

chapter **EIGHT**

GENERAL DISCUSSION

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Osteochondral defects are becoming an increasing burden for the future society due to aging of the population leading to chronic pain and functional disability. Different treatment strategies are so far employed to treat these defects based on the age of the affected patient and the extent to which the joint is injured, ranging from replacement with a prosthesis in elderly patients with disseminated osteoarthrotic joints to bone marrow stimulation techniques, the microfracture technique or subchondral drilling technique for defects up to 2 cm². Larger defects of around 3-4 cm² are mostly treated with mosaicplasty or allografting, whereas the biggest defects are more and more being replaced with tissue engineered constructs, such as the first generation autologous chondrocyte implants (ACI) or third generation matrix-associated chondrocyte implants (MACI). Disadvantages and disappointing results of the current treatment modalities lead to ongoing studies on tissue engineering of osteochondral defects.

In view of the above, the general aim of the research described in this thesis was to develop a challenging new concept in cartilage tissue engineering: the one-step surgical procedure (OSP) for cartilage repair using adipose-derived MSCs (see introduction and Helder et al¹ for details). Different issues of the procedure were studied *in vitro*. Regarding the cellular component, we compared the suitability of adipose tissue as cellular source with dermis, and investigated different adipose tissue sites for ASC harvesting. In order to establish the necessity of stem cell triggering towards the desired lineage, we compared growth factor induction with biophysical stimulation under micro-environmental conditions. Furthermore, we evaluated two different scaffolds for their compliance with the OSP (e.g. rapid adherence of ASC, chondrogenic differentiation). Finally, we performed an *in vivo* proof-of-concept study to get this concept closer to clinical application. The results of our findings are described in the various experimental chapters, and the major outcomes are summarized in chapter 9.

This OSP is only one concept in cellular therapies for cartilage regeneration. The use of adipose derived stem cells is increasing, but currently only explored at the preclinical phase. In comparison, at the time of writing this thesis, 26 clinical trials are being performed evaluating the use of chondrocytes in fourth generation MACI therapies. As the term implies, numerous improvements have been made so far to the MACI concept, from the addition of a periosteal flap to scaffold materials or hydrogels. Although this might finally result in a feasible cellular therapy, recent *in vitro* studies showed an inferior quality of aggrecan formed by native cartilage when compared with BMSC². We therefore believe that the use of undifferentiated cells are preferred over fully differentiated chondrocytes.

In chapter 2 we compared the use of adipose stem cells with dermal stem cells and showed that both cell types have similar stem cell phenotypes, chemokine receptor profiles and trilineage potential. Therefore these DSC might be an interesting complementary cell source when available during surgery (e.g. in abdominoplasty). However, trilineage potential was diminished in comparison to adipose stem cells. Furthermore, the isolation protocol for the procurement of these cells lasted significantly longer than for the isolation of adipose stem cells. Therefore we considered dermal stem cells not feasible for the OSP for cartilage tissue engineering and continued our studies with adipose derived stem cells.

Concerning the feasibility comparison between bone marrow- and adipose-derived stem cells in regenerative therapies, *in vitro* and *in vivo* studies show differentiation capacities varying from inferior to superior³⁻¹⁰. Whether those differences are due to donor or species variation, test model or intrinsic properties remains elusive. Additional studies, e.g. gene expression profiling, will likely provide more insight in this matter. Nevertheless, we can use the clinical experience with the bone marrow-derived MSCs (at least seven clinical studies are currently ongoing) to our advantage, and select the proper conditions for similar studies with ASCs. It is clear that until then, definite conclusions on the superiority of each cell source in these cellular therapies and the molecular and cellular mechanisms underlying these differences cannot be made. However, we expect that the current and future studies with BMSCs and ASCs will show the benefit of using undifferentiated cells in these regenerative therapies.

A very important issue regarding the development of new therapies is the cost-effectiveness of the product. If a tissue engineered product is too costly to produce, it has no market value. Especially products containing, for example, expensive growth factors or complex manufacturing, or procurement of cells via difficult processing methods and subsequent costly transfection to include one or more anabolic factor or inflammatory inhibitor, are much more expensive than the conventional easy to apply subchondral drilling technique, which is currently the first line treatment for focal cartilage defects as stated earlier. Concerning this issue our innovative one-step procedure strategy overcomes the need for expensive *in vitro* expansion and repeated surgeries, putting it well ahead of cellular therapies involving chondrocytes and bone marrow stem cells, which application involves these expansion steps.

FUTURE PERSPECTIVES

Cartilage tissue engineering strategies are becoming amazingly popular these days to treat cartilage defects which have a limited self-regenerative capacity. Although results are very promising so far and clinical studies are ongoing and/or the first results are emerging, still many aspects have to be unravelled, e.g. biological processes of cartilage development and the possible value of gene therapy. This thesis focussed on the development of a one-step surgical procedure with adipose derived stromal cells to treat articular cartilage defects and therefore I will focus on future aspects regarding this concept. We tested different *in vitro* aspects involved in the feasibility of this concept, like 1) the use of dermal stem cells as an alternative or complementary cell source, 2) preferential adipose tissue harvesting site, 3) the effect of the physiological environment on chondrogenic differentiation by mimicking the joint environment and 4) testing the suitability of two polymeric scaffold materials for application in osteochondral defects. Moreover, we evaluated this one-step surgical procedure *in vivo*. For this purpose, we seeded freshly isolated stromal cells onto a collagen type I/III scaffold and implanted this construct into a caprine knee defect. The results of this proof-of-concept study greatly enhance the feasibility of the concept towards clinical application.

In the *in vivo* study, osteochondral defects of 5 mm diameter with a depth of 3.5 mm were created in the knee of goats. Although this can be regarded as a critically sized defect^{11,12}, larger defects should be tested to demonstrate a) a similar regenerative effect and b) significant differences between the cell treated group versus the scaffold only group. Larger groups are required (based on power calculations) to demonstrate significant results, as wide donor variation is present in the caprine study groups. Long term follow-up results should be obtained, as persistent results of the applied treatment are of eminent importance in showing the additive value of the implanted SVF cells. Summarizing, these aspects should be addressed and covered before the one-step surgical concept for cartilage regeneration is ready for clinical implementation.

In addition, new cell sources are still being discovered and investigated for their potential application in cartilage tissue engineering, e.g. synovium derived cells, cord blood cells^{13,14}, induced pluripotent stem cells (iPS)¹⁵, and muscle-derived stem cells¹⁶. These cells might sound interesting due to similar ontogenic development (synovial cells), or pluripotency (in case of unrestricted somatic stem cell (USSC) from placental cord blood¹³ or iPS¹⁵). All three cell types have shown promising regenerative potential *in vitro*¹⁷ and/or *in vivo*^{7,9,18}. However, as holds true for the use of chondrocytes, due to limited supply and/or allogenic character of these cells their final use might be limited. The use of iPS might be limited due to teratoma formation¹⁸. As for the USSC, tissue banking might offer a solution to this problem, allowing storage of autologous cord blood MSC in the near future for later regenerative therapies. Moreover, since more and more recent reports indicate that allogeneic use of adult stem cells may be feasible due to the immunosuppressive actions attributed to these cells, off-the-shelf use of liquid nitrogen-stored ASC- or BMSC-preparations which are cultured to homogeneity may be an alternative option as well. Yet another source of stem cells may be fetal tissue-derived cells, of which our department, in collaboration with the Burn Centre Beverwijk, has shown that *in vitro* cartilaginous differentiation could easily be accomplished. Since these cells do not express tissue-specific surface markers, these cells will not be rejected by the host, and may therefore be widely applicable for use in a broad scala of tissues. It may be clear from the above that the debate about which cell source may be optimal is far from solved, and should be a core subject of intense investigations in the coming years.

To induce adipose derived stem cells into the desired lineage we showed in our OSP that submitting cell-loaded constructs directly to the physiological joint conditions without any preconditioning gives rise to similar or even superior chondrogenic differentiation, at least *in vitro*. The observation that the conditions present in the local microenvironment may already be sufficient for proper induction of the chondrogenic phenotype in and cartilage regeneration by freshly administered SVF cells, is strongly supported by our *in vivo* caprine knee defect study. These results can overcome the big hurdle that translation of a promising therapy into the clinical situation is often hampered by the use of inductive growth factors due to strict regulations by official authorities^{19,20}. It might be that preconditioning of the SVF cells before implanting them will have an additional effect on the regeneration of the damaged tissue. Bearing in mind the strict regulations, future research should focus on preconditioning of the implantable cells using biochemical cues

like hypoxia or hyperosmolarity, or biophysical cues like biomechanical or ultrasound stimulation instead of induction with growth factors.

Another interesting option might be the application of gene transfected cellular therapy for the treatment of cartilage disorders (for a review see Steinert et al.²¹). *In vitro* data have shown promising results regarding the chondrogenic potential of these transfected cells, but only few *in vivo* studies are yet conducted to confirm these promising results²²⁻²⁷. Since cartilage injuries are not life-threatening, the potential of this technology for clinical use strongly depends on the development of safe and efficient vectors, transgenes and delivery systems²¹. Therefore future research looking into the potential of these gene-transfer approaches have to address these topics before clinical studies can be performed.

The choice we have to make for which scaffold material to apply, should be tailored to the type of defect that has to be regenerated, i.e. chondral versus subchondral defects. Whereas in chondral defects only the cartilaginous part has to be reconstituted, subchondral defects are composed of a rigid osseous phase and a porous, highly elastic chondral phase. In our studies we tested the feasibility of two different scaffold materials for their possible use in the one-step surgical procedure to treat cartilage defects. Although these scaffolds both allowed chondrogenic differentiation of SVF cells from adipose tissue *in vitro* (Chapter 4 and 5), as well as regeneration of osteochondral defects in a caprine knee defect (Chapter 7), the use of more sophisticated biphasic scaffolds resembling both bone and cartilage characteristics might be preferable. To this end, analytical and computational modeling techniques designing the ultimate scaffolds^{28,29} might be applied, resulting in scaffolds with zonal architecture using novel fabrication techniques (including the solid free form (SFF) fabrication, formerly called rapid prototyping)³⁰. Furthermore, the functionalization of scaffolds might be enhanced by incorporation of agents that promote cell adhesion^{31,32} or growth factors^{31,33-35}, thereby serving as delivery vehicle. Geometrical modifications, like oriented pores and varying pore sizes³⁶⁻⁴³, or materials mimicking the extracellular matrix of cartilage like hybrid poly-(lactic-co-glycolic acid) (PLGA)-gelatin/chondroitin/hyaluronate (PLGA-GCH)^{31,35,44-46}, or the use of the natural acellularized extracellular cartilage matrix itself^{47,48}, have also been shown to enhance the differentiation potential of mesenchymal stem cells.

Hydrogels provide a promising starting point to create a scaffold that can be implanted in a one-step procedure with limited morbidity. They mimic the extracellular matrix of cartilage by their high water content creating a protective environment. In addition, growth factors and cell-signalling molecules can diffuse freely throughout the gel, in situ gelation allows the hydrogel to accurately and completely fill irregularly shaped defects and the injectable nature of hydrogels has introduced the possibility of arthroscopic implantation of engineered articular cartilage. Recently, promising *in vitro* experiments showed that these hydrogels can provide a suitable environment for MSC survival, chondrogenesis, and ECM production⁴⁹. Moreover these hydrogels allow for the release of growth factors from microspheres, or the addition of chondroitin sulphate (CS) which enhanced both chondrogenic gene expressions and cartilage specific matrix production, while inhibiting the further differentiation of MSCs into hypertrophic chondrocytes⁵⁰. In conclusion, hydrogels might become a very important biomaterial in the near future. To this end we are planning *in vivo* studies combining freshly isolated adipose derived stromal cells with

a patented, clinically approved fibrin hydrogel. Results from our *in vivo* pilot-study will not only be reproduced, but also expanded to larger (4 cm²) cartilage defects, and longer follow-up period. We expect that these preclinical studies will provide sufficient and sound information to support translation to clinical implementation.

In our *in vivo* study an osteochondral defect was created in the medial condyle and trochlear groove of the goat knee, allowing press-fitting of the implanted scaffold. Furthermore, the construct used in this study was sutured to guarantee containment of scaffolds in the defect site. However, suturing causes trauma to the adjacent 'healthy' cartilage leading to osteoarthritic degeneration⁵¹. Therefore, this method may be only used in preclinical *in vivo* studies, but in the clinical situation should be replaced by other methods like the use of hydrogels or sealant to glue the scaffold material to the defect site. This sealant should be autologous in nature, as the use of commercial fibrin sealant (Tissucol®) caused an immunological reaction to the cells leading to cell death in our pilot-study as was also described by others⁵². Another option to obtain better integration can be found in postoperative immobilization, allowing integration of the scaffold material in the surrounding tissue. To this end, we immobilized the goat knees for 4 weeks using special designed Softcast®. As a result no scaffolds were lost due to detachment. Whether this immobilization phase should be added in the clinical phase and more specifically for what time-frame, should be investigated into further detail, as this aspect is currently unknown for this specific application and was not tested in this study. Hopefully the problem of implant fixation can be overcome in the near future by the synthesis of new smart biological glues (e.g. from mussels or frogs) to fixate the scaffold material in the defect, or resolved by the introduction of sticky hydrogels, or the development of scaffoldless therapies with genetically transduced cells with sticky characteristics due to up-regulation of genes involved in cell adhesion (e.g. integrins, collagen receptors).

Despite the fact that the development of a new technique faces numerous challenges in design to improve the outcome, regulatory aspects should not be overlooked in the translation process, also known as advanced therapy medicinal products (ATMP) hurdles. Since the OSP can be regarded as medicinal product it has to meet strict conditions relating to product definition, manufacturing process (GLP, cGMP, and Standard Operating Procedures), and quality control. In this regard, questions which have to be addressed are e.g. 1) what is the bioactive substance, and 2) is the product for homologous (adipose to adipose) or heterogeneous (adipose to bone/cartilage) use? Furthermore the institution should obtain a licence for intended use. Recently, a review by Gimble et al. addresses these issues to make them transparent in order to accelerate the standardization and reproducibility of adipose-derived cell therapies with respect to their efficacy and safety⁵³. Next to these regulatory aspects, it should not be forgotten that in the end the clinician should embrace the newly developed therapy and add it to his armoury. Issues like user-friendliness, patient-friendliness and cost-effectiveness should thus be kept in mind. Although probably at least five generations OSP are ahead of us, the preclinical results thus far raise enough challenges to translate it into a safe and sound clinical product!

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