardized in lab-scale, leading to a consistent technology, verified in five independent runs performed during 1 year. Silk fibroin dissolution following a previously defined protocol, allowed the conformational transition from silk II (insoluble β-sheet conformation) to silk I (water-soluble structure). Alginate microcarriers (AMs) were prepared using a bead generator after parameter optimization (alginate flow rate, nozzle diameter, voltage magnitude, calcium chloride concentration, magnetic stirring rate, distance between the needle tip and gelling bath, data not shown). Subsequently, AMs were mild shaken into the silk fibroin solution and finally treated with ethanol to reconvert soluble fibroin (silk I) in a stable, insoluble homogeneous fibroin coating (Silk II) (Farago et al. 2016). The distribution of the fibroin coating has been investigated with confocal microscopy exploiting the fluorescence emitted by the fibroin when it was excited with laser.

In Figure 2, a representative picture showing a 3D reconstruction of the fibroin coating florescence of FAMs is shown. The fibroin completely covered the surface of the microcarrier, and numerous and scattered clots can be distinguished on the surface of the microcarrier.

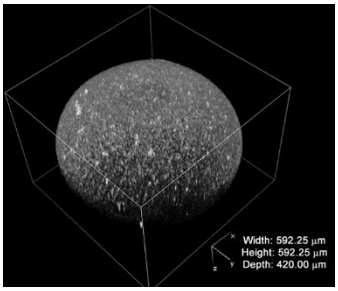


Figure 2. *Fibroin coating reconstruction by confocal microscopy (F) of FAMs.*

Physico-chemical and morphological characterization of microcarriers

Before and after fibroin coating process, the microcarriers were characterized in terms of particle size distribution, morphology, and fibroin molecular conformation. In Figure 3, the microcarrier size distribution curve is reported.

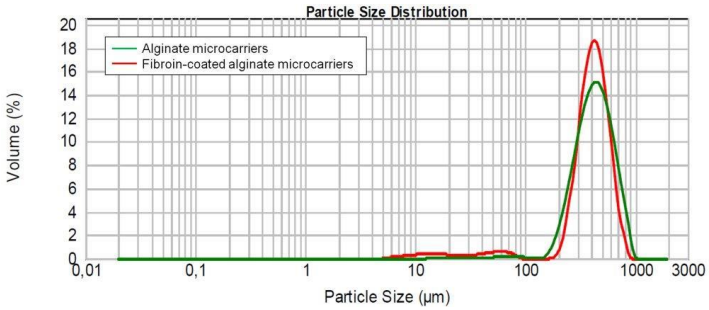


***Figure 3**. An illustrative particle size distribution of AMs (green line) and FAMs (red line). Data are reported as volume percentage values.*

The coating process did not influence the microcarrier size distribution as evidenced by the absence of a significant difference in volume weighted mean distribution, before and after coating ($p > 0.05$). In particular, AMs showed a mean diameter of 464.34 ± 62.190 µm (n = 5) respect to FAMs that showed a particle size of 421.94 ± 46.003 µm (n = 10). The morphological characterization of microcarriers were performed by electron scanning microscopy: AMs appeared as spherical structures with smooth surface (Figure 4, A and B at different magnification), whereas the fibroin coating formed a shell with a rough surface (Figure 4, E and F). The elemental analysis of microcarrier surface was performed by EDX analysis and a well-defined peak corresponding to nitrogen confirmed the presence of silk fibroin in FAMs (Figure 4G), with respect to AMs (Figure 4C). The fibroin molecular conformation was evaluated analyzing the FTIR spectra on both AMs and FAMs. The FTIR spectrum of AMs showed peculiar absorption bands regarding stretching vibrations of O-H bonds in the range 3000-3600 cm-1, stretching vibrations of aliphatic C-H at ~2900 cm-1 and asymmetric stretching vibration of the carboxylate group at ~1600 cm−1 (Figure 4D). The FTIR spectrum of FAMs confirmed the conformational transition of silk fibroin after ethanol treatment as showed by the main absorption bands detected at ~1620 cm-1 for Amide I and ~1520 cm-1 for Amide II, indicating that fibroin existed in its stable conformation (Figure 4H).

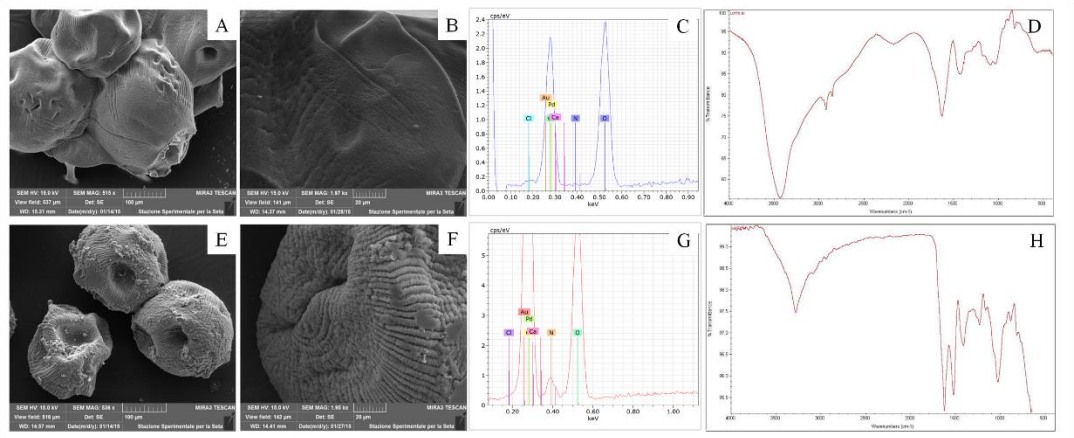


Figure 4. *Scanning Electron Microscope (SEM) images (A, B, E and F), Energy Dispersive X-ray (EDX) spectra (C and G) and FTIR spectra (D and H) of AMs (A-D) and FAMs (E-H).*

hASCs viability and proliferation

In order to evaluate their cytocompatibility, FAMs were cultured with hASCs. Cells were stained with Calcein-AM and Ethidium Homodimer-1 to evaluate live (green) and dead (red) cells, respectively (Figure 5).

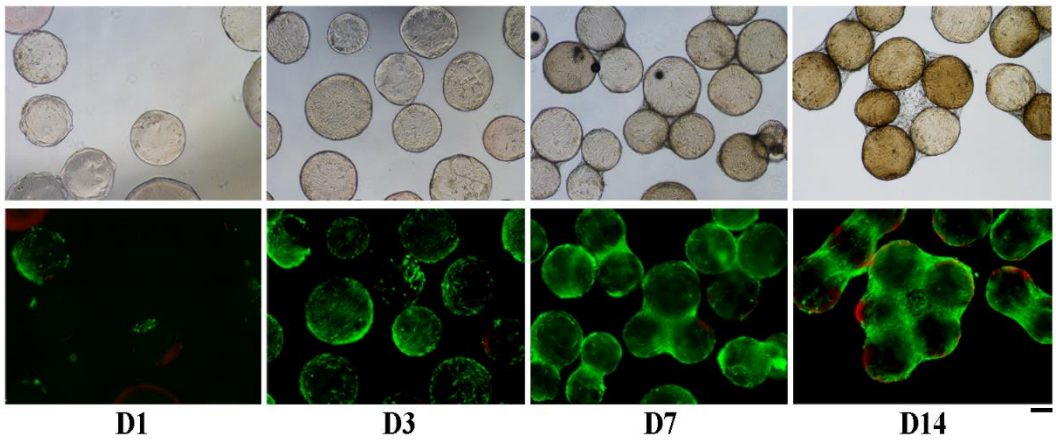


Figure 5*. Phase contrast (top) and fluorescence microscopy (bottom) images of Live&Dead stained cells with FAMs at 1, 3, 7, and 14 days from the cell seeding. Scale bar = 200 µm*

At day one, the adhesion of numerous cells was observed to the surface of FAMs, even if they were not homogeneously distributed. After 3 days of culture the cells almost completely covered the surface of the FAMs, whereas at day 7 cells started to build connections among FAMs. At 14 days from cell seeding, the most of FAMs were linked together by cells, and numerous tridimensional structures were created by interaction of adherent cells. No dead cells were detected on the FAMs surface along time in culture, thus confirming the good cytocompatibility of FAMs. The actin staining

of hASCs with fluorescent phalloidin after 7 days of culture allowed to observe that the cells strongly adhered to the fibroin coating, with a “stretching” shape morphology adapted to the curvature of the microcarrier surface (Figure 6).

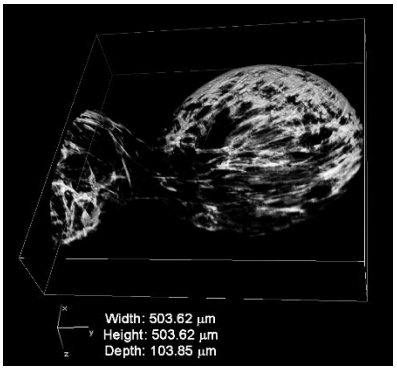


Figure 6. *Confocal microscopy image of hASCs F-actin cytoskeleton distribution on FAM surface after 7 days from cell seeding.*

These data were confirmed by the ultrastructural analysis of the transversal sections of FAMs, that showed a well-defined outline of alginate microcarriers and a silk fibroin shell (thickness about 0,2 µm, Figure 7). Human ASCs tightly adhered onto the FAM surface and exhibited their characteristic fibroblast-like shape. Moreover, a continuous and regular cell membrane has been appreciated and typical cytoplasm components such as nucleus, rough endoplasmic reticulum, vesicles, mitochondria, vacuoles and lysosomes were observed.

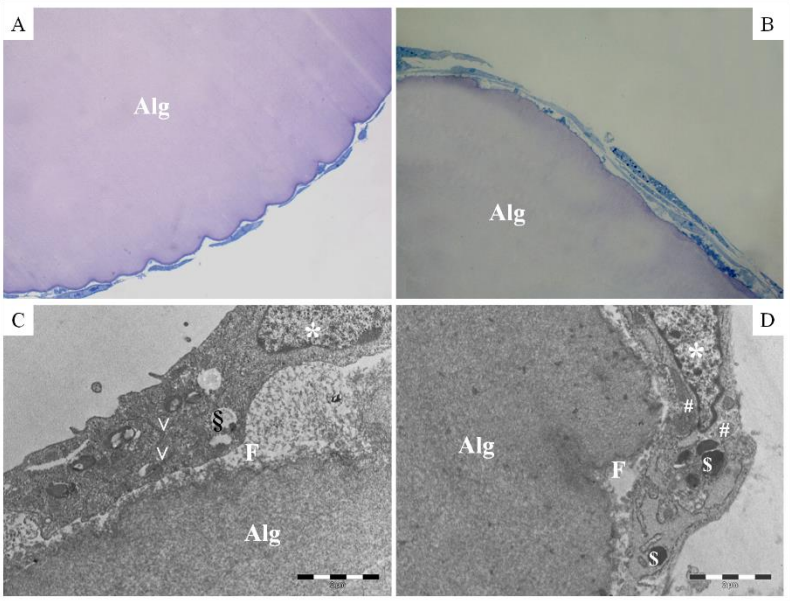
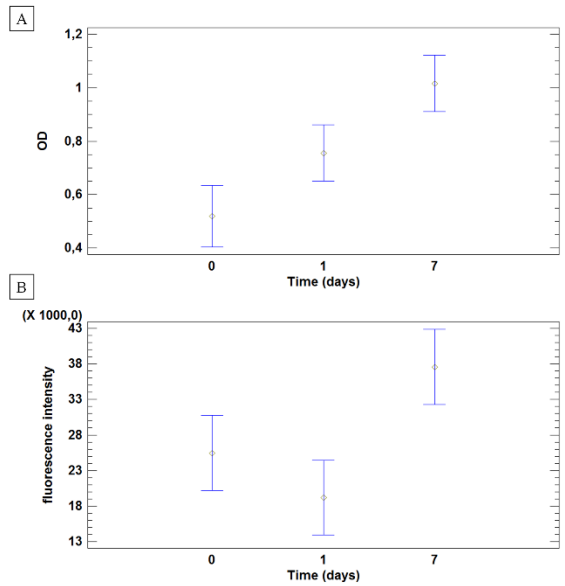


Figure 7. *Transversal sections of hASCs cultured on FAMs after 7 (A and C) and 14 (B and D) days of culture; A-B) light microscopy microphotographs, Toluidine Blue Staining, magnification 20x; C-D) Transmission electron microphotographs, 2 µm of scale bar: Alg alginate; F fibroin; * nucleus; v rough endoplasmic reticulum; # mitochondria; § vacuoles; $ lysosomes.*

Analysis of the hASCs metabolic activity (two cell lines) was performed by both MTT and AlamarBlue assays on five batches of FAMs at 3 time points: day 0 (2 hours after cell-microcarrier incubation), 1 and 7 days from cell seeding. Mean values and 95.0 % least significance difference (LSD) intervals are reported in Figure 8 as optical density (OD, Figure 8A) and fluorescence (Figure 8B) for MTT Assay and Alamar Blue, respectively, during the culture time.



Analyses revealed that both OD and fluorescence intensity depend on culture time (p-values: 0.0002 and 0.0038, respectively). No significant effect was observed between the two cell lines (p-values: 0.1024 and 0.6042, respectively for OD and fluorescence intensity). Moreover, the batch of FAMs did not influence the hASCs metabolic activity ($p > 0.05$). Results of both metabolic assays clearly suggest that no differences were observed after 2 hours of stirring (0 day) and 1 day of culture, but hASCs were able to proliferate on the FAMs.

Figure 8. *hASCs metabolic activity, performed by MTT (A) and Alamar Blue (B) assays. Results are reported as mean values and 95.0 % least significance difference (LSD) intervals of optical density (A) and fluorescence emission (B) of hASCs cultured on FAMs after 7 days of culture.*

Human ASCs multi-differentiative potential

Osteogenic lineage differentiation was assessed analyzing deposition of mineralized matrix and hydroxyapatite (HA) bone-like nodules. On day 28, untreated cells showed no mineralized matrix, whereas osteogenic-differentiated ones were able to produce mineralized matrix both in presence and in absence of FAMs (Figure 9A). When cultured in adipogenic condition, numerous lipid droplets were observed in hASCs cultured both in presence and in absence of FAMs (Figure 9B). Nile Red staining analysis was performed onto the FAM surface using confocal microscopy selecting only the fluorescence Ex/Em spectra of tryglicerides in order to exclude the Nile Red bound to phospolipides of the bilayer membranes. In Figure 9B a representative 3D reconstruction of microcarrier surface is reported where it is possible to distinguish the presence of triglycerides (red) in the treated sample respect to control.

The histological evaluation after chondrogenic differentiation revealed a higher amount of matrix deposited with higher compactness in presence of chondrogenic stimulus hASCs with FAMs compared to control condition (Figure 9C).

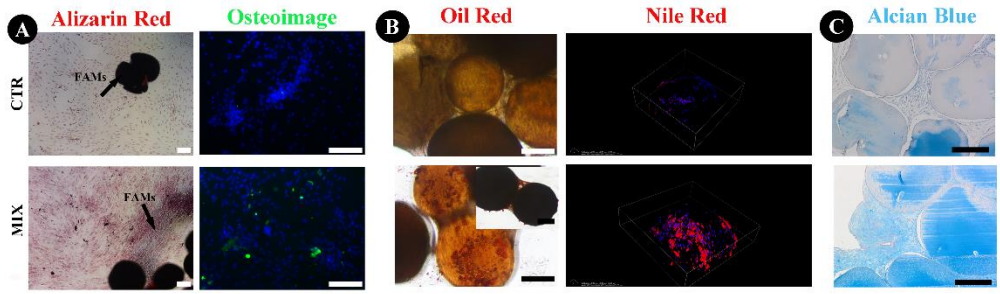


Figure 9. *Evaluations of osteogenic, adipogenic, and chondrogenic potential of hASC, cultured on FAMs. Alizarin Red and Osteoimage stainings for osteogenesis (A), Oil Red and Nile Red staining for adipogenesis (B), and Alcian Blue staining for chondrogenesis (C). Scale bar = 200µm.*

DISCUSSION

Microcarriers represent the ideal cell delivery systems in tissue engineering for their properties, such as an optimal surface area to volume ratio, promoting cell adhesion and proliferation; microcarriers can be directly injected in the affected site ensuring cell localization, remaining in the desired target tissue (A. K.-L. Chen, Reuveny, and Oh 2013), minimizing cell manipulation ex vivo, and reducing costs of cell expansion in GMP facilities. In the present work, a new composite microcarrier consisting of an alginate core and a silk fibroin shell was designed as a potential vehicle and delivery system for cells in regenerative medicine purposes. Alginate and fibroin, two natural polymers, were selected because they are biocompatible, biodegradable and clinical-grade available. Alginate was used as core giving stability to microcarriers, whereas fibroin was added to enhance cell adhesion and proliferation on the biomaterial, as previously reported (Chlapanidas et al. 2011, 2013). Our coating process did not modify the size (400µm) of microcarriers, similar to the ones of commercially available cell carriers (Rubin et al. 2007; Wu et al. 2003). Coating with fibroin was able to give a rough surface morphology to microcarriers that could be relevant to increase cell adhesion and thus proliferation. In fact, many authors have already highlighted the importance of mimicking the roughness nature of extra-cellular matrix during scaffold design and production, in order to improve the adhesion and the spreading of cells (Kim et al. 2007; Tang et al. 2014). A silk stable conformation consisting in crystalline β-sheets was observed by FTIR; confocal microscopy investigation showed a homogeneous coating on alginate surface, with some fluorescent clots related to marked roughness. Cell-FAMs samples analyzed by TEM showed tight contacts between hASCs and fibroin surface, while maintaining cell morphology unaltered. The hASCs adhesion on microcarrier substrate was also confirmed by immunofluorescent staining, labeling the F-actin with a fluorescent antibody after a cultivation time of 7 days (Figure 6) up to 14 days (data not shown). It is well-known that adhesion is a complex series of specific interaction-recognitions that occur between the ECM and integrins present on cell surface. A correct orientation of F-actin filaments determines the spreading-like morphology of the cell body that favor their normal phenotype and functionality. In this study, the hASCs adhesion on FAMs was clearly observed through confocal imaging showing dense and well elongated cell actin fibrils on the microcarrier surface. A few authors correlate these cytoskeletal distributions to the augment of cytoskeletal tension that should improve some cell abilities such as

proliferation and differentiation potential (Dumbauld et al. 2010; Tseng et al. 2012). FAMs also showed to be cytocompatible, since hASCs seeded on them were able to efficiently proliferate. Indeed they started to proliferate already after 24 hours after being seeded onto FAMs, and completely covered their surface after 7 days of culture, as reported by Schop et al, for dextran based microcarriers. Microcarriers were frequently used for MSCs expansion and several authors analyzed their proliferation and differentiation capabilities when cultured on these supports. In 2014, Caruso and colleagues used commercial Cytodex 3 microcarriers for MSCs expansion and they observed that cells maintained their metabolism as well as immunophenotypic and functional characteristics (Caruso et al. 2014). Similarly, MSCs were cultured on fibronectin-coated non-porous plastic microcarriers in spinner flasks, then detached and cryopreserved; after thawing, cell viability was higher than 70% and cells maintained their adherence ability to plastic surface and proliferation (Heathman et al. 2015). Similar results were published by Yuan and colleagues who demonstrated the maintenance of adipogenic and osteoblastic differentiation of MSCs after static or dynamic cultures on commercial CultiSpher-S microcarriers (Yuan et al. 2014). In our experiments, hASCs cultured on FAMs maintained their viability, and multipotential ability. Recently, a new approach in regenerative medicine is oriented to intraoperative solutions based on minimal cell manipulation, such as cell concentrate from bone marrow or adipose tissue (Enea et al. 2015; Gigante et al. 2011). In this context, fibroin has been employed as adipose stromal vascular fraction delivery system to support cell implantation in a murine model, (Vigani et al. 2016) we suppose that also fibroin coated microcarriers allow for cell adhesion, thus making them suitable for intraoperative use of cell concentrate.

CONCLUSIONS

In conclusion, our results suggest that the technological process is consistent for the production of fibroin/alginate microcarriers in lab-scale, and the products support hASCs adhesion and proliferation. Moreover, hASCs cultured in these 3D systems maintain their ability to differentiate. Therefore, technological resources are available for scale up production: these devices can be used for in vitro expansion/culture as well as for cell transplantation by a minimally invasive procedure (e.g. injection).

Acknowledgements

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Manuscript 6

In vivo biocompatibility and efficiency of silk/alginate microcarriers as delivery system of adipose-derived stem cells

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ABSTRACT

Background and objectives: the use of microcarriers for the delivery of mesenchymal stem cells is considered as a suitable approach for one-step surgical applications. The aim of this study was to test the biocompatibility of a fibroin coated alginate microcarriers (FAMs) previously developed in a rat model. Moreover, we evaluated the viability of the cells seeded on FAMs at the target site.
Materials and Methods: to test the biocompatibility of the device, 14 immunocompetent rats were used: 7 rats were treated with a small volume of un-seeded carriers (65 µl), 7 rats with a big one (275 µl). At 7d and 28d post-surgery the animals were analyzed by magnetic resonance imaging (MRI); the explants, obtained at 2d, 8d and 28d after surgery, were stained with Hematoxylin-Eosin. To evaluate the FAMs performance as cell carriers, FAMs were seeded with labeled hASCs and subcutaneously implanted in 10 athymic rats. Not-seeded FAMs were used as negative control, while positive controls were represented by a commercially-available scaffold seeded with the same cells. The animals were monitored with MRI (7d-14d) and in vivo fluorescence (ivF) imaging (1d-3d-7d-14d). The histological evaluation was also performed on samples retrieved at 8 and 15 days after surgery.
Results: MRI showed a consistent decrease of the implant size, confirming the biodegradability of the device. No adverse reaction was detected in any of the animals while evidences of tissue ingrowth within the implants were observed after 2 days from implantation. In the cell carrier assay, ivF showed specific fluorescence emission at the implant sites in all the cell-seeded implants, being the fluorescent signal more intense in FAMs than in the reference material. Histological analysis showed that hASCs were located on the tissue surrounding the FAMs, rather than on the carriers themselves.
Conclusion: FAMs represent a biocompatible and effective device for the delivery of MSCs in a rat model; these results represent the premise to verify the efficacy of FAMs in pathological conditions, like osteoarthritis.

INTRODUCTION

In the context of musculoskeletal disorders there is an increasing need to identify innovative treatments able to improve tissue healing, avoiding the use of invasive procedures.
Among musculoskeletal disorders, osteoarthritis (OA) is one of the most frequent conditions, affecting a large percentage of the population and thus representing a major social burden. Most of the current conservative treatments used in medical practice mainly act on symptoms relief and pain control rather than on the real cause of the disorder. As a consequence, an unavoidable further

degeneration of the whole joint occurs and in many cases joint replacement is the only possible alternative. Facing this scenario, regenerative medicine could represent a promising tool for the treatment of OA, offering the possibility to intervene where the current traditional drug therapies seem not to be effective.

In this context, mesenchymal stem cells (MSCs) play a crucial role due to their multiple properties able to promote restoration of tissue homeostasis. Firstly, MSCs have been considered for their ability to differentiate into cell types of the mesenchymal lineages such as osteocytes, adipocytes and chondrocytes (Dominici M et al, 2006). More recently, it has been demonstrated that the most important therapeutic property of MSCs rely on their anti-inflammatory and immunomodulatory properties exerted once they reach the site of injury (Chapel A et al 2003; Nasef A et al 2007; Franquesa M et al, 2012; Caplan AI and Correa D, 2011). Another great advantage in the use of MSCs is their considerable abundancy in several connective tissues. Indeed, a relevant amount of MSCs can be isolated from different adult tissues, such as bone marrow, adipose tissue, tendons, muscles (de Girolamo L et al, 2013). MSCs have been widely studied in several pre-clinical and clinical applications and currently more than 500 clinical trials are ongoing showing satisfactory preliminary results that strengthen the hypothesis of the safety and effectiveness of MSCs-based therapies (Ra JC et al, 2011; Lalu MM et al, 2012).

However, several open questions concerning the use of MSCs still remain unanswered, including the way of administration and the best strategy to improve their engraftment at the target site.

In spite of their homing capacity (de Becker A et al, 2016), the cell viability and survival of MSCs at the lesion site could be negative influenced by lack of nutrients and oxygen usually present at pathological sites (Sarkar D et al, 2011).

Even though they are generally used for vaccine production (Tree JA et al, 2001; Trabelsi K et al, 2012) microcarriers have been recently recognized as an effective substrate to culture and expand anchorage dependent cells, such as MSCs too (Merten OW, 2014), as they are able to promote their growth, proliferation, maintenance of viability and differentiation (Shop D et al, 2010; Goh TK et al, 2013; Lin YM et al, 2016; Sart S et al, 2016). Furthermore, since they are biodegradable and biocompatible in the majority of the cases, microcarriers may be considered as a suitable tool for cell delivery (Bouffi C et al, 2010). In this view the permanence of MSCs at the lesion site would be improved while maintaining the native features and potentialities of MSCs, providing a transient platform suitable for cell survival and improving their functions (Anderson HJ et al, 2016; Dolatshahi-Pirouz A et al, 2011).

We recently developed a cell-carrier system based on alginate-silk biomaterials (FAM, fibroin-alginate microcarrier) and we evaluated its in vitro potential suitability as a carrier for the delivery of MSCs in regenerative medicine applications (Chlapanidas T et al, waiting for submission). The results suggest that the technological process to produce FAMs is suitable and consistent to further scale up their production, providing an optimal vehicle for cell delivery, guarantying a good cytocompatibility and preserving the viability and the multi-differentiation potential of adipose-derived stem cells (ASCs). The aim of this study is to test the device an *in vivo* rat model, focusing on their biocompatibility, and ability to load and carry viable mesenchymal stem cells.

MATERIALS AND METHODS

Microcarriers preparation

Microcarriers were prepared as previously described (Chlapanidas T et al, waiting to be submitted). Briefly, alginate microbeads of about 450 µm-diameter were obtained by dropping a 1% w/v sodium alginate aqueous solution with an electrostatic bead generator (Encapsulator VAR V1, Nisco Engineering AG; Zurich, Switzerland) into a gelling bath consisting of a stirred 100 mM calcium chloride aqueous solution. Alginate microbeads were then coated with silk fibroin by immersing them for five minutes in a gently stirred 1.5 % w/v solubilized fibroin aqueous solution (previously obtained according to (Chlapanidas T et al, 2013), and subsequent bathing in 96% (v/v) ethanol (Carlo Erba, Italy) for 15 minutes to induce silk conformational transition and β-sheet formation. The latter step was repeated three times to ensure a homogeneous and complete fibroin coating. Finally, fibroin-coated alginate microcarriers (FAMs) were suspended in ethanol and stored at 4ºC until their use.

Cell Culture

Human adipose-derived mesenchymal stem cells (ASCs) were isolated from abdominal subcutaneous fat from patients undergoing liposuction at the Galeazzi Orthopedic Institute (Milan, Italy). Waste surgical samples were collected as under written consent of the patients (M-SPER-014.ver7 for the use of surgical waste). hASCs were isolated as previously described (de Girolamo L et al, 2009). Briefly, the adipose tissue was digested by 0.075% w/v type I collagenase (Worthington Biochemical Corporation, LakeWood, NJ, USA). The cells were then collected by centrifugation (1,200 g, 10 minutes) and seeded in complete medium (CM), consisting of Dulbecco's Modified Eagle's Medium-high glucose (DMEM-HG; Sigma-Aldrich, St. Louis, MO, USA), supplemented of 10% fetal bovine serum (FBS; HyClone, EuroClone), 50 U/ml Penicillin, 50 mg/ml Streptomycin, and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA). ASCs were maintained in standard culture conditions (37°C in 5% humidified CO2 atmosphere), with medium changes every 3 days. When cells reached 70-80% confluence, they were detached by 0.5% trypsin/0.2% EDTA (Sigma-Aldrich) and plated at a density of 3,000 cells/cm^2. ASCs were used for following experiments until passage 5.

Cell seeding and labelling on FAMs

Prior to cell seeding, FAMs were washed three times with sterile saline (0,9% NaCl aqueous solution) and conditioned overnight with cell culture medium (CM). The carrier suspension was divided into 500 µL aliquots and each aliquot was seeded with 500,000 cells suspended in 1 mL of CM, corresponding to a cells/carriers density of 5,000 cells/cm^2 of carrier surface. The tubes containing the seeded carriers were stirred for 2h at 70 rpm on an oscillating shaker (Rotamax 120, Heidolph) placed inside an incubator (37°C, 5% CO_2), in order to promote cell adhesion to all the carriers surface. Afterward, fresh CM was added to the tubes. The following day, cell-seeded FAMs were washed for 10 minutes with sterile saline and ASCs were labeled with DiD (Molecular Probes; Eugene, Oregon, USA) according to the manufacturer's instructions. Briefly, cells were incubated for 30 minutes at 37°C and 5% CO_2 in serum free CM supplemented with 5 µM DiD.

A set of carriers was treated alongside with seeded ones, but left unseeded as negative controls.
An injectable biocompatible scaffold approved for clinical use made of atelocollagen (CartiFill™; Regenerative Medical System; Hertfordshire, UK) was used as reference material and undergone to the same procedure steps as the carriers.
Prior to in vivo implantation, fluorescent emission from DiD-labelled cells (Excitation 650 nm/Emission 700 nm) was checked in an Optical and X-Ray imaging system (in vivo Xtreme; Bruker; Billerica, Massachusetts, USA).

Animal handling and implantation surgery

Immunocompetent rats were used to assess microcarrier biocompatibility, while athymic nude rats (rnu) were used to assess microcarrier-seeded cell survival upon in vivo implantation. The rats of both strains were housed in the animal house facility of BIONAND (Andalusian Centre for Nanomedicine & Biotechnology) at a constant temperature of 24 ℃ and a 12 hours light/darkness photoperiod, with free access to food and water. Animal handling was conformed to Spanish and European regulations (RD 53/2013 and Dir. 2010/63/EU).
To study carrier biocompatibility in vivo carriers were washed for one day in sterile saline with regular saline changes, and implanted subcutaneously into the back of 14 adult (8 week old) male Fischer 344 rats (Charles River Laboratories Inc, Wilmington, Massachusetts, USA). The anesthesia was induced by sevoflurane inhalation at 3% (SevoFlo®, Abbot Animal Health, USA) and the animals were maintained under anesthesia during the procedure (Figure 1).

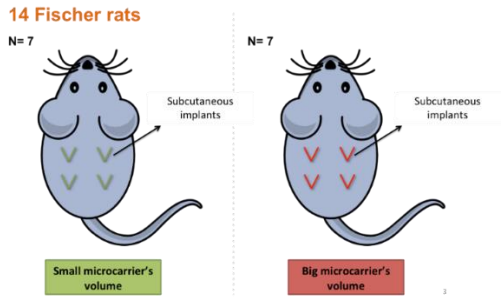

Figure 1: *schematic representation of the implants for the biocompatibility evaluation*

The rats received a single dose of 15 mg/Kg body weight of the antibiotic enrofloxacin (Baytril®, Bayer; Leverkusen, Germany) and 0.03 mg/Kg body weight of buprenorphine hydrochloride (Buprecare, DVF Group; Barcelona, Spain), both injected subcutaneously. After hair removal, the dorsal region was then disinfected with povidone-iodine and an incision was practiced with a sterile scalpel through the medial dorsal skin. Four subcutaneous pockets were opened at the sides of the incision using sterile blunt scissors and a volume of microcarriers was placed inside each pocket. The fourteen rats were divided in two group of treatment: seven rats received a big volume (275 µL) of microcarrier and the other severe a small volume (65 µL). The skin was closed with non-absorbable suture and the rats placed back into their cage until total awakening from anesthesia. Analgesia was maintained for at least 24 hours by buprenorphine hydrochloride dissolved in the drinking water (0.15 µg/mL water). The behavior of the animals was observed twice a day by their usual carers to

check for signs of stress or pain. On days 7th and 28th after microcarriers implantation, the rats were undergone to magnetic resonance as described below. Two, eight and twenty-eight days after implantation, the animals were anesthetized by sevoflurane inhalation and euthanized by CO_2 inhalation. The implants were dissected out and processed for histological analysis. Seven rats were used for each time-point.

To study their survival on the microcarriers after implantation in vivo, ASCs were seeded either on microcarriers or on Cartifill™ and labelled with DiD (Figure 2a).

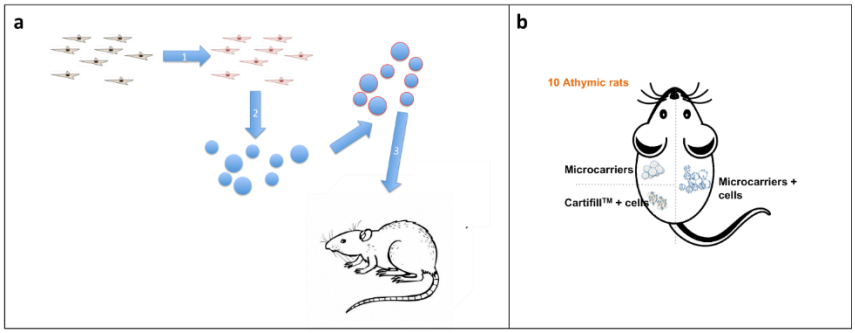

Figure 2: *schematic representation of the cell labeling before implantation (a) and implants disposition (b) in the cell-carrier assay.*

Following the same technical procedure reported above, 14 athymic nude rats (rnu, Charles River Laboratories Inc) received 3 subcutaneous dorsal implants, corresponding to non-seeded microcarriers, DiD-labelled ASC-seeded microcarriers and DiD-labelled ASC-CartiFill™, a biocompatible material clinically used as cartilage filler, used as reference material. After 1, 3, 7 and 14 days after implantation, the animals underwent to in vivo fluorescence detection and MRI (only day 7 and 14). Eight days and fifteen days after implantation surgery, the rats were anesthetized and euthanized as already described, and the implants dissected out and processed for histological analysis. Seven rats were used for each time-point.

Non-invasive imaging and analysis

For in vivo fluorescence imaging, the rats were anesthetized by isoflurane inhalation (induction with 4% IsoFlo in 100% oxigen at 1.0 mL/min; maintenance with 1% IsoFlo). They were then placed inside the chamber of an in vivo Xtreme instrument (Bruker; Massachusetts, USA), equipped with a warming system and anesthesia inhalator. Then DiD fluorescence was captured with the following settings: Excitation 650nm/Emission 700 nm; 20 seconds exposure time. X-ray acquisition was performed in the same equipment to obtain a background reference image. Acquisition and image analysis was performed with MI software (Bruker; Massachusetts, USA). Statistical analysis of quantitative data was performed with Numbers software (Apple; California, USA). The rats undergoing MRI were anesthetized by isoflurane inhalation (and placed in the cradle of a Bruker Biospec® 9.4T/20 MRI system for small animals (Bruker; Massachusetts, USA). While in the magnet,

respiratory rate was monitored, body temperature maintained using a temperature-controlled water circuit within the holder, and anesthesia maintained by isoflurane inhalation.
MRI images were acquired with the following parameters: TR/TE = 300/6 ms, 30 deg flip angle, 5 slices covering the lung, slice thickness1mm and gap of 0.5 mm,FOV = 6 cm x 4 cm, matrix 256 x 256, in plane resolution of 156 µm, 80 repetitions resulting in a 12 min acquisition; the navigator slab was 1cm wide excited with a 0.8 ms sinc10H pulse with a 1.5 deg flip angle. For reconstruction, 70% of the respiration and ECG period was used (Paravision 5.1, Bruker).

Histology

The implants were fixed in 4% formaldehyde (Panreac; Barcelona, Spain), or in Bouin Solution for 24 hours. Bouin-fixed samples was washed after fixation for 24 hours in a saturated aqueous solution of lithium carbonate to wash out picric acid excess. Paraformaldehyde-fixed samples was washed for six hours in 1 mg/mL sodium borohydride (Panreac; Barcelona, Spain), diluted in PBS buffer (Sigma-Aldrich; Missouri, United States), to block free amines. Fixed samples were dehydrated through a graded series of ethanol and embedded in paraffin. They were then cut into 8 µm-thick sections and mounted on silane-coated glass slides.
Tissue sections were stained with Haematoxylin-Eosin as follows: after deparaffination with xylene and rehydration through a negative gradient of ethanol, sections were rinsed in distilled water, stained in Harry's Haematoxylin for five minutes, washed in distilled water, stained with hydroxyalcoholic Eosin for 3 minutes and then dehydrated and mounted in Eukitt™.
To localize ASC in rat tissue sections, human nuclei were immunostained according to the following protocol: deparafffinated and rehydrated tissue sections were rinsed in PBS buffer and incubated overnight in mouse monoclonal anti-human nuclei antibody (Merck-Millipore; Massachusetts, USA), diluted 1:250 in PBS-BT buffer (PBS buffer supplemented with 1% bovine serum albumin and 0,5% Triton™ X-100). Bovine Serum Albumin (BSA) and Triton™ X-100 were purchased from Sigma-Aldrich (Missouri, USA). Afterwards, sections were washed with PBS buffer and incubated for one hour with a biotin-conjugated anti-mouse IgG (Merck-Millipore; Massachusetts, USA), diluted 1:2,500 in PBS-BT buffer. Then, sections were washed again with PBS and labelled for 30 minutes with ExtrAvidin®-Peroxidase complex (Sigma-Aldrich; Missouri, United States), diluted 1:200 in PBS buffer. Diaminobenzidine (DAB; Sigma-Aldrich, Missouri, USA) was used as a chromogenic substrate of the peroxidase reaction. Finally, sections were dehydrated in a graded ethanol series and mounted in Eukitt™.
Unless otherwise stated, all reagents and products were purchased from Electron Microscopy Sciences (Pennsylvania, USA)

RESULTS

Subcutaneous implants of microcarriers in immunocompetent rats

Behavioral analysis

After implantation surgery, and for all the duration of the experiments, the animals showed no signs of stress or discomfort, with no scratching, licking nor biting the skin over the implants or the surgical

wound. Their grooming behavior was normal, and they maintained a regular food and water intake. No other adverse reactions after surgery nor during the experiment follow up were recorded.

FAMs biodegradability detected by Magnetic Resonance Imaging

During the first week, the implants could be detected as small lumps under the skin of the animals, which could be clearly palpate with fingers. The MRI confirmed the subcutaneous location and allowed volume measurement for both implant types (Figure 3). In particular, the data showed a consistent decrease in term of size of all the implants between 7 and 28 days, confirming the biodegradability of the device.

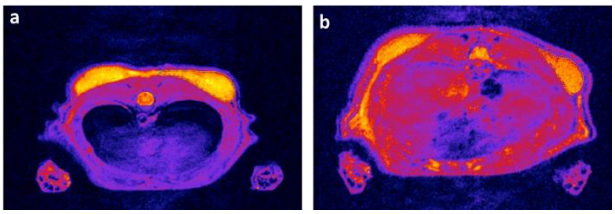

Figure 3: *Magnetic Resonance Imaging of the implanted carriers showing their subcutaneous location. Representative images of implants at 7days (a) and 28 days (b) after the surgery.*

Histological analysis

On the second day after surgery, the implants appeared enclosed in a light sheath of vascularized connective tissue, and they were infiltrated by the host's tissue up to the center of the implant (Figure 4), in both the small and the big sized implants. Tissue infiltrate consisted mainly of white blood cells and some fibroblasts (Figure 4). Some FAMs appeared broken and the host cells colonized their interior (Figure 4). The extracellular matrix was absent, with the exception of some scarce fibrous material (Figure 4).

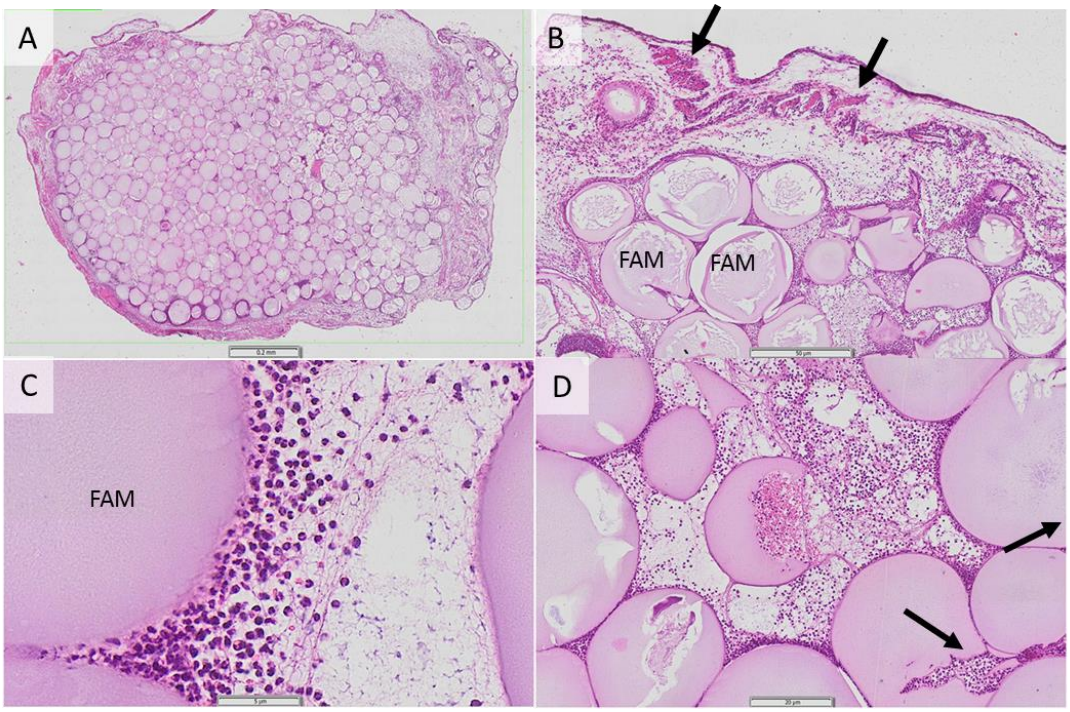

Figure 4: *Big-size FAM implants 2 days after surgery**. A:** Overview of the whole implant, where FAMs are visible as spheres. A sheath is visible around the mass formed by spheres. **B:** Detail of the FAMs and the sheath that envelopes the implant. Arrows point to blood vessels. **C:** Detail showing the infiltrate between the FAMs. Numoerous white blood cells are visible in around the FAM surface. No extracellular matrix apart of some fibres was observed. **D**: Details showing colonization of the FAMs by the host cell infiltrate. Arrows point to broken FAMs that are being colonized by cells.*

Eight days after implantation, a tissue rich in cell elements was present within the implants, in between the FAMs. It was composed by abundant fibroblastic cells and a certain amount of extracellular matrix (Figure 5). Mature blood vessels could be observed even in the most inner part of the implant, both in small as in big-sized implants. A certain difference as to tissue maturation could be appreciated between the small and the big size implants. The extracellular matrix between the FAMs was more abundant in the small implants, and a certain fiber alignment indicated a more advanced degree of ECM organization (Figure 5). In both types of implants, an initial FAMs resorption could be observed: numerous FAMs were broken and infiltrated by cells, - in many cases only fragments remained-, and in all cases, cells were attached to the FAMs surface (Figure 5).

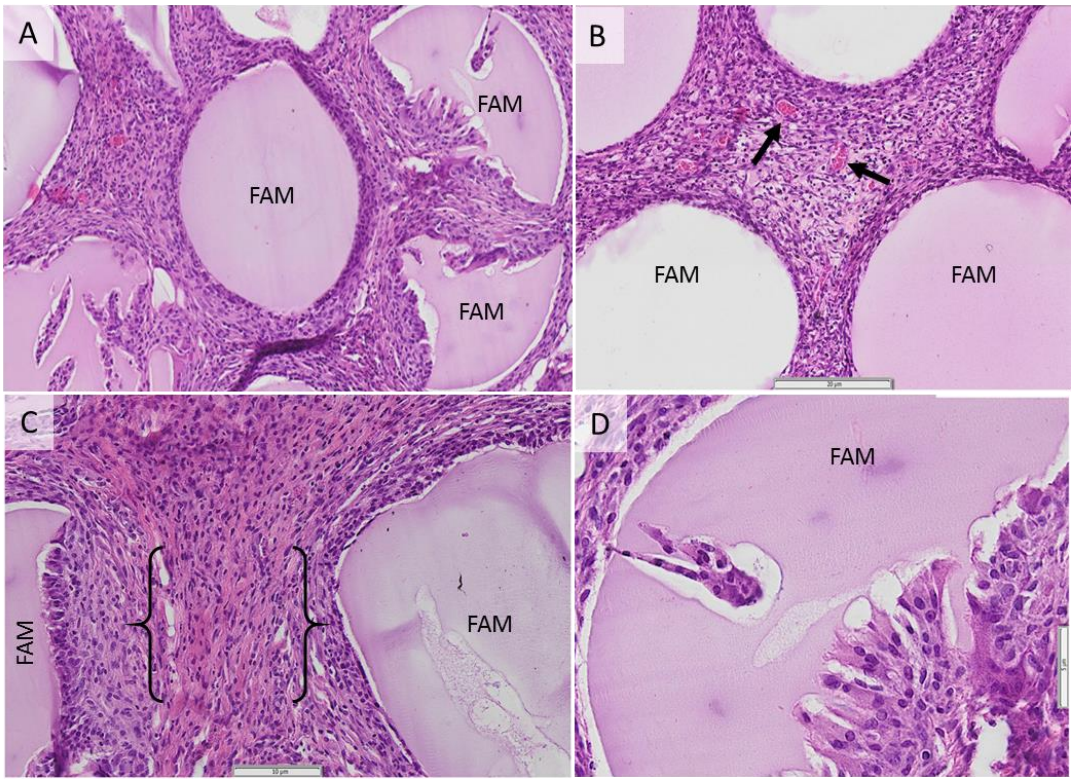

Figure 5: *FAM implants, 8 days after surgery. H-E staining A: Small size implant. B: Big size implant. In both cases a tissue rich in cell elements was present in between the FAMs, most of which are broken into fragments. Arrows point to blood vessels. C: Detail of a small size implant. Extracellular matrix showed signs of maturation, where fibers alignment was appreciable {}. D: Detail showing a fragment of a FAM that is being resorbed.*

On the 28th day after implantation, the small size implants were almost totally resorbed and were difficult to find them during the dissection. The big implants were not as resorbed, but they were not manually palpable any more. Histological analysis revealed that in both implant types the infiltration tissue between the FAMs was rich in extracellular matrix, which presented a more mature aspect: the extracellular component was more abundant than the cellular one and was rich in fibers (Figure 6).

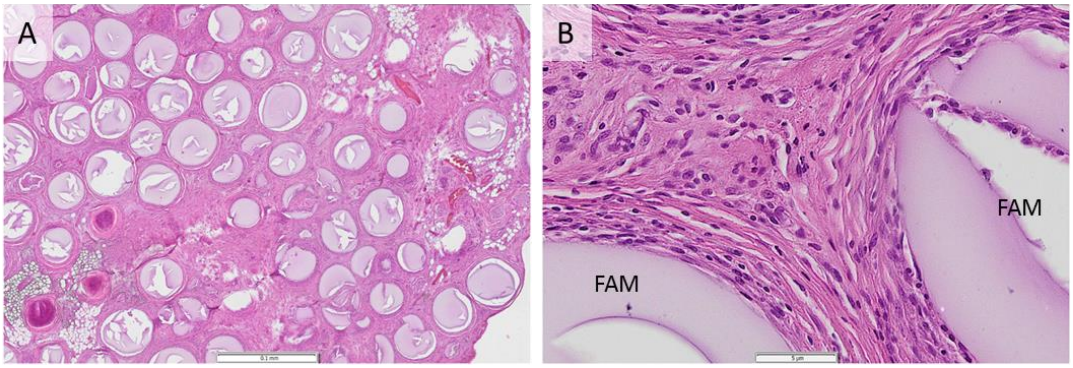

Figure 6: *FAM implants, 28 days after surgery. H-E staining A: Overview of a small size implant. An abundant amount of tissue was present in between the FAMs, expecially as compared to figure 4A. B: Detail of the ECM between the FAMs. An abundant extracellular matrix, rich in fibers, can be observed between the FAMs. FAMs surfaces are covered by cells.*

No fibrotic tissue formation was observed in any case. At all the time points, the presence of leukocytes and lymphocytes, index of an inflammatory response, was very scarcely seen around the residual scaffold, indicating that the material was highly biocompatible.

Subcutaneous implants of cell-seeded microcarriers on athymic rats

Before being implanted in vivo, the cell labelling efficiency was assessed under fluorescence light (Ex./Em. 650/700nm). DiD-labelled cells emitted a strong signal, thus confirming a correct labeling. No emission was detected in un-seeded scaffolds.

In vivo Fluorescence Detection of DiD-labelled hASCs seeded on FAMs and Cartifill

Fifteen days after the implantation, cells were detected by non-invasive in vivo fluorescence emission Imaging. In all the animals, the fluorescence emission was more intense in ASC seeded FAMs than in CartiFill^{TM} samples. Again, after implantation no fluorescence emission was detected in unseeded FAMs. As expected, fluorescence intensity decreased over time (Figure 7).

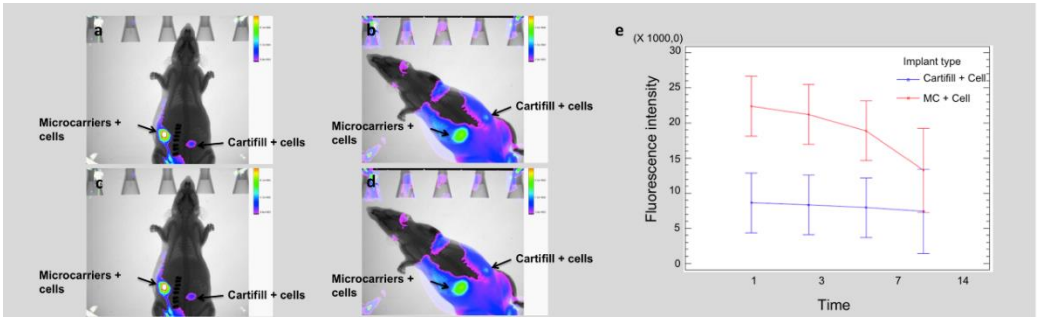

Figure 5: *In vivo Fluorescence Detection of DiD-labelled hASCs seeded on FAMs and on Cartifill, implanted subcutaneously. The labelled cells were followed up for 14 days after implantation. Imaging was performed on days 1 (a), 3 (b), 7 (c) and 14 (d) post-implantation. Relative fluorescence emission quantification are represented in the graph (e)*

Histological analysis

The tissue infiltration pattern was similar to the one observed previously in immunocompetent rats. The localization of implanted cells by immunohistochemistry at both 8 and 15 days confirmed the in vivo fluorescent data, showing the presence of human cells in the seeded FAMs and CartiFill scaffolds. Immunolocalization of human nuclei showed that implanted human ASCs were located on the tissue surrounding the scaffolds both for FAMs and CartiFill^{TM}, rather than adhered to their surface (Figure 8).

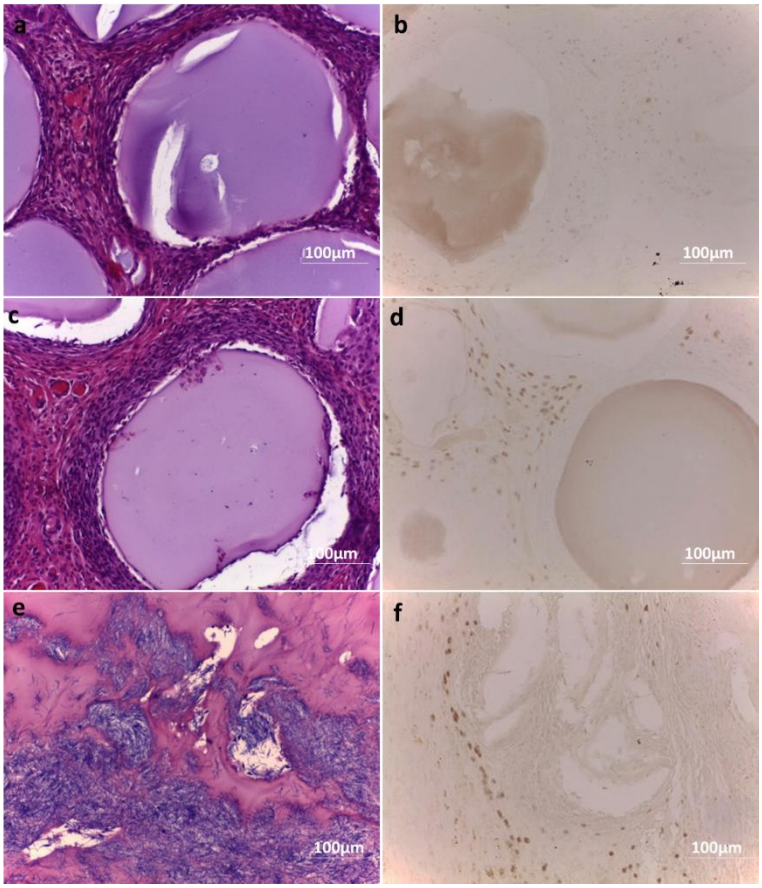

Figure 6: *Haematoxilin-Eosin (a-c-e) staining and human nuclei immunolocalization (b-d-f) on the FAMs without cells (a-b), cell seeded FAMs (c-d) and cell-seeded CartiFill™ (e-f) implants 15 days after the implantation. Scale bar: 100µm*

In one case, cells migrated from a cell-seeded implant site to a non-seeded implant one. This was detected by in vivo fluorescence and later confirmed by immunodetection of ASC. The cells were located in the extracerllular matrix around blood vessels of the non-seeded microcarrier implant.

DISCUSSION

The results of this study demonstrated that FAMs are able to keep viable cells at the target site for a long period after implantation into subcutaneous tissue of rats, thus confirming the previous in vitro results, and not to elicite any immunoreaction. These findings support the feasibility of the use of FAMs to locally deliver MSCs, thus potentially providing an improvement in term of therapeutic potential of cells. Indeed, it has been demonstrated that the way to deliver MSCs is it crucial to obtain the best results. Indeed, in cell therapy application a great challenge is the maintenance of cells at the target site. In this view the use of biodegradable and biocompatible and bioactive carriers could be seen as a valid approach to overcome this issue, thus enlarging the potential of these kinds of applications. In this regard, we developed a device composed of silk fibroin and sodium alginate

with the final aim to achieve a suitable tool to deliver locally MSCs *(Chlapanidas T et al,waiting to be submitted).*

Basing on the previous results derived from the in vitro characterization of FAMs, in this study we aimed to evaluate the suitability of the device in a rat model, in order to assess both the biocompatibility and the suitability of this approach to delivery MSCs and to maintain them at the target site.

Our results showed that FAMs progressively biodegraded during the follow-up, until their almost complete disappearance at later time points (28d) for the small volume samples. It is known that the biodegradability is a necessary condition *(Xie H et al, 2007*). However, the adequate time of biodegradability should be correlated to the conditions in which they are used and should be appropriate to the cell survival and maintenance, avoiding a too fast degradation that would actually nullify the usefulness of this approach. In this view, future researches will be needed to determine the time of biodegradability sufficient to permit a complete in vivo function of MSCs in a model of OA, where the environment could differently affect the biodegradability time.

Our findings also showed the lack of important inflammatory reactions; the slight signs of inflammation revealed by our histological analysis can be considered as a basal inflammatory reaction to any external bodies, as it was already described *(Tang L, Eaton JW. 1995)*, thus supporting the biocompatibility of FAMs.

Since FAMs have been developed to be used as delivery systems, in this context the use of a biomaterial possesses a different significance. In fact, FAMs is needed only for the delivery of cells and for the maintenance of them at the target site, thus moving away from the more traditional concept of scaffold in tissue engineering application that should also possess adequate properties in term of shapes and mechanical strength *(Rahaman MN et al, 2005; Choi YS et al, 2005).* However the properties of cell carriers still play a crucial role since in tissue engineering and cell therapy the cell anchorage-dependency is a key factor *(Thiery JP, 2003; Lakard et al. 2004)*. Indeed the adhesion to a support is a necessary condition to avoid the occurrence of apoptotic events *(Dalby MJ et al, 2004,*) and to allow cells to live in a more physiological conditions that should lead to better performances. It is known that the behavior and functions of cells can be deeply affected by the type of support and by the morphology that they acquire while on that. Our results showed that the viability of ASCs seeded on FAMs was always higher than the ones of cells seeded on the material used as control. Since the impants were done subcutaneously in an ectopic site, we cannot correlate any therapeutic efficacy to the length of permanence of viable cells; however, the longer permanence of MSCs at the target site should surely represent an advantage, expecially if we consider the longer duration of the MSCs paracrine activity. So, in our future study, we will investigate if the delivery of MSCs by FAMs will positively affect the secretory activity of cells, both due to the physical support provided by FAMs as well as by the increased length of permanence at the target site. Moreover these tests will be performed again in a pathological condition to assess any possible modifications induced by the pathology itself on the performances of the ASCs-FAMs construct.

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Manuscript 7

Optimization of cell adhesion rate on silk/fibroin microcarriers for one-step mesenchymal stromal cell delivery: Design of Experiment approach

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ABSTRACT

Background and objectives: the use of minimally manipulated MSCs in intra-operative approaches for local therapy is progressively increasing. To improve the efficiency of the treatment, cells can be delivered to the lesion site using specific delivery system. However, as one-step procedures imply short time of preparation, the time needed to achieve a satisfactory cell adhesion to carriers is crucial. In this view, the aim of the present work is to optimize and speed up the adhesion efficiency of ASCs on lyophilized fibroin coated alginate microcarriers (L-FAMs), using the Design of Experiment (DoE) approach, with the final aim to obtain a validated protocol for intra-operative ASCs administration.
Materials and methods: the DoE approach resulted in thirteen different parameters combinations to test the seeding conditions on lyophilized FAMs. The seeding efficiency was evaluated in term of speed and percentage of adhesion, as well as viability and metabolic activity of adhered cells.
Results: the best cell seeding protocol in term of seeding efficiency was the one involving the intermittent stirring of the cell-L-FAMs suspension, at a speed of 10 rpm in a seeding volume of 400 µl. These factors resulted to be crucial to obtain a satisfactory and homogeneous adhesion in less than two hours. The DoE outcomes confirm these results suggesting also that a rotation speed of 12 rpm had the best response.
Conclusions: Our results demonstrated that L-FAM are a good delivery system compatible with one-step local procedures, as they were able to guarantee a rapid cell adhesion and maintenance of MSCs features in a limited timeframe, properties that could be potentially exploited also for one step surgical applications.

INTRODUCTION

Thanks to the ability of Mesenchymal Stem Cells (MSCs) to direct participate to tissue regeneration as well as to restore tissue homeostasis through their paracrine activity (Caplan Al, 1995) the interest on MSCs applications in the clinical practice for the treatment of a wide range of musculoskeletal pathologies has progressively increased (Friedenstein et al, 1966).
To adequately target the injury site is crucial, so that many studies described different strategies to efficiently deliver MSCs, ranging from systemic to local injection, and in presence or absence of specific carriers. In fact, although the homing ability of MSCs allows them to migrate to the site of injury, it has been also demonstrated that in case of systemic administration only a low number of cells is able to actually reach the target tissue (De Becker A et al, 2016; Park JS et al, 2015; Steigen C

et al, 2008; Chapel A et al, 2003; Gao J et al, 2001). Hence, for site-specific pathologies, the local delivery is preferred.

A consolidated method to deliver MSCs to the injury site, especially for musculoskeletal disorders, is the use of tridimensional scaffolds. Among them microcarriers (MC) (Whang L et al, 201; Qazi TH et al, 2015; Pumberger M et al, 2016; Cezar CA et al, 2014; Shekaran A et al, 2015; Shekaran A et al, 2016; Navqi SM et al, 2016; Jin GZ et al, 2014), despite they don't provide any structural support, represent a valid tool to improve cell delivery at the target site, and thus to improve their therapeutic potential (Chen AK et al, 2013; Georgi N et al, 2014).

To overcome the regulatory restrictions regarding the advanced cell therapy products, the most innovative approaches are directed to the development of intra-operative protocols for minimally manipulated autologous MSCs local therapies, namely the administration of non-expanded cell concentrates from bone marrow and stromal vascular fractions (SVF) from adipose tissue. Another crucial point is the optimization of the carrier system in order to obtain a rapid and satisfactory delivery of cells to the target site, in compliance with the one-step surgical approach requirements, the maintenance of a minimal cell manipulation, and the preservation of the peculiar cell features. Since MSCs are adherent cells, a delivery system in which MSCs can maintain their adherent nature would preserve their physiological status representing the optimal condition for MSCs to exert their activity.

We previously developed a biocompatible silk fibroin-coated alginate microcarriers model (FAM), as a cell-carrier system for the delivery of adipose derived mesenchymal stromal cells (ASCs) for one-step surgical approach for the treatment of osteoarthritis and intervertebral disc degeneration (Chlapanidas T et al, waiting to be submitted; Perucca Orfei C et al, waiting to be submitted). This model allowed the cell adhesion in a short period (2 hours) thus making it a suitable approach for one step surgical applications. However, the cell adhesion can be further improved in term of homogeneity, maintenance of viability and time.

The aim of the present study is the optimization of the ASCs seeding procedure on lyophilized FAMs (L-FAMs) in order to obtain a rapid and satisfactory cell adhesion, in the less time possible. This research is intended to offer a model of ASCs seeding onto FAMs that could be suitable also for minimally manipulated procedures, with the final purpose to validate a protocol for intra-operative such as the bone marrow concentrate or the SVF administrations. To reach our goal we employed the Design of Experiment (DOE) approach that allows the quantitative analysis of a wide range of experimental conditions and the identification of synergistic mechanism that are not able to recognize with traditional statistic method. Future researches will be performed to confirm the suitability of the developed model also for the administration of SVF.

METHODS AND MATERIALS

L-FAMs preparation

Fibroin-coated alginate microcarriers were obtained as previously described (Chlapanidas T et al, waiting to be submitted). Briefly, sodium alginate (1% w/v, medium viscosity, Sigma-Aldrich, Milan, Italy) was solubilized in distilled water and then the solution was added dropwise into an aqueous solution containing calcium chloride (Sigma-Aldrich) 100 mM under magnetic stirring using a bead

generator (Encapsulator VAR V1, Nisco Engineering AG, Zurich, Switzerland) to obtain alginate microcarriers (AMs).
Bombyx mori cocoons were degummed and silk fibroin fibers were solubilized in phosphoric acid/formic acid (80:20 v/v) (Sigma-Aldrich) under magnetic stirring at room temperature; the obtained silk fibroin solution was dialyzed against distilled water (membrane cut off 12 kDa, Visking, London, UK) at RT.
AMs were shaken into fibroin solution (volume ratio alginate microcarriers: fibroin solution 1:2) and then immersed in 96% (v/v) ethanol (Carlo Erba Reagents, Milan, Italy) to induce silk conformational transition. The procedure was performed three times to assure the homogeneous and complete coating. Fibroin-coated alginate microcarriers (FAMs) were washed with distilled water and subjected to freeze-dried process. Lyophilized fibroin-coated alginate microcarriers (L-FAMs) were stored at room temperature.
Granulometric analysis of L-FAMs was performed with a laser light scattering analyzer (Mastersizer 2000, Malvern Instruments Ldt, Worcestershire, UK) equipped with Hydro SM wet sample dispersion unit.

Isolation of human adipose derived stem cells (ASCs)

Adipose tissue was obtained at our Institute, from 3 patients who underwent aesthetic liposuction. All the procedures involving the use of human biological material were carried out accordingly to our Institutional Review Board approval (M-SPER-014.ver7 for the use of surgical waste). The adipose tissue was washed with Phosphate Buffered saline (PBS) and centrifuged at 1200g for 2 minutes in order to remove blood and other contaminants. The ASCs was performed by enzymatic digestion with collagenase type I 0,075% w/V (0.075% w/v type I collagenase; Worthington Biochemical Corporation, LakeWood, NJ, USA) for 30 minutes at 37°C (de Girolamo L et al, 2009). The digested tissue was then filtered and centrifuged at 350 g for 4 minutes. The obtained cellular pellet was re-suspended in complete medium (CM), composed of Dulbecco's Eagle Modified Medium supplemented with 10% of Fetal Bovine Serum (FBS) and 1% of PSG and then seeded at a density of 5000 cells/ cm^2 at 37° C with 5% CO_2. When 90 % of confluence was reached, cells was washed with PBS and harvested adding Trypsin EDTA 0,025% and re-plated. Cells at passage 4 were used for the following experiments.

Cell seeding onto L-FAMs by DOE method

The Design of Experiment (DoE) was used to maximize the seeding efficiency and cell viability considering different parameters of seeding protocol (seeding volume, rotation speed, type of rotation and seeding time) (Table 1). A statistical software (JMP, SAS Institute Inc.) defined the optimal minimal experimental conditions to be performed, in order to identify the best cell seeding condition and therefore to obtain the higher cell adhesion rate on L-FAMs.

Fixed parameters	Cell density (15.000 cells/mg)	
	L-FAMs/sample (10 mg)	
Variable parameters	Seeding time	30 minutes
		60 minutes
		90 minutes
		120 minutes
	Seeding speed	5 rpm
		10 rpm
		20 rpm
	Pause	yes
	(10' ON and 10' OFF)	no
	Seeding volume	400 µl
		1000 µl
Outcome	cell adhesion rate	

Table 1. *Fixed and variable parameters and outcome of the experiments*

The set of experiments was performed on ASCs isolated from 3 donors. The software was set with a desirability coefficient of 0:1, representing the best and the worst experimental setting, respectively. For each cell population, 13 conditions were tested (Table 2) in triplicates.

Experimental conditions	RPM	Seeding Time (minutes)	Pause (10 min ON and 10 min OFF) With pause + Without pause -	Volume (µl)
1	20	30	+	1000
2	20	120	+	1000
3	10	60	+	400
4	10	120	+	400
5	5	30	+	400
6	5	90	+	1000
7	20	60	-	400
8	20	90	-	400
9	10	30	-	1000
10	10	60	-	1000
11	10	120	-	400
12	5	30	-	400
13	5	120	-	1000

Table 2*. Experimental conditions resulting by the combination of seeding speed (RPM), seeding time (minutes), presence (+) or absence (-) of pause and seeding volume (µl).*

L-FAMs were pre-reconstituted in complete medium then ASCs were added to microcarriers at a density of 15,000 cells/mg and maintained in incubator at 37°C and 5% CO_2 for a maximum time of two hours. A bioreactor system, previously described (Lovati AB et al, 2011), was used to perform a pre-settable dynamic culture. Briefly, the bioreactor is a custom-made tube roller, able to rotate at a programmable speed in continuous mode or with defined pause between rotation cycles.

Cells seeded on L-FAMs and cultured for 2 hours in an oscillating shaker (Rotamax 120, Heidolph) at 70 rpm were considered as control group (CTRL), as performed in our previous works (Chlapanidas T et al, wainting to be submitted; Perucca Orfei C et al; waiting to be submitted). L-FAMs without cells were prepared for each tested condition and considered as blank sample.

Evaluation of cell adhesion rate

Immediately after the stirring time, the medium was removed to exclude non-adherent cells. The Alamar Blue and CyQuant assays were performed to assess the metabolic activity of adherent cells and to quantify the total amount of DNA of each sample, index of adhesion rate onto L-FAMs. In details, each cell seeded L-FAM sample was incubated with a working solution 10% v/v Alamar Blue solution (Life Technologies) for 4 hours at 37°C. The fluorescence of the obtained solution was measured by a spectrophotomer (Victor X3, Perkin Elmer) at Ex/Em 560/590 nm. The same samples were then harvested and lysed for the DNA content evaluation (Triton X-100 0.1 % in ddH_2O as lysis buffer) with the CyQuant cell proliferation Assay Kit (Invitrogen) and the fluorescence was read at 520 nm (excitation 480 nm).
A qualitative evaluation of cell adhesion was also performed with Live&Dead Assay (Life Technologies): each sample was treated with 2 µM of Calcein-AM and 4 µM of Ethidium Homodimer (Invitrogen, Ltd.,Paisley, UK) in saline solution for 10 minutes at 37°C and 5% CO_2. Then, cells were observed under a fluorescence microscope (Microscope OLYMPUS IX71). Live cells stained green and dead cells red. Three randomly chosen fields were photographed for one sample of each population (n = 3).

Statistical analysis

Desing of Experiment (DoE) was performed by the JMP, SAS Institute software. Statistical analyses of data were performed by GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as the mean ± SD. The values distribution was assayed by Kolmogorov–Smirnov normality test. For normally distributed data, student T test or one-way analysis of variance (ANOVA) were performed to compare groups. Otherwise, Mann-Whitney test or Kruskal-Wallis test were applied. A linear regression model was used to assess the correlation of experimental data and conditions, assessing Pearson and Spearman r values in cases of normal and not normal distribution, respectively. p values <0.05 were considered statistically significant.

RESULTS

L-FAMs particle size evaluation

After the lyophilization process, L-FAMs were analyzed in terms of particle size distribution: results showed a mean diameter of 279.20 ± 27.837 µm (n = 5), lower than 380 µm for the 90% of microcarriers (Figure 1).

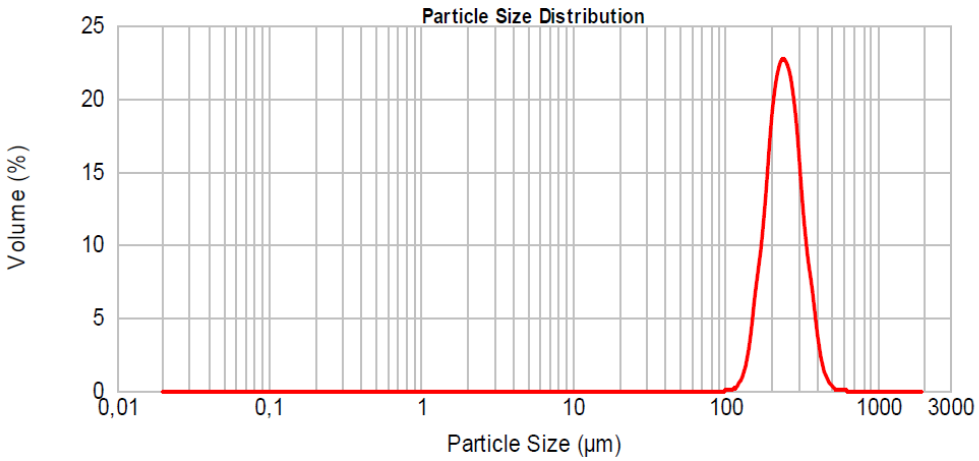

Figure 1. *An illustrative particle size distribution of L-FAMs. Data are reported as volume percentage values.*

Metabolic activity and DNA quantification as indicators of cell adhesion onto L-FAMs

The metabolic activity evaluation was performed by Alamar Blue assay. The highest fluorescence intensities were obtained with the third (10 rpm, 60', with pause, volume of 400 µl) and the fourth experimental conditions (10 rpm, 120', with pause, volume of 400 µl) (Figure 2a), indicating that the intermittent stirring of the cell-L-FAMs suspension, the stirring speed of 10 rpm, and the seeding volume of 400 µl are the main factors involved in the cell adhesion. The DNA quantifications confirmed the above mentioned results (figure 2b).

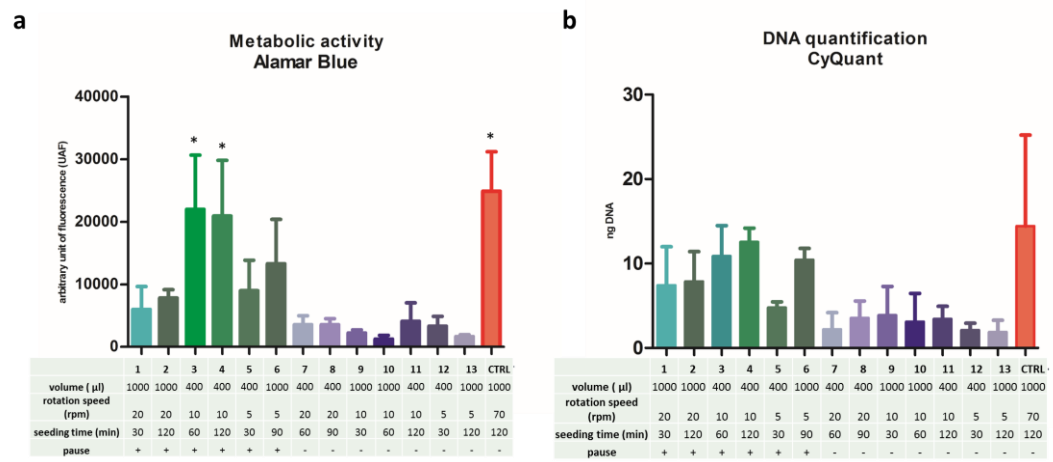

Figure 2*. Metabolic activity (a) and DNA quantification (b) of adhered cells for each condition. Data are expresses as mean ± SD. VS experimental condition 1, 7, 8, 9, 10, 11, 12, 13: *p<0.05*

Experimental conditions qualitative observation

In addition to the Doe approach that gives a significant outcome in terms of seeding efficiency and is able to identify the most performing condition for this purpose, we wanted to observe also which condition provides not only the higher cell adhesion in the less time possible but also the most homogeneous cell adhesion onto the surface of the microcarrier. Finally, for each experimental condition we performed a Live&dead staining. Despite the control sample (CTRL) resulted to be one of the best performing one, the picture clearly shows that the quantity of cells is probably higher than in the other conditions however the disposition of cells onto the L-FAM surface is the less homogeneous one, revealing the presence of several aggregates (Figure 3a).

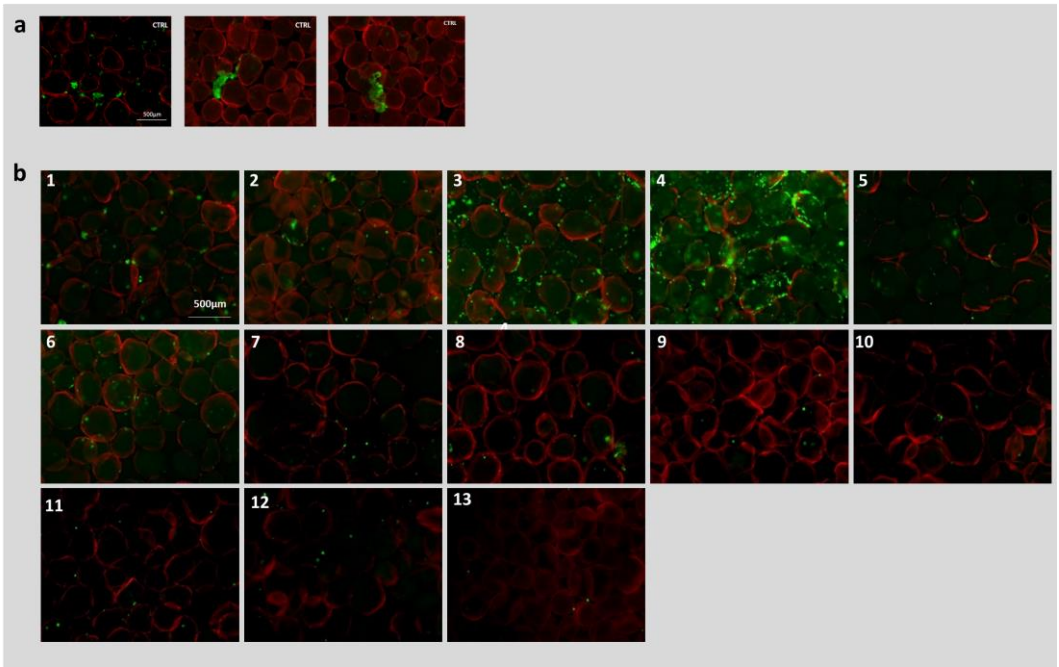

Figure 3*. Representative images of Live&Dead stained cells seeded on L-FAMs with different seeding conditions.* ***a****: representative micrographs of ASCs seeded onto L-FAMs after CTRL protocol. b: representative images of ASCs seeded with the DoE proposed conditions. Scale bar = 500 µmrepresentative micrographs of ASCs seeded onto L-FAMs after CTRL protocol.*

As previously showed, the third and the fourth experimental conditions are the best performing ones. The qualitative observation confirms this point offering also a clear view that cells are better distributed on the surface of microcarriers, with an elongated conformation too, after the seeding by these protocols (figure 3b).

Single parameters influences on cell adhesion rate

As already identified by the analyses showed above, in a linear regression model we confirmed a correlation between

the presence of a pause and the adhesion rate, thus entailing that consequent use of an intermittent stirring during the cell adhesion is the most incisive parameter (figure 3a; 3b).

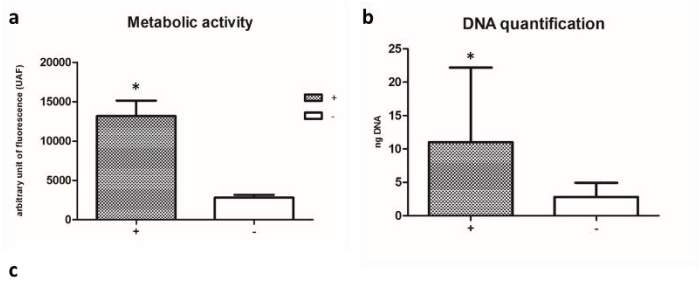

Figure 4. *Metabolic activity (a) and DNA quantification (b) of cells treated or not with an intermittent seeding (+: with pause; -: without pause). Spearman r values and p values for each parameter (c)*

c

		Volume	Stirring speed	Seeding time	pause
Metabolic activity	Spearman r values	-0,2742	0,02904	0,1312	0,7769
	p values (two-tailed)	0,0912	0,8607	0,4258	P<0.0001 *
DNA quantification	Spearman r values	0,04824	0,07590	0,1513	0,7419
	p values (two-tailed)	0,7706	0,6461	0,3580	P<0.0001*

As showed in the table, the *Spearman r values* and the *p values* obtained are statistically significant only for the pause, indicating that this parameter deeply influences the cell adhesion outcome.

Further correlation studies have been performed selecting only the most influencing factors in order to obtain a parameters hierarchy. Our results showed that the volume of 400 µl and the stirring speed of 10 rpm are the second and the third most influencing parameters, respectively. This analysis permitted to identify the time as the less influencing one.

Identification of the optimal experimental condition to maximize the cell adhesion rate onto L-FAMs

In our previous works we demonstrated that L-FAMS possess all the features to be used as a reliable cell delivery system of MSCs. However, to obtain a complete and a homogeneous cell adhesion in less than two hours, the seeding protocol should be further improved. In this study, we evaluated how multiple combinations of several parameters exert an influence on the time and on the amount of cells on microcarriers. Using the DoE approach, we tested 13 conditions for each cell population. The combination of the results deriving from all the 39 tests allowed to identify the best results in term of viability metabolic activity of adhered cells. A desirability coefficient (0:1) was used to rate the experimental conditions.

Figure 5 shows the optimal parameter configuration obtained maximizing the desirability in the statistical software. We evaluated the individual desirability which assesses how the settings enhance a single response, the metabolic activity (figure 5a) or DNA content (figure 5b) respectively, and the composite desirability which evaluates how the settings optimize the two responses in the same time (figure 5c).

We observed that an intermittent stirring (with a pause of 10 minutes), a rotation speed of 12 rpm and 400 µl of seeding suspension are determinant factors to improve both the metabolic activity's and DNA content's desirability, confirmed also by the composite one.

For what concern the seeding time, the DoE approach suggest that 84,23442 minutes are sufficient to optimize as much as possible the seeding efficiency, measured in term of metabolic activity of

adhered cells (figure 5a) whereas the seeding time of 87,1842 is a suitable time to obtain an higher seeding efficiency measured in term of DNA content (figure 5b). The outcome resulting from the combined desirability suggest an adhesion time of 85,61914 minutes as the one able to further maximize the response (figure 5c).

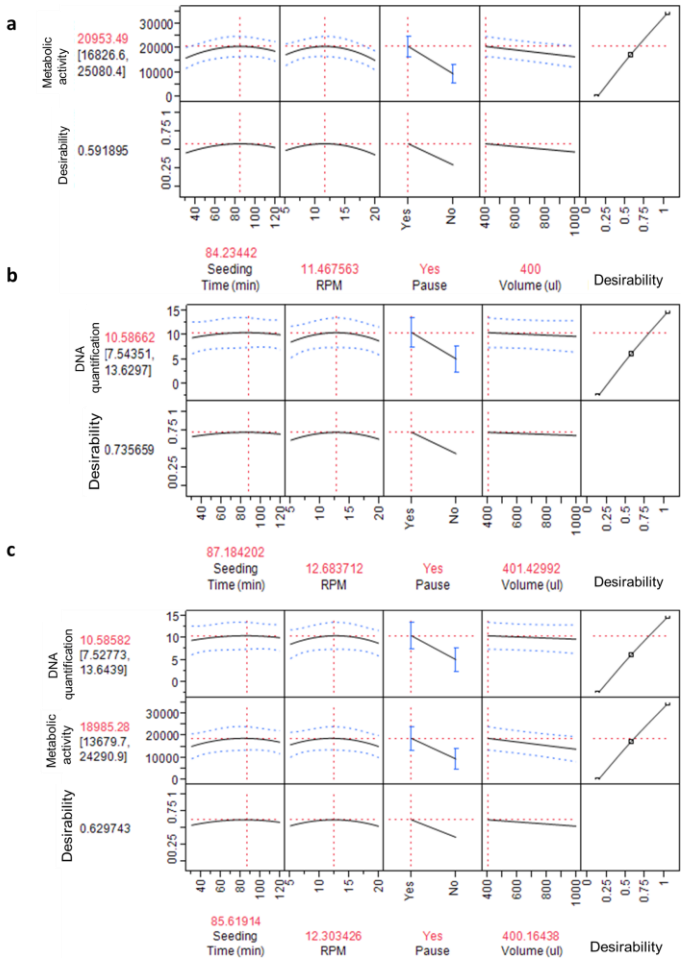

Figure 5. *The figure shows the Doe outcomes reporting the optimal parameter configuration obtained maximizing the desirability in the statistical software. The individual desirability (figure 5a-b) and the composite desirability (figure 5c), are reported.*

The used statistical approach allows also to analyze the obtained results by the single modification of a single parameter thus measuring the effects of this modification in terms of the desirable outcome. We observed that the use of the intermittent rotation cell seeding is the most incisive parameter for the maximization of the response.

DISCUSSION

Microcarriers have been widely used in several applications, especially regarding cell growth and expansion (Lam AT et al, 2016; Mizukami A et al, 2016; Tsai AC et al, 2016; Want AJ et al, 2012). The first interest in the use of microcarriers for cell culture was owed to the optimal surface area-volume ratio that represents a great advantage especially in term of cost-effectiveness, reducing the amount of the materials and the time needed for monolayer cell expansion (Jenkins MJ et al, 2015). Subsequently, the use of microcarriers as a support for cell growth has been considered a suitable approach for MSCs culture to provide not only a good cellular expansion but also a good maintenance of cell proliferation and differentiation capability (Caruso et al. 2014; de Soure AM et al, 2016; Badenes SM et al, 2016; Lin YM et al, 2016; Bertolo A 2015). Finally, microcarriers have been defined as a suitable cell delivery system since they can be directly injected in the affected site ensuring cell localization, remaining in the desired target tissue (Chen AKL et al, 2013), and minimizing cell manipulation *ex vivo*.

For these reasons we decided to develop and characterize a cell delivery system suitable for future uses in one-step applications, thus reducing cell manipulation, but still permitting a high cell viability (Chlapanidas T et al, waiting to be submitted; Perucca Orfei C et al, waiting to be submitted).

Results indicated that ASCs were able to adhere on silk/alginate microcarriers in a time lower than 2 hours and despite the amount of adhered cells is not high, it must be considered that this system respects the anchorage-dependency of MSCs. Other approaches that could be suitable for one-step applications, such as the direct injection of cells in the target tissue or the delivery of them by capsules or hydrogels, can obtain a more rapid cell administration (Stucky EC et al, 2015; Zeng Y, et al, 2015). However, they do not support all the peculiar features of MSCs, such as the anchorage-dependency (Merten O, 2015) and this could damage the final safety and efficacy of the system (Wang X et al, 2016).

In the context of one-step surgical applications, the identification of the most performing conditions to apply for the development of the cell carrier system still represents a great challenge. However, several evidences in literature confirm that a dynamic culture of cells promote both the cell adhesion and the cell viability on microcarriers in comparison with static cultures (Perez RA et al, 2014; Gupta P et al, 2016).

In specific, the agitation speed has to be monitored and measured to limit the cell aggregates formation, cell damage and cell detachment from the microcarriers. The speed and modality of the stirring have to be well-determined since they deeply influence the final cell adhesion and the subsequent expansion and differentiation. Indeed, the minimal agitation speed is necessary to permit the homogeneous maintenance in suspension of microcarriers and cells, but guaranteeing also the lowest hydrodynamic shear stress to the cells (Carmelo JG et al, 2015; Hewitt CJ et al, 2011; Rafiq QA et al, 2013). Hence, we decided to provide an intermittent agitation of the cell/microcarriers suspension to further improve the cell adhesion and to limit as much as possible the time needed. Our results demonstrated that this particular parameter is determinant in the improvement of the cell seeding efficiency, in term of time and homogeneity of the cell adhesion, thus confirming the hypothesis that the intermittent dynamic culture can improve the adhesion rate (Yuan Y et al, 2014).

One-step procedures represent a convenient and cost effective approach to exploit the MSCs potential for the treatment of many conditions. Among musculoskeletal ones, osteoarthritis, intervertebral disc degeneration and tendinopathy seem to be the most interested by this kind of treatment. However, although many studies have demonstrated the feasibility and effectiveness of MSCs in these conditions, many efforts should be still done to improve this method and achieve better results. For example, the local delivery of MSCs, namely of progenitor cell concentrate obtained mainly from bone marrow and adipose tissue, and their permanence at the injury site could be improved with the final aim to achieve better results. Indeed, the possibility to deliver MSC while adhered to a surface rather than in a liquid suspension would allow to maintain a more physiological situation, eventually resulting in a more performing activities of MSCs, especially in term of paracrine action. Moreover, the use of microcarriers like FAMs could lead to a progressive release of cells instead of simultaneous as it happens with a regular MSCs injection

CONCLUSIONS

The cell seeding protocol intended for the one-step procedure of adipose-derived MSCs on silk/alginate microcarriers has been optimized by DoE approach. It has been proved that an intermittent stirring, with a pause of 10 minutes, at a stirring rate of 10 rpm, the seeding volume of 400 µl permit to obtain optimal performances: in these conditions, ASCs are able to adhere on the microcarrier surface in a favorable time for intra-operative cell transplantation.
In conclusion, our results suggest that the lyophilization improves the technological process used for the production of FAMs: we obtained a well performing delivery system, ameliorating the preservation and the reproducibility of the process, and guaranteeing in the same time the obtainment of off the shelf ready to use product, with a subsequent decrease of costs. After this study we can confirm that L-FAMs can be successfully used not only for MSCs expansion and but also for one-step applications, permitting a rapid cell adhesion and the maintenance of the MSCs features.

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Conclusion remarks

The most recent evidences about MSCs anti-inflammatory and immunomodulatory properties, as well as their role in tissue healing support the hypothesis that they can exert a peculiar therapeutic response, thus representing an extremely valid tool in the treatment of many clinical conditions, including musculoskeletal diseases. Indeed, despite the huge and rapid scientific progress, many disorders that are characterized by tissue degeneration and chronic inflammation, such as tendinopathy and osteoarthritis, are still open issues as the existing therapies are not able to provide satisfactory results.

With this PhD research, we provided a further demonstration of the great potential of MSCs, supporting the hypothesis that cells can be considered as drugs able to produce a dynamic and peculiar reaction basing on the external stimuli that they receive.
During my doctoral path I was involved in a multifactorial study on the role of MSCs in tissue healing considering them from several persectives, due to the undeniable need to better clarify this issue for a better employment of this cell type in future clinical procedures. In this view, different *in vitro* and *in vivo* experimental conditions have been established in all the studies mentioned above, with the intent to observe any possible resulting influence on MSCs behavior.

The development of an adeguate *in vivo* model of tendon tissue degeneration permitted to highlight the disease progression and spontaneous healing, providing an increasing awareness about the best timing and way to use MSCs-based therapies.

Our *in vitro* findings confirmed that all the mesenchymal cell types involved in the study, bone marrow mesenchymal stem cells, adipose stem cells and tendon stem progenitor cells possess a good multi-differentiation potential, not only toward the traditional osteogenic, chondrogenic and adipogenic lineages, but also toward the tenogenic lineage: This opens new perspectives in the treatment of tendon degeneration which still lacks of adequate conservative treatments.
Moreover, the importance of the secretory activity of MSCs was also investigated to support the feasibility of cell-free applications. The first approach, aimed to the obtainement and evaluation of the MSCs secretome confirmed the ability of MSCs to secrete mediators with immunomodulatory and chondro-protective abilities. These results represent the basis for further in depth examination about the use of secretome as an innovative cell-free therapeutic tool, optimizing as much as possible their potential to better intervene on the surrounding microenvironment.

The second approach considered MSCs not only as a therapeutic agent but also as a target, studying the possibility to stimulate the secretory ability of resident progenitor cells, thus improving the endogenous regenerative process of the tissue. Basing on the findings obtained by the *in vivo* model of tendinopathy, this approach was based on the hypothesis that biophysisical stimulations, such as pulsed electromagnetic fields, could directly influence the resident multipotent progenitor population and thus their application should be tuned on this response. This approach can be considered as a feasible, non-invasive and cost-effective approach that may potentially improve the outcome of patients affected by tendinopathy.
Even though the final phases of both studies are still ongoing, the preliminary results are very encouraging and seem to demonstrate that the paracrine activity of MSCs is an even more powerful tool than the multidifferentiation potential, thus leading to the need of further studies and researches context.

Recognizing the importance of an accurate cell delivery in these kinds of approaches, a large part of my research activity was aimed to developed a cell delivery system suitable for local surgical application of MSCs. Thanks to the optimization of their surface that allowed for a fast cell adhesion and their biocompatibility, the carriers we developed represent an effective delivery system for local MSCs administration, thus making this approach economically sustainable and poorly invasive. Basing on the satisfactory results obtained from the pre-clinical study, future researches will be addressed to evaluate if the 3D support provided by the microcarriers could improve the MSCS anti-inflammatory and immunomodulatory properties.

The data collected in these years of my PhD path, as well as the results reported in literature, highlight that MSCs can be really considered as drugs, mainly thanks to their paracrine ability based on the release of a plethora of a variety molecules with different effects. What makes MSCs to be considered a potential step forward in comparison with traditional drugs, is their ability to respond adaptively to the different microenvironmental stimuli, thus making them a smart tool for the treatment of many disorders, both as a therapeutic agent and target. As they can be considered as drugs, the huge potential of MSCs has to be directed properly towards the correct clinical application, respecting indications of treatment, dosage and timing. The response to all these issues can just come from the combination of a strong basic research and an effective translational approach, and both of them have been played a consistent role in this collection of studies.

Despite the field of MSCs is continuously and rapidly evolving, many important issues still remain open questions. My PhD path has allowed me to increase the expertise and consciousness about MSCs therapeutic potentials and preparing the groundwork for future researches, with the final aim of an active collaborating between basic scientist and clinicians to increase the possibility for a near concrete application of cell therapy in clinical practice.

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"You never change things by fighting the existing reality. To change something, build a new model that makes the existing model obsolete"

R. Buckminster Fuller