$CD271^+$ MESENCHYMAL STROMAL CELLS FOR AN INTRAOPERATIVE THERAPY OF CHONDRAL DEFECTS

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Table of Contents

1 Introduction 1
1.1 Articular cartilage 1
1.2 Cartilage lesions 2
1.3 Self-healing capability of articular cartilage 3
1.4 Treatment option for cartilage lesions 4
1.5 Mesenchymal stromal cells in cartilage regeneration 6
2 Rationale 8
3 Publication manuscripts 9
Point-of-care treatment of focal cartilage defects with selected chondrogenic mesenchymal stromal cells - An *in vitro* proof-of-concept study 9
Single-stage preparation of human cartilage grafts generated from bone marrow-derived CD271+ mononuclear cells 21
4 Summary 33
5 References 35
6 Appendix 42
7 Declaration of Authorship 47
8 Curriculum vitae 48
9 Publications 49
10 Acknowledgements 49

List of abbreviations

CD	cluster of differentiation
CFU-F	fibroblast colony-forming units
ISCT	International Society for Cellular Therapy
MACS	magnetic cell separation
MNC	mononuclear cell
MSC	mesenchymal stromal cell
OA	osteoarthritis
sGAG	sulphated glycosaminoglycan
unsep	unseparated

1 Introduction

1.1 Articular cartilage

Articular cartilage is an avascular, alymphatic and aneural tissue that covers the ends of synovial joints and thereby represents a unique tissue of the human body. Due to its characteristics and matrix composition, this tissue is called hyaline cartilage, which is also present in the respiratory tract and the cartilaginous parts of the ribs [1]. Further types of cartilage are fibrocartilage and elastic cartilage. Fibrocartilage is characterized by a majority of unmasked collagen type I fibres with single cells or in a linear arrangement between these fibres. It is present in tissues such as the menisci or in the outer fibrous ring of intervertebral discs. Elastic cartilage can be found in parts of the auricle and the larynx and is similar to hyaline cartilage, but contains additional elastic fibres [2].

The main function of articular cartilage is to compensate for mechanical impacts and minimize the friction of opposing cartilage surfaces during movements. This functionality is provided by a complex composition of extracellular matrix (ECM) molecules consisting of collagens, proteoglycans and non-collagenous proteins. The ECM content in healthy cartilage is >95% of its dry weight [3].

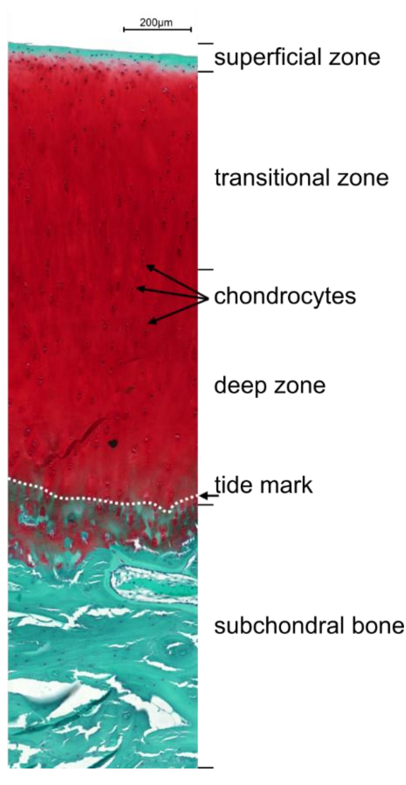

Figure 1: Cryosection of healthy adult ovine hyaline cartilage stained by Safranin O and Fast green. Cell nuclei appear black. The white dotted line indicates the tidemark; scale bar 200 µm.

Articular cartilage shows a unique architecture with different zonal structures distinguishable from each other by ECM composition, ECM orientation, cell shape and cell arrangement (Figure 1). The superficial zone contains flattened cells and horizontally orientated collagen fibres. In combination with lubricin from the synovial fluid, the friction of the cartilage surface is minimized [4].

The subjacent transitional zone is characterized by increased proteoglycan content and a round-shaped cell morphology. The deep zone shows the lowest cell density with the highest concentration of proteoglycans and vertically-arranged collagen fibres. The demarcation between the deep zone and calcified cartilage is called the tidemark. It provides a tethering structure for the collagen fibres above, thereby preventing cartilage detachment from the subchondral bone. The calcified cartilage defines the transition from cartilage to the subchondral bone below, in which bone marrow is situated [3].

There is only one cell type in articular cartilage, namely the chondrocytes. These cells are highly specialized to sustain the surrounding ECM. In humans, healthy articular cartilage contains about $9.6x10^3$ chondrocytes/mm^3 [5]. Chondrocytes are rarely capable of forming direct cell-cell contacts as they are completely surrounded by the ECM. The functional complex of a chondrocyte and the direct surrounding matrix is called the “chondron” and is responsible for the final ECM synthesis [6].

Since 1743, when William Hunter described defects in articular cartilage as “a very troublesome disease;… and that, when destroyed, it is never recovered” [7], the dogma of the absence of an insufficient, intrinsic repair mechanism in articular cartilage persists.

1.2 Cartilage lesions

Articular cartilage lesions remain one of the major problems in orthopaedic medicine. The most common cartilage disorders are osteoarthritis (OA) and rheumatoid arthritis [8]. The following chapter will focus on degenerative and traumatic cartilage lesions, prospectively treatable by regenerative tissue engineering approaches.

OA is the most common joint disease in the industrialised world with about “10% men and 13% … women aged 60 years or older” affected people in the United States [9] and 13.9% men and 21.8% women affected people in Germany in 2017 [10]. The prevalence of OA is increasing due to an aging population and obesity. It develops gradually over several years, whereby the symptoms, characterized by cartilage damage, changes of the subchondral bone and inflammation of the synovial tissue can progress in spurts [11]. Joint malalignment, mechanical stress and catabolic tissue enzymes induce the release of breakdown products of the ECM from cartilage, which further causes inflammation of the synovial membrane. This process initiates the production of proinflammatory cytokines, collagenases and other hydrolytic enzymes from synovial cells and local macrophages [12]. A vicious positive feedback loop involving cartilage breakdown and synovial inflammation occurs [13]. Since OA is characterized by a slow disease progression, the initial starting point of the disease is difficult to define, as symptoms arise late. A traumatic injury of the joint might be one of the main initiators of cartilage breakdown. Hence a therapeutic intervention for primary traumatic cartilage defects is seen as a promising approach to prevent progression to secondary OA (Figure 2) [14].

The risk of developing OA is >40% after ligament and meniscus tears as well as after cartilage surface injuries [15]. The more severe the initial damage, the earlier the progression of OA will start. The pathology of development of secondary OA after a traumatic cartilage defect is characterized by an initial inflammatory response and the onset of ECM restoration, which formally leads to a fibro-cartilaginous tissue with poor mechanical properties.

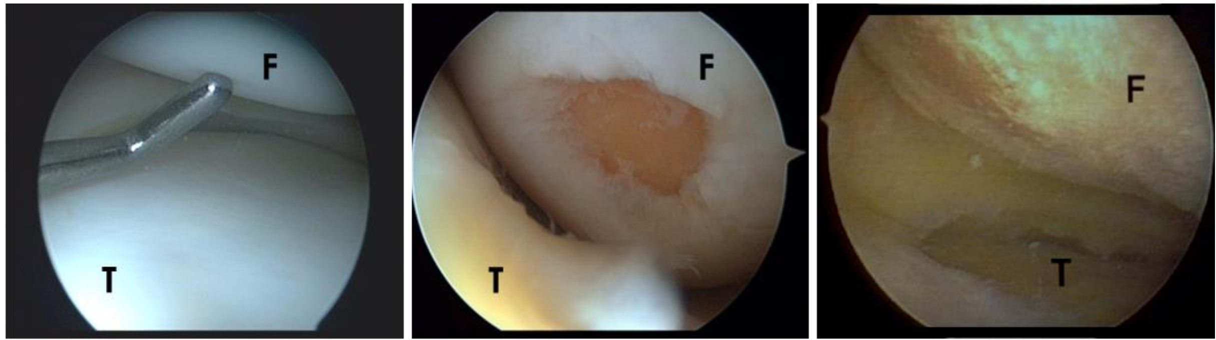

Figure 2: Arthroscopic photographs of the human knee (left) of healthy cartilage, (middle) a focal cartilage lesion and (right) secondary osteoarthritis. The femur and tibia are indicated by capital letters “F” and “T”.

The inflammation process is not only restricted to cartilage tissue, but also spreads to the bone, synovial membrane, ligaments and meniscus. Following this, proinflammatory mediators are detectable in the synovial fluid. The pathologic progression leads to fissures in the cartilage, which are accompanied by cartilage destruction until it reaches the subchondral bone. The disease procession is than comparable with the pathologic stages of OA [16].
Depending on the defect depth and the involvement of the subchondral bone, an intrinsic cartilage regeneration process arises, providing a promising target cell population for regenerative medicine.

1.3 Self-healing capability of articular cartilage

The self-healing capability of cartilage defects can be driven by mesenchymal progenitor cells from within the articular cartilage [17] or mesenchymal stromal cells (MSC) which are infiltrating the defect site from the subchondral bone [18].
From human embryonic limb buds, it is known that chondrogenesis harbours two different subpopulations of multipotent cartilage stem cells and oligopotent cartilage progenitor cells [19]. In 2004, two research groups published in parallel findings on adult “mesenchymal progenitor cells” in osteoarthritic cartilage. These progenitor cells can be characterized by the expression of CD166 (cluster of differentiation 166) and the co-expression of CD105 [20] or either CD90 [21] and their *in vitro* adipogenic, osteogenic and chondrogenic (trilineage) differentiation potential. Chondrocytes derived from OA-affected tissue also showed increased chondrogenic potential, predominantly by higher expression of sulphated glycosaminoglycans [22]. In 2011 mesenchymal progenitor cells with a comparable phenotype were also identified in biopsies of macroscopically healthy cartilage [23].
Another self-healing mechanism of cartilage defects arises from MSCs from the subchondral bone marrow, as cartilaginous deposition has been detected within the subchondral bone in patients with severe OA. It is hypothesised that these aggregates arise from MSCs and might

support resurfacing of destroyed articular cartilage [24,25] by migrating through the tidemark [18,26]. The synovial fluid is known to support such a migration [27]. However, the progression of degenerative cartilage diseases shows, that these intrinsic repair mechanisms alone cannot lead to a full recovery. Hence, surgical treatment options or novel regenerative procedures are required to sustain or improve the patients' welfare.

1.4 Treatment option for cartilage lesions

The treatment of a cartilage defect in order to restore joint function and improve patient wellbeing is the ideal goal. A variety of possible treatment options are currently available depending on the defect size, the patient´s activity level and the patient´s age [28]. An overview is given in Figure 3.

The most frequent conventional treatment option for small focal cartilage defects is the microfracture or Pridie drilling, as it is a single-stage procedure with a minimal morbidity of healthy surrounding cartilage [29]. In the intervention, the defect site is cleared of loose cartilage fragments and the subchondral bone is drilled multiple times to provoke a bleeding of bone marrow and a formation of a bone marrow clot. This clot contains MSCs which induce spontaneous formation of scar-like tissue. Microfracture is recommended as first-line treatment for isolated defects of <2.5 cm². particularly in younger patients [29–31]. Despite a short-term improvement in functionality, the fibrous cartilage lacks of mechanical durability and long-term stability [32].

An improvement in long-term stability can be achieved with the Osteoarticular Transfer System (OATS). With this technique, osteochondral plugs from a non- or lesser-weight-bearing region

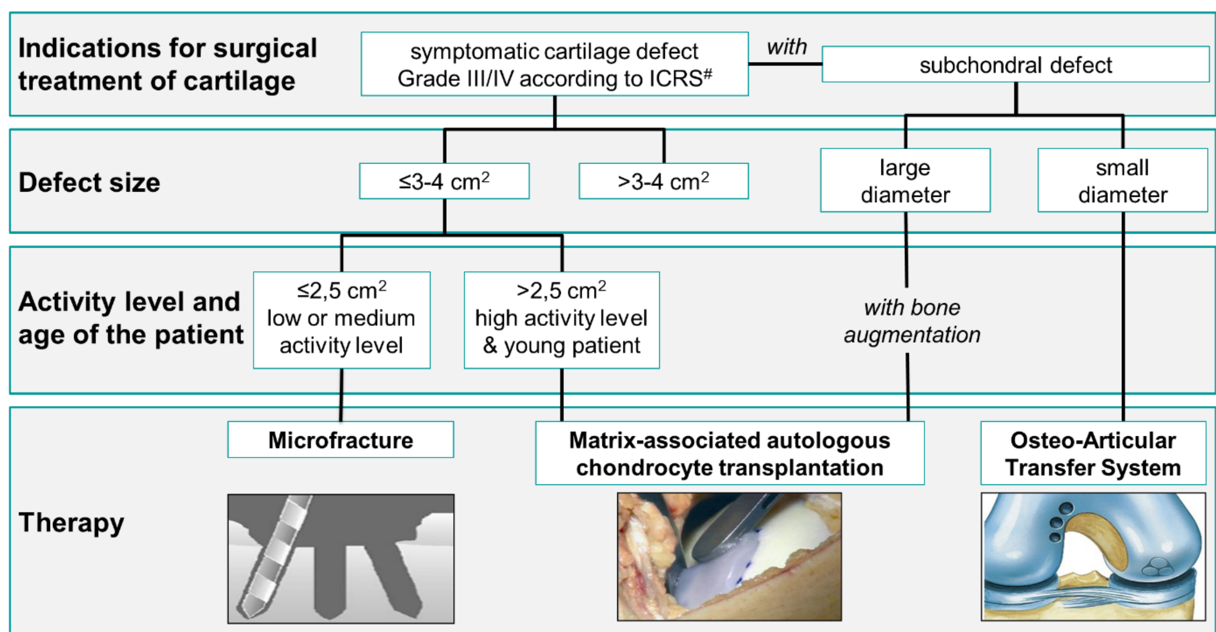

Figure 3: Treatment schedule for cartilage defects according to defects size, patients level of activity and age adopted from [28]. # International Cartilage Repair Society classification [33,34].

of the joint are transferred to the former defect site. The advantage is an immediate restoration of the mechanical function of the tissue [35]. The donor-site morbidity limits the size of treatable defects and the integration of implanted cartilage pieces is poor. To overcome the donor-site morbidity, allograft osteochondral plugs are frequently used. With this, the application of fresh donor material is highly recommended as stored plugs show poor chondrocyte viability and worse biomechanical properties [35,36]. A further limitation of allografts is the risk of disease transmission and immunological reactions [37].

Cell-free approaches are a faster, cheaper, off-the-shelf and easy-to-use alternative. The basic principle addresses the support of the defect side by stabilizing the defect borders, providing a mechanically stable structure to cope with load-bearing and shear forces, limiting the ongoing degenerative process and supporting the intrinsic regeneration. A broad range of medical products are commercially available. Further product developments are focusing on "smart materials" and "stimulant-combined" products. Smart materials can include zonal layered structures to mimic the natural structure of the ECM of hyaline cartilage (e.g. 3D-printed scaffolds), while stimulant-combined scaffolds are a combination of a matrix structure with incorporated growth factors or pharmaceuticals [38]. The included substances can either stimulate the proliferation and differentiation of adjacent cells or treat local inflammations and degenerative processes. However, the clinical benefit of cell-free approaches must be examined in high-quality studies [39].

In 1994 Brittberg *et al*. described a regenerative approach in cartilage defect treatment, the autologous chondrocyte transplantation (ACT). In this technique, chondrocytes from a biopsy of an unaffected area of the joint were isolated and expanded *in vitro* for 14-21 days. The expanded chondrocytes were injected in the defect area and covered with a periosteal flap from the tibia [40]. This first-generation ACT was associated with limitations such as leakage of cell suspension after periosteal flap detachment, periosteal hypertrophy and chondrocyte dedifferentiation after extensive monolayer expansion [36]. To overcome some of the disadvantages of this technique, the cell suspension was combined with scaffolds to provide a homogenous distribution and retention of cells within the transplant. This technique is known as second-generation ACT, or matrix-assisted ACT (MACT) [41]. The clinical application of MACT techniques show superior results when compared to other interventions such as microfracture [42]. However, MACT is limited by donor site morbidity, the available cell pool of chondrocytes, their dedifferentiation during extensive *in vitro* expansion and their reduced ability to re-differentiate [43,44]. Major complications after implantation such as cartilage hypertrophy, insufficient bonding to the surrounding healthy cartilage, and formation of insufficient regenerative cartilage tissue or delamination from the subchondral bone are still seen in clinical practice [45], despite optimization of application techniques [46] and the selection of potent chondrocytes [14]. Major improvements can be expected from the

substitution of the chondrocytes as stated by the editors in chief of the journal *Arthroscopy*: "The ultimate goal is a single-step, tissue-engineered solution to focal cartilage defects, and elimination of the morbidity of the donor defect." [47].

Therefore, MSCs seem to be a promising candidate to fulfil the requirements of an optimal cartilage defect treatment procedure [48].

1.5 Mesenchymal stromal cells in cartilage regeneration

Friedenstein *et al.* were the first to describe fibroblast colony-forming units (CFU-Fs) from bone marrow [49]. Caplan *et al.* named these cells MSCs in the early 1990s [50]. These cells have a native non-haematopoietic, nonendothelial character with topographically diverse niches in bone marrow [51] and several other tissues including synovial membrane, muscle, fat, dental pulp and others [37]. They are located in perivascular or bone-lining niches [52] and their native phenotype can be described based on the expression of CD271 [53] and CD140b [54], while they are negative for CD34, CD14, CD45, CD11b, CD49d, CD106, CD10 and CD31. The frequency of native MSCs in bone marrow aspirate ranges from 0.0017% to 0.0201% of the mononuclear cells [55], but only about 10% of these show a true colony formation under *in vitro* monolayer conditions [56], which makes them extraordinarily rare cells in bone marrow. However, their high expansion potential, anti-inflammatory and immunomodulatory paracrine effects [57] as well as multipotent differentiation potential, including the potential to differentiate into chondrocytes [58], make them an ideal candidate for regenerative medicine [59].

The characterization of isolated, *in vitro* expanded MSCs was defined by the current guideline of the International Society for Cellular Therapy (ISCT) based on three compliance criteria: "First, MSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Second, >95% of the MSC population must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (≤2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Third, the cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions." [60].

Numerous preclinical studies have been performed using MSCs with promising results on cartilage regeneration [61–63]. Minor side effects of the first attempts were reported as calcification of implanted tissue, fibrogenesis, and heterotopic tissue formation in the cartilage [45]. These drawbacks could be reduced by a preconditioning of expanded MSCs to chondrogenic differentiation such as with supplementation with transforming growth factor β3 [63–65].

Several case studies, randomized and comparative clinical studies showed the general ability of autologous, bone marrow-derived, expanded MSC transplantation to repair articular

cartilage defects [48]. Early applications of MSCs in human cartilage repair have reported follow-up of up to 11 years without infection or tumour formation, proving the safety of the therapeutic application of MSCs [66]. However, the potential for malignant changes during clinical use of MSC is still an important aspect [67,68]. Therefore, stringent safety, purity and potency measures are required to ensure patient safety [69,70]. In particular, *in vitro* cell expansion strategies present a risk of contamination, transformation or chromosomal aberrations [71]. Appropriate risk minimization could be achieved by elimination or reduction of cultivation time, limitation of the number of population doublings, monitoring of cytogenic aberrations and testing sterility, phenotype and viability [59,72].

Multiple approaches highlight the application of allogenic, *in vitro* expanded MSCs as an "off-the-shelf" product. A major drawback for allogenic MSC approaches is an increased immunogenic potential of MSCs after chondrogenic differentiation. This might be due to increased expression of MHC-I and MHC-II receptors [73]. Furthermore, chondrogenically differentiated MSCs lose their ability to suppress dendritic cell function [74] as well as to suppress activated $CD4^+$ and $CD8^+$ T cells [73], which makes them detectable by the host immune system [57]. Therefore, autologous cell sources should be preferred to allogenic approaches.

The major drawbacks of an *in vitro* expansion based two-stage procedure could be bypassed by single-stage preparation of cartilage graft with highly potent, non-expanded MSCs. One of the most promising marker candidates for prospective separation of these potent, non-expanded MSCs is CD271 [56,75–83]. CD271, also known as low-affinity nerve growth factor receptor, was first described by Chesa and Thomson *et al.* in 1988. It is involved in survival and developmental signalling in neuronal cells. Histological analysis revealed additional expressions in epithelial, mesenchymal and lymphoid tissues [84]. The function of CD271 on MSCs is currently unknown, although it affects the morphogenesis, growth factor stimulation and the prevention of cells from apoptosis [81]. However, $CD271^+$ MSCs contain the majority of CFU-Fs [81] with an increased chondrogenic potential compared to non-separated MSCs [79,85,86]. The marker is downregulated during monolayer expansion; therefore, it is only a potent marker for native MSC isolation [87]. $CD271^+$ cells were already found to be involved in spontaneous cartilage repair in joint explant cultures [88]. Jones *et al.* proved the ability of a clinical feasible separation strategy based on magnetic cell separation (MACS) for non-union bone fractures [89]. This procedure could be transferable to cartilage defects. However, to the author´s best knowledge, no single-stage procedure for $CD271^+$ separated cells from bone marrow for cartilage defect therapy has been reported previously in the literature.

2 Rationale

Regenerative treatment of hyaline cartilage focal defects could prevent the development of secondary OA. The common use of bone marrow stimulating techniques results partly in formation of mechanically inferior fibrous cartilage, which increases the need for improved interventions with a long-term perspective. Chondrocyte-based procedures like MACT present the disadvantages of donor site morbidity, dedifferentiation of chondrocytes due to *ex vivo* expansion and reduced re-differentiation potential.

The self-healing capacities of injured and degenerated cartilage revealed a promising target cell population for a regenerative, autologous single-stage procedure for the treatment of these defects using non-expanded MSCs from the bone marrow. Currently available clinical approved cell separation devices enable for intraoperative purification of CD271$^+$ cells, which contain the majority of colony-forming MSCs, by MACS technology. By providing a hydrogel with non-expanded CD271$^+$ cells, the advantages of a MACT approach could be combined with the need for a “single-step, tissue-engineered solution to focal cartilage defects, and elimination of the morbidity of the donor defect” [47].

The preliminary experiment regarding CD271$^+$ cells from bone marrow of adult sheep was published in the *Journal of Tissue Engineering and Regenerative Medicine* in May 2018 [90]. Therein, four single marker candidates were tested for their effectiveness of separating ovine MSCs via MACS and the feasibility to generate cartilage grafts from non-expanded CD271$^+$, CD271$^-$ and unseparated ovine MNCs.

The present *in vitro* study investigated the feasibility of generating cartilage grafts from human CD271$^+$ bone marrow cells in a CE-marked collagen type I hydrogel without initial monolayer expansion. Cell viability, DNA content, chondrogenic differentiation capacity, extracellular matrix secretion, and graft properties were monitored for up to 5 weeks to investigate the single-stage therapeutic approach for human focal cartilage defects.

3 Publication manuscripts

Point-of-care treatment of focal cartilage defects with selected chondrogenic mesenchymal stromal cells - An *in vitro* proof-of-concept study

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RESEARCH ARTICLE

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Point-of-care treatment of focal cartilage defects with selected chondrogenic mesenchymal stromal cells—An in *vitro* proof-of-concept study

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Abstract

Due to the poor self-healing capacities of cartilage, innovative approaches are a major clinical need. The use of in vitro expanded mesenchymal stromal cells (MSCs) in a 2-stage approach is accompanied by cost-, time-, and personnel-intensive good manufacturing practice production. A 1-stage intraoperative procedure could overcome these drawbacks. The aim was to prove the feasibility of a point-of-care concept for the treatment of cartilage lesions using defined MSC subpopulations in a collagen hydrogel without prior MSC monolayer expansion. We tested 4 single marker candidates (MSCA-1, W4A5, CD146, CD271) for their effectiveness of separating colony-forming units of ovine MSCs via magnetic cell separation. The most promising surface marker with regard to the highest enrichment of colony-forming cells was subsequently used to isolate a MSC subpopulation for the direct generation of a cartilage graft composed of a collagen type I hydrogel without the propagation of MSCs in monolayer. We observed that separation with CD271 sustained the highest enrichment of colony-forming units. We then demonstrated the feasibility of generating a cartilage graft with an unsorted bone marrow mononuclear cell fraction and with a characterized CD271 positive MSC subpopulation without the need for a prior cell expansion. A reduced volume of 6.25% of the CD271 positive MSCs was needed to achieve the same results regarding chondrogenesis compared with the unseparated bone marrow mononuclear cell fraction, drastically reducing the number of nonrelevant cells. This study provides a proof-of-concept and reflects the potential of an intraoperative procedure for direct seeding of cartilage grafts with selected CD271 positive cells from bone marrow.

KEYWORDS

ATMP, bone marrow-derived mesenchymal stem cells, cartilage, cell separation, hydrogel, mesenchymal stromal cells, point-of-care

Abbreviations: 3D, three dimensional; AC, articular chondrocytes; ACT, autologous chondrocyte transplantation; Agc, aggrecan; ATMP, advanced therapy medicinal product; BM, bone marrow; BMC, bone marrow concentrate; CD146, melanoma cell adhesion molecule; CD271, low-affinity nerve growth factor receptor; CFU-F, colony-forming unit-fibroblasts; Col, collagen type; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; DMMB, dimethylmethylene blue assay; FCS, fetal calf serum; GMP, good manufacturing practice; MACS, magnetic cell sorting; MACT, matrix-associated autologous chondrocyte transplantation; MNC, mononuclear cell; MSC, mesenchymal stromal cell; MSCA-1, mesenchymal stem cell antigen-1; neg, negative; PE, phycoerythrin; PoC, point-of-care; pos, positive; sGAG, sulphated glycosaminoglycan; unsep, unseparated; W4A5, neural progenitor cell marker.

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1 | INTRODUCTION

Due to the limited intrinsic regeneration potential of articular cartilage, it is still an ambitious clinical challenge to regenerate traumatic cartilage lesions (Minas, 2012). Chondrocytes or multipotent mesenchymal stromal cells (MSCs) from different origins, especially from bone marrow (BM), play the mayor role in regeneration (Filardo et al., 2013).

Chondrocyte-based therapies, such as autologous chondrocyte transplantation (ACT) and matrix-associated ACT (MACT), have the disadvantages of donor-site morbidity, a limited number of primary chondrocytes and subsequent loss of phenotype with induced dedifferentiation during the ex vivo monolayer expansion (Huang, Hu, & Athanasiou, 2016; Hunziker, Lippuner, Keel, & Shintani, 2015; Park, Jin, Chi, & Taylor, 2004; Roelofs, Rocke, & de, 2013; Schulz et al., 2014). The use of MSCs instead of chondrocytes has been proposed to overcome these disadvantages. Several clinical case reports, case series, and clinical studies reported promising outcomes for autologous MSC-based repair of cartilage lesions (Chahla et al., 2016; Gobbi & Whyte, 2016; Goldberg, Mitchell, Soans, Kim, & Zaidi, 2017). However, a limitation in the use of MSCs is their low initial isolation frequency of about $1{:}1 \times 10^4$ to $1{:}1 \times 10^6$ MSCs in human BM mononuclear cells (MNCs; Sarugaser, Hanoun, Keating, Stanford, & Davies, 2009). Hence, the challenge is to acquire an adequate number of MSCs for a promising treatment approach.

There are two different strategies for obtaining sufficient numbers of MSCs. One of these is the two-stage approach, which isolates MSCs of various sources via plastic adherence and subsequent in vitro expansion under good manufacturing practice (GMP). The GMP conditions allow for consistent monitoring of the quality of the cell product, including cellular stability, identity, and potency. However, this strategy is time-consuming, cost-intensive, strictly supervised and inspected by regulatory authorities, and requires two surgical interventions for harvesting of the cells/tissues and later implantation of the graft (Grässel & Lorenz, 2014).

Second, a point-of-care (PoC) approach utilizes concentrated cells, such as BM concentrate (BMC), without an in vitro expansion. This technique may overcome the limitations of two-stage approaches by avoiding substantial in vitro cell manipulation, eliminating or minimizing the usual time, cost, and personnel efforts involved in cell expansion and differentiation procedures in GMP facilities, and by minimizing the risk of contamination (Giannini, Buda, Vannini, Cavallo, & Grigolo, 2009).

Although the PoC approach, for example, BMC, is currently not associated with major regulatory obstacles, it represents an undefined mixture of a variety of cells and active substances including MSCs, hematopoietic stem cells, platelets, red blood cells, growth factors, and cytokines (Grässel & Lorenz, 2014). Furthermore, the underlying mode of action is not yet fully understood. Thus, the popular use of BMC could be defined as "unproven cell therapy" according to the International Society for Cellular Therapy (Srivastava et al., 2016).

Novel PoC approaches for cartilage treatment have focused on obtaining a highly clonogenic, proliferative, and potent chondrogenic MSC subpopulation by either removing undesired cell populations or by positive selection of target cells based on surface markers (Coelho, Cabral, Joaquim, & Karp, 2012; Roelofs et al., 2013). However, due to the current lack of a unique surface marker for MSCs, a number of surface molecules have been suggested for prospective isolation of MSCs (Calloni, Cordero, Henriques, & Bonatto, 2013). Among these surface markers, mesenchymal stem cell antigen-1, neural progenitor cell marker (W4A5), melanoma cell adhesion molecule (CD146), and low-affinity nerve growth factor receptor (CD271) have been used efficiently to enrich the colony-forming unit fraction (CFU-F), which is considered to be the precursors or stem cells from which monolayer-expanded MSCs originate and are able to show their multilinage potential (Battula et al., 2009; Busser et al., 2015; Flores-Figueroa, Varma, Montgomery, Greenberg, & Gratzinger, 2012; Lv, Tuan, Cheung, & Leung, 2014; Rozemuller et al., 2010; Sivasubramaniyan et al., 2012; Tormin et al., 2011). CD271 is of particular interest because all CFU-F in human BM reside in the CD271 positive fraction (Quirici et al., 2002). It has been shown that a human CD271 pos BM subpopulation ($CD271^{pos}/CD140a^{low/neg}$ MSCs) represents a population of primary cells with an MSC phenotype fulfilling stringent functional stem cell criteria in vivo (Ghazanfari, Li, Zacharaki, Lim, & Scheding, 2016; Li, Ghazanfari, Zacharaki, Lim, & Scheding, 2016). In addition, Mifune et al. (2013) indicated in a rat model that CD271 pos MSCs have greater chondrogenic potential than plastic adherent MSCs in both in vitro and in vivo.

The aim of this study was to prove the feasibility of a PoC approach in vitro by forming a cartilage graft for the treatment of cartilage lesions based on defined MSC subpopulations without the need for an expansion phase.

2 | METHODS

To prove the feasibility of a PoC approach for the regeneration of injured cartilage tissue, the study was divided into two studies (see Figure 1). In the first study, the above-mentioned four cell surface marker candidates were tested for their effectiveness of separating CFU-Fs of ovine BM (Donor 1) that were accompanied by both high purity of the selected cell fraction and a high enrichment factor.

In the second study, the most promising cell fraction was directly used for the generation of a cartilage graft, consisting of a clinically approved collagen type I hydrogel with a defined, nonexpanded MSC subpopulation. Therefore, positive separated MNCs (pos MNCs) were compared with the negative separated MNCs (neg MNCs), unseparated MNCs (unsep MNCs) and primary articular chondrocytes (ACs) from two animal donors. The cellular quality, including cell viability, DNA content, chondrogenic differentiation capacity, extracellular matrix (ECM) secretion, and graft properties, was evaluated to prove the feasibility of the envisaged PoC approach for the treatment of focal cartilage lesions.

2.1 | Cell isolation

As the sheep is a well-known large animal model for cartilage regeneration (Marquass et al., 2011), BM and articular cartilage samples were taken from adult Merino sheep. The aspiration of BM and tissue harvesting was approved by the local ethics committee for experimental animal studies (State directorate of Saxony, N13/13). Briefly,

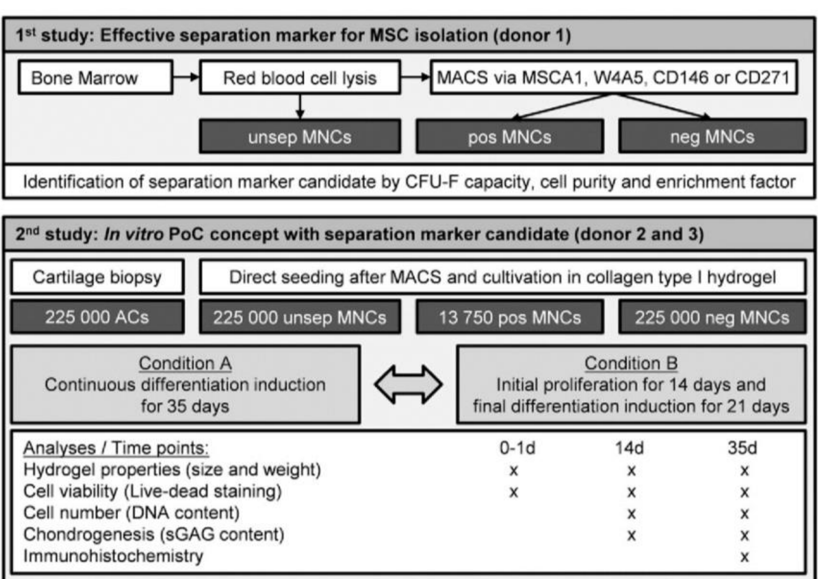


FIGURE 1 Overview of the study design for the marker identification and application of a separation marker for the generation of a cartilage graft. Abbreviations: ACs = articular chondrocytes; CD146 = melanoma cell adhesion molecule; CD271 = low-affinity nerve growth factor receptor; CFU-F = colony-forming unit-fibroblasts; MACS = magnetic activated cell sorting; MSC = mesenchymal stromal cell; MSCA-1 = mesenchymal stem cell antigen-1; MNCs = mononuclear cells; PoC = point-of-care; sGAG = sulphated glycosaminoglycan; W4A5 = neural progenitor cell marker

at least 30 ml of BM was collected into a sterile heparinized syringe (500 IE/ml BM; Ratiopharm, Ulm, Germany) from the iliac crest of the sheep with a 15-gauge needle (HVM Medical Products) under anaesthesia. MNCs including the MSC fraction were isolated from ovine BM aspirate by red blood cell lysis within 12 hr after aspiration. Whole BM was filtered with 70 μm mesh, diluted 1:3 with phosphate-buffered saline without Ca^{2+}/Mg^{2+} (PBS, Biochrom, Berlin, Germany) and centrifuged (630 *g* for 5 min at room temperature). Subsequently, the resuspended cells were incubated with ammonium chloride solution (Riedel-de-Haen, Seelze, Germany) for 5 min and centrifuged (235 *g* for 5 min at room temperature). The MNCs were washed once with PBS and resuspended with AutoMACS Running Buffer (Miltenyi Biotec). The remaining cell suspension was split for subsequent magnetic activated cell sorting (MACS), whereby unsep MNCs served as the reference population in both experimental studies.

For a positive reference group in the second study, healthy full-depth articular cartilage from the medial condyle of both stifle joints was freshly isolated post mortem from the animal donors. The cartilage biopsies were minced and digested for 12 hr in collagenase A solution (2 mg/ml in Dulbecco's Modified Eagle Medium [DMEM], Roche, Basel, Switzerland). The obtained cell suspension was washed twice with PBS, and the primary ACs were processed for direct seeding into the 3D collagen type I hydrogel for subsequent culture at two defined medium supplementation conditions for up to 35 days (see Section 2.3).

The number of viable MNCs among the unseparated cells and the four separated cell fractions as well as the ACs was determined by DAPI staining (Miltenyi Biotec) for dead cell exclusion with the MACSQuant Analyser 10 (Miltenyi Biotec).

2.2 | MACS separation

The unsep MNCs were labelled either indirectly with MSCA1-PE (20 μl/10^7 MNC) or W4A5-PE (10 μl/10^7 MNC) or CD146-PE (20 μl/10^7 MNC) followed by anti-PE-MicroBeads (Miltenyi Biotec) or directly with CD271-MicroBeads (20 μl/10^7 MNC) according to the manufacturer's protocol with minor modifications (for detailed list of antibodies, see Table 1). Briefly, unspecific binding sites were blocked with FcR-blocking reagent (Miltenyi Biotec) for 5 min and afterwards incubated with the respective conjugated PE antibody solution or with the CD271-Microbeads for 30 min at room temperature in the dark. The cell suspension was washed with AutoMACS Running buffer and labelled either with anti-PE-MicroBeads with subsequent washing and subsequent MACS or, for the CD271-MicroBeads, directly transferred onto the MACS column within a

TABLE 1 Antibodies used for separation and phenotypic characterization

Antibody	Clone	Isotype	Label	Source
MSCA-1	W8B2	IgG1	PE	Miltenyi Biotec
W4A5	W4A5	IgG1	PE	Biolegend
CD146	P1H12	IgG1	PE	BD Bioscience
CD271	ME20.4-1.H4	IgG1	APC	Miltenyi Biotec
CD271	ME20.4-1.H4	IgG1	MicroBead	Miltenyi Biotec
Anti-IgG1 mouse	X56	IgG1	FITC	Miltenyi Biotec
Anti-IgG1 mouse	X56	IgG1	PE	Miltenyi Biotec
Anti-IgG1 mouse	X56	IgG1	APC	Miltenyi Biotec

MACS Separator (Miltenyi Biotec). Unlabelled cells were washed out (neg MNCs). After the removal of the column, positive, enriched cells (pos MNCs) were eluted from the column. Afterwards, the viable cell yield was determined by DAPI staining for dead cell exclusion as referred in Section 2.1.

2.3 | 3D cultivation and chondrogenic differentiation

In the second study, the unsep MNCs, pos, and neg MNCs, separated by the most effective marker obtained from the first study, were directly seeded into a clinically approved 3D collagen type I hydrogel medical device (Arthro Kinetics Biotechnology, Krems, Austria) in parallel to the gold standard primary ACs without a common in vitro expansion phase. The cells were initially seeded into the gels according to the manufacturer's protocol. Briefly, defined numbers of cells as given in Figure 1 were mixed with gel neutralization buffer on ice. A collagen type I solution was added in bubble-free fashion into a well. For each gel, 550-μl gel suspension was transferred into a 24 multiwell plate with a diameter of 15.7 mm and a resulting height of approximately 2.8 mm and polymerized for 20 min at 37 °C.

Due to a higher clonogenic capacity of the pos MNCs (based on preliminary experiments, data not shown), they were only seeded with a lower cellularity of the 16th part (13,750 CD271 pos MNCs) of the unsep MNCs group per gel, whereas for the neg MNC, the unsep MNC, and the AC groups, 225,000 cells were seeded per graft. Due to the necessity of the initial digestion of cartilage, primary ACs were seeded into the gel with a delay of 12 hr when compared with the other groups. Subsequently, the 3D gels of all four groups were differentiated into chondrogenic lineage either overlaid with 1-ml serum-free chondrogenic media (Lonza, Switzerland) supplemented with 10 ng/ml recombinant human transforming growth factor β3 (Miltenyi Biotec; Condition A) or cultivated for the initial 14 days with DMEM (Thermo Scientific) supplemented with 10% fetal calf serum (Gibco, Germany) and 1% penicillin/streptomycin (Biochrom), followed by serum-free chondrogenic media (Condition B). The media was exchanged completely twice a week. Analyses of the gels were carried out after 14 and 35 days of cultivation.

2.4 | CFU-F evaluation

To determine the number of CFUs, a CFU-Fs assay (fibroblast CFUs) was performed for both studies. Therefore, 78,000–152,000 of unsep MNC/well, 6,000–9,000 of pos MNCs/well, or 152,000 of neg MNCs/well were plated in 12-well plates and incubated with DMEM media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 5% O_2 and 5% CO_2. The medium was fully changed once a week. After 9–10 days of culture, the cells were washed twice with PBS, fixed with methanol and colonies of more than approximately 50 cells were counted using light microscopy (Zeiss Axiovert 200, Zeiss Microscopy, Oberkochen, Germany). The number of CFU-Fs was calculated as the mean of up to 12 wells based on the respective cell seeding densities.

2.5 | Flow cytometry analysis

To analyse the purity of cell fractions and effectiveness of the cell separation by MACS, the pos and neg MNCs as well as unsep MNCs were characterized by flow cytometry. Therefore, doublets, cell debris, and dead cells, counterstained with DAPI, were excluded and only MNCs were used for the flow cytometry analyses with a minimum of 50,000 events. The negative/positive boundary was determined by using fluorescence minus one and isotype controls. The cells were stained according to the manufacturer's instructions (for detailed list of antibodies, see Table 1). Samples were then evaluated by MACSQuant Analyser 10 and FlowJo V10 software.

2.6 | 3D gel properties

The wet weight and the gel area of each respective gel per group and time point were evaluated from the 3D gel. The area was calculated based on scanned images of the grafts by using ImageJ V1.42q.

2.7 | Live-dead staining

The cell viability within the 3D gels in the second study was visualized by staining with ethidium homodimer and calcein AM (Live/Dead® Viability/Cytotoxicity Kit, Invitrogen, Germany, each 1:1,000 in PBS for 15 min at 37 °C). After three final washing steps with PBS, confocal images of the superficial layers of the grafts were taken with an inverted confocal laser scanning microscope TCS SP5 (Leica Microsystems, Wetzlar, Germany) and a HC PL FLUOTAR 10×/0.3 DRY objective and lasers at 488 and 561 nm wavelength.

2.8 | DNA-(Quant-iT™ PicoGreen®) and sGAG content (DMMB assay)

The hydrogels were digested with papain (50 μg/ml; Sigma-Aldrich) at 60 °C overnight. Subsequently, the samples were analysed by Quant-iT™ PicoGreen® assay for the determination of the DNA content and the content of freshly secreted sulphated glycosaminoglycans (sGAG) was measured by DMMB assay as described previously (Zscharnack, Poesel, Galle, & Bader, 2009). All measurements were performed in duplicates and according to the manufacturer's instructions.

2.9 | Immunohistochemical staining

Sections of the 3D gels were shock frozen and embedded in TissueTEC® (VWR). Cryosections were prepared with a thickness of 8 μm. The sections were stained immunohistochemically for aggrecan (Acris, Herford, Germany, clone HAG7D4) and collagen type II (MP Biomedicals, Eschwege, Germany, clone II-4C11) as chondrogenic differentiation markers and for collagen type X (Sigma-Aldrich, clone COL-10) as a hypertrophy marker. Nuclei were counterstained with haematoxylin (DakoCytomation, Hamburg, Germany). Digital scans of the stained sections were prepared by Pannoramic Slides scanner (Sysmex, Norderstedt, Germany). Images are displayed in 200× and 400× magnifications. Human adult hyaline cartilage was used for positive and isotype controls for each antigen staining (see Figure S1).

3 | RESULTS

BM aspirate was collected from the iliac crest of three adult Merino sheep and processed individually for each donor. The yield of unsep MNCs from BM after red blood cell lysis were 7.96 (first study), 3.96, and 2.38 million MNC/ml BM with a viability of more than 95%.

3.1 | First study—Effective separation marker for MSC isolation

The BM from Donor 1 was used to identify one potent single marker to isolate a clonogenic MSC subpopulation with a high cell purity and high enrichment factor for the respective separation marker. The resulting pos MNCs and neg MNCs were analysed according to their purity, which was measured by flow cytometry and compared with unsep MNCs and their CFU capacity and their enrichment factor.

As Table 2 shows, although MSCA1 showed the highest enrichment factor (194.16-fold) and W4A5 the highest cell purity (68.9%) of enriched cells compared with the other separation strategies, neither MSCA1 nor W4A5 positive cells showed enrichment of the number of clonogenic MSCs. In addition, the CFU-F frequency in the MSCA1 and W4A5 depleted fractions were only slightly reduced (31.11 and 20.00 CFU-F/10^6 MNC, respectively) when compared with unsep MNCs (49.44 CFU-F/10^6 MNC). Moreover, using MSCA1 as a separation marker was more likely to enrich granulocytes than MNCs (data not shown).

Separation with either CD146 or CD271 showed a comparable enrichment factor of the respective markers (42.24- and 47.39-fold) in comparison with unsep MNC, but CD271 pos MNCs displayed an eightfold higher enrichment of clonogenic MSCs than CD146 pos MNC.

In addition to measuring the cell purity of each single marker, the frequency of CD271 pos MNCs was investigated independently of the separation marker that was used. Interestingly, CD146 pos MNCs indicated a sevenfold capacity for CFU-F enrichment compared with unsep MNCs, so the percentage of CD271 pos MNCs was indirectly enriched by 10.63-fold (data not shown). In contrast, MSCA1 and W4A5 enriched fractions showed no or marginal indirect enrichment of CD271 pos MNC frequency.

However, the CD146 and CD271 neg fractions still contained CFU-Fs indicating that both separation markers alone are not able to separate all MSCs from ovine BM.

In summary, the marker CD271 seems to be the most effective separation marker to enrich the number of clonogenic MSC from ovine BM. MSCA1 and W4A5 are not suitable markers for MSC isolation from sheep due to a lack of clonogenic cell enrichment. Interestingly, the enrichment of CFU-Fs per 10^6 MNC gained via the CD146 separation might be based on the indirect parallel enrichment of CD271 pos MNCs.

3.2 | Second study—In vitro PoC with separation marker candidate

Based on the findings of the first study, CD271 was used as a separation marker for MSCs to investigate the use of this cell population to produce a PoC graft for treating focal cartilage defects. This approach was compared in vitro with the gold standard of a MACT graft that contains primary ACs. Primary ACs and BM were obtained from two further animal donors, and the unsep MNCs were separated by CD271 MACS. The subsequently enriched and depleted cell fractions as well as unsep MNCs and ACs were directly seeded into a clinically approved collagen type I gel according to the study design (see Figure 1).

Cells of both donors showed high individual variation after separation with CD271 antibody, resulting in 10.8- and 21.3-fold enrichment of CFU/10^6 MNCs (see Table 3) compared with unsep

TABLE 2 Results of the first study for the identification of effective separation marker

Separation marker	Percentage of positive cells for the respective separation marker [% of viable MNC]			Clonogenic capacity [CFU-F/10^6 MNC]		
	unsep MNCs	pos MNCs	neg MNCs	unsep MNCs	pos MNCs	neg MNCs
CD146	1.10	46.42 (42.24x)	0.65 (1.69y)	49.44	324.07 (6.55x)	3.33 (14.83y)
MSCA1	0.21	41.55 (194.16x)	0.17 (1.25y)	49.44	41.67 (0.84x)	31.11 (1.59y)
W4A5	20.03	68.90 (3.44x)	9.50 (2.11y)	49.44	48.15 (0.97x)	20.00 (2.47y)
CD271	1.38	65.40 (47.39x)	0.51 (2.71y)	57.02[a]	3 125.00 (54.81x)	21.38 (2.67y)

Note. Numbers in brackets indicate x-fold enrichment and y-fold depletion factor of viable MNCs, respectively. CFU-F = colony-forming unit fraction; MNCs = mononuclear cells.

[a]The bone marrow was split into two halves, and magnetic activated cell sorting separation was performed separately.

TABLE 3 CD271 separation for proof of concept of point-of-care therapy

Separation with CD271 Donor	Percentage of positive cells for respective separation marker [% of viable MNC]			Clonogenic capacity [CFU-F/10^6 MNC]		
	unsep MNCs	pos MNCs	neg MNCs	unsep MNCs	pos MNCs	neg MNCs
2	0.67	30.60 (45.67x)	0.25 (2.68y)	192.98	2 097.22 (10.87x)	3.29 (58.66y)
3	0.79	70.20 (88.86x)	0.44 (1.80y)	163.38	3 486.11 (21.34x)	2.74 (59.63y)

Note. Numbers in brackets indicate x-fold enrichment and y-fold depletion factor of viable MNCs, respectively. CFU-F = colony-forming unit fraction; MNCs = mononuclear cells.

MNCs from both donors, respectively. Moreover, the cell purity between both donors differed between 30.6% and 70.2%, whereas only 3.3 and 2.7 CFU-Fs per 10^6 MNCs were found in the negative fractions, indicating an effective separation of MSC with the surface marker CD271 from BM aspirate.

In consequence, the seeded pos MNC grafts of Donors 2 and 3 contained 4,207 and 9,652 CD271+ MNCs, including about 29 and 48 CFU-Fs, respectively.

3.2.1 | 3D gel properties

The macroscopic evaluation of stem cell-loaded collagen hydrogels (initial area: 3.80 cm^2) showed progressive contraction from Day 7 till Day 25 of culture for the gels seeded with cells of the CD271 pos fraction and the unsep MNCs (both 0.20 cm^2), whereby gels loaded with CD271 neg cells exhibited less contraction (1.82 cm^2). Hydrogels seeded with ACs showed an initial contraction until Day 21 and remained constant in size (range from 0.73 to 0.95 cm^2) regardless of the medium condition (see Figure 2). Both the extent and the kinetics of the observed graft shrinkage were similar in all four groups irrespective of the medium supplementation condition. Further, the gel contraction correlated well with the decline of the overall wet weight of the grafts over the entire duration (data not shown).

3.2.2 | Live-dead staining of cell-seeded gels

The seeding of unsep MNCs, MACS sorted CD271 pos, and CD271 neg MNCs as well as primary ACs in the collagen type I hydrogel caused a minimal decrease in cell viability, as observed by live-dead staining after 24 hr (see Figure 3). After 2 weeks of 3D cultivation, homogenous colony formation was observed in the gels with unsep MNCs and with CD271 pos MNCs, whereas grafts seeded with CD271 neg MNCs showed little to no viable cells or colony formation in Condition A and Condition B. However, gels loaded with primary ACs showed a higher number of viable, proliferative cells when compared with the above-mentioned BM-derived fractions.

With progressive cell proliferation and gel maturation, the hydrogels of the unsep MNCs, CD271 pos MNCs, and AC group become opaque and therefore were not scannable by laser scanning microscopy later than 14 days of 3D cultivation. However, no influence of the cultivation condition was seen on viability or cell distribution in any group.

3.2.3 | Immunohistological staining of direct-seeded constructs

Cryosections of the cell-seeded hydrogels of Day 35 were stained for the chondrogenic differentiation markers aggrecan and collagen type II in addition to the hypertrophy marker collagen type X. The immunohistological sections of unsep MNC, CD271 pos MNCs, and AC-seeded gels revealed a homogenous cell distribution throughout the entire gel with a dense circumferential cell layer with a depth of two to 10 cells. In contrast, gels loaded with the CD271 neg MNC fraction displayed an inhomogeneous presence and distribution of these cells, with rare solitary colonies within the graft.

Although grafts with unsep MNC, CD271 pos MNCs, and primary ACs also showed a homogenous intense aggrecan and collagen type II staining, especially in peripheral regions and sporadic cell colonies, the gels loaded with the CD271 neg MNC fraction exhibited only marginal secretion of both chondrogenesis markers around the sporadic cell colonies.

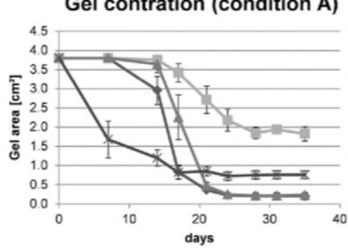
Further, it also became obvious that medium supplementation during the initial 14 days of the proliferation and terminal differentiation phase (Condition B) resulted in a stronger secretion of cartilage markers such as aggrecan and collagen type II when compared with their counterparts of Condition A, in which there was an initial and steady induction of chondrogenic differentiation.

Regarding the collagen type X staining, minimal positive staining was observed in gels seeded with the primary AC when cultured under medium Condition A, whereas there was no positive staining signal under Condition B.

Grafts loaded with unsep MNC and CD271 pos MNC-seeded gels, regardless of medium condition, showed rare positive hypertrophic cells, dominantly in the peripheral regions, whereas cryosections of gels loaded with CD271 neg fraction remained negative for this terminal differentiation marker.

3.2.4 | DNA and sGAG content of cell-seeded gels

The DNA content in cell-seeded gels was measured by Quant-iT™ PicoGreen® after papain digestion. As Figure 4 illustrates, gels seeded with CD271 pos MNCs, with unsep MNCs, or with primary ACs of both donors showed increasing DNA content from Day 14 to Day 35 of cultivation irrespective of the medium cultivation condition. However, Condition B resulted in a higher DNA content (higher cell



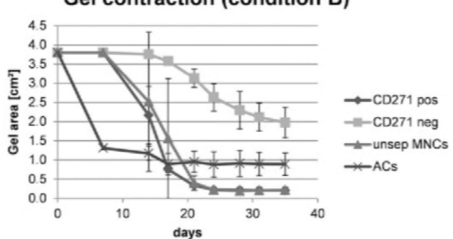


FIGURE 2 Gel contraction kinetics of cell-seeded gels from Donors 2 and 3. The surface area of the gels was measured via ImageJ software from scanned multiwell plates. AC-seeded gels showed an initial contraction until Day 17 and remained constant from there on. Unsep MNC and CD271 pos-seeded gels showed a comparable decrease in the gel area, whereby CD271 neg-seeded gels showed minimum contraction. Data are shown as mean with standard deviation

FIGURE 3 Representative live-dead staining and immunohistochemical staining of Donor 2 for both medium supplementation conditions. First two rows show live-dead staining after 1 and 14 days of 3D cultivation either under Condition A (top) or B (bottom). Lower four rows displayed immunohistochemical staining for aggrecan (Agc), collagen type II (Col II), and the hypertrophy marker collagen type X (Col X) of cell-seeded 3D gels after 35 days of cultivation, respectively. Scale bars indicate 200 μm [Colour figure can be viewed at wileyonlinelibrary.com]

numbers) when compared with Condition A. In contrast, gels loaded with CD271 neg MNCs showed a declining trend of DNA content under both conditions and both donor samples, which is comparable with the findings of live-dead staining showing marginal cell presence and proliferation at both time points. Irrespective of the medium condition, the hydrogels of the reference group with primary ACs showed higher DNA and cell content after 35 days in vitro when compared with grafts laden with CD271 pos MNCs or unsep MNCs, respectively (Figure 4).

To confirm and quantify the findings of the immunohistochemical staining, freshly synthesized sGAG was measured by DMMB assay after papain digestion. Progressive sGAG secretion was observed for the three groups of CD271 pos MNCs, unsep MNCs, and AC-seeded gels between Days 14 and 35 under both medium supplementation regimes.

In contrast to this observation, gels of the CD271 neg MNC group revealed sGAG content at baseline levels of approximately 2-μg sGAG per gel that is comparable with acellular reference hydrogels (Figure 4). This finding is in line with the observations of the immunohistochemistry, which also showed the lack of notable production of cartilage-specific markers. The sGAG per DNA ratio reveals comparable values for the gels of all cell populations that were cultivated under Condition B, whereby gels of CD271 pos MNCs showed a 3.5-fold increased sGAG per DNA ratio at Day 14 in comparison with grafts of the unsep MNCs or the AC group. The total amount of sGAG was dependent on the cultivation condition. The secretion of sGAG from ACs was directly correlated with the supplementation of chondrogenic differentiation media, thus the sGAG amount under Condition B after 14 days remained the lowest for ACs (3.4- and 3.3-μg sGAG per gel, respectively). In contrast, after 35 days, the sGAG level was increased up to 12.7- and 14.0-μg sGAG per gel with the AC group. Within the CD271 pos MNCs and unsep MNCs groups, the level of sGAG was correlated with the DNA amount (number of cells) and therefore depended on the culture condition. Irrespective of both medium supplementation conditions and the donor, the gels seeded with CD271 pos and unsep MNC-seeded gels showed similar sGAG levels. In summary, cell-seeded gels initially cultivated with expansion medium for 2 weeks and terminal differentiation phase of 3 weeks (Condition B) possessed higher sGAG per gel when compared with gels that were permanently cultivated with differentiation medium (Condition A).

In summary, the results of the second study demonstrated that it would be technically feasible to generate chondrogenic differentiated MSC-based cartilage grafts while avoiding a time- and cost-consuming initial monolayer expansion of MSC populations. The grafts seeded with the ovine CD271 pos and the unsep MNC cell populations showed the most promising results with respect to cell proliferation, viability, and homogenously cell distribution over 5 weeks in vitro independent of the medium condition. In contrast, gels with CD271 neg MNCs exhibited minimal cell proliferation after 2 and 5 weeks of cultivation. Further, live-dead staining also revealed that comparable amounts of dead cells were observable in gels of unsep MNCs and CD271 neg MNCs at both time points, whereas grafts of the CD271 pos MNCs group exhibited homogenously distributed viable cells. This CD271 pos MNC gels also showed comparable DNA amounts (cell numbers) after 2 and 5 weeks with the unsep MNCs

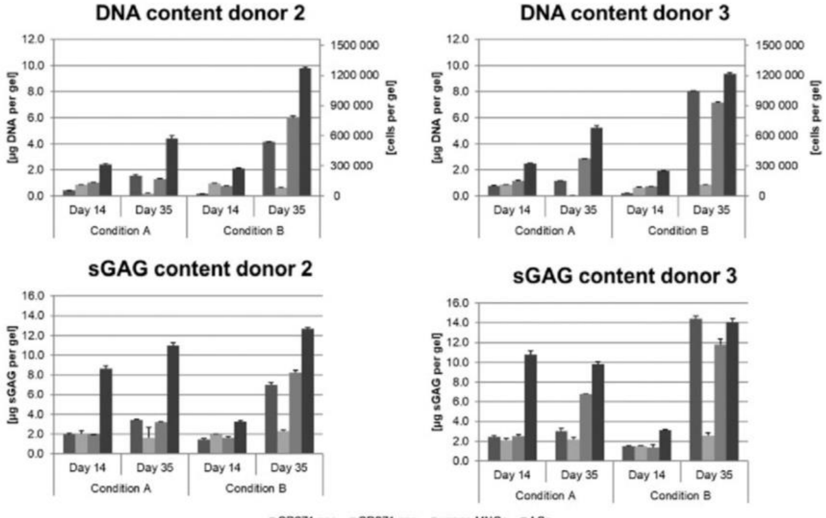


FIGURE 4 DNA content [µg], respectively, no. of cells, and µg sGAG per gel for each donor after 14 and 35 days of cultivation. ACs = articular chondrocyte; MNCs = mononuclear cells; sGAG = sulphated glycosaminoglycans

gel group, even though the CD271 pos MNCs were initially seeded with a 16-fold lower cell number (6.25% of unsep MNCs) compared with their unseparated counterparts.

The positive reference group of primary ACs showed the highest DNA and sGAG content after 2 and 5 weeks in nearly all cases irrespective of the medium conditions when compared with the other three cell subpopulations. The immunohistochemical staining of CD271 pos MNCs-loaded gels after 35 days showed strong chondrogenic differentiation via positive accumulation of the differentiation markers aggrecan and collagen type II. Grafts with unsep MNCs had stronger chondrocytic differentiation in sporadic cell colonies accompanied with a more homogenous marker expression throughout the rest of the gels. Minimal positive signals for collagen type X were observed in grafts made of ACs.

4 | DISCUSSION

This work sought to prove the feasibility of a PoC concept for the treatment of cartilage lesions based on a defined MSC subpopulation in shape of a cartilage graft without the need for an in vitro cell expansion phase. After identifying the most promising cell marker (CD271) for this purpose in the first study, this separated cell fractions were seeded into a 3D hydrogel. The properties of this graft were evaluated for suitability as a treatment modality for cartilage defects. Grafts seeded with CD271 pos selected MNCs showed promising results regarding the cell viability and proliferation with homogeneous distribution over a 5-week culture period compared with CD271 neg MNCs and unsep MNCs, irrespective of the media condition. In addition, a chondrogenic differentiation is not limited by a prior proliferation induced by serum-containing media (Condition B) as a continuous differentiation (Condition A) showed already a high chondrogenic differentiation. However, the resulting cell number and sGAG content was higher in gels after a 2-week proliferation phase in 3D.

Regardless of whether intraoperative or traditional cell therapy approaches are used, a limitation in the application of MSCs is their low frequency of 0.001–0.01% in freshly isolated human BM-MNCs (Muschler, Nitto, Boehm, & Easley, 2001; Sarugaser et al., 2009). These results are challenging in acquiring an adequate number of MSCs for cellular products. To overcome this disadvantage, it is either possible to use a relatively large amount of BM, for example, in form of a BMC, or to start with a limited volume of BM and expand the cells over several passages in vitro. The in vitro cell expansion is associated with substantial cell manipulation, as well as time-, cost-, and personnel-consuming processes in GMP facilities, accompanied by the potential risk of contamination, as well as a high level of regulatory oversight. Another option is to separate the MSCs by selective, intraoperative isolation. The separation of MSCs with a PoC strategy counteracts the low natural frequency of MSCs in BM, reduces the applicable volume for the affected joint, and avoids the mayor disadvantages of the existing gold standard (MACT), including donor site morbidity, chondrocyte dedifferentiation, and GMP-related costs. Moreover, depending on the (national) regulatory framework, the application of minimally or nonsubstantially manipulated cells and their homologous use could lead to a cellular product either classified as an advanced therapy medicinal product or not. The interested reader is referred to the relevant draft guidance of the U.S. Food and Drug Administration (U.S. Food and Drug Administration, 2014), the Annex I of the Regulation (EC) No 1394/2007 EU (*Official Journal of the European Union*), and a review article on the current legal basis for PoC therapies (Faltus & Schulz, 2015).

The absence of a unique surface marker for the separation of all MSCs makes it a challenge to choose the right surface marker or marker combinations with regard to MSC yield versus MSC purity (Coelho et al., 2012). The present study has shown that CD271 is a suitable separation marker to isolate MSCs. Consistently, CD271 has been considered to be one of the most specific markers in terms of the yield of CFU-Fs from human BM, as all CFU-F reside in the

CD271 pos fraction (Quirici et al., 2002). In addition, it has been shown that the CD271 pos population represents primary stem cells with MSC phenotype fulfilling stringent functional stem cell criteria in vivo (Ghazanfari et al., 2016). The cells have been shown to exhibit the essential trilineage potential and were phenotypically identical to plastic adherence-selected MSCs following expansion culture (Colosimo et al., 2015; Cuthbert et al., 2015). In contrast to the results of Rozemueller et al., who demonstrated an enrichment of CFU-Fs for flow-sorted ovine W4A5 pos and MSCA1 pos cells, it was not possible to enrich clonogenic MSCs by using these markers for separation in the present study. However, a separation via CD146 and CD271 was able to enrich clonogenic MSCs from ovine BM, whereas CD271 appeared to be the most promising marker to isolate MSCs, comparable with the findings of Rozemueller et al. (2010). The 7-fold enrichment of CFU-Fs by separation via CD146 can also be correlated by the parallel 10-fold enrichment of CD271 pos cells. In contrast, the MSCA1 and W4A5 pos fractions showed little to no marginal parallel enrichment of CD271 pos MNCs. This experiment was performed with one donor, thus making it impossible to determine any statistical significance. However, considering the robustness of the separation approach, each individual case should provide the same effect regardless of the size of the effect. Although, the direct MicroBead-mediated MACS in contrast to the two-stage PE-mediated MACS separation could positively influence the purity of separation, the cell purity of all four markers in the enriched pos MNC fractions was higher than 40%. However, a high purity of a marker does not guarantee efficient separation of MSCs if it is not the proper marker. In addition, one should bear in mind that the increase of purity comes at the expense of the cell yield.

The high variation of CD271 purity (30.6–70.2%) among the three donors might be explained by the different initial, individual donor-specific frequency of CD271 in the BM. In addition, the cell frequency also depends on the aspiration technique; a higher BM aspirate volume has been shown to diminish the frequency of MSCs by dilution with peripheral blood (Fennema, Renard, Leusink, van Blitterswijk, & de Boer, 2009). However, our purity was comparable with other studies, showing 38.8 ± 10% CD271 purity after direct magnetic cell separation (Busser et al., 2015).

Despite the relative enrichment of highly clonogenic and chondrogenic MSCs using one-stage separation, it should be taken into account that the sum of CFU-Fs of pos and neg fractions after separation is lower than the total number of CFU-Fs in unsep MNCs. However, the MACS® technique allows for the enrichment of clonogenic cells to a usable volume and also depletes nonchondrogenic differentiable cells. Thus, cell subpopulations of therapeutic relevance can be separated and this technique provides the basis for further studies on one-stage cellular therapy regarding the mode of action. This might be particularly useful in the relevant orthotopic large animal model (Marquass et al., 2011). If an enhanced purity of native MSCs were necessary, a depletion of CD45 pos cells would further reduce hematopoietic cells (Tormin et al., 2011).

With regard to the mode of action of CD271 pos MNCs, these cells were examined in an in vitro PoC study for one-stage cartilage repair. The second study showed that nonmonolayer expanded MSCs were capable of producing a cartilage graft either with the strongly heterogeneous fraction of unsep MNCs or with selected CD271 pos MNCs in combination with a collagen hydrogel. As little to no colony forming was evident in CD271 neg MNC hydrogels, these cell fractions could be excluded from chondrocytic-relevant cell populations. On the contrary, a large number of lost and dead cells were observed in CD271 neg MNC hydrogels that could be detrimental for clinical application, because necrotic and apoptotic cellular debris promote the development of autoimmune disease (Ishii et al., 2001). It has also been shown that exposure of cartilage to whole blood has negative effects on the maturation of chondrocyte-like cells in a graft, at least in vitro (Sosio et al., 2011).

In contrast to equal cell distribution and aggrecan staining in CD271 pos MNCs and unsep MNCs hydrogels, collagen type II was only punctual expressed at the outer areas of the hydrogels. A non-uniform deposition of ECM is well known from the literature and is based on a required proliferation phase of MSC colonies for a subsequent collagen type II deposition (Dexheimer, Frank, & Richter, 2012). This could be observed either at the outer zones of the cartilage grafts or within highly proliferative cell colonies. The lack of cartilage marker expression in the centre of the gels may be due to low nutrient and oxygen conditions.

Because collagen type II is expressed later during chondrogenesis (Xu et al., 2008), Bartz et al. (2016) showed in an ex vivo human cartilage repair model that aggrecan protein expression levels can potentially be used as surrogate potency marker to predict the regenerative capacity. Here, a high aggrecan protein expression of spheroids before implantation correlates positively with the level of formed repair tissue.

In contrast, the control group of grafts with primary chondrocytes seems to be superior regarding their ECM production when compared with the other fractions. However, the clinical use of chondrocyte-based grafts requires a biopsy of healthy cartilage, resulting in donor site morbidity. Furthermore, such chondrocyte-based advanced therapy medicinal products for cartilage regeneration primarily use expanded cells, which are known to demonstrate a fibro-cartilaginous phenotype (Park et al., 2004).

The limited seeding numbers of CD271 pos MNCs 3D grafts were accepted related to the multiple culture conditions and time points examined within this study. However, the number of CD271 can be easily increased to the maximum for therapeutic use.

Nonetheless, grafts of Donor 2 seeded with 13,750 MNCs that consist of 4,207 CD271 positive MNCs that in turn contained of about 29 CFU-Fs showed that these minimal numbers were able to populate the construct with viable cells that exhibits adequate chondrogenic potential.

The chondrogenic potential of CD271 pos derived, in vitro expanded cell fractions was shown by Mifune et al. (2013) with increased chondrogenic potential and greater healing capacities in a cartilage defect model in rats after 4 weeks compared with in vitro expanded MSCs isolated by plastic adherence. Moreover, in vitro expanded CD271 pos cells of the synovial membrane of osteoarthritic altered joints were capable of repairing cartilage spontaneously in an in vitro model (Hermida-Gómez et al., 2011).

A limitation of this study was the use of defined in vitro conditions, which do not reflect the actual conditions at the defect site in vivo. In consideration, the mode of action of MSCs is the secretion

of immune-modulatory and regenerative agents rather than the replacement of damaged tissue by differentiated MSCs (Caplan & Correa, 2011). Hence, further studies such as coculture models and, especially, in vivo proof of concept studies are necessary to prove the concept of CD271 pos separated MSCs as a viable PoC treatment.

5 | CONCLUSION AND FUTURE PERSPECTIVE

Due to the poor self-healing capacities of cartilage, innovative, regenerative approaches are a major clinical need. The use of in vitro expanded MSCs is accompanied by cost-, time-, and personnel-intensive GMP production. A one-stage operative procedure could overcome these drawbacks. However, it remains unclear whether the use of highly heterogeneous BMC or unsep MNCs provides the highest regenerative effect. With regard to the mode of action, separated cell fractions allow for direct comparison of different cell populations with accompanied cell fractions. This study is the first to demonstrate direct generation of a cartilage graft with the defined CD271 pos MNC fraction without the need for prior in vitro expansion. To determine the utilization of targeted separation of MSCs by the marker CD271 against the unsep MNCs, a seeding density of the same number of CFU-Fs might be necessary. Because the numbers of CFU-Fs in the separated fractions are not predictable, this would be challenging for subsequent experimental studies. This could be bypassed by halving the CD271 pos MNCs and putting one half into a gel and the other half to CD271 neg MNCs, which represent unsep MNCs with comparable CFU-Fs. Further studies, especially in vivo proof of concept studies, are necessary to examine the in vivo regenerative effects of separated MNC fractions.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1: Positive and negative controls for immunohistochemical staining. Human adult hyaline cartilage was used as a control for each antigen staining. Images are displayed in 100× magnification.

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Single-stage preparation of human cartilage grafts generated from bone marrow-derived CD271+ mononuclear cells

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Single-Stage Preparation of Human Cartilage Grafts Generated from Bone Marrow-Derived CD271$^+$ Mononuclear Cells

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Due to the limited self-healing capacity of articular cartilage, innovative, regenerative approaches are of particular interest. The use of two-stage procedures utilizing in vitro-expanded mesenchymal stromal cells (MSCs) from various cell sources requires good manufacturing practice-compliant production, a process with high demands on time, staffing, and financial resources. In contrast, one- stage procedures are directly available, but need a safe enrichment of potent MSCs. CD271 is a surface marker known to marking the majority of native MSCs in bone marrow (BM). In this study, the feasibility of generating a single-stage cartilage graft of enriched CD271$^+$ BM-derived mononuclear cells (MNCs) without in vitro monolayer expansion from eight healthy donors was investigated. Cartilage grafts were generated by magnetic-activated cell sorting and separated cells were directly transferred into collagen type I hydrogels, followed by 3D proliferation and differentiation period of CD271$^+$, CD271$^-$, or unseparated MNCs. CD271$^+$ MNCs showed the highest proliferation rate, cell viability, sulfated glycosaminoglycan deposition, and cartilage marker expression compared to the CD271$^-$ or unseparated MNC fractions in 3D culture. Analysis according to the minimal criteria of the International Society for Cellular Therapy highlighted a 66.8-fold enrichment of fibroblast colony-forming units in CD271$^+$ MNCs and the only fulfillment of the MSC marker profile compared to unseparated MNCs. In summary, CD271$^+$ MNCs are capable of generating adequate articular cartilage grafts presenting high cell viability and notable chondrogenic matrix deposition in a CE-marked collagen type I hydrogel, which can obviate the need for an initial monolayer expansion.

Keywords: cartilage, point of care, bone marrow-derived mesenchymal stem cells, mesenchymal stromal cells, hydrogel, cell separation

Introduction

Based on the chondrogenic differentiation potential, bone marrow (BM)-derived mesenchymal stromal cells (MSCs) are an ideal cell candidate for cartilage graft production [1–4]. In this study, MSC-based treatment options can be divided into two- or one-stage procedures.

In two-stage procedures, monolayer-expanded MSCs are produced under good manufacturing practice (GMP) conditions, similar as performed in a chondrocyte-based therapeutic approach. The quality assurance and quality control, including testing of identity, purity, potency, stability, and sterility of the MSCs, have to be recorded according to European Regulation 1394/2007 [5,6], ensuring the maximum safety for these advanced therapeutic medicinal products. However, cellular expansion was defined as a ''substantial manipulation'' by the European Medicines Agency [6] as it influences the differentiation capacity of the cells and enhances the risk for contamination and genetic instabilities in these cellular products [7,8]. Apart from the accompanied challenges, the time to treatment is prolonged. In single-stage procedures, the articular cartilage defect can be evaluated during arthroscopy or open surgery intraoperatively, and a subsequent therapeutic intervention by isolation and enrichment of potent MSCs can be simultaneously addressed. Thus, the challenges of the time- and cost-

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consuming GMP expansion of MSCs and the inherent contamination risk might be reduced.

The common single-stage procedure is the microfracture of the subchondral bone leading to the infiltration of BM, forming a blood clot at the defect site and stimulating the recruitment of local MSCs from subchondral BM and synovial membrane [9,10]. Alternatively, the lesion can be coated with a concentrated BM aspirate containing autologous MSCs and chemokines. Nonetheless, both techniques provide only few potent MSCs contaminated with high numbers of hematopoietic cells in a frequency between 1 and 1.8×10^5 [2,11]. Moreover, only the treatment of small defects is recommended by microfracture (<2.5 cm^2) or concentrated BM (<4 cm^2) [12], and in the aftermath, the formation of mechanically unstable fibrocartilage in mid- to long-term follow-up is not uncommon [13].

Hitherto, a procedure overcoming time- and cost-consuming GMP expansion of MSCs, reducing the inherent contamination risk, avoids the (substantial) cell manipulation, and may fulfill the requirements for quality control and quality assurance of the cellular product is still absent.

The consideration of both single- and two-stage procedures underlines the advantages and disadvantages and additionally, the need for a single-stage procedure combining the opportunity of the direct treatment and the safety for the formation of a stable cartilage graft. A possible attempt to address this problem might be the intraoperative enrichment of MSCs in BM aspirates using surface markers.

A number of surface markers have been suggested for prospective isolation of MSCs from BM [14]. Among these surface markers, the low-affinity nerve growth factor receptor, $CD271^+$ BM-derived MSCs, yielded excellent proliferation rate in monolayer culture and differentiation potential compared to MSCs selected by plastic adherence [15–21].

Furthermore, $CD271^+$ cells contain the majority of fibroblast colony-forming units (CFU-F) [16,22] and displayed an enhanced chondrogenic differentiation potential after monolayer expansion in a rat cartilage defect model [19].

Jones et al. in 2010 described CD271 as a suitable surface molecule to enrich MSCs from BM aspirates intraoperatively [21].

This in vitro study investigated the feasibility of generating cartilage grafts from human $CD271^+$ BM cells in a CE-marked collagen type I hydrogel without initial monolayer expansion. Cell viability, DNA content, chondrogenic differentiation capacity, extracellular matrix secretion, and graft properties were monitored for up to 5 weeks to investigate the single-stage therapeutic approach for focal cartilage defects.

Material and Methods

The flow chart in Fig. 1 gives an overview of this study and is described in detail in the following sections:

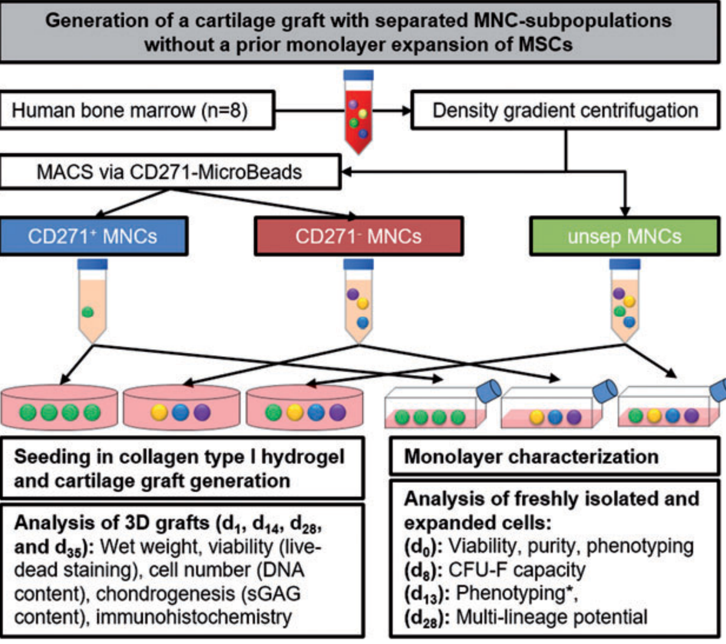
Ethics statement

The BM was aspirated as part of BM donations from eight donors without hematopoietic diseases, approved by the local ethics committee (State directorate of Saxony, EK-BR-86/14-1), and performed in accordance to Declaration of Helsinki. Macroscopically healthy cartilage was obtained during total knee arthroplasty for control immunohistochemistry. Tissue harvesting was approved by the local ethics committee (University of Leipzig, 190-14-02062014) and performed in accordance to Declaration of Helsinki.

All donors gave written informed consent. Baseline data were collected regarding donor's age and gender.

Cell isolation

Thirty to 60 mL of BM from the iliac crest was collected into a sterile heparinized syringe (170 IE/mL BM; Ratiopharm, Ulm, Germany) under anesthesia according to standard surgery



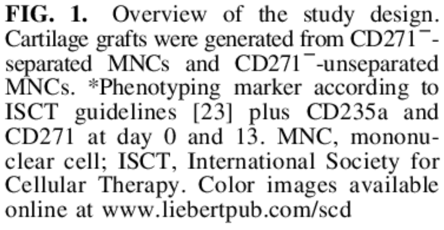


FIG. 1. Overview of the study design. Cartilage grafts were generated from $CD271^-$-separated MNCs and $CD271^-$-unseparated MNCs. *Phenotyping marker according to ISCT guidelines [23] plus CD235a and CD271 at day 0 and 13. MNC, mononuclear cell; ISCT, International Society for Cellular Therapy. Color images available online at www.liebertpub.com/scd

protocol. Within 20–24 h after aspiration, whole BM was filtered with 70 μm mesh, diluted 1:3 with phosphate-buffered saline without Ca^{2+}/Mg^{2+} (phosphate-buffered saline [PBS]; Biochrom, Berlin, Germany), and centrifuged through Ficoll paque premium ($\rho = 1.077$ g/mL; GE Healthcare, Buckinghamshire, United Kingdom) in Leucosep® tubes (Greiner Bio One) to obtain mononuclear cells (MNCs), including the MSCs. MNCs were washed twice with PBS and resuspended in DMEM (DMEM, high glucose, GlutaMAX™ supplement, pyruvate; Thermo Fisher Scientific, Dreieich, Germany). The number of viable cells was determined at each isolation step by dead cell exclusion by 4′, 6-diamidino-2-phenylindole (DAPI) staining (0.5 μg/mL in PBS; Sigma Aldrich, Munich, Germany) in the MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch-Gladbach, Germany). 1.2×10^7 MNCs were taken as unseparated (unsep) MNCs, as a reference cell population. The majority of the MNCs were subjected to separation by magnetic cell separation (MACS).

Magnetic cell separation

The MNCs were labeled with CD271 MicroBeads according to the manufacturer's protocol (all reagents Miltenyi Biotec) with minor modifications. First, nonspecific binding sites were blocked with 20 μL FcR-blocking reagent per 10^7 MNCs for 5 min. Afterward, cells were incubated with 20 μL CD271 MicroBeads per 10^7 MNCs for 30 min at room temperature in the dark. MNCs were washed with autoMACS Running Buffer and transferred onto the MACS column within a MACS Separator. Unlabeled cells were washed out and represent the group of CD271$^-$ MNCs. After removal of the column from the separator, CD271$^+$-enriched MNCs were eluted with an autoMACS Running Buffer.

The cell fractions were used for the generation of 3D cartilage grafts without a prior monolayer expansion (see chapter 3D cultivation and chondrogenic differentiation) as well as expanded (2D) for 12–14 days for the characterization of cells according to the specifications of the International Society for Cellular Therapy (ISCT) [23].

The cells should fulfill "minimal criteria for defining multipotent mesenchymal stromal cells." MSCs are defined by plastic adherence in monolayer cultivation as well as expression of CD73, CD90, and CD105 and deficiency of CD14, CD34, CD45, or CD11b, CD79α or CD19, and HLA-DR markers. In addition, MSCs must show multilineage potential by differentiating into adipocytes, osteoblasts, and chondroblasts [23]. These monolayer culture-based minimal criteria exclude a prospective isolation of MSCs from BM, as it would be essential for intraoperative single-stage therapy approaches. Nonetheless, these criteria enable a standardized definition of monolayer-expanded MSCs. To highlight the difference of monolayer-expanded MSCs and single-stage optimized enriched cell populations, all used cell fractions are named MNC fractions, as they also contain non-MSCs, for example, lymphocytes and monocytes.

Flow cytometry analysis

To characterize the cells and to control separation, freshly isolated and primary expanded MNCs were examined for the presence of CD271, CD73, CD90, and CD105 and the absence of CD14, CD19, CD34, CD45, HLA-DR, HLA-DP, HLA-DQ, and CD235a using direct labeled antibodies (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). The cells were stained according to the manufacturer's protocol. Briefly, at least 2×10^4 freshly isolated or 2×10^5 expanded cells were washed with autoMACS Running Buffer and incubated with FcR blocking reagent. Afterward, the cells were stained either with a panel of antibodies or with a combination of, respectively, isotype controls. The cells were measured by using the MACSQuant Analyzer 10 and FlowJo X10.0.7r2 Software (FlowJo, Ashland). Fluorescence minus one and isotype controls were used to compensate the MACSQuant Analyzer and to set the positive/negative gates. Doublets, debris, and dead cells, stained by DAPI, were excluded from further analysis.

Evaluation of CFU-F

Due to the different clonogenic capacity of cell fractions examined in preliminary experiments and reported previously [18], the initial seeding cell numbers were adjusted to 6.7×10^2 CD271$^+$ MNCs, 1.0×10^5 CD271$^-$ MNCs, and 2.0×10^4 unsep MNCs/cm^2 to determine the number of colony-forming cells from freshly isolated and separated cells. The cells were plated in 12-well plates and incubated with 1 mL DMEM/10% allogeneic AB-serum (Biowest, Nuaillé, France) at 5% pO_2 and 5% pCO_2. The medium was changed once a week. After 7 days, the cells were washed twice with PBS, fixed with methanol for 5 min, and colonies of more than ~ 50 cells were counted using light microscopy (Zeiss Axiovert 200; Zeiss Microscopy, Jena, Germany). The number of CFU-Fs was calculated as mean from 6 to 12 wells.

Evaluation of osteogenic and adipogenic potential

To determine the osteogenic potential by mineralization, cells were seeded in a density of 3.0×10^4 cells/cm^2 in 12-well plates in DMEM/10% allogeneic AB serum. The next day, the medium was exchanged to the osteogenic induction medium StemMACS OsteoDiff (Miltenyi Biotec) and renewed every 2–3 days. After 14–21 days, the cells were fixed with 4% formaldehyde, stained by von Kossa, and analyzed as described [24].

To determine the adipogenic potential through lipid deposition, cells after passage 1 were seeded into a well of a 12-well plate at a cell density of 5×10^2 cells/cm^2 in 1 mL DMEM/10% allogeneic AB serum. The next day, the medium was exchanged to the adipogenic medium StemMACS AdipoDiff (Miltenyi Biotec) and replaced twice a week. On days 14 and 21, accumulated lipids in the cells were stained by Oil Red O and analyzed as described [24].

3D cultivation and chondrogenic differentiation

MNCs were seeded into a CE-marked, clinically approved 3D collagen type I hydrogel (Arthro Kinetics Biotechnology, Krems, Austria) according to the manufacturer's protocol without an initial in vitro monolayer expansion. Briefly, cells were mixed with a gel neutralization buffer (Arthro Kinetics Biotechnology) on ice and the collagen type I solution (2 mg/mL; Arthro Kinetics Biotechnology) was added. For each gel, 550 μL gel suspension was transferred into a well of a 24-well plate and polymerized for 20 min at 37°C. Due to the higher clonogenic capacity of the CD271$^+$ MNCs determined in

preliminary experiments, different cell numbers of each MNC fraction were seeded into the gels: 9.0×10^3 $CD271^+$ MNCs, 1.125×10^6 $CD271^-$ MNCs, and 2.25×10^5 unsep MNCs/graft.

In BM of four donors of preliminary experiments, in mean, 2.7×10^6 MNCs per mL BM were obtained after density gradient centrifugation (Supplementary Table S2). Following CD271 MACS separation in this BM, 2.67×10^5 $CD271^+$ MNCs were separated. Based on this observation, the study was conducted, leading to a seeding density of 9,000 $CD271^+$ MNCs per graft due to the different time points, replicates, and the required cells for CFU assay, flow cytometry analysis, and 2D expansion for evaluation of osteogenic and adipogenic potential (1.4×10^5 $CD271^+$ MNCs lost for gel production).

On the basis of previously published reports and approved cell therapeutics for cartilage repair [25] for the control group (unsep MNCs), a seeding density of 2.25×10^5 MNCs ($\triangleq 1 \times 10^6$ cells/cm^2) was chosen.

The grafts were then initially cultivated for 14 days in DMEM/10% allogeneic AB serum, followed by serum-free chondrogenic differentiation media (Lonza, Basel, Switzerland) supplemented with 10 ng/mL recombinant human transforming growth factor β3 (Miltenyi Biotec) for up to 21 days at 5% pO_2 and 5% pCO_2. The media were changed twice a week. The grafts were analyzed after 1, 14, 28, and 35 days.

The usage of human AB serum instead of fetal calf serum as supplement increased the proliferative activity of $CD271^+$ MSCs at least in monolayer expansion [26]. Therefore, human AB serum was used in this study.

Analysis of 3D grafts

Cell viability was visualized by staining the grafts with ethidium homodimer and calcein AM (Live/Dead® Viability/Cytotoxicity Kit; Invitrogen, Karlsruhe, Germany) for 15 min at 37°C. After three washing steps with PBS, images of the grafts were taken with a fluorescence microscope (Zeiss Axiovert 200, Zeiss Microscopy) and analyzed.

To determine the DNA and secreted sulfated glycosaminoglycan (sGAG) content, the grafts were weighed and subsequently digested with 50 μg/mL papain (Sigma-Aldrich) at 60°C overnight. The DNA content in the samples was quantified with the Quant-iT PicoGreen assay (Thermo Fisher Scientific). The cell number/graft was calculated as 6.4 pg DNA per cell [27]. The sGAG content was measured with the dimethylmethylene blue (Sigma-Aldrich) assay [28]. All measurements were performed in triplicate according to the manufacturer's instructions.

For immunostaining, the grafts were shock frozen, bisected, and embedded in Tissue-Tek OCT Compound (VWR, Darmstadt, Germany). Eight micrometer cross-sections were stained for classical chondrogenic differentiation markers with monoclonal antibodies for aggrecan and collagen type II (Supplementary Table 1), detected by BrightVision goat-anti-mouse/rabbit/rat horseradish peroxidase (medac, Wedel, Germany) and stained with 3-amino-9-ethylcarbazole (AEC; Sigma Aldrich) substrate. Nuclei were counterstained with hematoxylin (DakoCytomation, Groß Lindow, Germany). Moreover, macroscopically healthy cartilage obtained during total knee arthroplasty was used as positive control for immunohistochemistry. Digital scans of the stained sections were taken with a Pannoramic Slides scanner (Sysmex, Norderstedt, Germany) and analyzed.

Statistical analysis

Data are presented as mean ± standard deviation. Comparative analyses of the non-Gaussian distributed data were performed by Kruskal-Wallis tests of the three cell fractions at the same time point. The level of significance was defined with P value <0.05. If measurements of individual fractions of a donor could not be performed due to the limited cell number, all values were withdrawn from the analysis of these time points. These events are indicated by varying n-values within the respective tables and figures. The statistical analyses were performed with Statistica version 12.7 (www.statsoft.de).

Results

Identity and purity of the MNC fractions

BM aspirates (mean volume 43.3 ± 10.8 mL) were collected from the iliac crest of eight healthy adults (mean age: 28.9 ± 3.4 years; one female and seven male donors) and processed. The yield of unsep MNCs from BM after density gradient centrifugation was $5.24 \pm 2.95 \times 10^6$ MNC/mL BM (range: $2.01–9.75 \times 10^6$ MNC/mL BM) with a viability of >98.5%. The majority of unsep MNCs were $CD45^+$ leukocytes (95.42% ± 2.49%, Table 1). Only 0.05% ± 0.04% of the unsep MNCs were $CD45^-/CD271^+$, that is the putative MSC-containing fraction [21].

The MACS separation by CD271 MicroBeads resulted in $1.32 \pm 0.82 \times 10^4$ $CD271^+$ MNCs/mL BM and $4.14 \pm 2.00 \times 10^6$ $CD271^-$ MNCs/mL BM. The $CD45^-/CD271^+$ fraction in $CD271^+$ MNCs was enriched by the factor 165.0 from unsep MNCs, with a purity of 31.72% ± 26.14% $CD271^+$ cells. In parallel, $CD45^+$ cells were reduced in $CD271^+$ MNCs from 95.42% to 89.57%.

In the CFU-F assay, the number of colony-forming units was enhanced 66.8-fold in $CD271^+$ MNCs (4551 ± 2833 CFU-F/10^6 cells) compared to unsep MNCs (68 ± 33 CFU-F/10^6 cells). $CD271^-$ MNCs contained 58.0 times less CFU-Fs (1 ± 1 CFU-F/10^6 cells).

Analysis of cell characteristics and multilineage potential

Monolayer expansion was performed to simultaneously characterize the MNC fractions according to the minimal criteria on MSCs reported by the ISCT [23]. Monolayer expansion was calculated as the ratio of gained cell number per 10^6 MNCs initially plated. After 13 days of monolayer expansion, the $CD271^+$ MNCs contained the majority of plastic-adherent, proliferating cells (Table 2). $CD271^-$ MNCs showed no or minimal proliferation, although 25-fold higher initial cell numbers were initially plated compared to $CD271^+$ MNCs. The unsep MNCs showed a reduced cell yield after primary monolayer expansion compared to $CD271^+$ MNCs.

By cultivating monolayer-expanded cells with specific differentiation media, both adipogenic and osteogenic differentiation were seen in each MNC fraction. However, $CD271^+$ cells showed a higher adipogenic lipid and osteogenic matrix deposition than $CD271^-$ MNCs, while unsep MNCs were comparable to $CD271^+$ cells (Fig. 2).

Table 1. Cell Surface Marker Expression of the Mononuclear Cell Fractions After Isolation

	CD271$^+$ MNCs (%)	*CD271$^-$ MNCs (%)*	*unsep MNCs (%)*	$P_{CD271+vs.\ CD271-}$	$P_{CD271+vs.\ unsep}$	$P_{CD271-vs.\ unsep}$
CD45$^-$/CD271$^+$	8.83 ± 6.17	0.13 ± 0.31	0.05 ± 0.04	**0.0004**	**0.0089**	1.0000
CD271$^+$	40.56 ± 22.91	0.70 ± 0.51	0.59 ± 0.53	**0.0011**	**0.0039**	1.0000
**CD73$^+$	23.29 ± 25.87	6.18 ± 4.33	5.80 ± 4.46	**0.0005**	**0.0001**	1.0000
**CD90$^+$	14.63 ± 12.36	3.10 ± 3.25	2.90 ± 3.54	**0.0011**	**0.0019**	1.0000
**CD105$^+$	20.62 ± 13.37	7.51 ± 3.66	7.14 ± 3.62	**0.0025**	**0.0006**	1.0000
CD14$^+$	22.98 ± 6.72	9.56 ± 9.96	7.94 ± 4.86	0.2691	**0.0175**	0.8665
CD19$^+$	14.36 ± 14.29	7.38 ± 3.04	6.82 ± 2.06	1.0000	1.0000	1.0000
*CD34$^+$	29.57 ± 20.89	6.83 ± 2.08	6.40 ± 2.21	**0.0175**	**0.0175**	1.0000
CD45$^+$	89.57 ± 5.29	92.95 ± 1.00	95.42 ± 2.49	0.3988	0.1373	1.0000
*HLA-DR,-DP,-DQ$^+$	47.81 ± 17.81	19.66 ± 1.56	17.38 ± 1.79	0.1752	0.1363	1.0000
CD235a$^+$	8.29 ± 3.70	13.51 ± 7.19	15.88 ± 7.73	0.3594	0.1373	1.0000

Mean % viable singlet MNCs ± SD and *P* values; significant *P* values are highlighted in *bold*; $n=8$; *$n=6$; **$n=16$.
SD, standard deviation; MNC, mononuclear cell.

Flow cytometric characterization after monolayer expansion showed that the CD271$^+$ MNCs fulfilled the ISCT criteria for MSCs, except for the slightly increased percentage of 4.56% ± 0.49% HLA-DR, HLA-DP, and HLA-DQ+ cells (Table 3). In the monolayer-expanded CD271$^-$ MNCs, the percentage of CD45$^+$ and HLA-DR, HLA-DP, and HLA-DQ+ cells was increased. The monolayer-expanded unsep MNCs could not fulfill the MSC criteria concerning CD90 expression; however, the percentage of CD90$^+$ cells was below the postulated 95% threshold (Table 3).

In summary, CD271$^+$ MNCs showed a distinct MSC phenotype with a spindle-shaped cell morphology after primary monolayer expansion regarding to plastic adherence, colony formation, and ISCT marker expression, as well as an adipogenic and osteogenic differentiation potential, thus they can be considered primary MSCs. CD271$^-$ MNCs showed a minor proliferation in monolayer culture with a flattened, globular cell morphology and did form no or only marginal colonies. Moreover, they displayed an increased expression of hematopoietic markers and nearly no multilineage potential, and therefore they did not fulfill minimal MSC criteria. Although the properties of the unsep MNCs were comparable to CD271$^+$ MNCs, a higher amount of these cells are necessary to achieve similar effects related to the high amount of non-MSC cells in this fraction.

3D cultivation and chondrogenic differentiation

In preliminary experiments, a rapid cell death within the first 48 h was noticed without a proliferation phase, independent of the used fraction (CD271$^+$ MNCs, CD271$^-$ MNCs, and unsep MNCs). Therefore, an initial cultivation period of 14 days in DMEM/10% allogeneic AB serum was necessary and performed in this study.

Generated grafts of CD271$^+$ and unsep, but not CD271$^-$ MNCs yielded proliferating cell colonies after 14 days (data not shown), corresponding to the CFU-F formation. After 1 day of culture, the cell numbers corresponded to cell numbers at the time of seeding (CD271$^+$ MNCs: 9.0×10^3; CD271$^-$ MNCs 1.12×10^6; and unsep MNCs: 2.25×10^5). The cell number increased within the grafts of CD271$^+$ MNCs during cultivation (Fig. 3A; Supplementary Table S2). After the total cultivation time of 35 days, the cell number was 2.8-fold higher compared to the first day for CD271$^+$ MNCs grafts. In contrast, CD271$^-$ MNCs did not proliferate in the grafts and unsep MNCs showed only a slight proliferation. On day 35, the cell number in grafts with CD271$^+$ MNCs was 2.2-fold higher compared to grafts with unsep MNCs. These data were confirmed by qualitative live/dead staining of the cells within the grafts (Fig. 4). After seeding, only few viable CD271$^+$ MNCs were present, whereas CD271$^-$ and unsep MNCs showed a high number of viable cells. Proliferation and colony formation could be especially seen in grafts with CD271$^+$ MNCs and at a lower level, also in grafts with unsep MNCs during cultivation. In contrast, most of the CD271$^-$ MNCs died; after 35 days, nearly no viable cells were present.

To evaluate the chondrogenic differentiation potential and the amount of freshly produced extracellular matrix, the sGAG content was quantified within the grafts (Fig. 3B; Supplementary Table S3). The sGAG content of an acellular graft was 1.5–2.0 µg. In grafts with CD271$^+$ MNCs, sGAG production increased over time and reached its maximum on day 35, whereas grafts with CD271$^-$ MNCs showed no measurable sGAG deposition. The amount of sGAG in unsep MNC grafts increased only slightly over the whole cultivation period.

After change to and supplementation with the chondrogenic differentiation medium on day 14, the grafts of

Table 2. Cell Yield (Cells/cm^2) and the Ratio Gained Cell Number/10^6 Mononuclear Cells Initially Plated After Analysis-Related Monolayer Expansion of CD271$^+$, CD271$^-$, and Unsep Mononuclear Cells

	CD271$^+$ MNCs	*CD271$^-$ MNCs*	*Unsep MNCs*
Yield (cells/cm^2)	$2.18 \pm 1.16 \times 10^4$	$2.19 \pm 2.52 \times 10^3$	$1.66 \pm 0.79 \times 10^4$
Cell number/10^6 MNCs initially plated	$2.35 \pm 1.22 \times 10^7$	$1.61 \pm 1.80 \times 10^4$	$6.65 \pm 3.10 \times 10^5$

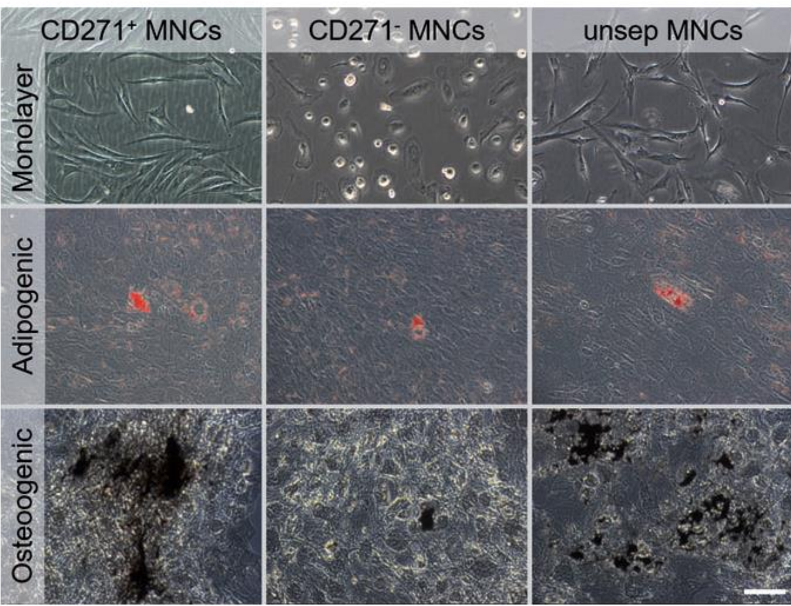


FIG. 2. Characterization of monolayer-expanded MNCs. Phase contrast microscopy of all MNC fractions after analysis-related monolayer expansion (day 13). Oil Red O staining of the adipogenic differentiation and von Kossa staining of the osteogenic differentiation of the MNC fractions; scale bar 100 μm. Color images available online at www.liebertpub.com/scd

$CD271^+$ and unsep MNCs immediately began to shrink, while $CD271^-$ MNC grafts remained unchanged with a wet weight of about 241 mg each. In minimum, the $CD271^+$ MNC grafts weighted 17±4 mg/graft, while unsep MNC grafts weighted 31±11 mg each after 35 days (Fig. 3C). Thus, proliferating cells induced shrinkage of collagen-based grafts in vitro.

Cryosections of the grafts after 35 days of culture were stained for the chondrogenic differentiation markers aggrecan and collagen type II (Fig. 5). The cryosections of grafts with $CD271^+$ and unsep MNCs showed a homogenous distribution of the cells within shape of dominant cell colonies. In contrast, $CD271^-$ MNC grafts only rarely showed solitary colonies within the graft. Aggrecan and collagen type II staining varied considerably between the MNC donors (Fig. 5). Collagen type II positive staining was observed in $CD271^+$ MNC grafts (5/8 donors) and unsep MNC (2/8) grafts. Due to individual grafts sizes, varying sizes of cryosections were present. In comparison to macroscopically healthy cartilage, 3D grafts of the $CD271^+$ group yielded a proceeding extracellular matrix production (Supplementary Fig. S1).

As mentioned in the Material and Methods section, different seeding densities were used, possibly causing varying cell numbers and sGAG contents in the grafts related to differences in CFUs. Thus, donor-specific analyses of CFU contents and their relationship ($CD271^+$ to unsep MNCs) were performed (Table 4). Indeed, higher CFUs in the $CD271^+$ MNCs compared to the unsep MNCs resulted in an obvious higher cell number and sGAG content in the graft of $CD271^+$ MNCs (eg, donor 1, Table 4). However, even if the

Table 3. Cell Surface Marker Expression of the Mononuclear Cell Fractions After Analysis-Related Monolayer Expansion According to International Society for Cellular Therapy Minimal Criteria for Mesenchymal Stromal Cells

	CD271⁺ MNCs (%)	*CD271⁻ MNCs (%)*	*Unsep MNCs (%)*	$P_{CD271+vs.\ CD271-}$	$P_{CD271+vs.\ unsep}$	$P_{CD271-vs.\ unsep}$
$CD45^-/CD271^+$	11.74±12.87	6.80±9.58	7.68±12.02	0.5831	1.0000	1.0000
$CD271^+$	12.03±13.13	11.71±10.10	7.85±12.06	1.0000	1.0000	1.0000
*$CD73^+$	**97.51 ± 4.34**	78.93±11.6	**97.13 ± 4.44**	0.0002	0.5251	0.0252
*$CD90^+$	**96.77 ± 3.54**	79.32±14.13	93.66±2.30	0.0001	0.3109	0.0291
*$CD105^+$	**97.63 ± 2.68**	93.56±11.66	**98.58 ± 5.28**	0.1379	1.0000	0.0238
$CD14^+$	**0.18 ± 5.59**	7.56±17.52	**0.88 ± 1.94**	0.0062	0.5038	0.2655
$CD19^+$	**0.50 ± 0.21**	**0.22 ± 7.31**	**0.21 ± 1.24**	1.0000	1.0000	1.0000
$CD34^+$	**0.37 ± 0.84**	**0.34 ± 0.18**	**0.26 ± 0.12**	1.0000	1.0000	1.0000
$CD45^+$	2.17±4.54	18.04±6.67	**1.51 ± 3.49**	0.0916	0.8385	0.8385
HLA-DR,-DP,-DQ$^+$	4.56±0.49	10.43±0.28	3.27±0.27	0.9912	1.0000	0.3143
$CD235a^+$	0.22±0.13	1.41±2.17	0.26±0.12	0.1547	1.0000	0.3143

For the fulfillment of the criteria, CD73, CD90, and CD105 should be expressed ≥95%, while CD14, CD19, CD34, CD45, and HLA-DR, HLA-DP, and HLA-DQ should be ≤2% (mean % viable singlet MNCs±SD and *P* values $n=6$; $*n=16$; ISCT criteria matches are marked in *bold*).

ISCT, International Society for Cellular Therapy.

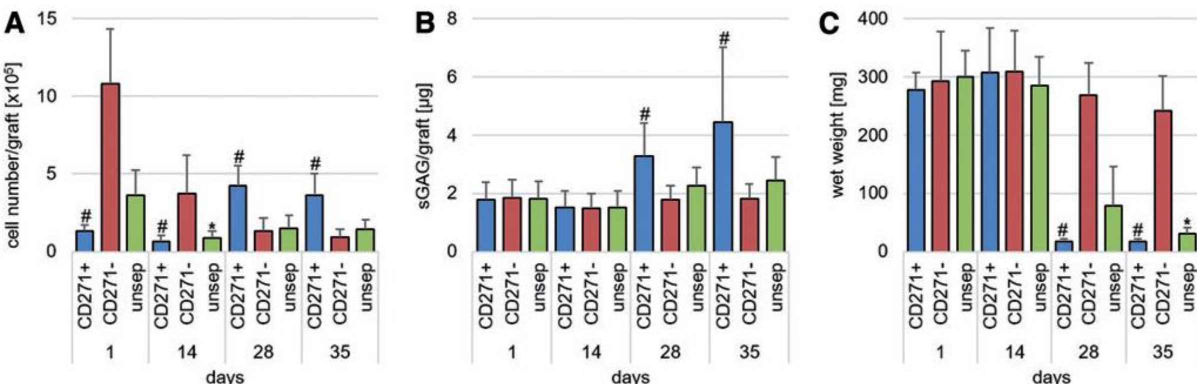


FIG. 3. Temporal characteristics of the MNC-seeded collagen I hydrogel grafts regarding cell number **(A)**, sGAG content **(B)**, and wet weights **(C)**. **(A)** Time course of the number of cells/graft after an initial seeding of 9.0×10^3 CD271$^+$, 1.125×10^6 CD271$^-$, and 2.25×10^5 unsep MNCs at the indicated days (#$P < 0.01$ CD271$^+$ compared to CD271$^-$, *$P < 0.05$ unsep MNCs compared to CD271$^-$). **(B)** Amount of freshly produced sGAG/graft; #$P < 0.05$ CD271$^+$ compared to CD271$^-$. **(C)** Wet weights of the grafts show the shrinking of grafts with CD271$^+$ and unsep MNCs after culture in chondrogenic differentiation media; #$P < 0.01$ CD271$^+$ compared to CD271$^-$ and *$P < 0.05$ unsep MNCs compared to CD271$^-$. sGAG, sulfated glycosaminoglycan. Color images available online at www.liebertpub.com/scd

CFUs in donors for CD271$^+$ MNCs and the unsep MNCs were quite similar (eg, donor 2, Table 4), cell number and sGAG in grafts of CD271$^+$ MNCs were higher compared to the unsep MNCs. Surprisingly, if the CFUs of the unsep MNCs were twofold higher than CD271$^+$ MNCs (eg, donor 4, Table 4), cell number and sGAG content in the grafts of the CD271$^+$ MNCs were still higher compared to the unsep MNCs. Therefore, the different seeding densities and/or resulting CFUs might have only a slight impact on the observed results.

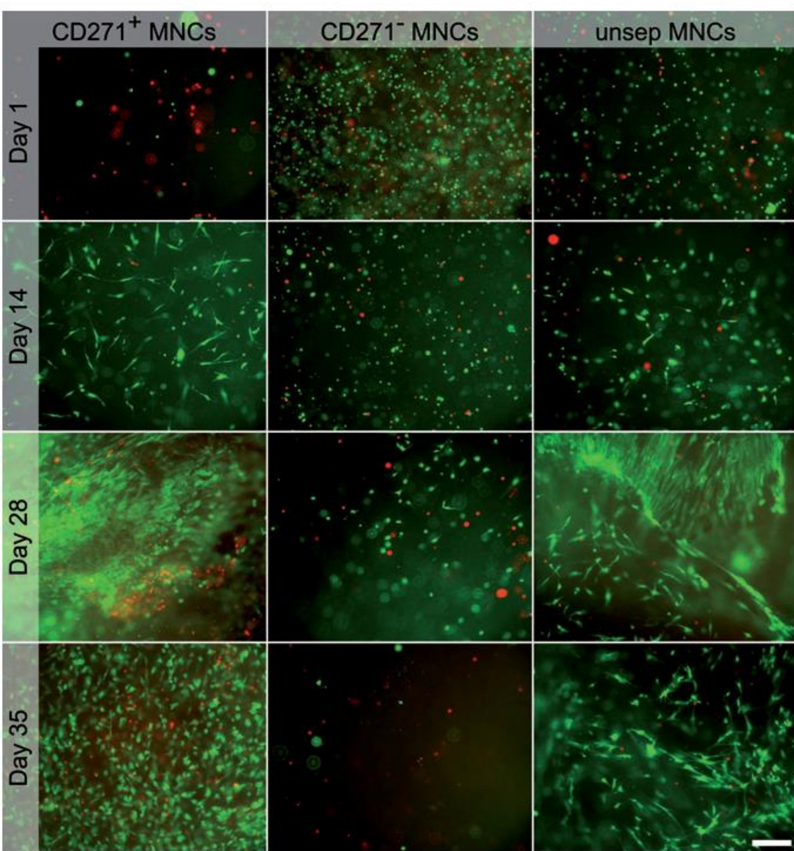


FIG. 4. Live/dead staining of CD271$^+$, CD271$^-$, or unsep MNC-seeded collagen I hydrogel grafts after 1, 14, 28, and 35 days. Viable cells are stained in *green*, while dead cells appear in *red*, scale bar 200 µm. Color images available online at www.liebertpub.com/scd

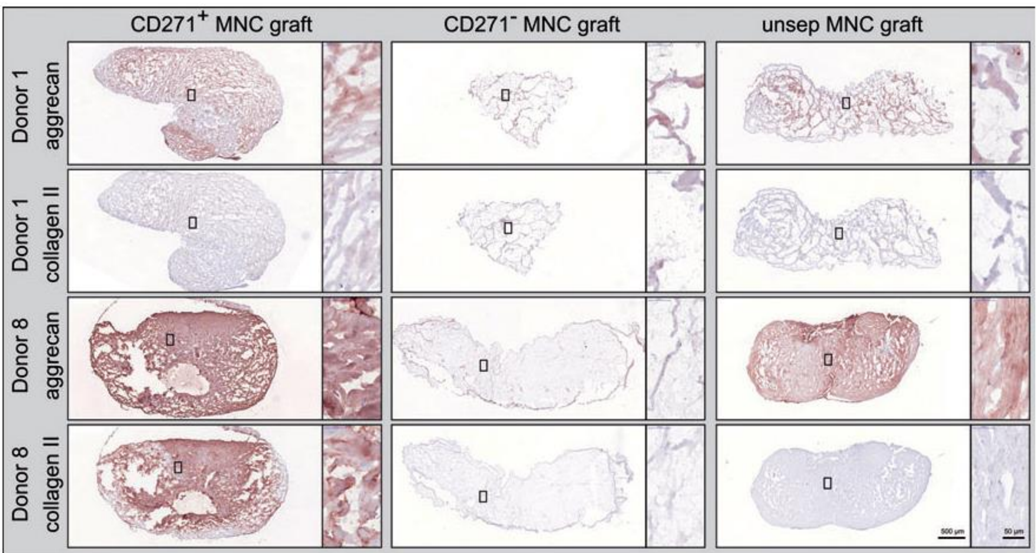


FIG. 5. Immunohistochemical staining for aggrecan and collagen type II of CD271^{+}, CD271^{-}, or unsep MNC-seeded collagen I hydrogel grafts. Shown are the donor (1) with the weakest and the donor (8) with the strongest staining intensity within the grafts, respectively, after 35 days of culture. Complete cross-sections through the shrunken grafts of CD271^{+} and unsep MNCs are shown. CD271^{-} grafts, which did not shrink, were divided into one-eighth sections; scale bar cross-section 500 μm, scale bar-magnified image 50 μm. Color images available online at www.liebertpub.com/scd

Discussion

Intraoperative strategies for the application of non-expanded MSCs or MNC subpopulations from BM already exist [21] and may lead the way to broad clinical application beyond their homologous use for bone regeneration and repair.

Native MSCs are widely available in vivo, for example, from adipose tissue, umbilical cord blood, synovial membrane, synovial fluid, periosteum, skin, trabecular bone, infrapatellar fat pad, and muscle [1,29]. Apart from BM-derived MSCs, adipose tissue-derived MSCs are assumed as a promising alternative for cartilage repair related to their large availability and the easy access [30].

In contrast to other tissues sources, such as the umbilical cord, BM and adipose tissue CD271 can be utilized to isolate MSCs [20]. Moreover, adipose-derived MSCs displayed similar characteristics as BM-MSCs regarding their potential for chondrogenic differentiation in 3D culture.

Nevertheless, their quality in tissue engineering of cartilage is discussed controversial due to differing results regarding their chondrogenic potential in comparison to BM-MSCs [31–35]. In addition, the outline translational single-stage preparation of MSCs for a cartilage graft might avoid substantial manipulating procedures, for example, enzymatic digestion of adipose tissue.

The utilization of a CD271^{+} MSC fraction (from BM) instead of unsep MSCs for cartilage repair strategies possibly has several benefits in vivo. The therapeutic superiority of CD271^{+} MSCs was recently shown in a rat cartilage defect model [19]. In this study, CD271^{+} MSCs showed a higher histological score in cartilage staining and fewer apoptotic cells when compared to the outcome of plastic-adherent unsep MSCs. In addition, intrinsic cartilage repair

Table 4. Colony-Forming Units Seeded/Graft and the Resulting Cell Number and Sulfated Glycosaminoglycan Content on Day 35 in the Cartilage Grafts for Each Donor

	CFUs seeded/graft			*Cell number/graft (×10^{5}) on day 35*			*sGAG/graft (μg) on day 35*		
Donor	*CD271^{+} MNCs*	*CD271^{-} MNCs*	*Unsep MNCs*	*CD271^{+} MNCs*	*CD271^{-} MNCs*	*Unsep MNCs*	*CD271^{+} MNCs*	*CD271^{-} MNCs*	*Unsep MNCs*
1	54.5	0.2	9.6	1.67	0.25	0.54	2.01	1.25	1.37
2	16.4	3.8	20.4	3.25	0.64	2.34	3.48	1.26	3.59
3	14.4	2.2	4.1	1.75	1.24	0.76	1.72	1.93	1.54
4	9.8	0.0	22.6	5.09	0.47	1.70	3.26	1.17	1.74
5	51.1	2.9	13.2	4.44	0.86	1.47	5.23	2.45	3.01
6	38.4	1.0	8.4	5.11	1.16	1.75	8.07	2.21	3.11
7	79.0	0.5	22.4	2.98	1.59	0.88	3.22	2.04	2.57
8	64.0	0.0	21.9	4.73	1.45	1.97	8.45	2.16	2.67

sGAG, sulfated glycosaminoglycan; CFU, colony-forming units.

mechanism can also be accompanied by proliferating and matrix-deposing CD271$^+$ cells either derived from the surrounding synovial membrane [36] or from the BM. Histological studies of knee and femoral heads from patients undergoing total knee or hip arthroplasty yielded the migration of CD271$^+$ cells from BM through the tidemark in cases of full-thickness cartilage defects [37,38].

In this study, we show the feasibility of generating a cartilage graft from CD271$^+$ BM-derived MNCs from eight donors in a CE-marked collagen type I hydrogel without previous monolayer expansion.

In the BM of the herein investigated eight healthy human donors, 0.01%–0.12% of the unsep MNCs were CD45$^-$/CD271$^+$. These observed frequencies were threefold higher when compared to published data [21,39], probably due to the young and uniform age of all donors in our study. Indeed, the frequency of native MSCs in BM depends on age [40] and various clinical comorbidities such as osteoarthritis [38] or myelodysplastic syndrome [41,42].

The direct MACS enriched the frequency of CD45$^-$/CD271$^+$ cells 165-fold and the number of CFU-F 66.8-fold in the CD271$^+$ MNC fraction compared to unsep MNCs. These data are in line with previous studies reporting a 37-fold and a 54-fold CFU-F enrichment in CD271$^+$ MNCs, respectively, and nearly no CFU-F formation in the CD271$^-$ MNC fraction [16,26,43]. As reported previously, CD271$^+$ separation using indirect MACS sorting or clinical-grade CD271 MicroBeads resulted in a purity of 40%–50%, comparable to the here-obtained purity using direct MACS [43,44].

After seeding into collagen type I hydrogels, the CD271$^+$ MNCs showed the highest proliferation rate, cell viability, sGAG deposition, and cartilage marker expression compared to CD271$^-$ and unsep MNC fractions. In grafts of CD271$^-$ MNCs, the cell number decreased continuously and cartilage matrix was absent. Final cell numbers and cartilage matrix deposition were higher in grafts from the CD271$^+$ MNC fraction compared to grafts from unsep MNCs. For cartilage grafts, a 0.26 μg sGAG/mg wet weight value was determined. Previous studies measured sGAG contents of 20–50 μg sGAG/mg wet weight of the femoral head cartilage in elderly patients with a femoral neck fracture using the Alcian Blue staining [45]. Nevertheless, it should be emphasized that differences occur by differing locations, age groups, and measurement methods [46]. Moreover, the varying sGAG contents and their comparison might be limited comparing in vivo cartilage and in vitro cartilage grafts. In addition, sGAG released into the supernatant was not measured, leading to an underestimation of the sGAG content produced by the cells seeded in collagen type I grafts.

A variety of materials have been utilized as potential scaffolds for cartilage repair. Due to an appropriate biodegradability, biocompatibility, control over cell–matrix interactions, and similar mechanical behavior to articular cartilage, hydrogels are widely used [47,48]. Based on previous obtained preclinical and clinical results for this study, the collagen type I hydrogel (CaReS™, Arthro Kinetics, Esslingen) was chosen [49,50].

Indeed, nonsimilar seeding densities might be a pitfall of this study, but the obtained results yielded similar cell numbers, viability, sGAG content, and histological structure compared to the higher seeded unsep MNCs. These differences might be most plausible caused by the threefold higher CFU content of the CD271$^+$ group leading to the here-presented data. Nevertheless, in donor-specific analyses of CFU contents, in cases of similar CFU contents of one donor, the CD271$^+$ MNCs displayed better results (Table 4). Moreover, the CD271$^+$ cell fraction was capable to produce similar cell numbers, even the CFU content of the unsep MSC in a donor was twofold higher.

In parallel to graft seeding, we expanded the various obtained MNCs by monolayer culture to evaluate them according to the minimal criteria of MSCs of the ISCT guideline [23]. CD271$^+$ and unsep MNCs fulfilled these criteria in almost all points. HLA-DR, HLA-DP, and HLA-DQ expression were slightly higher as the ISCT limit in both cell fractions. However, the CD90 expression was reduced in unsep MNCs. Based on their marker expression, colony-forming and multilineage potential CD271$^-$ MNCs showed a non-MSC phenotype.

This study has several experimental limitations besides the above-mentioned variation of differing seeding densities. First, we could not correlate the numbers of CFU-Fs with the CD271$^+$ MNC amount, because of donor-dependent variations with regard to separation frequency, cell purity, and yield. Second, as found in preliminary experiments, culturing gels without a proliferation phase were marked by a massive cell death within the first 48 h. Dexheimer et al. highlighted the close link of proliferation and differentiation in chondrogenesis. Therefore, a proliferation stimulating environment is a crucial basis for an efficient chondrogenic differentiation of MSCs [51].

However, for the use of a single-stage approach in vivo, the question arises to what extent a simultaneous proliferation and differentiation would take place. For this purpose, in vivo tests are necessary in future studies. This effect is well known from standard chondrogenic pellet culture [51] and only proliferating MSC colonies are capable to differentiate into chondrocytes. Third, the presented data focus only on BM-derived MSCs. Further MSC sources are known to give rise to potent MSC populations. Finally, it remains unclear which particular, minimal, dose of CD271$^+$/CD45$^-$ cells ensures the optimal generation of a cartilaginous graft, which in turn induces effective and sustainable cartilage regeneration in vivo. However, the identification of the ideal cell dose was typically conducted in a phase 2 clinical trial [52].

In summary, because of the limited self-healing capacity of cartilage defects, the need for potent, safe, and easily applicable cellular therapies is underlined. In this study, MSCs are a promising cell source for cartilage regeneration. An intraoperative procedure to enrich MSCs without a previous monolayer expansion as shown here, will overcome a cost-, time-, and personnel-intensive GMP production of cartilage grafts. This is the first study demonstrating that CD271$^+$ MNCs are capable of forming appropriate cartilage grafts with high cell viability and chondrogenic matrix deposition, in combination with a clinically approved collagen type I hydrogel without a monolayer expansion.

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Author Disclosure statement

No competing financial interests exist.

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4 Summary

Regenerative treatment of focal hyaline cartilage defects could prevent or delay the development of secondary osteoarthritis. Current surgical techniques result partly in i) the formation of mechanically inferior fibrous cartilage or ii) present the disadvantage of the donor site morbidity from harvesting cartilage biopsy as well as iii) the dedifferentiation of chondrocytes due to *in vitro* expansion and iv) the reduced re-differentiation potential of *in vitro* expanded chondrocytes.

The self-healing capacities of injured and degenerated articular cartilage revealed a promising target cell population for a regenerative, autologous treatment of these defects using mesenchymal stromal cells (MSCs). Several case studies, randomized and controlled clinical studies showed the general ability of autologous, bone marrow-derived, expanded MSC transplantation to regenerate articular cartilage lesions [48]. However, these two-stage approaches are based on time- and cost-consuming expansion of MSCs under good manufacturing practice (GMP) conditions and hold a risk of contamination during this process.

In 2010, CD271, the low-affinity nerve growth factor receptor, was described as a suitable surface marker to enrich MSCs from human bone marrow aspirate intraoperatively [56].
The aim of the present dissertation was to investigate the feasibility of generating cartilage grafts from either ovine (study no. 1) and human (study no. 2) non-expanded $CD271^+$ bone marrow cells in a collagen type I hydrogel.

Study no. 1 ("Point-of-care treatment of focal cartilage defects with selected chondrogenic mesenchymal stromal cells - An *in vitro* proof-of-concept study") investigated several surface marker candidates for the prospective MSC separation and examined their potential of resulting colony-forming units, respective their yield of potent MSCs [90]. This study was conducted with ovine bone marrow samples. CD271 was the most effective surface marker to isolate the target cell population. Subsequently, $CD271^+$, $CD271^-$ and unseparated mononuclear cells (MNCs), containing the MSCs, were used to generate cartilage grafts without an expansion of these cells in monolayer culture. It could be proven, that ovine $CD271^+$ cells were able to generate a potent hyaline cartilage graft.

Study no. 2 ("Single-stage preparation of human cartilage grafts generated from bone marrow-derived $CD271^+$ mononuclear cells") was performed as the final translational step from animal-derived bone marrow to human donor material and is therefore strengthening the therapeutically focus of the entire work [91].

Briefly, eight bone marrow aspirates were used for MNC isolation and subsequent magnetic cell separation (MACS). The resulting $CD271^+$ and $CD271^-$ MNCs were compared to unseparated MNCs. Subsequently, they were seeded in a clinically approved collagen type I hydrogel and cultivated for up to 5 weeks to investigate the progression of the chondrogenic differentiation processes. Graft analysis included cell viability visualization by live/dead staining, determination of the DNA and the secreted sulphated glycosaminoglycan (sGAG) content as well as the immunohistochemical staining for typical chondrogenic differentiation markers and the extracellular matrix molecules aggrecan and collagen type II.

A proliferation of cells in the generated grafts was shown of $CD271^+$ and unsep, but not $CD271^-$ MNCs. Hence, the cell number was 2.8-fold higher after 35 days compared to the first day for $CD271^+$ MNCs grafts, while $CD271^-$ MNCs did not proliferate in the grafts and unsep MNCs showed only a slight increase in cell number.
The chondrogenic potential was measured by quantification of freshly produced sGAGs and the expression of chondrogenic markers. In grafts with $CD271^+$ MNCs, sGAG production increased over time and reached its maximum at day 35, whereas grafts with $CD271^-$ MNCs showed no measurable sGAG deposition. The amount of sGAG in unsep MNC grafts increased only slightly over the whole cultivation period. Aggrecan and collagen type II staining varied considerably between the MNCs donors. Collagen type II positive staining was observed in $CD271^+$ MNC grafts (5/8 donors) and unsep MNC (2/8) grafts. In comparison to macroscopically healthy cartilage, three-dimensional grafts of the $CD271^+$ group yielded a proceeding extracellular matrix production.
In summary, $CD271^+$ MNCs showed the highest proliferation rate, cell viability, sGAG deposition and cartilage marker expression compared to the $CD271^-$ or unseparated MNC fractions in *in vitro* generated three-dimensional cartilage grafts.
Therefore, the presented work demonstrated the feasibility of generating a cartilage graft from $CD271^+$ bone marrow-derived MNCs in a clinically approved collagen type I hydrogel without a previous monolayer expansion of these cells. This will enable the intraoperative purification of $CD271^+$ MNCs, which contain the majority of colony-forming MSCs, by MACS technology. The clinical application will be possible with currently available and clinical approved cell separation devices.

Providing a cartilage graft with non-expanded $CD271^+$ MNCs by a fast and simple intraoperative therapeutic approach fulfils the need for a “single-step, tissue-engineered solution to focal cartilage defects, and elimination of the morbidity of the donor defect” as requested by the editors of the journal *Arthroscopy* [47].

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6 Appendix

Appendix to manuscript “Point-of-care treatment of focal cartilage defects with selected chondrogenic mesenchymal stromal cells - An *in vitro* proof-of-concept study”:

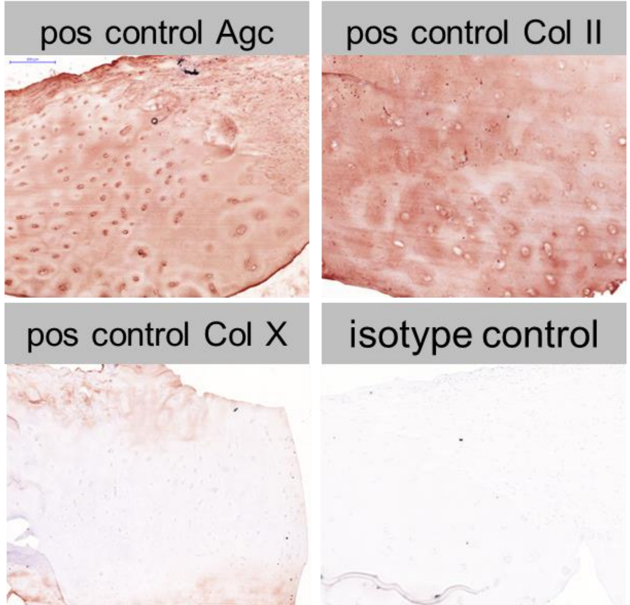
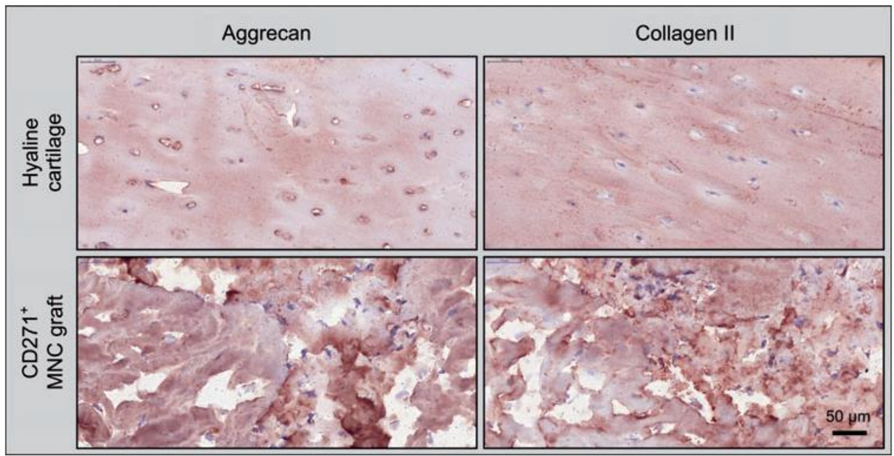


Figure S1: Positive and negative controls for immunohistochemical staining. Human adult hyaline cartilage was used as a control for each antigen staining. Images are displayed in 100× magnification.

Appendix to manuscript "Single-stage preparation of human cartilage grafts generated from bone marrow-derived CD271+ mononuclear cells":

Supplementary Data



SUPPLEMENTARY FIG. S1. Aggrecan and collagen type II immunohistochemistry of human hyaline cartilage and CD271^{+} MNC-seeded collagen I hydrogel grafts of donor eight revealing hyaline cartilage-specific produced extracellular matrix. Scale bar 50 µm.

SUPPLEMENTARY TABLE S1. ANTIBODIES USED FOR PHENOTYPIC CHARACTERIZATION AND IMMUNOHISTOCHEMISTRY

Antigen	*Clone*	*Isotype*	*Label*	*Source*	*Panel*
CD271*	ME20.4-1.H4	IgG1	APC	Miltenyi	3
CD73	AD2	IgG1κ	PerCP-Cy5.5	BD	1, 2
CD90	DG3	IgG1	PE-Vio770	Miltenyi	1, 2
CD105	43A4E1	IgG1	PE	Miltenyi	1, 2
CD14	TÜK4	IgG2a	FITC	Miltenyi	1
CD19	LT19	IgG1	APC-Vio770	Miltenyi	1
CD34	AC136	IgG2a	FITC	Miltenyi	2
CD45*	5B1	IgG2a	PE	Miltenyi	3
HLA-DR,-DP,-DQ	REA332	rh IgG1	APC-Vio770	Miltenyi	2
CD235a*	HIR2	IgG2b	FITC	Acris	3
Mouse IgG2a isotype control	S43.10	IgG2a	FITC	Miltenyi	—
Mouse IgG1 isotype control	IS5-21F5	IgG1	APC-Vio770	Miltenyi	—
Mouse IgG1 isotype control	IS5-21F5	IgG1	PE	Miltenyi	—
Mouse IgG1κ isotype control	MOPC-21	IgG1κ	PerCP-Cy5.5	BD	—
Mouse IgG1 isotype control	IS5-21F5	IgG1	PE-Vio770	Miltenyi	—
Mouse IgG1 isotype control	IS5-21F5	IgG1	APC	Miltenyi	—
REA control	REA293	—	APC-Vio770	Miltenyi	—
Mouse IgG2b isotype control	TEN-0	IgG2b	FITC	Acris	—
Mouse IgG2a isotype control	S43.10	IgG2a	PE	Miltenyi	—
Aggrecan	HAG7D4	IgG1	—	Acris	—
Collagen type II	II-4C1	IgG	—	MP Bio-medicals	—

*Negative gates are set by fluorescence minus one control.

Supplementary Table S2. Data from Preliminary Experiments on $CD271^+$ Mononuclear Cell Yield

Donor of preliminary experiments	*mL BM/donor*	*$\times 10^6$ MNCs/mL BM*	*$\times CD271^+$ MNCs yield after MACS*
A	30	1.51	0.86
B	55	1.79	2.78
C	60	3.44	5.41
D	30	2.94	1.61
Mean	43.8	2.42	2.67

MACS, magnetic cell separation.

Supplementary Table S3. Temporal Characteristics of the MNC-Seeded Collagen I Hydrogel Grafts Regarding Cell Number, sGAG Content, and Wet Weights Presented as Mean ± Standard Deviation

		Cell number/graft ($\times 10^5$)	*sGAG/graft (μg)*	*Wet weight (mg)*
1 day	$CD271^+$	#1.31 ± 0.43	1.77 ± 0.61	277 ± 31
	$CD271^-$	10.80 ± 3.50	1.83 ± 0.63	292 ± 85
	Unsep	3.61 ± 1.66	1.80 ± 0.62	300 ± 44
14 days	$CD271^+$	#0.63 ± 0.39	1.51 ± 0.56	307 ± 77
	$CD271^-$	3.73 ± 2.49	1.48 ± 0.53	308 ± 71
	Unsep	*0.90 ± 0.41	1.52 ± 0.55	284 ± 49
28 days	$CD271^+$	#4.24 ± 1.27	#3.29 ± 1.11	#18 ± 4
	$CD271^-$	1.35 ± 0.80	1.79 ± 0.48	269 ± 54
	Unsep	1.51 ± 0.80	2.25 ± 0.63	79 ± 67
35 days	$CD271^+$	#3.63 ± 1.42	#4.43 ± 2.59	#17 ± 4
	$CD271^-$	0.96 ± 0.48	1.81 ± 0.51	241 ± 61
	Unsep	1.43 ± 0.64	2.45 ± 0.81	*31 ± 11

Cell number: Time course of the number of cells/graft after an initial seeding of 9.0×10^3 $CD271^+$, 1.125×10^6 $CD271^-$, and 2.25×10^5 unsep MNCs at the indicated days (#$P < 0.01$ $CD271^+$ compared to $CD271^-$, *$P < 0.05$ unsep MNCs compared to $CD271^-$).

sGAG: Amount of freshly produced sGAG/graft; #$P < 0.05$ $CD271^+$ compared to $CD271^-$.

Wet weight: Wet weights of the grafts show the shrinking of grafts with $CD271^+$ and unsep MNCs after culture in chondrogenic differentiation media; #$P < 0.01$ $CD271^+$ compared to $CD271^-$ and *$P < 0.05$ unsep MNCs compared to $CD271^-$.

Declaration of the doctoral student's scientific contribution to the publication

Declaration of the scientific contribution to the publication "Point-of-care treatment of focal cartilage defects with selected chondrogenic mesenchymal stromal cells - An in vitro proof-of-concept study" published 2018 in the Journal of Tissue Engineering and Regenerative Medicine.

The lead authorships Oliver Pelters and Christian Schmidt contributed equally to this work.

The contribution of Oliver Pelters included the experimental study setup (50%), the practical lab work (50%) (with technical assistance by Christian Schmidt, Christian Thümmler and Frank Peinemann), the data analysis (100%), the statistical analysis (100%), and the discussion (100%) to the study part for direct chondrogenic differentiation of ovine $CD271^+$ mesenchymal stromal cells in a 3D hydrogel.

The contribution of Christian Schmidt included the experimental study setup (50%), the practical lab work (50%) (with technical assistance by Oliver Pelters, Christian Thümmler and Frank Peinemann), the data analysis (100%), the statistical analysis (100%), and the discussion (100%) to the study part for marker candidate testing for ovine mesenchymal stromal cells isolation without a prior monolayer expansion.

Both lead authors contributed equally in the literature research (50%), manuscript writing (50%) and manuscript submission (40%).

Dr. Matthias Zscharnack, Dr. Jeremy Somerson and Dr. Ronny Schulz performed the proposal approval of the study for the local ethics committee, assisted with scientific advices, proof reading and manuscript submission.

24.08.2018	
Date	Signature – Christian Schmidt
24.08.18	
Date	Signature – Christian Thümmler
31.08.'18	
Date	Signature – Dr. Matthias Zscharnack
17.8.2018	
Date	Signature – Dr. Ronny Schulz (Corresponding author)

Declaration of the doctoral student's scientific contribution to the publication

Declaration of the scientific contribution to the publication "Single-stage preparation of human cartilage grafts generated from bone marrow-derived CD271+ mononuclear cells" published 2018 in Stem Cells and Development.

The contribution of the lead author included the experimental study setup (100%), the practical lab work (75%) (with technical assistance by Christian Schmidt, Christian Thümmler and Frank Peinemann), the data analysis (100%), the statistical analysis (100%), the literature research (100%), manuscript writing (90%), the discussion (100%) and manuscript submission (90%).

Dr. Ralf Henkelmann and Dr. Bastian Marquaß performed the collection of human cartilage biopsies and wrote the proposal approval of the study for the local ethics committee. PD Dr. Gero Hütter performed the collection of human bone marrow samples and assisted with scientific advices.

24.08.2018 — Date

Signature – Christian Schmidt

20.08.2018 — Date

Signature – Dr. Ralf Henkelmann

20.08.18 — Date

Signature – Dr. Philipp Pieroh

31.08.18 — Date

Signature – Prof. Dr. Gabriela Aust

17.8.2018 — Date

Signature – Dr. Ronny Schulz (Corresponding author)

7 Declaration of Authorship

Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

______________________________ ______________________________

Ort, Datum Unterschrift

8 Curriculum vitae

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Education and Training

04/2012-03/2017	Research associate at the University of Leipzig, Centre for Biotechnology and Biomedicine Leipzig, Saxon Incubator for Clinical Translation (formerly Translational centre for Regenerative Medicine) Leipzig
03/2012-03/2017	Visiting scientist at the Fraunhofer Institute for Cell Therapy and Immunology IZI Leipzig
07/2011-03/2012	Master thesis at the Translational centre for Regenerative Medicine Leipzig
10/2009-03/2012	University of Lübeck Molecular Life Science, M.Sc.
05/2009-08/2009	Bachelor thesis at the Centre for Biotechnology and Biomedicine Leipzig, Professorship Cell Techniques and Applied Stem Cell Biology
10/2006-08/2009	University of applied Science Zittau/Görlitz Biotechnology, B.Sc.

9 Publications

Petters O, C Schmidt, R Henkelmann, P Pieroh, G Hütter, B Marquass, G Aust and RM Schulz. (2018). Single-Stage Preparation of Human Cartilage Grafts Generated from Bone Marrow-Derived CD271^{+} Mononuclear Cells. Stem Cells Dev 27:545–555.

Petters O*, C Schmidt*, C Thuemmler, F Peinemann, M Zscharnack, JS Somerson, and RM Schulz. (2018). Point-of-care treatment of focal cartilage defects with selected chondrogenic mesenchymal stromal cells – an *in vitro* proof-of concept study. J Tissue Eng Regen Med
**These authors contributed equally to this work*

Wallenborn M*, **O Petters***, D Rudolf, H Hantmann, M Richter, P Ahnert, L Rohani, JJ Smink, GC Bulwin, W Krupp, RM Schulz and H Holland. (2018). Comprehensive high-resolution genomic profiling and cytogenetics of human chondrocyte cultures by GTG-banding, locus-specific FISH, SKY and SNP array. Eur Cell Mater 35:225–241.
**These authors contributed equally to this work*

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