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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Masterthesis

Dental pulp stem cells and leukocyte- and platelet-rich fibrin as candidate therapies for articular cartilage and tendon repair

Joël Beaumont

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Clinical Molecular Sciences

SUPERVISOR :
Prof. dr. Ivo LAMBRICHTS

CO-SUPERVISOR :
dr. Pascal GERVOIS

MENTOR :
Mevrouw Melissa LO MONACO

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Joël Beaumont; June 7th, 2018

2. Contents

3. List of abbreviations

α-MEM	Alpha Modification Minimum Essential Medium	**L-PRF**	Leukocyte- and Platelet- Rich Fibrin
AC	Articular Cartilage	**MMPs**	Matrix MetalloProteinases
ACI	Autologous Chondrocyte Implantation	**MSC**	Mesenchymal Stem Cell
ADSC	Adipose tissue Derived Stem Cell	**NSAIDs**	Nonsteroidal Anti-Inflammatory Drugs
ALP	Alkaline Phosphatase	**OA**	Osteoarthritis
BM-MSC	Bone Marrow derived Mesenchymal Stem Cell	**PBS**	Phosphate-Buffered Saline
CM	Conditioned Medium	**PC**	Platelet Concentrate
DAB	3,3'-Diaminobenzidine	**PDGF**	Platelet Derived Growth Factor
DAPI	4',6-diamidino-2-phenylindole	**PDL-SCs**	Periodontal Ligament Stem Cells
DMEM - F12	Dulbecco's modified eagle's medium nutrient mixture F-12 ham	**PES**	Polyethersulfone
DMEM - HG	Dulbecco's Modified Eagle's Medium – high glucose	**PFA**	Paraformaldehyde
DMEM - LG	Dulbecco's Modified Eagle's Medium – low glucose	**PI**	Propidium Iodide
DPSC	Dental Pulp Stem Cell	**RPL13a**	Ribosomal protein L13a
ECM	Extra Cellular Matrix	**RPM**	Rounds Per Minute
EGR-1	Early Growth Response protein 1	**RT-qPCR**	Reverse Transcriptase Quantitative Polymerase Chain Reaction
FGF	Fibroblast Growth Factor	**Runx2**	Runt-related transcription factor 2
GAGs	Glycosaminoglycans	**SDF-1**	Stromal cell-Derived Factor-1
hiFBS	Heat Inactivated Fetal Bovine Serum	**SM-MSC**	Synovial Membrane derived Mesenchymal Stem Cell
ICC	Immunocytochemistry	**TEM**	Transmission Electron Microscopy
IGF-1	Insulin-Like Growth Factor 1	**TGF-β**	Transforming Growth Factor β
IHC	Immunohistochemistry	**TNF-α**	Tumor Necrosis Factor α
IL-1β	Interleukin 1β	**VEGF**	Vascular Endothelial Growth Factor
iMAC	Imature Murine Articular Chondrocyte	**YWHAZ**	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

4. Abstract

Introduction: Articular cartilage defects and tendon lesions affect millions of patients each year and are associated with a high economic burden. Furthermore, articular cartilage defects often progress into osteoarthritis (OA), a degenerative and inflammatory condition of synovial joints with associated loss of cartilage matrix. Current treatments are unable to provide long-term regeneration of the damaged tissue, stressing the need for alternative therapeutic options including stem cell-based approaches. Human dental pulp stem cells (DPSCs) can differentiate into cartilage-producing cells and secrete numerous growth factors associated with tissue repair. Moreover, leukocyte- and platelet-rich fibrin (L-PRF), an endogenous blood-derived biomaterial, has recently emerged as a promising treatment strategy due to its growth factor content and supportive fibrin matrix. We hypothesize that human DPSCs and L-PRF improve the outcome in articular cartilage defects via cell replacement and stimulation of cartilage regeneration. Furthermore, we hypothesize that human DPSCs contribute to the repair of tendon lesions via cellular replacement.

Methodology: The effect of L-PRF on the chondrogenic differentiation of human DPSCs was examined and compared to human bone marrow-mesenchymal stem cells (BM-MSCs) using a micromass chondrogenic differentiation system, with or without L-PRF conditioned medium (CM) or exudate. The differentiation was evaluated via (immuno)histology. Next, the secretome-mediated effects of DPSCs and L-PRF on the survival and proliferation of murine chondrocytes was evaluated using a propidium iodide assay. Additionally, the migration of human DPSCs towards chondrocytes was examined using a transwell assay. Lastly, the ability of DPSCs to form tendon tissue was evaluated and compared to periodontal ligament stem cells (PDL-SCs) using a 3D differentiation system with uniaxial tension and TGF-β3 stimulation during 7 and 14 days.

Results: L-PRF does not enhance the chondrogenic differentiation of DPSCs and BM-MSCs as demonstrated by collagen type 2, aggrecan and glycosaminoglycan (GAG) production. Furthermore, differentiated DPSCs do not produce aggrecan, in contrast to BM-MSCs. Human DPSC- and L-PRF CM displayed a proliferative and a pro-survival effect on chondrocytes *in vitro*. Additionally, DPSCs were able to migrate towards chondrocytes. Lastly, human DPSCs and PDL-SCs formed tendon-like tissues characterized by the production of collagen and the parallel alignment of cells.

Discussion and conclusion: The results of this study support the hypothesis that human DPSCs and L-PRF show promise for the treatment of cartilage defects via a paracrine effect and for the treatment of tendon lesions via cellular replacement. Future experiments will focus on these paracrine effects of DPSCs and L-PRF on OA chondrocyte proliferation, survival and protein expression. Furthermore, the effect of L-PRF on the tenogenic differentiation of DPSCs will be examined. Eventually, the reparative potential of human DPSCs and L-PRF will be examined in ovine models of cartilage and tendon lesions.

5. Samenvatting

Introductie: Articulaire kraakbeendefecten en peeslaesies treffen elk jaar miljoenen patiënten en worden geassocieerd met een hoge economische last. Bovendien leiden kraakbeendefecten vaak tot osteoartritis (OA), een degeneratieve en inflammatoire aandoening van synoviale gewrichten die gepaard gaat met verlies van kraakbeen matrix. De huidige behandelingen zijn niet in staat om de beschadigde weefsels voldoende te herstellen, wat de noodzaak voor de ontwikkeling van nieuwe therapieën, zoals stamcel gebaseerde benaderingen, benadrukt. Humane dentale pulpa stamcellen (DPSCs) kunnen differentiëren naar kraakbeen producerende cellen en secreteren verschillende groeifactoren die geassocieerd worden met weefsel regeneratie. Leukocyt- en plaatjes-rijk fibrine (L-PRF), is recentelijk naar voren gekomen als een veelbelovende behandeling dankzij zijn groeifactoren en ondersteunende fibrine matrix. We veronderstellen dat humane DPSCs en L-PRF kraakbeen herstel kunnen bevorderen via differentiatie en via de stimulatie van kraakbeen regeneratie. Bovendien veronderstellen we dat humane DPSCs bijdragen aan pees herstel via differentiatie.

Methodology: Het effect van L-PRF op de chondrogene differentiatie van humane DPSCs werd onderzocht en vergeleken met de differentiatie van beenmerg mesenchymale stamcellen (BM-MSC) door middel van een 3D differentiatie systeem, met of zonder toevoeging van L-PRF geconditioneerd medium (CM) of L-PRF exudaat. De differentiatie werd geëvalueerd door middel van (immuno)histologische kleuringen. Vervolgens werden de secretoom-gemedieerde effecten van DPSCs en L-PRF op de overleving en proliferatie van muis chondrocyten geëvalueerd door middel van een propidium iodide assay. Ook werd de migratie van DPSCs naar chondrocyten getest met een transwell migratie assay. Het vermogen van DPSCs om *in vitro* peesconstructen te vormen werd getest en vergeleken met periodontaal ligament stamcellen (PDL-SCs) door middel van een 3D differentiatie systeem waarbij de cellen blootgesteld werden aan uni-axiale spanning en TGF-β3 gedurende 7 en 14 dagen.

Resultaten: Quantificatie van de collageen type 2, aggrecan en glycosaminoglycaan (GAG) productie toonde aan dat L-PRF de chondrogene differentiatie van DPSCs en BM-MSCs niet bevorderde. Bovendien produceerden de DPSCs geen aggrecan, in tegenstelling tot BM-MSCs. Humane DPSCs en L-PRF stimuleerden de proliferatie en overleving van chondrocyten *in vitro*. Ook werd de migratie van DPSCs naar chondrocyten aangetoond. Bovendien vormden humane DPSCs en PDL-SCs pees-achtige constructen gekarakteriseerd door de productie van collageen en de parallelle oriëntatie van de cellen.

Discussie en conclusie: De resultaten van deze studie ondersteunen de hypothese dat DPSCs en L-PRF kraakbeen regeneratie kunnen bevorderen door middel van paracriene effecten en gebruikt kunnen worden voor het herstel van pees laesies omwille van hun differentiatie vermogen. In de toekomst zullen we kijken naar het effect van DPSCs en L-PRF op de overleving, proliferatie en proteïne productie van OA chondrocyten. Bovendien zullen de effecten van L-PRF op de tenogene differentiatie getest worden. Uiteindelijk zullen DPSCs en L-PRF getest worden in een schaap model van kraakbeen defecten en pees laesies.

6. Introduction

6.1 Synovial joint

Bones are connected to each other via joints which can be divided in two large groups: joints that allow only limited movement of the bones, such as the intervertebral discs, and synovial joints, which allow free movement of the bones, for example the knee joint (Figure 1) (1). Synovial joints consist of internal and external ligaments which connect and stabilize the bones and prevent the over-flexion or -extension of the joint (1). The external ligaments surround a joint capsule of which the inner cell layers, called the synovium, are responsible for the production of synovial fluid. This fluid acts as a lubricant between the bones (1). Articular cartilage (AC) covers the ends of the bones and allows, together with the synovial fluid, a frictionless movement. Furthermore, it acts as a shock absorber, protecting the underlying bone from compressive forces (1, 2). Tendons attach skeletal muscles to bones and translate contraction of the muscle into movement of the joint (3). Degenerative diseases and overloading of the joint may damage the articular cartilage and tendons and result in pain and disability. Unfortunately, these tissues have a limited self-healing capacity, caused by the low vascularity and (stem) cell number (1, 2, 4, 5). Furthermore, treatments are not able to provide a full and stable recovery of the damaged tissue (6). Numbers from the United States show that injuries of the AC and tendons are associated with an annual economic burden of $33 billion and $10 billion respectively (7-9). Therefore, there is an increasing need for the development of new treatment strategies for AC defects and tendon lesions.

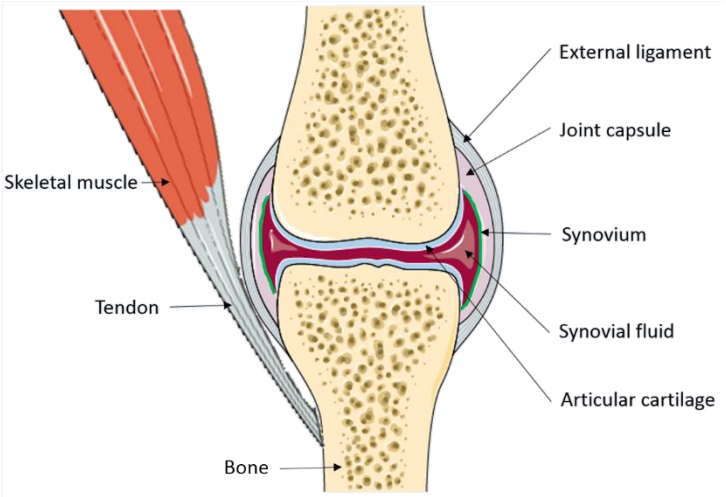

Figure 1: Schematic overview of the synovial joint. *The synovial joint consists of ligaments which prevent over-flexion or -extension. The synovial fluid, produced by the synovium, together with the articular cartilage allow the frictionless movement of the bones over one another. Tendons attach skeletal muscles to bones and translate the contraction of the muscle into movement of the joint. This image was created using Servier Medical Art.*

6.1.1 Articular cartilage

Approximately 95% of the cartilage consists of extracellular matrix (ECM) which is produced by sparsely distributed chondrocytes, which make up the remaining 5% of the cartilage volume (5). The ECM of the AC is mainly composed of collagen type 2 and proteoglycans (5, 10). Proteoglycans consist of long polysaccharide chains composed of repeated disaccharides units, called glycosaminoglycans (GAGs), which are anchored to a protein core (1, 2). The most predominant proteoglycan in the AC is aggrecan (5). Four distinct zones can be distinguished in the AC, namely the superficial-, the transitional-, the deep-, and the calcified zone (5). Each of these zones differs in collagen orientation and thickness, proteoglycan content and cellular distribution (2, 5).

Cartilage defects can be classified in three types of lesions (Figure 2). Partial thickness lesions only affect the superficial cartilage layers. Full thickness cartilage lesions reach up to the subchondral bone, but do not extend into the underlying bone marrow whereas osteochondral lesions extend into the bone marrow (2, 11). The low reparative capacity of the AC is most obvious in partial thickness lesions, in which no spontaneous regeneration of the damaged cartilage occurs (11). However, in full thickness- and osteochondral lesions repair is observed, caused by the migration and the subsequent differentiation of bone marrow mesenchymal stem cells (BM-MSCs) and synovial membrane mesenchymal stem cells (SM-MSCs) into the lesion (6, 11). In partial thickness lesions these cells are not involved for multiple reasons. Firstly, the intact subchondral bone hinders the migration of BM-MSCs into the lesion (6, 11). Secondly, the subchondral bone, containing collagen type 1, has been shown to be much more suitable for stem cell adhesion compared to the collagen type 2 of the AC (11). Furthermore, stromal cell-derived factor-1 (SDF-1), expressed in subchondral bone and in the bone marrow but not in the AC, has been shown to attract SM-MSCs to the lesion (11). Unfortunately, the intrinsic healing reactions result in the production of mechanically inferior fibrocartilage tissue which integrates poorly with the surrounding tissue (6).

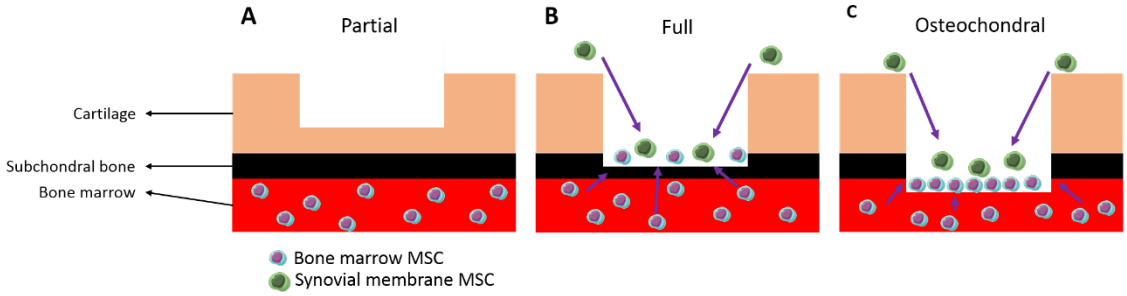

Figure 2: Classification of cartilage lesions. *(A) Partial thickness lesions affect only the articular cartilage surface. No spontaneous healing is seen in these lesions. (B) Full-thickness cartilage lesions extent into the subchondral bone, but do not reach into the underlying bone marrow. Some spontaneous regeneration is observed. (C) Osteochondral defects reach into the bone marrow. Spontaneous healing occurs as a result of migration and differentiation of BMSCs and SMSCs. This image was adapted from Zhang et al. (2013) and was created using Servier Medical ART.*

AC defects are often risk factors for the development of osteoarthritis (OA), which is a degenerative and inflammatory condition of synovial joints with progressive loss of articular cartilage (8, 12, 13). OA is one of the ten most disabling diseases and is estimated to affect over 37% of all people over 60 years old in the United States (9). It is caused by a disturbance in chondrocyte homeostasis, resulting in the upregulation of proteolytic proteins such as matrix metalloproteinases (MMPs) and aggrecanases, which in turn lead to the degradation of cartilage (14). Several factors can induce this chondrocyte dysfunction. Firstly, mechanical stresses, such as wear and tear, overloading of the joint and AC lesions, disturb normal chondrocyte activity (13, 14). Secondly, genetic factors play a role in the development of OA (8, 14). Specific polymorphisms in genes encoding for proteins involved in signal transduction and normal chondrocyte functioning, such as frizzled-related protein 3, asporin and calmodulin 1, have been shown to increase the risk for the development of OA (15). Furthermore, dysregulation of the Nuclear Factor-κβ (NF- κβ) pathway, caused by inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), causes the chondrocytes to upregulate their MMP expression and downregulate the expression of collagen type 2 and aggrecan (14, 16-19). Loss of ECM eventually induces chondrocyte apoptosis which in turn leads to a further disturbance in cartilage homeostasis, resulting in a self-sustaining pathological cycle (20).

Several therapies for the repair of cartilage lesions have been developed. Firstly, production of micro fractures extending into the bone marrow is used for the treatment of partial thickness lesions. These microfractures stimulate intrinsic healing responses, i.e. the migration and differentiation of stem cells into the lesion site. As mentioned earlier, these repair mechanisms are unable to provide a lasting repair tissue (2, 6). Secondly, autologous osteochondral transfer involves the removal of cartilage from non-weight bearing regions and its implantation into the defect site. This technique is associated with a high donor site morbidity. Furthermore, the limited amount of donor sites poses a problem for large chondral defects (6). Autologous chondrocyte implantation (ACI) is another approach that is available. ACI has several drawbacks as it requires a two-step surgery and only yields a small quantity of harvested cells, resulting in a long cell culture time before sufficient numbers are reached for implantation (6). Additionally, chondrocytes cultured as a monolayer undergo dedifferentiation, limiting the potential for *in vitro* expansion (6, 21). Nonetheless, increase in clinical outcome is observed after ACI. However, clinical scores deteriorate significantly in time (22). Nonsteroidal anti-inflammatory drugs (NSAIDs) are used in OA to inhibit NF-κβ signalling and to relieve pain but are associated with an increased risk for cardiovascular diseases, renal failure and gastro-intestinal problems including nausea and heartburn (23-25). Alternatives which are widely used are glucosamine and chondroitin sulfate which have virtually no side effects. However, their effectiveness is still under debate (23). Exercise to strengthen the muscles around the joint in order to stabilize the joint has been shown to be effective in reducing pain (26). In severe cases of OA, joint replacement is needed (8). Unfortunately, none of these treatments are able to restore the damaged cartilage to satisfactory levels.

In conclusion, current treatments are unable to provide a full recovery of AC lesions and OA, signifying the need for the development of better therapies. The use of innovative biological tissue engineering techniques using mesenchymal stem cells could provide an alternative to current therapies for cartilage defects. Additionally, Leukocyte- and Platelet- Rich Fibrin (L-PRF) has emerged as a promising autologous bio-material in cartilage repair due to its growth factor content and supportive fibrin matrix (27, 28). Both candidate therapies will be discussed into detail later.

6.1.2 Tendons

Skeletal muscles are attached to bones via tendons which translate muscular contractions into joint movement (3). Furthermore, tendons act as a mechanical buffer, temporarily storing generated energy before it is transferred in order to prevent injuries (29, 30). Tendons consist of densely packed collagen type 1 fibres (approximately 70% of the dry weight) which form fascicles, orientated in the direction of force application (3, 4, 29). Furthermore, they contain small amounts of proteoglycans and glycoproteins which are produced by cells called tenocytes (3, 4, 29). Elastin in the interfascicular matrix is responsible for the energy storing properties of tendons (29). Degradation of elastin with age might explain the higher incidence of tendon lesions in elderly people (29). The connection between the tendon and the bone is called the enthesis. It is made up of a fibrocartilaginous tissue, rich in collagen type 2, which is produced by chondrocyte-like cells (4). Tendons are surrounded by a tendon sheath which protects them from damage caused by friction against the bone. The cavity between the tendon and the tendon sheath, called the tenosynovium, is filled with fluid which allows the frictionless movement of the tendon within its sheath (1). Tenocytes are characterized by the expression of tenomodulin (4). This glycoprotein is involved in maintaining a healthy functioning

tissue and its loss results in distorted collagen fibrillogenesis (31). Little is known about how tenocytes produce the specific parallel organization of collagen in tendons. Proteins known to be involved in this process are Transforming Growth Factor β (TGF-β), Fibroblast Growth Factor (FGF) and the transcription factors scleraxis, Mohawk and early growth response protein 1 (EGR-1) (4).

Tendon ruptures affect millions of people worldwide and cause pain, decreased strength, reduced movement and are associated with a high economic burden as stated previously (4, 7, 27, 32). Chronic lesions are caused by repetitive abnormal loading and are characterized by disorganized collagen fibrils and increased non-collagenous ECM (4). Acute lesions are usually caused by a single overloading incident and are characterized by macroscopic tearing of the tendon (4). The limited healing capacity of tendons is caused by their low number of tenocytes and blood vessels (1, 4). Some intrinsic healing reactions are observed, however, these result in a mechanically inferior scar tissue which has only one-third of the strength of the native tissue (4). Furthermore, this scar tissue causes the adherence of the tendon to the surrounding tendon sheath, thereby disrupting tendon gliding (3). NSAIDs and corticosteroids are given for the management of pain and to suppress inflammation in tendon defects (33). However, the effectiveness of these treatments is still under debate (33). Other strategies include surgical techniques, laser- and ultrasound therapies and mechanical stimulation (3, 33). Unfortunately, evidence of the effectiveness of many of these techniques is lacking and structural repair is often incomplete, signifying the need for the development of improved therapeutic strategies (3, 28, 33). The use of mesenchymal stem cells and L-PRF in innovative tissue engineering techniques could provide an alternative to current therapies for tendon lesions, as will be discussed later.

6.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multi-potent stem cells and can be isolated from a variety of tissues including, but not limited to, the bone marrow, adipose tissue and the dental pulp (34-36). They are characterized by their adherence to plastic, expression of CD44, CD73, CD90 and CD105, absence of CD45, CD34, CD14, CD19 and HLA class II, and differentiation into osteoblasts, chondroblasts and adipocytes (37). They have been shown to differentiate into cells from other germ layers such as neural cells and epithelial cells, although the success rate of these trans-differentiations *in vivo* is very low (38, 39). Interestingly, MSCs have angiogenic properties and they produce a range of growth factors and extracellular vesicles (EVs) which are involved in tissue repair (40-43). Furthermore, MSCs have been shown to be immunomodulatory, inhibiting the maturation of dendritic cells, natural killer cells, T-cells and B-cells and increasing the amount of regulatory T-cells (44). EVs released from MSCs are able to induce a phenotype switch in macrophages from the inflammatory M1 phenotype towards the reparative M2 phenotype (45). These properties make MSCs interesting for therapeutic application in a variety of diseases.

6.2.2 MSCs as promising therapy in cartilage regeneration

MSCs could aid in the repair of cartilage lesions via multiple mechanisms (46). Firstly, they may be used as a replacement therapy, as they are able to differentiate into cartilage producing chondrocytes (47). Secondly, their secretion of growth factors and EVs can stimulate intrinsic repair mechanisms (40-42). Additionally, suppression of the inflammatory reaction and macrophage phenotype switch

by MSCs could inhibit catabolic protein production by osteoarthritic chondrocytes and stimulate tissue regeneration (14, 43-45).

To study the chondrogenic differentiation *in vitro*, both 3D pellet- and monolayer- cultures have been developed (46). The 3D pellet differentiation system shows superior chondrocytic phenotype, owing to increased cellular interactions which mimic the *in vivo* micro-environment during the initiation phase of chondrogenesis (46, 48). MSCs isolated from various tissues, including bone-marrow (BM-MSCs) and dental pulp (DPSCs), have been successfully differentiated using this system as demonstrated by the expression of collagen and aggrecan (47, 49, 50). However, the chondrogenic potential was found to be donor-site dependent, with BM-MSC displaying superior differentiation over other MSC types (51). *In vitro* evaluation of the paracrine effects of MSCs, demonstrated an increased expression of collagen type 2 and aggrecan when chondrocytes were co-cultured with MSCs (52). Scaffolds functionalized with MSC conditioned medium showed similar effects on cartilage specific ECM production (53). Furthermore, MSC-derived exosomes were shown to stimulate chondrocyte proliferation, reduce apoptosis and promote the production of collagen type 2 and aggrecan (43, 54). MicroRNAs present in MSC-derived EVs, such as miR-320 and miR-125b, were shown to target aggrecanases and MMPs, thereby downregulating cartilage breakdown by inflamed chondrocytes (43, 55-57).

Promising results were also observed in animal models of cartilage lesions. Transplantation of MSCs into rat and rabbit models of cartilage lesions resulted in an increased cartilage regeneration, improved clinical scores and better collagen fibre arrangement (58, 59). Improved regeneration was also observed in rats when cartilage defects were treated with MSC-derived EVs (42, 54). Injection of these EVs into the lesion site resulted in the deposition of hyaline cartilage with a high expression of collagen type 2 and GAGs (42, 54). Furthermore, treated groups, in contrast to control groups, produced only low levels of collagen type 1, the main component of mechanical inferior fibrocartilage (42). A combination of BM-MSCs and micro fractures was used to treat cartilage lesions in horses and was shown to result in higher clinical scores and increased collagen type 2 and GAG production compared to micro fractures alone (60).

Phase 1 clinical trials demonstrated the safety of using MSCs for cartilage regeneration as no adverse effects were observed (61-63). Furthermore, treatment of the defects with MSCs resulted in improved clinical scores, complete filling of the defects with cartilage tissue and no recurrence of symptoms during a two year follow-up period (64, 65). Comparison between MSC injection and ACI in the patellofemoral joint showed similar improvements in clinical scores in both groups (66, 67). However, the use of MSCs requires one less knee surgery and is associated with less donor site morbidity (67). Intra-articular injection of MSCs in patients with OA showed reduction in pain and better quality of the regenerated hyaline cartilage (62, 63, 68).

Most studies investigating the regenerative potential of MSCs in cartilage lesions focus on BM-MSCs. However, this type of MSCs is associated with several drawbacks. Firstly, the isolation of BM-MSCs is invasive and is associated with chronic pain and scarring (69). Secondly, BM-MSCs are known to differentiate towards hypertrophic chondrocytes, characterized by the expression of runt-related transcription factor 2 (Runx2), MMP13, collagen type 10, alkaline phosphatase (ALP) and Indian Hedgehog (70, 71). Hypertrophic differentiation is responsible for the conversion of the temporary

hyaline skeleton into mineralized bone via endochondral ossification in fetal development, and induces the growth of bones in later development (1, 70). However, hypertrophic differentiation in the context of cartilage regeneration is detrimental as it causes the breakdown of collagen type 2 and aggrecan and induces the mineralization of the matrix (70). Furthermore, due to the low proliferation rate of BM-MSCs, a long culturing time is required before sufficient cell numbers are reached (72).

A suitable alternative stem cell source might be found in the dental pulp tissue. Human DPSCs were first isolated by Gronthos *et al.* and have been shown to differentiate into chondrocytes (34, 47). A first advantage of these stem cells is their ease of isolation from third molars, which are often extracted for orthodontic reasons and are thus considered as 'waste-material' (47). Their extraction is associated with only limited donor site morbidity and a low risk of complications (47). Furthermore, because of their higher proliferative capacity compared to BM-MSCs, DPSCs require less culturing time to reach sufficient cell numbers (72). Another advantage of these cells in comparison to other MSC types is their superior immunomodulatory capacity, which might reduce cartilage loss in OA (46, 72). Additionally, they have been shown to produce a range of growth factors involved in cartilage regeneration including Transforming Growth Factor (TGF-β), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) (73, 74).

Several studies already demonstrated the potential of DPSCs in cartilage regeneration *in vitro* and *in vivo* (58, 75, 76). Their chondrogenic differentiation was enhanced when they were co-cultured with chondrocytes (76). Furthermore, AC defects treated with DPSCs displayed improved regeneration with an organized cellular and fibrillar arrangement (58). Additionally, dogs suffering from OA displayed improved clinical outcome and reduced pain scores when treated with DPSCs (75).

6.2.3 MSCs as promising therapy in tendon regeneration

Both monolayer- and 3D- culturing systems have been developed to study the tenogenic differentiation of MSCs (32, 77, 78). Monolayer differentiations, which are solely based on the addition of growth factors from the TGF-β superfamily, only partially induce a tenocyte phenotype, with transient expression of essential tenocyte markers and atypical proteoglycan production (78). The poor differentiation capacity in monolayer systems is mainly caused by lack of tension, which has been shown to be essential for both normal tenocyte functioning and tenogenic differentiation of MSCs (77, 79, 80). In 3D differentiation systems, a fibrin gel containing MSCs is subjected to uniaxial tension, which induces TGF-β expression, cellular alignment and differentiation of MSCs into tenocytes (77).

In vivo studies observed an accelerated healing with an increase in collagen type 1 expression, improved fibre orientation and increased strength when tendon lesions were treated with MSCs (81-88). Furthermore, inflammatory infiltrates were decreased in the treated groups and progression of the lesion was prevented (81). Additionally, walking distance, speed and time increased in the MSC treated groups and re-injuries were less frequent compared to conventional therapies (88, 89). Also DPSCs have been shown to stimulate tendon healing. Horses displayed clinical improvements with less re-injuries after injection of DPSCs in the lesions site (90). Despite the positive pre-clinical results, no clinical trials investigating the effect of MSCs on tendon lesions have been carried out.

6.3 Platelet concentrates

Platelet concentrates (PCs) are blood-derived bio-materials which are emerging in various clinical applications including maxillofacial surgeries, wound healing and treatment of elbow tendinosis (91-93). The main asset of platelet concentrates are the point-of-care availability, their growth factor content, the autologous origin and the related absence of rejection risks (74). PCs can be subdivided in first- and second-generation concentrates. First generation PCs, such as platelet-rich plasma (PRP), are associated with several disadvantages, as their preparation is often labor intensive, associated with a high cost, includes the use of anti-coagulantia which suppress wound healing and requires large volumes of blood to produce sufficient amounts of concentrate (91, 94, 95). Leukocyte- and platelet-rich fibrin (L-PRF) is a second generation PC developed by Choukroun *et al.* (95). It is superior compared to first generation PCs in many respects. Firstly, its preparation does not require anti-coagulants or other additives (95). Blood is collected in glass coated tubes and is immediately centrifuged. The formation of the fibrin clot is based on natural blood clotting, in which the Hageman factor is activated by the negative surface of the glass, resulting in the activation of various downstream factors, eventually leading to the polymerization of fibrin monomers (96). Furthermore, the production of L-PRF is easy, cost-effective, fast and platelets are collected with high efficiency (95). Additionally, the leukocytes present in L-PRF produce antimicrobial proteins, thereby reducing the risk of post-operative infections (97).

6.3.1 Platelet concentrates as promising therapy in cartilage regeneration

In vitro examination of the effects of PCs on chondrocytes demonstrated their therapeutic potential in both cartilage lesions and OA. Stimulation of chondrocytes with PRP resulted in an increase in cartilage specific ECM production (98). Furthermore, chondrocyte proliferation and viability were increased upon PRP stimulation (98-100). Additionally, PRP was shown to increase the migration, proliferation and chondrogenic differentiation of subchondral mesenchymal progenitor cells (101). Interestingly, NF-κβ activity in osteoarthritic chondrocytes could be reduced with PRP, thereby partially restoring the dysregulated expression of collagen type 2, aggrecan and MMPs (16, 24). Hepatocyte growth factor was shown to be responsible for the reduction in NF-κβ activity (16).

Intra-articular injection of PRP in rabbit AC defect and OA models resulted in an improved repair, owing to an increase in chondrocyte proliferation and cartilage specific ECM production (94, 102, 103). Additionally, PRP and L-PRF treatment showed a better integration of the reparative tissue into the native tissue and a more normal cellular distribution in porcine-, canine-, ovine- and leporine-models of cartilage defects (74, 104-107). Combining micro fracturing with PRP resulted in an improved regeneration with more complete defect coverage, integration into native tissue and collagen type 2 expression compared to either micro fracture or PC treatment alone in both murine- and ovine- models of chondral defects (108, 109). In rabbit and horse OA models, PRP has been shown to increase proteoglycan production, stimulate cartilage regeneration and reduce lameness and disease severity (102, 103, 110).

Clinical trials using PRP for the regeneration of AC lesions demonstrated a significant reduction in pain and stiffness and increased functionality (111, 112). The combination of micro fracture treatment with PRP injection also resulted in better pain reduction and functional outcome compared

to patients treated with micro fracture alone (113). Similar results were observed when OA patients were treated with PRP (114-117).

6.3.2 Platelet concentrates as promising therapy in tendon regeneration

Several studies have shown the ability of PRP to increase tenocyte proliferation, viability and metabolic activity *in vitro* (118-120). Furthermore, PRP caused an upregulation in tendon specific ECM production (119-122). However, this effect was concentration dependent and adverse reactions in metabolic activity and apoptosis were observed when tenocytes were stimulated with higher PRP concentrations (118, 121). Murine and porcine models of tendon lesions have demonstrated tendons with higher stiffness and a higher loading capacity upon PRP treatment (123, 124). The combination of MSCs and PRP resulted in higher histological scores and was able to prevent the progression of the tendon lesions in horses (81). Several clinical trials already demonstrated the effectiveness of PRP and PRF for tendon regeneration, with better and faster functional recovery and pain reduction compared to control groups (125-127). Furthermore, intra-articular injection of PRP was shown to yield significant better results compared to conventional corticosteroid therapy (128).

6.3.3 L-PRF for articular cartilage and tendon regeneration

Several studies have shown the superiority of L-PRF over other platelet concentrates in AC and tendon regeneration. Cartilage defects treated with L-PRF demonstrated better regeneration and integration of reparative tissue into the healthy cartilage compared to other PCs (74, 104, 105). Also repair of the Achilles tendon was shown to be accelerated with better tissue organization when treated with L-PRF compared to PRP treatment (27). Growth factors released from L-PRF, including TGF-β, Basic Fibroblast Growth Factor (bFGF), Insulin Like Growth Factor I (ILGF-I), PDGF and VEGF, have been shown to be involved in cartilage and tendon regeneration (28, 74, 129-131). Furthermore, these growth factors are released over a longer time-period compared to first generation PCs, resulting in better tissue regeneration (28). Additionally, the fibrin matrix of L-PRF serves as a scaffold to support the healing process (28).

6.4 Research project

We hypothesize that human DPSCs and L-PRF improve the outcome in articular cartilage defects via cell replacement and stimulation of cartilage regeneration. Furthermore, we hypothesize that human DPSCs contribute to the repair of tendon lesions via cellular replacement. Firstly, the chondrogenic differentiation of DPSCs is examined and compared to BM-MSCs *in vitro*. Additionally, the effect of L-PRF on the chondrogenic differentiation is evaluated. Secondly, we investigate the paracrine effects of DPSCs and L-PRF on the survival and proliferation of healthy chondrocytes *ex vivo*. For future *in vivo* studies, we examine the ability of DPSCs to migrate towards healthy chondrocytes. Furthermore, the tenogenic differentiation capacity of DPSCs is evaluated via a 3D differentiation system and is compared to PDL-SCs.

7. Materials and methods

7.1 L-PRF isolation

Blood samples were obtained from healthy male and female donors (n=6) with written informed consent. The study was conducted in accordance with the Belgian law on human bodily materials of 19 December 2008 and approved by the Medical Ethics Committees of Hasselt University and KU Leuven (S58789/B322201628215/I/U). Blood was drawn by venipuncture and was collected in glass-coated plastic tubes (VACUETTE 9 ml Z Serum Clot Activator Tubes, Greiner Bio-One, Vilvoorde, Belgium) without anti-coagulants. Samples were immediately centrifuged for 12 minutes at 2700 rpm (400g) without additives (IntraSpin Centrifuge, Intra-Lock, Boca Raton, Florida). Afterwards, the L-PRF clot was removed from the tube using sterile forceps and separated from the red blood cell phase with a straight iris spatula (Fine Science Tools, Heidelberg, Germany, 10094-13).

7.1.1 L-PRF conditioned medium and exudate

For L-PRF CM production, individual L-PRF clots were placed in a 6 well plate of which each well was filled with 6 ml of the standard culture medium of the targets cells (Table 1). After 96 hours, the CM was collected, centrifuged for 6 minutes at 1200 RPM (161g) and filtered through a 0.2µm polyethersulfone (PES) filter (VWR, Radnor, Pennsylvania , 28145-501). Conditioned medium was stored at -80°C until use. The L-PRF exudate was collected using the Xpression kit (Intra-lock). This kit compresses the clots resulting in the release of exudate, which was collected, filtered through a 0.2µm PES filter and stored at -80°C until use.

7.2 Stem cell isolation and culture

Human third molars were obtained with written informed consent from patients (n=6, 15–20 years of age) undergoing an extraction procedure for orthodontic or therapeutic reasons at Ziekenhuis Oost-Limburg, Genk, Belgium. Written informed consent of minor patients was acquired via their custodians. The study was accepted by the medical ethical committee of Hasselt University (13/0104u). Periodontal ligament was isolated and dental pulp tissue was obtained with forceps after mechanically fracturing the teeth with surgical chisels. Tissues were kept in standard culture medium (Table 1) supplemented with 10% heat-inactivated fetal bovine serum (hiFBS) (Gibco, Thermo Fisher Scientific, Erembodegem, Belgium, 10270-106) at 37°C for transportation to the lab. Tissues were washed thoroughly with Alpha Modification Minimum Essential Medium (α-MEM) to remove contaminants. Next, the tissues were minced into small pieces (1 – 2 mm^3) and DPSCs and PDL-SCs were isolated via the explant method (132). Shortly, minced tissue was seeded in standard culture medium with 10% hiFBS in a six-well plate and was incubated at 37°C with 5% CO_2 to allow the outgrowth of cells from the tissue. Cells were subcultured when confluency was reached, approximately 14-21 days later. BM-MSCs were kindly provided by Stem Cell Institute Leuven, of the KU Leuven. Cells were seeded at a density of 2.6 x 10^3 cells/cm^2 and maintained at 37°C with 5% CO_2 in standard culturing medium supplemented with 10% hiFBS (Table 1). The culture medium was changed every 3-4 days. Passaging occurred at 80% confluency. Briefly, cells were detached using Trypsin (Sigma life science, Sigma Aldrich, St-Louis, United states of America, T3924) for 5 minutes which was deactivated with 10% hiFBS in standard culture medium. Each experiment was performed with cells from passage 2–5. Stem cells were routinely screened for MSC markers. For immunocytochemical (ICC) stainings, 6.6 x 10^3 cells/cm^2 were seeded on glass coverslips for 48 hours. The presence of MSC markers CD29, CD44, CD90, CD105 and CD146 was then demonstrated via ICC stainings (see below).

7.3 iMAC isolation and culture

Immature Murine Articular Chondrocytes (iMACs) were isolated as previously described by Gosset *et al.* (133). In short, 5-6 day old mice were euthanized under general anesthesia. Femoral heads, femoral condyles and tibial plateaus were isolated from the hind limbs and placed in Phosphate Buffered Saline (PBS) (Lonza, Basel, Switzerland BE17-516F). The cartilage was then incubated twice in digestion solution (3 mg/ml collagenase D (Sigma-Aldrich, 11088866001) in iMAC standard culturing medium (Table 1)) for 45 minutes at 37°C in 5% CO_2. Cartilage was then incubated in diluted digestion solution (0.5 mg/ml collagenase D in standard culture medium) overnight at 37°C in 5% CO_2. Next, cartilage fragments were passed through 25-ml, 10-ml, 5-ml and 2-ml pipettes to disperse cell aggregates. After passing through a 48µm cell strainer, the cells were centrifuged at 400g for 10 minutes at 20°C. Cells were resuspended in standard culture medium supplemented with 10% hiFBS and seeded at a density of 8 x 10^3 cells/cm². The culture medium was changed twice a week. Passaging occurred when confluency reached 80%. Cells were detached using Trypsin (Sigma life science, T3924) for 5 minutes which was deactivated with 10% hiFBS in standard culture medium. Phenotypic characterization was performed via ICC and histological stainings. In short, 26.5 x 10^3 cells/cm² were seeded on glass cover slips for 96 hours in standard culture medium (Table 1) supplemented with 10% hiFBS, after which they were fixed using 4% PFA for 20 minutes. Immune-reactivity for collagen type 2 was demonstrated by ICC (see below). The presence of proteoglycans was demonstrated via Alcian blue and Safranin O stainings (see below). Culture purity was assessed by manual counting of at least 600 cells using ImageJ software (The National Institute of Health, Maryland, USA). Each experiment was performed with cells from passage 0-1.

7.4 Chondrogenic differentiation

Chondrogenic differentiation of DPSCs and BM-MSCs was performed using the Human Mesenchymal Stem Cell Functional Identification Kit (R&D, Abingdon, UK, SC006) according to the manufacturer's guidelines. In brief, a pellet containing 2.5 x 10^5 cells in a 15-ml conical tube (Greiner bio-one, 188285) was cultured in chondrogenic differentiation medium (Table 2). The chondrogenic supplement contained dexamethasone, ascorbate-phosphate, proline, pyruvate and transforming growth factor-β3. Differentiation medium was changed every 3 days with soft pipetting to detach the pellet from the tube. To evaluate the effect of L-PRF on the chondrogenic differentiation, L-PRF exudate (3%) and L-PRF CM (5%, 25%) were added to the differentiation medium. Positive and negative controls contained standard differentiation medium with or without the chondrogenic supplement respectively. After 21, 28 or 35 days, the pellets were either fixed in 4% paraformaldehyde (PFA) for 1.5 hours for immunohistochemical (IHC) analysis, used for RNA isolation or prepared for transmission electron microscopy (TEM) (see below).

7.5 Tenogenic differentiation

Six-well plates were coated with a 2mm-thick layer of SYLGARD (World Precision Instruments, Sarasota, Florida, SYLG184) and incubated overnight at 60°C to induce polymerization. Two silk sutures (SMI, St Vith, Belgium) of 8mm long were fixed 10mm apart in the middle of the plate with minutien insect pins (Fine Science Tools, 0.1mm diameter). The plates were sterilized by submersion in 99% ethanol while exposed to ultraviolet radiation for one hour. In each well, 6 x 10^5 cells were mixed with 1.93 mg/ml fibrinogen (Sigma-Aldrich, F8630) and 6.76 U/ml thrombin (Sigma-Aldrich, T4648) in a total volume of 1035µl tenogenic differentiation medium supplemented with 10% hiFBS (Table 2). The suspension was then quickly spread to cover the surface of the well and was incubated at 37°C for 10 minutes to allow fibrin polymerization. Afterwards, 2.5ml tenogenic differentiation medium was added per construct and adherences of the fibrin gel to the well were detached as these adherences generated additional lines of stress which interfered with the contraction of the gels. The medium was changed every 3 days and adherences were detached if necessary. After 7 and 14 days, the constructs were fixed with 4% PFA for 12 hours for IHC analysis (see below).

Table 1: Standard culture media of DPSCs, PDL-SCs, BM-MSCs and iMACs.

Cell type	Standard culture medium	Company	Reference
DPSCs & PDL-SCs	α-MEM	Sigma life science	M4526
	+ 2mM L-glutamine	Sigma life science	G7513
	+ 100 U/ml Penicillin	Sigma life science	P4333
	+ 100 µg/ml streptomycin	Sigma life science	P4333
BM-MSCs	DMEM-HG	Sigma life science	D6429
	+ 100 U/ml Penicillin	Sigma life science	P4333
	+ 100 µg/ml streptomycin	Sigma life science	P4333
iMACs	DMEM-LG	Sigma life science	D5546
	+ 2mM L-glutamine	Sigma life science	G7513
	+ 50 U/ml Penicillin	Sigma life science	P4333
	+ 50 µg/ml streptomycin	Sigma life science	P4333

Table 2: Differentiation media used for chondrogenic and tenogenic differentiation of MSCs

Differentiation	Medium	Company	Reference
Chondrogenic	DMEM-F12	Sigma life science	D6421
	+ 1% insulin transferrin selenite	R&D systems	AR014
	+ 1% chondrogenic supplement	R&D systems	SC006
	+ 2mM L-glutamine	Sigma life science	G7513
	+ 50 U/ml Penicillin	Sigma life science	P4333
	+ 50 µg/ml streptomycin	Sigma life science	P4333
Tenogenic	DMEM-LG	Gibco	11880-028
	+ 20 µg/ml aprotinin	Sigma life science	A1153
	+ 58 µg/ml L-ascorbic-acid	Sigma life science	A4544
	+ 1x MEM Non-essential amino acid solution	Sigma life science	M7145
	+ 1 ng/ml TGF-β3	R&D systems	243-B3
	+ 2mM L-glutamine	Sigma life science	G7513
	+ 100 U/ml Penicillin	Sigma life science	P4333
	+ 100 µg/ml streptomycin	Sigma life science	P4333

7.6 DPSC conditioned medium

Conditioned medium of DPSCs was prepared by seeding human DPSCs at a density of 2 x 10^4 cells/cm² in standard culture medium supplemented with 10% hiFBS. Cells were allowed to attach overnight. Afterwards, cells were rinsed twice with PBS and the culture medium was changed to 1 ml/5 cm² iMAC standard culturing medium (Table 1) with 0% hiFBS. After 48 hours, the medium was collected, centrifuged at 1200 RPM (161g) for 6 minutes and stored at -80°C.

7.7 Cell proliferation and survival assay

The effect of L-PRF and DPSCs on the proliferation and survival of iMACs was evaluated with a propidium iodide (PI) assay, taking the amount of PI intercalating between the DNA as a measure for the total number of cells. iMACs were seeded in triplicate wells in clear flat bottom 96 well plates (greiner bio-one, 655180) at a density of 18.5 x 10^3 cells/cm² and were allowed to attach for 24 hours. Afterwards, they were washed once with PBS and culture medium supplemented with L-PRF exudate (1%, 3%, 5%), L-PRF conditioned medium (5%, 25%, 50%) or DPSC CM was added. To evaluate iMAC proliferation, conditions were supplemented with 2% hiFBS. For survival assays, the cells were cultured in serum-free conditions. Negative and positive controls consisted cells cultured in serum-deprived medium(0% or 2% for survival and proliferation respectively) or medium supplemented with 10% hiFBS respectively. After 24, 48 or 72 hours, cells were lysated using 75µl Reagent A100 (Chemometec, Lillerød, Denemarken 910-0003). Next, cell lysate was incubated with PI (diluted 1/50 in 75µl Reagent B (Chemometec, 910-0002)) for 15 minutes in the dark. Solutions were transferred to a black 96 well plate with clear bottom (Greiner bio-one) and fluorescence was measured at an excitation wave length of 540nm and an emission wave length of 612nm (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany).

7.8 Transwell migration assay

iMACs were seeded in 24 well plates in standard culturing medium supplemented with 10% hiFBS at a density of 25 x 10^3 cells/cm², 50 x 10^3 cells/cm² and 62,5 x 10^3 cells/cm² and allowed to attach overnight. Afterwards, cells were washed with PBS and the medium was changed to serum-free standard culture medium. Positive and negative controls consisted of iMAC standard culturing medium supplemented with 10% or 0% hiFBS respectively. Twenty-four hours later, inserts (8 µm pore size, Greiner bio-one, 662638) were coated with 0.01 mg/ml poly-L-lysin (Sigma-Aldrich) for 1.5 hours and washed with MilliQ and PBS. DPSCs, suspended in iMAC standard culturing medium supplemented with 0% hiFBS, were seeded in the inserts at a density of 297 x 10^3 cells/cm². After 24 hours, migrated cells were washed with PBS, fixed with 4% PFA for 20 minutes and stained with 0.1% crystal violet in 70% ethanol for 30 minutes. Pictures were taken using the Nikon eclipse TS100 inverted microscope (Nikon, Tokyo, Japan) with a Jenoptik ProgRes C3 camera (Jenoptik, Jena, Germany) and quantified using AxioVision software (Carl Zeiss NV-SA, Zaventem, Belgium).

7.9 RT-qPCR

RNA was isolated using the PicoPure RNA isolation kit (Thermo Fisher Scientific, KIT0204) according to the manufacturer's guidelines. RNA was then converted into cDNA using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, USA, 95048-100) according to the manufacturer's protocol using 100ng RNA. Polymerase chain reactions were performed in the Biorad Thermal Cycler (Biorad, Hercules, California). Purity and RNA/cDNA concentrations were measured using the nanodrop 2000 spectrophotometer (Thermo fisher scientific). All samples were kept on ice as much as possible to prevent RNA/cDNA degradation.

Quantitative PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, California). The reaction mixture contained 5µl Fast SYBR Green Master Mix (Applied Biosystems, 4385612), 0.3µM forward primer, 0.3µM reverse primer and 0.75ng/µl cDNA sample with a total volume of 10µl. Gene expression levels were normalized using tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) and ribosomal protein L13a (RPL13a) (most stable housekeeping genes as measured by Genorm) and calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 3: Primer sequences for RT-qPCR. *Summary of the primer sequences used for the analysis of the gene expression in differentiation pellets exposed to L-PRF CM (5%, 25%) and L-PRF exudate (3%). Gene expression was analysed by means of RT-qPCR.*

Gene	Forward primer 3′ – 5′	Reverse primer 5′ – 3′
ACAN	AGG CAG CGT GAT CCT TAC C	GGC CTC TCC AGT CTC ATT CTC
Collagen type 1	GGC CCC ATT GGT AAT GTT GG	GGA CCT TTG CCG CCT TCT TT
Collagen type 2	CGT CCA GAT GAC CTT CCT ACG	TGA GCA GGG CCT TCT TGA G
Collagen type 10	GCT TCA GGG AGT GCC ATC ATC	CTC ACA TTG GAG CCA CTA GGA ATC
Runx2	TGA TGA CAC TGC CAC CTC TGA	GCA CCT GCC TGG CTC TTC T
RPL13a	AAG TTG AAG TAC CTG GCT TTC C	GCC GTC AAA CAC CTT GAG AC
YWHAZ	CTT GAC ATT GTG GAC ATC GG	TAT TTG TGG GAC AGC ATG GA

7.10 TEM imaging

Samples were fixed with 2% glutaraldehyde (Laborimpex, Brussels, Belgium) in 0.05M cacodylate buffer (pH 7.3; Aurion, Wageningen, the Netherlands) at 4°C. The fixative was gently aspirated with a glass pipette, and pellets were postfixed in 2% osmium tetroxide (Aurion) for 1 h. Next, the they were put through a dehydrating series of graded concentrations of acetone and embedded in araldite according to the popoff method (134). Ultrathin sections (0.06 µm) were mounted on 0.7% formvar-coated copper grids (Aurion), contrasted with 0.5% uranyl acetate and a stabilized solution of lead citrate (both from Laurylab, Saint-Fons Cedex, France), and examined in a Philips EM 208 transmission electron microscope (Philips, Eindhoven, The Eindhoven) operated at 80 kV. The microscope was provided with a Morada Soft Imaging System (SIS; Olympus, Tokyo, Japan) camera to acquire high-resolution images of the evaluated samples. The images were processed digitally with iTEM-FEI software (Olympus SIS).

7.11 Immunohistochemical staining

Fixated pellets (chondrogenic differentiation) and constructs (tenogenic differentiation) were embedded in paraffin and 7µm thick sections were cut. Samples were deparaffinized in xylene and ethanol baths (xylene: 2 times 5 minutes, ethanol: 100%, 100%, 95%, 80%, 70%, 50%, 2 minutes each). Antigen retrieval was performed by heating the samples three times in 1x Target Retrieval Solution (DAKO, Heverlee, Belgium, S2031) for 5 minutes with a resting period of two minutes between the sessions. Afterwards, the samples were left to cool down at room temperature for 30 minutes. In case of 3,3'-Diaminobenzidine (DAB) (DAKO, K3468) stainings, endogenous peroxidases were blocked with Peroxidase Block (DAKO, K4007) for 20 minutes. Next, nonspecific binding of the antibodies was inhibited with Protein Block (DAKO, X0909) for 30 minutes. Samples were then incubated with the primary antibodies (Table 3, Abcam, Cambridge, UK – Santa Cruz, Dallas, Texas – Biolegend, San Diego, California) diluted in 10% Protein Block in PBS for one hour at room temperature or overnight at 4°C. Afterwards, samples were incubated with the appropriate secondary

antibodies (Table 4) diluted 1/500 in PBS for 30 minutes. Immunofluorescent stainings were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes and mounted using Fluorescence Mounting Medium (DAKO, S3023). Samples stained with HRP-labeled antibodies (DAKO, K4007) were incubated with DAB for 5 minutes and counterstained with haematoxylin for 7 minutes after which they were washed twice with running tap water for 10 minutes. Samples were dehydrated in ethanol and xylene (ethanol: 70%, 80%, 95%, 100%, 100%, 100% xylene, 100% xylene, 2 minutes each) and mounted using DPX mountant (Leica Biosystems, Diegem, Belgium, H4010). Pictures were taken using a Leica DM400B LED fluorescence microscope equipped with a Leica DFC450C camera. Analysis were performed using ImageJ software. The integrated density (mean fluorescence intensity x area) within the pellet was measured and divided by the amount of cells within the area. Next, the fluorescence intensities of negative controls (not incubated with primary antibodies) were subtracted in order to remove background signal caused by autofluorescence.

Table 3: Overview of primary antibodies used for immunological stainings.

Antigens	Concentration IHC	Concentration ICC	Company	Reference number	Origin
Aggrecan	1/500	N.A.	Abcam	AB186414	Rabbit
Collagen type 1	1/500	N.A.	Santa Cruz	SC59772	Mouse
Collagen type 2	1/100	1/200	Abcam	AB34712	Rabbit
CD44	N.A.	1/200	Abcam	AB65829	Rabbit
CD90	N.A.	1/200	Biolegend	328101	Mouse
CD105	N.A.	1/50	Biolegend	323202	Mouse
CD146	N.A.	1/50	Biolegend	361001	Mouse
CD34	N.A.	1/50	Abcam	AB6330	Mouse
CD45	N.A.	1/100	Abcam	AB63390	Rabbit

Table 4: Overview of secondary antibodies used for immunological stainings.

Antibody	Company	Reference number
Donkey α Rabbit Alexa Fluor 555	ThermoFisher	A31572
Goat α Mouse Alexa Fluor 555	ThermoFisher	A21422
Goat α Rabbit Alexa Fluor 488	ThermoFisher	A11008

7.12 Immunocytochemical staining

Fixated cells were washed with PBS. In case of intracellular targets, the cells were permeabilized with 0.05% Triton in PBS for 30 minutes. Nonspecific antibody binding was blocked with Protein Block (DAKO, X0909) for 20 minutes. For iMACs, permeabilisation and blocking occurred in one step, using 10% protein block and 0.2% Triton in PBS for one hour. Cells were then incubated with the primary antibodies (Table 3) diluted in PBS for one hour at room temperature or overnight at 4°C. Afterwards, they were incubated with the appropriate secondary antibodies (Table 4) diluted 1/500 in PBS for 30 minutes. Nuclei were stained with DAPI for 10 minutes. Samples were mounted using Fluorescence Mounting Medium (DAKO, S3023). Pictures were taken using a Leica DM400B LED fluorescence microscope with a Leica DFC450C camera.

7.13 Histological staining

7.13.1 Safranin O

Samples were incubated with haematoxylin for 10 minutes, after which they were washed with running tap water for 10 minutes. Next, they were incubated with 0.05% Fast Green solution for 5 minutes and rinsed with 1% acetic acid solution for 10 seconds. Thereafter, the samples were incubated in 0.1% Safranin O solution (Merck, Overijse, Belgium CI50240) for 5 minutes. Finally, samples were dehydrated in alcohol and xylene baths (70% Ethanol, 80% Ethanol, 95% Ethanol, 100% Ethanol, xylene, 2 minutes each) and mounted using DPX mountant. Pictures were made using the Leica DM2000 LED microscope equipped with a Leica MC170 HD camera.

7.13.2 Alcian Blue

Samples were incubated with Alcian Blue solution for 30 minutes at room temperature after which they were washed with running tap water for 10 minutes and with distilled water for 1 minute. Afterwards, they were incubated with nuclear fast red solution for 10 minutes. They were then washed for 1 second with distilled water and were dehydrated in alcohol and xylene baths. Samples were mounted using DPX mountant. Pictures were made using the Leica DM2000 LED microscope equipped with a Leica MC170 HD camera.

7.13.3 Hematoxylin-eosin

Samples were incubated in hematoxylin solution for 5 minutes and washed with running tap water for 10 minutes. Afterwards, they were incubated in eosin solution for 3 minutes, after which they were dehydrated in alcohol and xylene baths and mounted using DPX mountant. Samples were visualized with the Mirax slide scanner (Carl Zeiss NV-SA) using the Mirax scan software.

7.13.4 Masson's trichrome

Tissues were incubated in hematoxylin for 10 minutes. Afterwards, they were rinsed with running tap water for 10 minutes and incubated in Ponceau/Fuchsine solution for 5 minutes. Next, samples were incubated in 1% phosphomolybdic acid and in Aniline blue solution for 5 minutes each. After another incubation in 1% phosphomolybdic acid for 5 minutes, samples were placed in acetic acid for 2 minutes. Between each incubation, samples were rinsed with distilled water for 2 minutes. Samples were dehydrated in alcohol and xylene baths, mounted using DPX mountant and visualized with the Mirax slide scanner.

7.13.5 Sirius red

Samples were deparaffinized and stained with hematoxylin for 8 minutes. Next, they were incubated in Phosphomolybdic acid for 2 minutes, Picrosirius red stain for 60 minutes and hydrochloride acid for 2 minutes (Polysciences, Warrington, Pennsylvania, 24901). Afterwards, samples were dehydrated, mounted using DPX mountant and visualized with the Mirax slide scanner.

7.14 Statistical analysis

Statistical analysis were performed using Graphpad Prism 7 software (Graphpad, San Diego, California). Experimental groups were compared using a Kruskal-Wallis test, followed by a Dunn's test for multiple comparisons. Differences were considered significant when $p \leq 0.05$. All data were presented as mean ± SEM.

8. Results

8.1 Immunophenotype of human dental pulp stem cells

The immunophenotype of DPSCs was assessed by means of immunofluorescence (Figure 3). Human DPSCs displayed a high immuno-reactivity for stem cell markers CD44 and CD90 and to a lesser extent CD105 (Figure 3 A, B, D). Furthermore, DPSCs showed a uniform expression of CD146, an endothelial cell marker (Figure 3 E). There was no expression of CD34 and CD45, two hematopoietic markers (Figure 3 C, F). Together, these stainings confirm the DPSC immunophenotype as described previously (47).

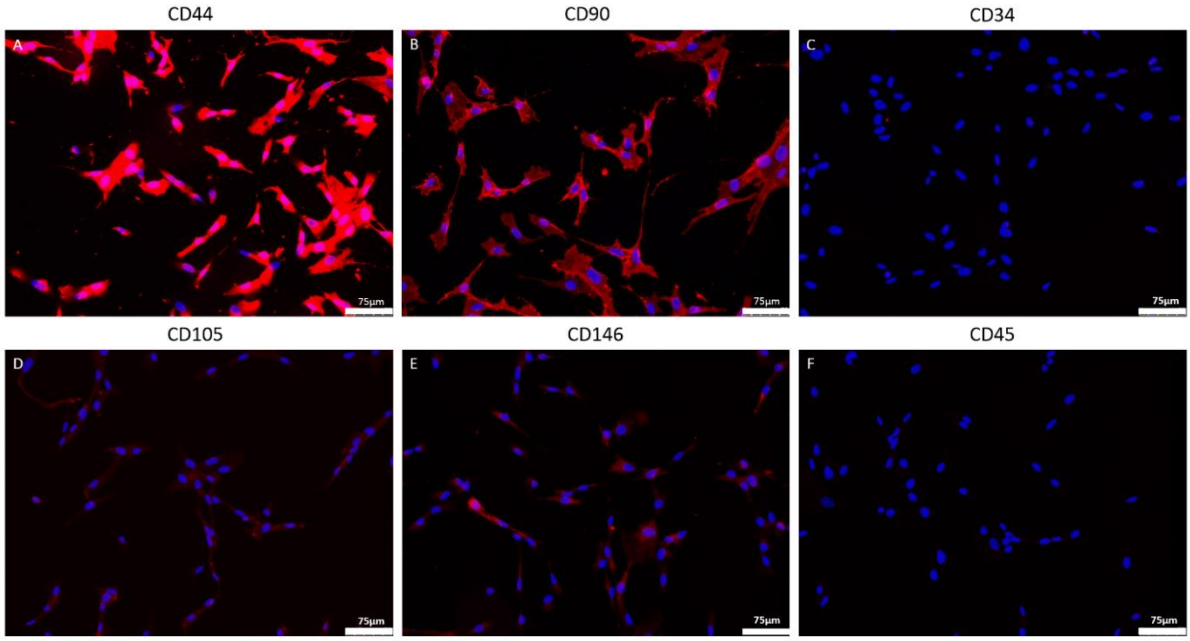

Figure 3: Immunophenotype of human DPSCs. *Human DPSCs immunophenotype was routinely screened by means of immunofluorescence. Representative immunofluorescent stainings of (A) CD44, (B) CD90, (C) CD34, (D) CD105), (E) CD146 and (F) CD45 are shown. Human DPSCs expressed the markers CD44, CD90, CD105 and CD146, but did not express the hematopoietic markers CD34 and CD45, confirming the immunophenotype of human DPSCs as previously described. Nuclei were stained with DAPI (blue). Scale-bar = 75µm*

8.2 Chondrogenic differentiation of human DPSCs and BM-MSCs

In order to evaluate the chondrogenic differentiation, DPSCs and BM-MSCs were subjected to a 3D chondrogenic differentiation system for 21 days. Following three weeks of chondrogenic differentiation, both DPSCs and BM-MSC formed compact cell spheres, surrounded by a thin layer of fibroblast-like cells (n=1). Both cell types expressed the hyaline cartilage marker collagen type 2 (Figure 4 A, F). Aggrecan, the most abundant proteoglycan in the articular cartilage, was only expressed in differentiated BM-MSCs (Figure 4 B, G). The differentiation time of DPSCs was extended from 21 days to 28 and 35 days, however, the expression of aggrecan remained absent at each time point (data not shown). Alcian blue staining demonstrated the presence of GAGs in the ECM of both differentiated DPSCs and BM-MSCs (Figure 4 C, H). Cartilage-like fragments (Figure D, E, I) and matrix-filled vesicles (Figure 4 J) were observed in both differentiated DPSCs and BM-MSCs.

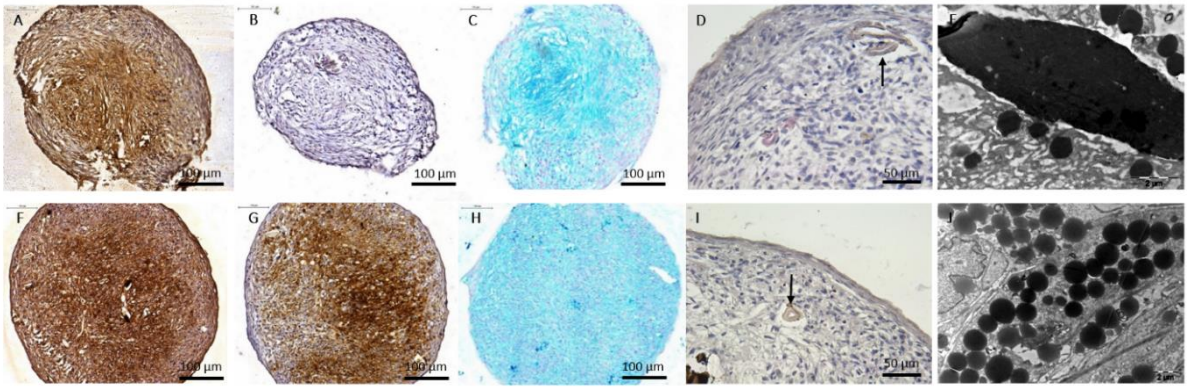

Figure 4: Chondrogenic differentiation of human DPSCs and BM-MSCs. *Following 21 days of differentiation, (A-C) human DPSCs and (F-H) BM-MSCs were stained for chondrocyte markers (A, F) collagen type 2 and (B, G) aggrecan. BM-MSCs expressed both collagen type 2 and aggrecan, whereas differentiated DPSCs only expressed collagen type 2. (C, H) Alcian blue staining demonstrated the production of GAGs in both cell types. Cartilage-like fragments could be detected in both differentiated (D) DPSCs and (I) BM-MSCs (black arrow). (E) Transmission electron microscopy (TEM) of a cartilage-like fragment in differentiated DPSCs. (F) Matrix-filled vesicles could be detected in both cell types via TEM, representative image of differentiated DPSCs. (A-C, F-H) Scale-bar = 100µm, (D, I) Scale-bar = 50µm, (E, J) Scale-bar = 2µm, n=1*

8.4 The effect of L-PRF on the chondrogenic differentiation

8.4.1 Cartilage-specific gene expression

Following three weeks of differentiation, RNA was extracted from differentiated DPSCs cell spheres for RT-qPCR analysis. To determine whether L-PRF could induce transcriptomic alterations in differentiating DPSCs, we evaluated the mRNA expression of hyaline cartilage markers collagen type 2 and aggrecan, hypertrophic markers Runx2 and collagen type 10 and fibrocartilage marker collagen type 1 (n=1). The expression of collagen type 1 and 2 seemed to decrease upon L-PRF CM and L-PRF exudate exposure (Figure 5 A, B) whereas the expression of aggrecan was not detected in any of the conditions (data not shown). Furthermore, exposure of the differentiation pellets to L-PRF CM and L-PRF exudate seemed to increase the expression of hypertrophic markers collagen type 10 and Runx2 (Figure 5 C, D).

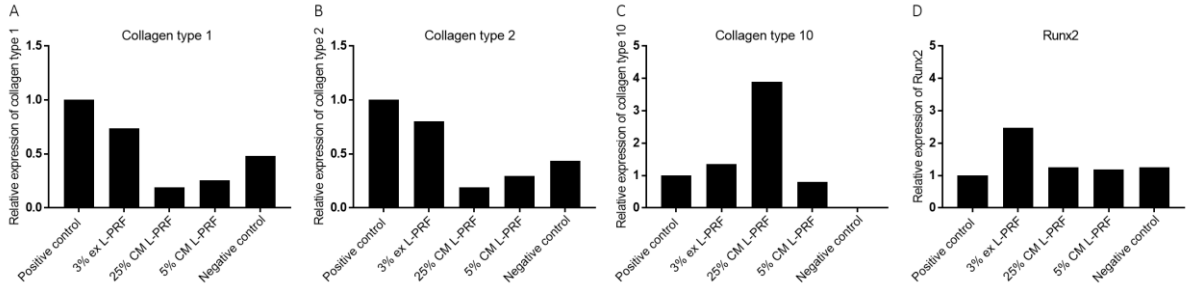

Figure 5: The effect of L-PRF on gene expression in differentiated human DPSCs. *Differentiation pellets were exposed to L-PRF CM (5%, 25%) and L-PRF exudate (3%). After 21 days (A) collagen type 1, (B) collagen type 2, (C) collagen type 10 and (D) Runx2 gene expression was measured via RT-qPCR. L-PRF CM seems to inhibit collagen type 1 and 2 expression and stimulate collagen type 10 expression whereas L-PRF exudate increased Runx2 gene expression, suggesting that L-PRF does not enhance the chondrogenic differentiation of DPSCs. n=1*

8.4.2 Cartilage-specific protein expression

In order to evaluate the effect of L-PRF on the chondrogenic differentiation, human DPSCs and BM-MSCs were exposed to a 3D differentiation system, supplemented with L-PRF CM (5%, 25%) or L-PRF exudate (3%) for 21 days (n=1). The expression of collagen type 2 and aggrecan was evaluated via immunofluorescence (Figure 6 A-D), while GAG production was evaluated via Alcian blue stainings. Quantification of fluorescence intensity showed no influence of either L-PRF CM or L-PRF exudate on the collagen type 2 production per cell (Figure 6 G). Aggrecan expression remained absent in the DPSC pellets whereas its expression per cell seemed to decreased in the BM-MSC pellets exposed to L-PRF (Figure 6 H). Fluorescent stainings of the differentiation pellets displayed a high level of autofluorescence at the edges of the pellet, probably caused by the presence of phenol red in the differentiation medium. Alcian blue stainings were performed to evaluate the effect of L-PRF on the GAG production by differentiated DPSCs and BM-MSCs (Figure 6 E, F). Both cell types produced GAGs, however, quantification of alcian blue showed that L-PRF seemed to decrease the GAG area percentage (Figure 6 I). Taken together, these data suggest that L-PRF does not enhance the chondrogenic differentiation of either DPSCs or BM-MSCs. However, experiments need to be repeated to show statistical relevance.

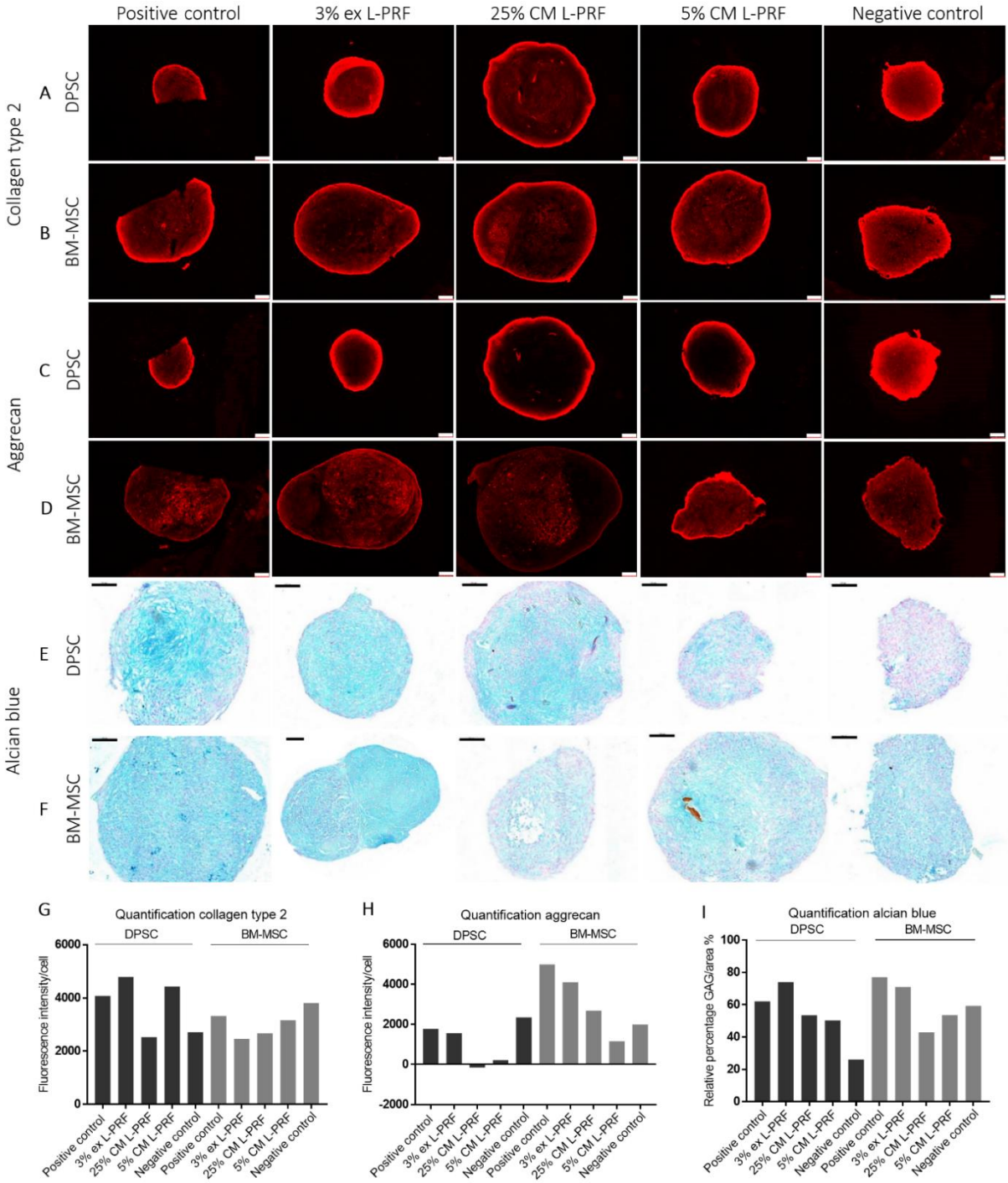

Figure 6: The effect of L-PRF on cartilage specific protein production in differentiated human DPSCs and BM-MSCs. *After 21 days of exposure to L-PRF CM (5%, 25%) or L-PRF exudate (3%), protein expression in the differentiation pellets was evaluated using (immuno)histological stainings. (A, B, G) Collagen type 2 expression per cell did not seem to be altered by L-PRF stimulation. (C, D, H) Aggrecan expression remained absent in the DPSCs pellets, whereas its production per cell appeared to be suppressed by L-PRF. (E, F, I) GAG production was assessed by means of an Alcian blue staining. L-PRF stimulation seemed to lower the GAG area percentage. (A-D) Scale-bar = 75µm, (E, F) Scale-bar = 100µm*

8.5 The effect of DPSCs and L-PRF on iMACs

8.5.1 iMAC characterization

iMACs were isolated from femoral heads, condyles and tibial plateau from the hind limbs of five day old mice. Cultures mainly displayed cells with typical polygonal morphology, only few cells displayed a fibroblast-like morphology (Figure 7 A, B). Phenotypic characterization of primary iMACs was performed via (immuno)histology. Alcian Blue and Safranin O revealed the presence of proteoglycan components (Figure 7 C-F). Immune-reactivity of iMACs for collagen type 2 was demonstrated via ICC (Figure 7 G, H). The average culture purity was 71% ± 8% (n=3), as assessed by manual counting. Together, these stainings provide evidence of functional chondrocytes.

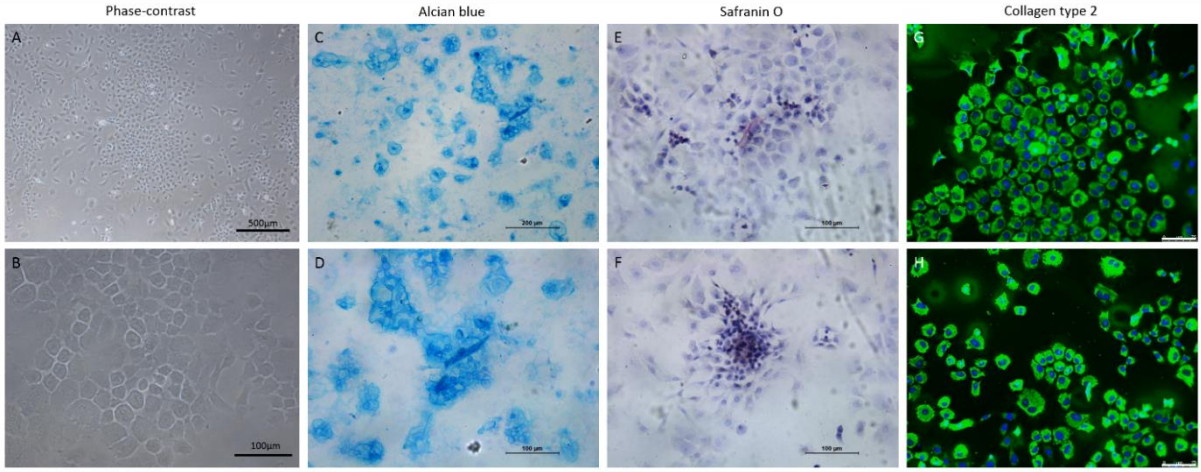

Figure 7: Characterization of iMACs. *iMACs were characterized via ICC and histological stainings. (A, B) Cells mainly displayed a small polygonal morphology. Proteoglycan production was demonstrated by (C, D) Alcian blue and (E, F) Safranin O stainings. (G, H) ICC staining demonstrated the production of collagen type 2 by iMACs. Together, these stainings provide evidence of functional chondrocytes. (A) Scale-bar = 500µm, (C) Scale-bar = 200µm, (B, D, E, F) Scale-bar = 100µm, (G, H) Scale-bar = 75µm*

8.5.2 Effect of DPSCs and L-PRF on iMAC survival

Serum-deprived healthy iMACs were cultured in standard culturing medium, supplemented with L-PRF CM (5%, 25% or 50%), L-PRF exudate (1%, 3% or 5%) or DPSC CM (n=5). The effect on iMAC survival after 24h, 48h and 72h was evaluated by means of a PI assay. A decrease in mean fluorescence intensity in the negative control was observed over time (56 ± 26 after 24 hours compared to 17 ± 16 after 72 hours), representing the cell death caused by the lack of serum (Figure 8, dotted line represents the fluorescence intensity of the negative control after 24 hours). This could not be prevented by addition of L-PRF exudate to the culture medium (Figure 8 A-C). However, addition of 25% and 50% L-PRF CM to the culturing medium significantly ($p<0.01$) increased fluorescence intensity and thus cellular survival (219 ± 30 and 226 ± 43 respectively after 48 hours compared to 1.3 ± 22 in the negative control after 48 hours; 328 ± 66 and 393 ± 43 respectively after 72 hours compared to 17 ± 16 in the negative control after 72 hours) (Figure 8 A-C). This effect was shown to be concentration dependent (after 72 hours: 124 ± 38, 328 ± 66 and 393 ± 43 for 5%, 25% and 50% L-PRF CM respectively) (Figure 8 A-C). DPSC CM also seemed to stimulated cell survival (100 ± 35 for DPSC CM compared to 32 ± 17 for negative control 72 hours), although not reaching significance ($p=0.31$) (Figure 8 D-F). These data demonstrate a significant survival stimulating effect of L-PRF CM on iMACs and suggest the same effect, although less pronounced, of DPSC CM.

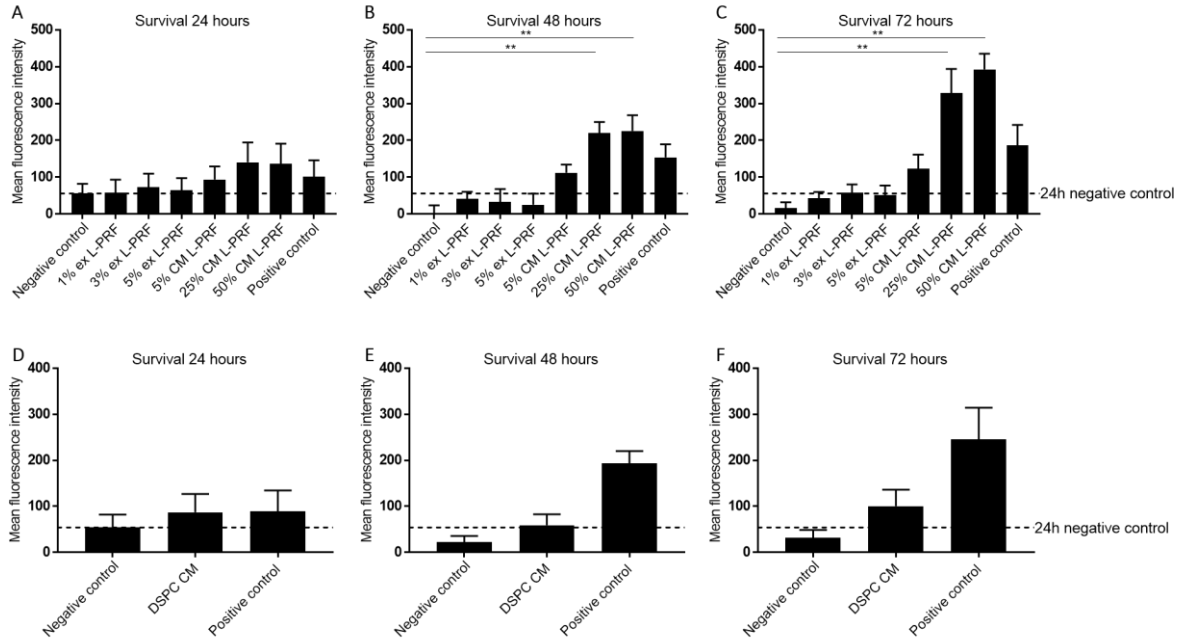

Figure 8: Effect of human DPSCs and L-PRF on the survival of iMACs. *iMACs were cultured in serum-free medium. The effect of (A-C) L-PRF exudate and L-PRF CM and (D-F) DPSCs CM on cell survival was assessed after (A, D) 24 hours, (B, E) 48 hours and (C, F) 72 hours by means of a PI assay. L-PRF exudate did not influence cell survival, whereas L-PRF CM was shown to significantly promote cellular survival ($p<0.01$). DPSC CM also seemed to promote survival, although not reaching statistical significance. n=5, **$p<0.01$*

8.5.3 Effect of DPSCs and L-PRF on iMAC proliferation

iMACs were cultured in serum-poor medium containing 2% hiFBS, supplemented with L-PRF CM (5%, 25% or 50%), L-PRF exudate (1%, 3% or 5%) or DPSC CM (n=2). The effect on iMAC proliferation after 24h, 48h and 72h was evaluated by means of a PI assay (Figure 9). The serum concentration was chosen based on previous optimization, in an attempt to prevent both cell death and proliferation in the negative control. Despite the reduced serum concentration, an increase in fluorescence intensity was observed in the negative control over time (26 ± 38 after 24 hours compared to 186 ± 3.5 after 72 hours) (Figure 9 A-C, dotted line represents the fluorescence intensity of the negative control after 24 hours). Nevertheless, the data show a significant ($p<0.05$) increase in iMAC proliferation upon stimulation with 50% L-PRF CM (824 ± 18 compared to 186 ± 3.5 in the negative control after 72 hours) (Figure 9 C). Furthermore, DPSC CM also seemed to increase iMAC proliferation (248 ± 80 compared to 142 ± 46 in the negative control after 72 hours) (Figure 9 D-F). However, these experiments need to be repeated in order to demonstrate statistical relevance.

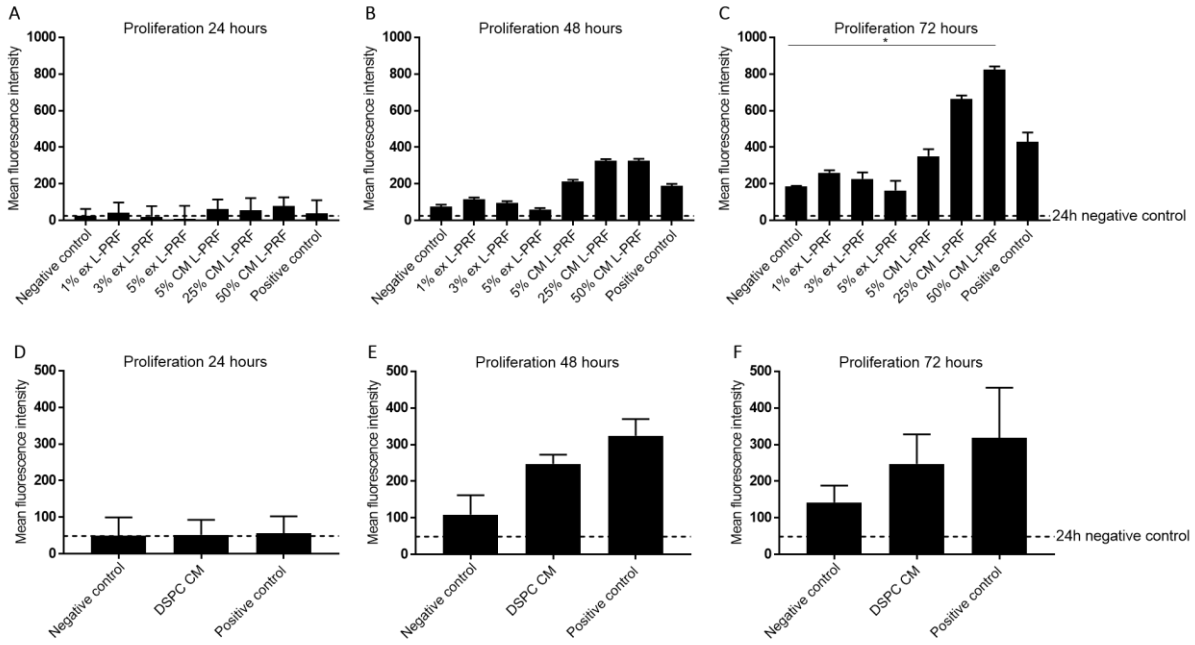

Figure 9: Effect of human DPSCs and L-PRF on the proliferation of iMACs. *iMACs were cultured in serum-deprived medium containing 2% hiFBS. The effect of (A-C) L-PRF exudate and L-PRF CM and (D-F) DPSCs CM on iMAC proliferation was assessed after (A, D) 24 hours, (B, E) 48 hours and (C, F) 72 hours by means of a PI assay. L-PRF CM was shown to significantly promote cell proliferation (P<0.05). DPSC CM also seemed to promote proliferation, although not reaching statistical significance. n=2, *p<0.05*

8.6 Migration capacity of human DPSCs towards healthy iMACS

The ultimate goal of this study will be to evaluate whether DPSCs and L-PRF can improve cartilage regeneration in an ovine model of cartilage lesions and OA. For intra-articular injection, it is important that stem cells migrate towards chondrocytes. Therefore we investigate whether DPSCs are able to migrate towards iMACs using a transwell migration assay. iMACs were seeded at a density of 25 x 10^3 cells/cm², 50 x 10^3 cells/cm² and 62.5 x 10^3 cells/cm² in the lower compartment, while 100 x 10^3 DPSCs were seeded in the upper compartment of a transwell. Our results demonstrate that DPSCs migrate towards healthy iMACs (migration area percentage of 31% ± 11.5%, 22% ± 10% and 38% ± 22% for 25 x 10^3 iMACs/cm², 50 x 10^3 iMACs/cm² and 62.5 x 10^3 iMACs/cm² respectively, compared to 1.9% ± 1.9% for the negative control) (Figure 10).

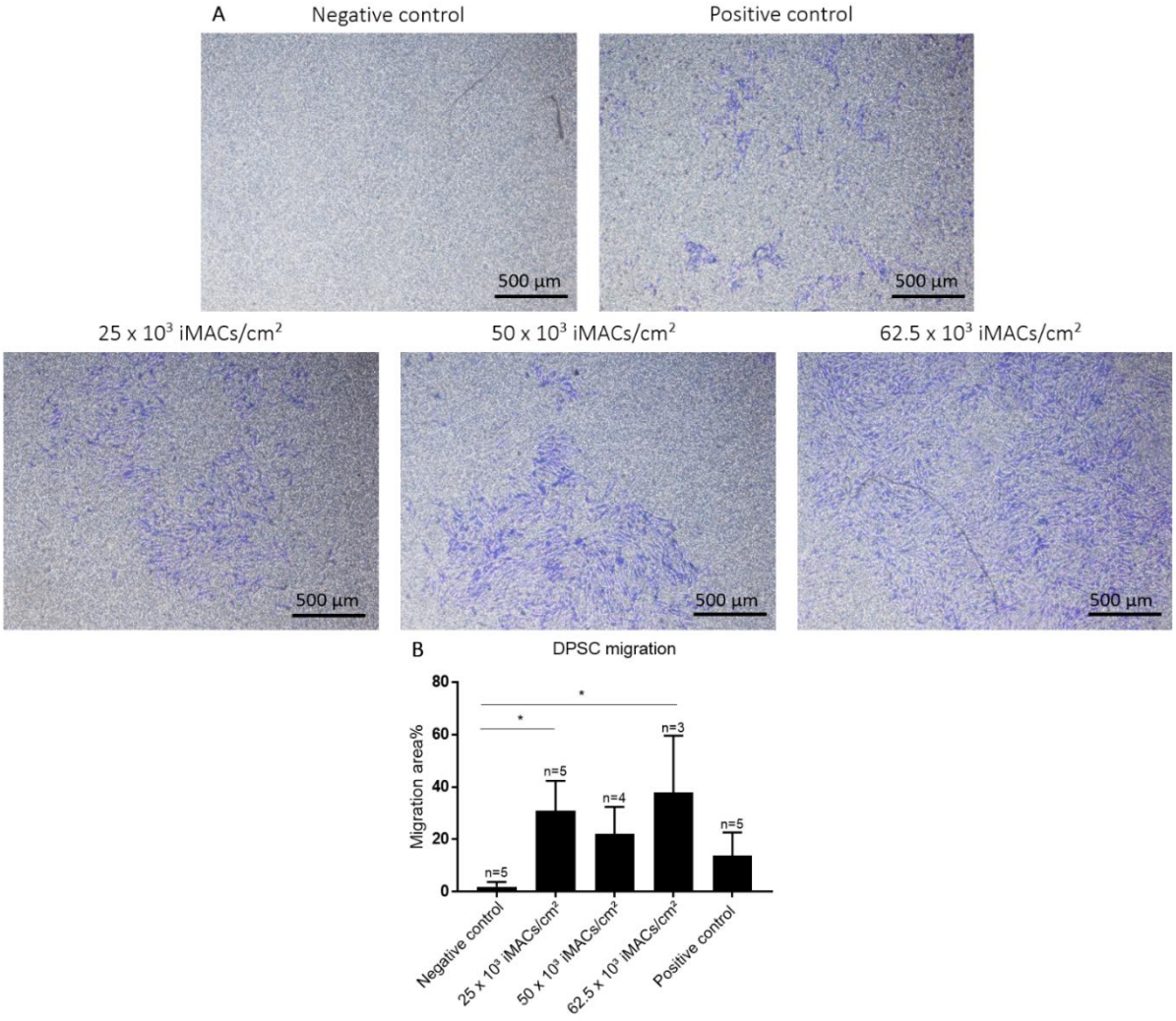

Figure 10: The migration capacity of DPSCs towards healthy iMACs. *The migration of DPSCs towards iMACs was evaluated using a transwell migration assay. iMACs were seeded in a 24 well plate (25 x 10^3 cells/cm², 50 x 10^3 cells/cm² and 62.5 x 10^3 cells/cm²), 100 x 10^3 DPSCs were seeded in the upper compartment of a transwell. (A) Representative pictures of DPSC migration towards iMACs. (B) Quantification of the migration area demonstrated the migration of DPSCs towards iMACs. Scale-bar = 500µm, *p<0.05, n=5 for negative control, positive control and 25 x 10^3 cells/cm², n=4 for 50 x 10^3 cells/cm², n=3 for 62.5 x 10^3 cells/cm²*

8.7 Tenogenic differentiation of human DPSCs and PDL-SCs

In order to investigate whether DPSCs could be used in the treatment of tendon lesions, we first evaluated their ability to form tendon construct *in vitro*. Human DPSCs and PDL-SCs were cultured in fixed-length fibrin gels stimulated with TGF-β3 for 7 and 14 days, as previously described by Kapacee *et al.* (n=1) (77, 80). After seven days in culture, the cells had contracted the gels and formed a tendon-like construct between the fixed placed minutien pins (Figure 11 A, B). Hematoxylin-eosin staining of the constructs after 7 and 14 days demonstrate a dense matrix with parallel alignment of both cell types (Figure 11 C - H). Masson's trichrome staining demonstrated the production of collagen fibres in the direction of the applied tension in both cell types after 7 days (Figure 11 K, O). After 14 days in culture, the constructs displayed a more even staining for collagen throughout the complete construct (Figure 11 L, P). The production of collagen was also demonstrated via a Sirius red staining (Figure 11 I, J, M, N). Taken together, these data demonstrate that both DPSCs and PDL-SCs can form tendon-like constructs *in vitro*.

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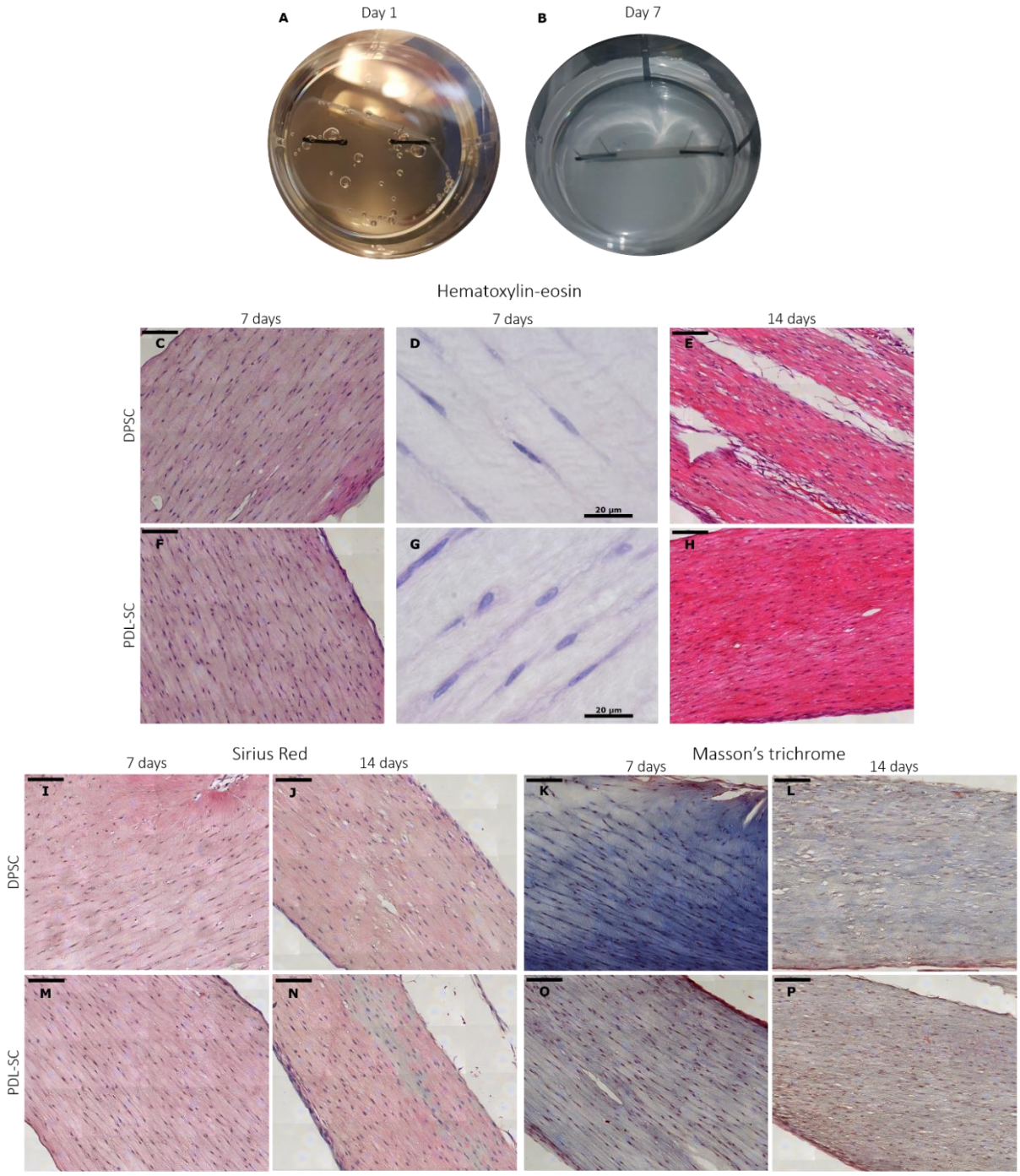

Figure 11: Formation of tendon-like constructs by human DPSCs and PDL-SCs. *(A) Human DPSCs and PDL-SCs were cultured in fixed-length fibrin gels. (B) Within 7 days, cells completely contracted the gel between the fixed minutien pins. (C - H) Hematoxylin-eosin staining of the constructs containing (C - E) DPSCs and (F - H) PDL-SCs. Collagen production was demonstrated via (I, J, M, N) Sirius red staining and via (K, L, O, P) Masson's trichrome staining of constructs containing (I – L) DPSCs and (M – P) PDL-SCs (C, E, F, H, I-P) Scale-bar = 100µm, (D, G) scale-bar = 20µm, n=1*

9. Discussion

Articular cartilage defects affect millions of people each year and numbers from the united states demonstrate an annual economic burden of approximately $33 billion (7-9). These lesions often progress to OA, an inflammatory joint disease which is characterized by progressive loss of cartilage tissue (8, 12, 13). The limited intrinsic healing capacity and the failure of current therapies to provide a lasting regeneration of the damaged tissue signify the need for the development of new treatment strategies (2, 6, 21, 22). MSCs are a promising alternative to current treatment strategies due to their differentiation capacity, production of growth factors and immunomodulatory properties (14, 40-45, 47). Their potential in cartilage repair has already been proven in various studies (42, 58, 59). In this project, we investigated the therapeutic potential of human DPSCs in cartilage repair, as these cells possess several advantages over other MSC types, including less invasive isolation, increased proliferation rate and a higher immunomodulatory capacity (46, 47, 72). Furthermore, we investigated whether L-PRF, an autologous biomaterial rich in growth factors, can enhance the potential of stem cell-based cartilage repair (27, 28).

Our first aim was to evaluate the chondrogenic differentiation of human DPSCs and BM-MSCs. After 21 days of differentiation, both DPSCs and BM-MSC formed compact 3D spheres rich in collagen type 2 and GAGs. However, the presence of aggrecan, the most predominant proteoglycan in the articular cartilage, was only demonstrated in BM-MSCs. These findings are conflicting with recent studies reporting a complete chondrogenic differentiation of DPSCs with expression of both collagen type 2 and aggrecan (47, 50). The absence of aggrecan expression in the DPSC pellets might be caused by several factors. Firstly, DPSCs might require more time to fully differentiate into chondrocytes. This was tested by extending the differentiation time from 21 to 28 and 35 days. However, in contrast to a recent study reporting an improved chondrocyte phenotype upon prolongation of the differentiation, the expression of aggrecan remained absent at each time point (135). In future experiments, we could try to use other 3D chondrogenic differentiation systems in order to induce a better differentiation of human DPSCs. For example, instead of pellet culturing, cell seeding onto 3D collagen- or polyethylene glycol-based scaffolds has been used in previous studies (135, 136). Hypoxic culturing conditions have been demonstrated to significantly enhance chondrogenic differentiation of MSCs (137, 138). Furthermore, addition of specific carbohydrates to the differentiation medium has been reported to enhance cartilage-specific matrix deposition (139). Another explanation for the lack of aggrecan production by differentiated human DPSCs might be found in the type of cartilage which is produced. It is possible that differentiated DPSC produce fibrous or elastic cartilage, rather than hyaline cartilage. This was tested via (immuno)histological stainings. The absence of collagen type 1, examined by IHC, suggests that the differentiated pellets do not produce fibrous cartilage. Furthermore, the absence of elastic fibres, demonstrated via an Orcein staining, suggests that they do not produce elastic cartilage either. The lack of aggrecan expression in our study might also have been caused by interpatient variability, as for example donor age and gender have been reported to influence MSC differentiation capacities (140, 141). Therefore, future experiments will focus on the chondrogenic differentiation of human DPSCs isolated from a range of different donors.

The transdifferentiation of chondrocytes into osteoblasts has been reported previously (142). Further evaluation of the differentiation pellets by means Alizarin red staining and IHC stainings for bone specific proteins such as osteocalcin need to be performed in order to prove that the absence of aggrecan was not caused by the transdifferentiation into osteoblasts. Lastly, DPSCs might be unable to completely differentiate into chondrocytes as the differentiation capacity of MSCs has been shown to be source dependent (51). A pre-chondrocyte specific phenotype (i.e. $CD166^{low/neg}$, $CD146^{low/neg}$, $CD73^{+}$, $CD44^{low}$, bone morphogenetic protein receptor 1 $beta^{+}$) has been previously described (143). Phenotypic analysis of the differentiation pellets is needed in order to evaluate whether the lack of aggrecan is caused by an incomplete chondrogenic differentiation. Despite the absence of aggrecan, cartilage fragments and matrix-filled vesicles could be detected in both human DPSC and BM-MSC differentiation pellets, as previously described by Hilkens *et al.* and Struys *et al.* (47, 50).

New treatment strategies for the repair of articular cartilage defects might arise if we succeed in differentiating human DPSCs into collagen type 2 and aggrecan producing chondrocytes. They might for example provide an alternative source of chondrocytes for ACI. Clinically relevant numbers of human DPSCs can be reached by *in vitro* expansion within a short period of time. Upon differentiation, DPSC-derived chondrocytes can be easily isolated and be immediately transplanted into the lesion site. In this way, the drawbacks of ACI, i.e. donor-site morbidity, long culturing times and chondrocyte dedifferentiation, are circumvented.

The chondrogenic differentiation of MSCs has been shown to be enhanced by the addition of PRP in several studies (92, 144, 145). However, the PRP preparation method has been reported to be important for its differentiation promoting effect, as different commercially available PRP preparation kits resulted in different effects on the chondrogenic differentiation (101). The effect of L-PRF on the chondrogenic differentiation of MSCs has remained unstudied (146). Therefore, we investigated the effect of L-PRF on the chondrogenic differentiation of DPSCs and BM-MSCs. On the transcriptional level, we found that L-PRF inhibited the expression of collagen type 1 and 2 and was not able to induce the expression of aggrecan in human DPSCs. Furthermore, L-PRF appeared to induce hypertrophic differentiation, as collagen type 10 and Runx2 were upregulated in differentiation pellets exposed to L-PRF CM and L-PRF exudate respectively. However, these experiments should be repeated in order to reach statistical significance. On the translational level, L-PRF did not seem to influence collagen type 2 expression in either cell type, whereas it decreased the expression of aggrecan in differentiated BM-MSCs. Furthermore, the area percentage of GAGs was shown to be reduced upon L-PRF CM exposure in both differentiated DPSCs and BM-MSCs. The observed upregulation of hypertrophic markers collagen type 10 and Runx2 on transcriptional level in response to L-PRF stimulation should still be confirmed on protein level in the future. The effect of L-PRF on the survival of cells in the chondrogenic pellets was assessed by means of a propidium iodide assay. However, due to the dense matrix formed after 21 days in culture, we were unable to dissolve the pellet. Addition of collagenase and slight modifications to the protocol might solve this problem in future experiments.

These data reject our theory that L-PRF could enhance the chondrogenic differentiation of MSCs. The difference between the previously reported differentiation promoting effect of PRP and our own data on L-PRF could be caused by the difference in growth factor concentration released by these

concentrates. L-PRF has been shown to secrete higher levels of TGF-β1, epidermal growth factor (EGF), IGF and bFGF, whereas PRP was shown to release more VEGF and PDGF-BB (147-149). Especially the higher concentration of EGF released by L-PRF could be detrimental for the chondrogenic differentiation, as EGF has been shown to inhibit cartilage formation and the expression of collagen type 2 and Sox9, an early marker of chondrogenesis (150). Furthermore, the difference in differentiation-stimulating effects of platelet concentrates between this and previous studies might be explained by the difference in used MSC types, as cell source has been shown to be important for the differentiation capacities of MSCs (51). Additionally, interpatient variability in L-PRF growth factor content has been reported and might also influence the study results (151). It should be noted that the experiments need to be repeated in order to confirm these preliminary results.

Taken together, these data do not support the use of human DPSCs as a cell replacement therapy in articular cartilage lesions, as was initially the idea behind stem cell-based therapies (46). However, recent studies investigating the fate of transplanted MSCs in both mouse models and clinical trials demonstrated that 12 months after injection, none of the transplanted cells could be detected, even though clinical improvements were observed (61, 152). Their findings suggest that the increased clinical outcomes seen upon MSC treatment are caused by their paracrine effects, rather than direct cellular replacement (152).

The paracrine effects of MSCs, predominantly BM-MSCs, and PRP have been shown to increase chondrocyte proliferation, survival and matrix production in numerous studies (43, 52-54, 98-100). However, as mentioned previously, both BM-MSCs and PRP are associated with several drawbacks, as the isolation of BM-MSCs is invasive and PRP preparation requires the addition of anti-coagulants, is often labor intensive and is associated with a high cost (69, 91, 94, 95). Therefore, our next goal was to examine the paracrine effects of human DPSCs and L-PRF, as their isolation is easy, less invasive and cost-effective (47, 95). iMACs were cultured in serum-deprived medium, supplemented with L-PRF CM, L-PRF exudate or DPSC CM. The survival and proliferation of iMACs was examined by means of a PI assay. We demonstrated L-PRF CM to significantly enhance iMAC survival in a concentration dependent manner *in vitro*. DPSC CM also seemed to enhance iMAC survival, although not reaching statistical significance. Furthermore, we demonstrate that both DPSC CM and L-PRF CM have a proliferative effect on iMACs *in vitro*. These proliferation and survival stimulating effects were not observed in cells cultured in the presence of L-PRF exudate. This difference in effect between the two L-PRF derivatives might be explained in the difference in growth factor content, as L-PRF CM has been shown to contain more VEGF and EGF (153). Both these growth factors have been reported to enhance chondrocyte survival and proliferation (154-157). The presence of VEGF in DPSCs has been reported previously, whereas preliminary data from our group demonstrated the absence of EGF in DPSCs (73). This might explain why DPSC CM demonstrates a tendency towards stimulating proliferation and survival, without reaching statistical significance. Future experiments might use VEGF- and EGF-receptor specific antagonists in order to confirm their role in chondrocyte survival and proliferation. However, it should be noted that L-PRF contains, in addition to VEGF and EGF, a range of other growth factors which are involved in chondrocyte functioning, including TGF-β, bFGF, IGF and PDGF, and comparison of their concentration in L-PRF CM and exudate has not yet been reported (28, 74, 129-131). Future experiments will need to evaluate their expression profiles in L-

PRF CM and exudate, as differences in their concentration might also influence chondrocyte survival and proliferation (158-160).

These results support the hypothesis that DPSCs and L-PRF can improve the repair of cartilage lesions via their paracrine effects. On one hand, they can stimulate endogenous cells to proliferate and replace the lost tissue, on the other hand, they could prevent the progression of the lesion by stimulating chondrocyte survival (12, 74). Furthermore, the proliferation stimulating effects of DPSCs and L-PRF could be used to stimulate *in vitro* chondrocyte expansion in ACI. The paracrine effects of DPSCs and L-PRF on chondrocyte matrix production have yet to be studied. This could be examined by addition of L-PRF CM, exudate or DPSC CM to micromass cultures of iMACs. The advantage of these micromass cultures compared to monolayer cultures is that the 3D environment closely resembles the *in vivo* environment, thereby increasing the translational value of these experiments towards future application in animal models, and ultimately clinical application (161). Furthermore, the paracrine effects of DPSCs could be evaluated by means of a co-culture, which allows the direct crosstalk between both cell types, more closely resembling the *in vivo* situation of intra-articular injection of DPSCs. Both pellet co-cultures and transwell co-culture systems have been previously described (52). Both these systems demonstrated the ability of MSCs to induce an upregulation of GAG and collagen type 2 production in chondrocytes (52). Future experiments will evaluate whether the same effect can be induced by human DPSCs. Furthermore, stimulation of chondrocytes with IL-1β has previously been used as an *in vitro* model for OA, mimicking the *in vivo* inflammatory environment in OA and inducing the production of catabolic proteins and apoptosis (162, 163). Future experiments will focus on the effect of human DPSCs and L-PRF on the survival, proliferation and matrix production of these *in vitro* inflamed chondrocytes.

One of the struggles of ACI as a treatment for articular cartilage defects, is the dedifferentiation of chondrocytes when cultured in monolayer (6, 21). Our own data showed a drop in culture purity of approximately 45% after the first passage, with an increase in multi-nucleated giant cells and a decrease in collagen type 2 production (data not shown). As both DPSCs and L-PRF contain a range of growth factors involved in cartilage repair and homeostasis, it might be interesting for future experiments to evaluate their paracrine effects on chondrocyte dedifferentiation (28, 73, 74, 129-131). Preventing or slowing down the chondrocyte dedifferentiation could significantly ameliorate ACI.

The ultimate goal of this research is to test the regenerative potential of DPSCs in an *in vivo* model of cartilage lesions and OA. Therefore, we evaluated the migratory capacity of human DPSCs towards iMACs by means of a transwell migration assay. We demonstrated that human DPSCs were able to migrate towards iMACs. This migratory capacity of human DPSCs towards chondrocytes is important as it allows simple intra-articular injection of stem cells in future applications instead of developing scaffolds to keep the DPSCs in the proximity of the cartilage. DPSCs have been shown to migrate towards TGF-β, which is produced by chondrocytes (164, 165). Future migration assays will need to confirm the importance of TGF-β in DPSC migration by using selective TGF-β receptor antagonists, such as for example SB-431542 (77). Furthermore, we will evaluate the migration of DPSCs towards *in vitro* inflamed chondrocytes as a model for OA. Additionally, the effect of L-PRF on the migratory capacity of DPSCs will be evaluated.

Tendons attach skeletal muscles to bone and translate their contraction into joint movement (3). Additionally, they act as a mechanical buffer in order to prevent injuries (29, 30). Tendon ruptures affect millions of people worldwide, causing pain and disability and resulting in a high economic burden (4, 7, 8, 27, 32). Their limited intrinsic healing capacity is caused by the limited number of tenocytes and blood vessels and results in a mechanically inferior scar tissue (1, 4). Unfortunately, current therapies fail in providing a complete structural repair, signifying the need for the development of new therapeutic strategies (3, 28, 33). Therefore, we investigated the potential of human DPSCs and PDL-SCs to form tendon constructs *in vitro*.

The tenogenic differentiation of MSCs using 3D differentiation systems was reported previously (77, 166). Their superiority over 2D monolayer differentiations is mainly due to the generation of tension in the 3D constructs, which has been shown to be essential for both normal tenocyte functioning and tenogenic differentiation of MSCs (77, 79, 80). Kapacee *et al.* demonstrated the formation of fibripositors in BM-MSCs cultured in fixed-length fibrin gels (77). Fibripositors are collagen fibril-containing plasma membrane protrusions, typical for embryonic tendon cells, and are speculated to be responsible for the parallel organization of collagen fibres in tendons (80). Kapacee and colleagues found that tension was required for the formation of these fibripositors (80). Furthermore, they demonstrated a significant upregulation in TGF-β3 expression by BM-MSCs at the time of maximum contraction of the gel, which was essential for the tendon-construct formation (77). Blocking the TGF-β receptor resulted in the inability to form tendon-constructs, eventually leaving cells in monolayer (77). Equine embryonic stem cells also showed an upregulation in tendon specific gene expression, including tenascin-C, collagen type 1 and tenomodulin, upon culturing in fixed-length collagen gels (166). Additionally, a synergistic effect of 3D culturing and TGFβ3 supplementation on the expression of these markers was demonstrated (166).

In this study, DPSCs and PDL-SCs were cultured in fixed-length fibrin gels stimulated with TGF-β3 for 7 and 14 days. We demonstrated that already after 7 days in culture, these constructs formed a dense, tendon-like matrix with cellular alignment. Collagen fibres, orientated in the direction of the applied tension, were produced. However, after 14 days in culture, orientation of the collagen fibers was less obvious and a more even staining pattern was observed. In future experiments, TEM imaging or polarized light microscopy might be used in order to confirm the collagen orientation after longer differentiation periods. Further analysis of the constructs, either by IHC staining or a combination of Sirius red with polarized light microscopy, is needed to confirm that the deposited collagen is indeed collagen type 1, the main collagen type in tendons (4). Additionally, the presence of other tendon ECM components and differentiation markers, including decorin, tenascin-C, scleraxis, thrombospondin-4 and tenomodulin, should be demonstrated in order to confirm the differentiation of DPSCs and PDL-SCs into tenocytes (4, 167, 168). Furthermore, as the elastic properties of the interfasciculary matrix has been shown to be important for the energy storing function of tendons, future experiments will evaluate the presence of elastic fibres in the tenogenic constructs (29). In addition to the tenogenic differentiation, TGF-β has been described to play a role in both bone and cartilage formation (169, 170). Therefore, the absence of cartilage and bone components should also be demonstrated.

Future experiments will focus on the effect of L-PRF on the tenogenic differentiation capacity of DPSCs and PDL-SCs. Furthermore, experiments will also examine the biomechanical properties of the different constructs, such as elasticity, stiffness and maximum loading capacity (171, 172). As 14 days is a relatively short differentiation time, the culture time will be extended towards 28 days and the tenocyte specific marker expression, ECM production and biomechanical properties at different time points will be evaluated. Eventually, these constructs will be tested in an ovine model of tendon lesions.

10. Conclusion

Articular cartilage defects and tendon lesions affect millions of people worldwide, resulting in a high economic burden. The limited intrinsic healing reactions of these tissues, together with the inability of current treatments to provide lasting tissue repair, signify the need for the development of new and improved therapeutic strategies. MSCs have been shown to increase clinical outcome and tissue regeneration. In this study, we investigated human DPSCs as a candidate therapy for cartilage and tendon lesions as they possess several advantages over other MSC types, including a less invasive isolation and the secretion of a range of growth factors involved in tissue repair. Furthermore, we investigated the paracrine effects of L-PRF, an endogenous blood-derived biomaterial which has recently emerged as a promising treatment strategy due to its growth factor content, on the chondrogenic differentiation of human DPSCs and BM-MSCs and on the survival and proliferation of iMACs.

The results of this study do not support the theory that DPSCs could enhance articular cartilage regeneration via cellular replacement. Furthermore, the chondrogenic differentiation of DPSCs seemed to be hindered by the addition of L-PRF to the differentiation, although the experiments need to be repeated in order to reach statistical significance. However, the paracrine effects of both human DPSCs and L-PRF show great promise for their future application in cartilage repair, as both L-PRF CM and DPSC CM were shown to enhance the survival and proliferation of iMACs. Future experiments will focus on the paracrine effects of DPSCs and L-PRF on the cartilage specific protein expression of iMACs. Additionally, their effect on the proliferation, survival and matrix production of OA chondrocytes will be evaluated. Eventually, the therapeutic potential of both DPSCs and L-PRF will be examined in an ovine model of cartilage lesions and OA.

Furthermore, DPSCs and PDL-SCs also demonstrated promising results for application in the repair of tendon lesions. Within 7 days, they formed tendon-like constructs characterized by the alignment of cells and the production of collagen, orientated in the direction of the applied force. However, additional characterization of the constructs is needed in order to confirm tenocyte phenotype. Furthermore, experiments should be repeated in order to reach statistical relevance.

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