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The use of mesenchymal stem cells for chondrogenesis

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Summary[1] The application of autologous chondrocytes in cartilage repair procedures is associated with several disadvantages, including injury of healthy cartilage in a preceding surgery frequently resulting in formation of inferior fibrocartilage at defect sites. In order to improve the quality of regeneration, adult mesenchymal stem cells (MSC) are regarded as a promising alternative. The great challenge, when considering MSC for articular cartilage repair, is to generate cells with features of stable chondrocytes which are resistant to hypertrophy and terminal differentiation, as found in hyaline articular cartilage. Common *in vitro* protocols for chondrogenic differentiation of MSC successfully induce expression of multiple cartilage-specific molecules, including collagen type II and aggrecan, and result in a chondrocyte-like phenotype. However, *in vitro* chondrogenesis of MSC additionally promotes induction of fibrocartilage-like features such as expression of collagen type I, and hypertrophy, as demonstrated by up-regulation of collagen type X, MMP13 and ALP-activity. As a consequence, differentiated MSC pellets undergo mineralisation and vascularisation after ectopic transplantation in a process similar to endochondral ossification. This review discusses the complexity and entailed challenges when considering MSC from various sources for clinical application and the necessity to optimise chondrogenesis by repressing hypertrophy to obtain functional and suitable cells for cartilage repair.

Cartilage repair by autologous chondrocytes

Due to its limited capacity for regeneration and self-repair as well as the scarcity of available therapeutic options, degeneration of articular cartilage may have severe consequences [11]. Osteoarthritis, as the most common joint disease, often results in total joint replacement, whereas size-limited injuries can be treated by autologous chondrocyte transplantation (ACT). Thanks to ACT technologies, the more than 250-year-old statement of William Hunter regarded as an axiom for a long time, that once cartilage is injured, it cannot be reconstituted, has been challenged [26]. This cell-based treatment is currently the only cartilage repair procedure approved by the US Food and Drug Administration and was first described by Brittberg in 1994 [10] for treatment of deep articular cartilage defects.

The principle of this method is the introduction of *in vitro* expanded autologous chondrocytes as a cell suspension or—in an improved version—in association with a supportive matrix (matrix-assisted ACT, MACT), into the defect site where the cells are supposed to regenerate the destroyed cartilage. Results of this cell-based procedure are acceptable as they lead to some kind of pain relief for the patient [5, 32, 44].

[1] Abstracts in German, French, Italian, Spanish, Japanese, and Russian are printed at the end of this supplement.

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A limitation of ACT, however, is that newly synthesised cartilage often consists of fibrous instead of hyaline tissue, which cannot be compared to the physiological and biomechanical properties of healthy articular cartilage [9, 19, 25, 46, 53]. In addition, the prerequiste injury of healthy cartilage in a preceding surgery to obtain cartilage biopsies from a non weightbearing region of the joint is not only an additional burden for the patient, it is also suggested that inflicting this injury may increase the long-term risk of developing osteoarthritis [24, 27, 37].

Compared to other techniques like microfracture and osteochondral plug transplantation, the effectiveness of ACT/MACT methods is still under debate [5, 25, 32, 59]. Furthermore, in addition to the disadvantages of harvesting the cells, the *in vitro* cultivation of chondrocytes is associated with difficulties as the required monolayer expansion to achieve adequate cell numbers is known to result in a phenotypic derangement to a fibroblast-like phenotype referred to as dedifferentiation [6]. We and others have shown that cells isolated from the extensive extracellular matrix by enzymatic digestion, results in decreased mRNA levels for cartilage-specific molecules; a phenomenon that becomes more pronounced when cells are expanded in culture [6, 7, 52, 54]. Although a rounded cell morphology and partial redifferentiation may be achieved in 3D culture, the gene expression levels revert only partially [7]. As a consequence of dedifferentiation and loss of phenotypic cell traits during *in vitro* expansion, a progressive loss of cell ability to form stable ectopic cartilage *in vivo* has been observed [15, 16]. It is conceivable that a regenerate composed of poorly differentiated fibrocartilage may arise from the application of such dedifferentiated cells in ACT or MACT therapy.

Taken together, the current state of the art demands the improvement of therapeutic options for treating articular defects. The aim is to develop new cell- and tissue-engineering-based methods that may also overcome the size limitations of current ACT technologies and be applied to larger defects, probably even including treatment of osteoarthritis-caused cartilage lesions. Besides the fact that chondrocytes can be applied in cartilage repair procedures and that they are the only cell source for these purposes by now, the previously mentioned disadvantages associated with the use of chondrocytes require us to find alternative solutions.

Mesenchymal stem cells as a source for chondrogenesis

Stem cells have recently evoked interest as a promising alternative cell source for treating articular cartilage defects. The easy availability of mesenchymal stem cells (MSC) from various tissues such as bone marrow [12, 17], adipose tissue [62], synovial membrane [13], trabecular bone [42] and other tissues, together with their high proliferation capacity, make them attractive as a distinguished cell substitute for chondrocytes in cartilage regeneration [3]. MSCs can fulfil the requirements demanded of cells for tissue engineering of cartilage, as they can be conveniently manipulated *in vitro* to differentiate to chondrocytes for these purposes.

The best characterised population of MSC are those originating from bone marrow. Given that the bone marrow is not an optimal source for MSC due to the painful and risk-containing sampling procedure, isolation of stem cells from other sources would bring an attractive alternative. Adipose tissue is particularly considered to be an equally attractive source for MSC to bone marrow, as it is easily accessible in large quantities and adipose-derived MSCs show a proliferation and multilineage capacity comparable to those from bone marrow [14, 58]. MSC from synovial membrane [13], muscle [8], periosteum [40] and many other mesenchymal tissues are in experimental use in the field of regenerative medicine.

Natural cartilage development

During embryonal development MSC give rise to two different kinds of cartilage: permanent and transient [33, 34, 45]. The permanent hyaline cartilage arises from MSCs exclusively at the distal ends of the developing bones. After initial condensation, the stem cells differentiate towards stable chondrocytes that synthesise the typical hyaline extracellular matrix of articular cartilage.

In addition to permanent cartilage, a second form also develops from MSCs: the transient cartilage. Prior to skeletal bone formation, chondrocytes originating from MSCs build up a transitional cartilaginous model of the skeleton that is later replaced by mineralised bone in a process called endochondral ossification. After the cartilaginous scaffold is formed, chondrocytes in the middle of the diaphysis cease to proliferate and become hypertrophic, implicating that they enlarge in size and start expressing the hypertrophy marker molecule collagen type X. After further differentiation, hypertrophic chondrocytes start calcifying the surrounding matrix and either transdifferentiate towards osteoblasts or undergo apoptosis [1]. Matrix mineralisation is followed by vascularisation, which initialises immigration of matrix-degrading osteoclasts and bone-depositing osteoblasts. Between this primary and an epiphyseal

secondary ossification centre, the growth plate is formed where the transient cartilage in the process of endochondral ossification is responsible for postnatal length growth of the bone.

When using MSCs for articular cartilage repair, the great challenge during chondrogenesis is to generate chondrocytes comparable to articular cartilage-derived chondrocytes that do not undergo hypertrophy as a terminal differentiation stage.

Induction of *in vitro* chondrogenesis in MSC

As there are no pronounced marker molecules identified for MSCs so far, the cells are commonly selected by plastic adhesion. As a result, a heterogeneous mixture of cells including—but not solely consisting of—MSCs serves as the starting population. This implies the risk, for instance regarding bone marrow-derived MSC populations, that osteoprogenitor cells might be present and favour differentiation towards the osteogenic lineage. Today, the chondrogenic differentiation potential of MSCs has been demonstrated [4, 30, 49, 58] and induction of *in vitro* chondrogenesis displays a well-established standard procedure.

After expansion, chondrogenesis is induced in high-density culture *in vitro* in the presence of transforming growth factor-β (TGF-β) resulting in the appearance of a chondrocyte-like phenotype [30] characterised by upregulation of cartilage-specific molecules such as collagen type II and IX, aggrecan, versican, biglycan, and decorin [58]. Because of the high sensitivity of polymerase chain reaction (PCR) for identifying mRNA, it is not unusual to detect the transcript long before protein expression is visible. For definite confirmation of successful chondrogenic differentiation, the histological detection of collagen type II and proteoglycans is imperative. However, there are publications showing that the application of common *in vitro* conditions for chondrogenic differentiation of bone-marrow-derived MSCs induces features characteristic of fibrocartilage such as the expression of collagen type I and osteopontin [51]. In addition, features typical of hypertrophy of chondrocytes are induced, such as expression of collagen type X and MMP13 [4, 28, 39, 41, 58] and an increase in alkaline phosphatase (ALP) activity [23, 43].

Adipose-derived MSCs showed a reduced chondrogenic differentiation capacity under standard induction conditions [47, 58]. Hennig et al [23] demonstrated that adipose-tissue-derived MSCs reveal an altered bone morphogenic protein (BMP) profile compared to MSCs from bone marrow and required exogenous application of BMP, in addition to TGF-β, to compensate for the reduced endogenous expression of BMP2, -4, and -6. Application of BMP6 in combination with TGF-β completely eliminated the reduced chondrogenic differentiation potential of MSC derived from adipose tissue. This demonstrated that MSCs isolated from different tissues do not represent identical cell populations, but vary in the expression profile of some growth factors relevant for chondrogenesis. Similar to MSCs from bone marrow, however, chondrocytes derived from adipose MSCs also revealed premature deposition of collagen type X and upregulation of ALP enzyme activity.

No stable arrest of chondrogenesis before hypertrophy

We recently demonstrated that upregulation of hypertrophy-associated marker molecules such as matrix metalloproteinase 13 (MMP13), collagen type X and activity of ALP during *in vitro* chondrogenesis, leads to strong matrix calcification accompanied by vascular invasion and even microossicle formation after ectopic transplantation into subcutaneous pouches of SCID-mice [43]. Thus, common protocols of chondrogenesis produce MSC-derived chondrocytes that undergo premature hypertrophy and develop into a transient, endochondral cartilage, instead of stable, articular cartilage-like tissue. As observed for bone-marrow derived counterparts, subcutaneous transplantation of adipose-derived-MSC spheroids resulted in comparable mineralisation and microossicle formation [23]. This indicated that predisposition of bone-marrow-derived MSCs for osteogenesis and matrix calcification is not due to their origin from bone, since MSCs from adipose tissue mineralised their surrounding matrix *in vivo* to a similar extent and these cells should not be predetermined to form bone according to their background.

Observations of further differentiation of MSC-derived chondrocytes towards calcification and mineral deposition were phenomena only seen in experimental settings *in vivo*. *In vitro*, no signs of mineralisation and no further differentiation as in the growth plate were detectable, independent of the expression of hypertrophy-associated molecules, even after long-time chondrogenic induction for up to 13 weeks. Most likely, terminal differentiation *in vitro* is repressed by TGF-β, as this main growth factor of chondrogenesis is known to retain chondrocytes in the prehypertrophic state [61]. However, when removing the growth-factor stimu-

lation after 5–7 weeks of chondrogenic induction, a further cultivation in chondrogenic medium for 4 weeks without TGF-β *in vitro* was not followed by calcification in the absence of a phosphate donor such as ß-glycerophosphate (unpublished data). This indicates that vascularisation of transplants may be an important factor for calcification, since only the *in vivo* environment supported vascularisation and replacement of cartilage by bone [18].

Not only the appearance of hypertrophy in chondrocytes derived from MSCs, but also the observation of vascularisation is a major concern when considering stem cells for cartilage regeneration—a tissue that is generally avascular. While MSCs not only mineralised *in vivo*, but also initiated vascular invasion, articular chondrocytes cultured under identical conditions showed a completely different behaviour: They formed stable cartilage at ectopic sites, negative for collagen type X, and were resistant to calcification without any signs of blood vessel development [43]. Based on these observations, intrinsic fundamental differences between articular and MSC-derived chondrocytes are likely to exist and will have to be further characterised. Possible means to arrest MSC-chondrocyte differentiation earlier in their developmental cascade to obtain stable articular cartilage formation should, therefore, be developed *in vitro* and *in vivo*. In conclusion, standard *in vitro* conditions for chondrogenesis will have to be improved—for instance by supplementation with other factors such as inhibitors of hypertrophy, or by application of mechanical loading—in order to enable stable articular cartilage formation in the absence of hypertrophy. In addition, the use of a scaffold may possibly enhance the capacity of MSC-chondrocytes to build up stable cartilage instead of undergoing hypertrophy. A recent publication of Mwale et al demonstrated that the *in vitro* expression of collagen type X, bone sialo protein, osteocalcin mRNA and the activity of ALP of expanded undifferentiated MSCs could be successfully repressed when using a nitrogen-rich plasma polymer scaffold [39].

Matrix production by MSC-derived chondrocytes

In MSC-derived chondrocytes, the gene expression levels for cartilage-typical matrix molecules like collagen type II, aggrecan, decorin, fibromodulin or COMP were below the level of articular cartilage and closer to intervertebral disc tissue [51]. These data were supported by an analysis reported by Mauck et al [36] who showed that chondrogenesis occurred in MSC-laden hydrogels, but the amount of the forming matrix and measures of its mechanical properties were lower than that produced by chondrocytes under the same conditions. Both authors suggested that further optimisation during differentiation of MSC along the chondrogenic lineage or a longer induction period is required to achieve levels similar to those produced by articular chondrocytes.

In contrast to MSCs, chondrocytes have experienced mechanical loading at their natural site in the joint. Studies demonstrated that mechanical stress strongly influences the maintenance of hyaline cartilage [20] and that cyclic, mechanical compression enhances the expression of chondrogenic markers in mesenchymal progenitor cells differentiated *in vitro*, resulting in an increased cartilaginous matrix formation [2, 35, 48]. Therefore, mechanical loading of MSCs could be considered to improve stable cartilage formation.

Application of MSCs for cartilage repair

For clinical application of MSCs as a cell source for the repair of cartilage defects, it is imperative to guarantee phenotypic stability and functional suitability of these cells. For the safety of the patient, any risk of graft instability has to be excluded before clinical application of MSCs into cartilage defects. Obviously, the ectopic environment of a subcutaneous pouch in mice is not representative of the clinical situation. In the natural joint environment, MSCs may behave in a different way, which has to be investigated in animal models.

Repair of osteochondral defects

Rabbits were used most frequently as model organisms, which restricted the investigation to the repair of osteochondral defects since the cartilage layer is too thin for articular defects. Wakitani et al treated full-thickness articular cartilage defects in the weightbearing surface of the medial femoral condyle with cultured MSCs isolated from periosteum or bone marrow. Cells were seeded into a type-I collagen gel and transplanted into the defect. Two weeks after implantation in the defect, cells had differentiated into chondrocytes and at 24 weeks the subchondral bone was completely repaired without loss of overlaying cartilage. The mechanical testing at 24 weeks after surgery yielded a repair tissue that was stiffer and less compliant than empty defects, but still less stiff and more compliant than normal cartilage tissue [55].

In a comparable rabbit study with MSCs isolated from bone marrow, significantly higher histological scores were observed in the experimental group than the control group 14 weeks after surgery. These results were accompanied by a more intense type-II collagen staining in the newly formed cartilage matrix and the detection of Col2a1 mRNA in mature chondrocytes [29]. In addition, Yan and Yu published that MSCs and chondrocytes embedded in a polylactid acid matrix placed into a full-thickness cartilage defect in rabbits showed a hyaline cartilage-like histology [60]. The histology scores in these groups were significantly higher than the groups where the defect was filled with fibroblasts or without cells. These studies suggested that the repair of osteochondral defects may be enhanced by implanting cultured MSCs.

Katayama et al reported that the cartilage regeneration in a full-thickness articular defect in the knee joint of rabbits was further enhanced if MSC were transfected with the cartilage-derived morphogenetic protein (CDMP1)-gene before implantation [31]. The histological score of this group was significantly better, with defects filled with hyaline cartilage up to the surface zone, than those of the MSC control group, showing repair tissue that contained hyaline cartilage but a surface zone that showed fibrous structure, and the empty control, where the defects were filled with fibrous tissue only.

In contrast to these publications, Solchaga and colleagues reported that the application of bone marrow, without the isolation of MSC, in a fibronectin-coated hyaluron-based sponge did not result in statistical differences in the overall histological score between cell-treated and bone-marrow free groups [50]. This result indicated that either the number of mesenchymal progenitor cells might be too low when unpurified bone marrow was applied, or there might be some constituents within these samples that inhibit effective chondrogenic differentiation.

MSC-derived chondrocytes for cartilage repair in chondral defects

In a large animal model, goats were used to analyse the repair of an articular cartilage defect in the femoral surface of weightbearing areas. Guo et al seeded culture-expanded autologous bone-marrow-derived MSC into bioceramic tricalcium-phosphate-scaffolds in an attempt to repair articular cartilage defects. They described that 12–24 months after implantation, the modification of neocartilage occurred accompanied by the increase in proteoglycan levels. At 24 weeks after surgery, the defects were resurfaced with a hyaline-like tissue and a good interface between engineered cartilage, normal cartilage and underlying bone [21, 22].

In a large animal model, osteoarthritis was induced by medial menisciectomy and resection of anterior cruciate ligament of goat knees [38]. The local injection of autologous MSCs from bone marrow resulted in the appearance of a meniscal-like repair tissue six weeks after injection of cells. The degeneration of articular cartilage, osteophyte remodelling and subchondral sclerosis were reduced in cell-treated joints compared to the cell-free control group at this time point. However, 20 weeks after cell injection, there were significant osteoarthritic (OA) lesions in both the cell-treated and control joint that the authors explained by the fact that no repair of the ligament was evident.

Application of MSCs in human cartilage

At present there are some rare reports of expanded autologous bone marrow stem cells transplanted for repair of articular cartilage defects in human injured or OA knees [56, 57]. In one study, 24 patients with OA underwent a tibial osteotomy. MSCs from bone marrow were expanded from twelve patients, embedded in collagen gel and transplanted into articular defects in the medial femoral condyle and covered with a periosteal flap. To implant the constructs, 2 mm of the eburnated bone was abraded, and to further facilitate bleeding, multiple perforations were performed on the abraded area. 42 weeks after implantation, the defects were covered with white soft tissue and hyaline cartilage-like tissue was partially observed in the cell-transplanted group. The arthroscopic and histological grading was better in the cell-transplanted group than the control group, although the clinical results were not significantly different [56]. In a case report on two patients with patella defects, cultured MSCs were embedded in collagen gel and placed in the patellae covered with autologous periosteum. Clinical results 2 years post transplantation revealed that clinical symptoms—including pain and walking ability—had improved, but histologically, the repaired tissue consisted of fibrocartilage [57].

One major critical point concerning all these animal or human studies is that up to now it remains unclear whether the implanted cells directly contribute to the functional repair of articular cartilage. The applied cells could influence regeneration indirectly, via secreted cytokines, growth factors, inhibitors or other bioactive factors, by acting on the

subchondral bone, especially when surgical treatments involved bleeding from the subchondral bone, and this tissue could then be the primary source of repair cartilage. There is still a lack of controlled, appropriately designed animal models with quantitative outcome measures addressing follow-up of cells and their role in cartilage regeneration. However, these studies require new methods and new study designs, and are much more difficult to perform than simply implanting cell-loaded carriers into a defect and analysing, mainly based on non-quantitative histology, the outcome after several weeks compared to cell-free carriers or empty defects.

Conclusion

The presented observations clearly demonstrate the complexity and the herewith entailed challenges in considering MSCs for clinical application. For safe application of MSCs, it is crucial to better understand the mechanisms of stem cell development. Gained knowledge in the regulation of hypertrophy in the growth plate will help to design improved *in vitro* conditions that could repress hypertrophy to obtain functional and suitable MSCs for cartilage repair. Of great interest is also the further characterisation of differences between articular and MSC-derived chondrocytes to understand what distinguishes these two cell populations and to resolve the developmental pathway enabling hyaline articular cartilage to become a permanent tissue. Most importantly, new study strategies to address the role and contribution of the transplanted cells to the repaired cartilage tissue have to be designed to provide a sound basis for applying MSCs in cartilage repair and regeneration.

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