Predifferentiated mesenchymal stem cells for osteochondral defects [Letter]

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Letter to the Editor

Dear Editor:

We read the recent article "Repair of Chronic Osteochondral Defects Using Predifferentiated Mesenchymal Stem Cells in an Ovine Model" by Zscharnack et al[10] with great interest.

Clinical results of matrix-associated chondrocyte transplantation (MACT) in the treatment of chondral defects are satisfying and this technique can be seen as gold standard in the present therapy of large chondral defects. In comparison with microfracture, it offers the possibility of regeneration with hyaline-like tissue.[5] However, advantage toward microfracture (MFX) is dearly bought by the necessity of invasive harvesting of autologous chondrocytes, leading to defect healing of donor sites and requiring an additional surgical procedure. However, recent efforts combining mesenchymal stem cells (MSCs), obtained by MFX, with artificial matrices to improve results of the MFX procedure, resulted in inferior regeneration compared with MACT.[2]

The current study directly addresses the subsequent and, in our opinion, absolutely relevant question, if in vitro predifferentiation of MSCs helps improve results. The authors attached importance to designing a study, with results that are transferable to humans, employing a chronic osteochondral defect model in large animals. Furthermore, they worked out a convincing treatment procedure that seems to be transferable to clinical application in patients. The extensive in vitro part of the work, investigating the conditions for the subsequent in vivo part, contributes markedly to the quality of the article.

However, the work of the in vivo part disappointed us with regard to a few facts.

All conclusions from the costly in vivo study were drawn from histologic examination. In our opinion, a biochemical, and even more a biomechanical, evaluation of the in vivo regenerate would have based the conclusions on a far more stable fundament. These additional data would characterize function of the regenerate and thereby give hints about its long-term survival.

One of the most critical points in the present study is the press-fit anchoring of the gels during the surgical procedure. It is doubtable that the gels are securely prevented from dislocation during mobilization of the specimens under full weightbearing. Previous studies in comparable models have reported that a critical number of gels dislocate even when they are additionally fixed by fibrin clue or sutures.[9] Even in clinical application of MACT, where patients avoid weightbearing for 6 weeks after surgery, delamination of the matrix is one of the major complications.[7] Furthermore, Figure 1 of the article shows that cartilage of the sheep's condyle is rather thin compared with that of humans.[9] This even more brings into question press-fit stability under full weightbearing. Also, if the authors have shown successful survival of implants in situ for 6 weeks in single sheep employing quantum dot-labeling (Figure 6), toluidine staining in Figure 8 of the article supports the assumption that some gels were dislocated after the surgical procedure. Here, the worst results of each treatment group show no residual matrix, resulting in persistence of full cartilage defects. This finding surely contributes to the general high standard deviation in the present study.

On the other hand, the best results shown in Figure 8, with full defect regeneration in all groups, including untreated controls, make it questionable if the 7-mm diameter represents a critical-size defect. Here, the result of the predifferentiated MSC-seeded gel group appears as good as native cartilage, raising the question if the histological slide has missed the regenerate.

Another critical limitation of the study design is creation of 2 randomized treated defects in 1 knee. Application of 2 different treatments in 1 joint cavity creates danger of their interaction. Although the authors state that they are conscious of that danger and quote that no obvious influence between the different defects was observed, it is well known that hydrogels loaded with cells release cytokines and cells to their environment and therewith to the joint cavity.[3,4] Here, it is likely that stem cells, either differentiated or undifferentiated, released from gels into the joint critically interacted with healing of defects, either treated with unloaded gels or even if left empty.[6] On the other hand, anabolic cytokines released from MSCs may have improved healing in defects treated by other methods in the same compartment.[3] Additionally, chondrogenic differentiated MSCs incorporated to matrices may have paracrinely influenced undifferentiated MSCs applied to the same joint cavity.[1] Thus, it is likely that interactions of the different treatment methods have contributed to the high standard deviation observed within the groups. Regarding these facts, we are wondering why the authors have not randomized the knees (instead of randomizing the single defects), meaning same treatment in both defects of 1 knee and thereby avoiding uncontrolled interaction.

An additional factor surely influencing the different treatment regimens is the depth of the set defect. How did the authors confirm, that each defect was 2 mm in depth? Did the drill have a mechanical stop? The authors note that the subchondral bone layer was not opened during drilling the procedure to avoid migration of MSCs to the defect. Nonetheless, in Figure 1 the subchondral layer seems to be affected, which is in line with hypertrophy of subchondral bone in several treatments represented in Figure 8. This bone hypertrophy is frequently occurring

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after microfracture as a result of MSC migration from bone marrow into the defect.[5] This fact seems to be critical, as it is known that previously performed MFX impairs outcome of autologous chondrocyte transplantation by 30%.[5] Furthermore, the "untreated defects," representing the negative control, would more likely represent conditions of a "microfracture control." Regarded critically, the best result of the untreated control group shown in Figure 8 shows an appearance that would be expected after MFX.

A problem that is frequently seen after in vivo implantation of chondrogenic differentiated MSCs is terminal differentiation of the cells. This results in a process similar to enchondral ossification, with cell hypertrophy, calcification of the cartilage regenerate, and vascularization.[8] It is amazing that no cartilage mineralization occurred within the present study in the group of predifferentiated MSCs, as indicated from the ICRS (International Cartilage Repair Society) score. Here, the announced 1-year results of the study will be of particular interest. Can terminal differentiation of the MSCs be prevented under the specified study conditions also in long-term follow-up?

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Authors' Response: Thank you very much for your interest in our study on the repair of chronic osteochondral defects using predifferentiated mesenchymal stem cells (MSCs) in an ovine model, published in *The American Journal of Sports Medicine*.[11] This work describes our findings that chondrogenic predifferentiation of MSCs in vitro improves MSC-based matrix-associated autologous chondrocyte transplantation for treatment of focal, chronic osteochondral defects after 6 months in sheep.

To begin with, this study is to be considered a proof of concept trial. As mentioned by Schmitt, Imhoff, and Vogt, one of the primary objectives of the study was to determine appropriate in vitro production parameters for MSC-based cartilage implants. In addition, the focus of the in vivo animal model was verification of cartilage regeneration with well-acknowledged end points. This was intended to show a causal relationship between in vitro predifferentiation of MSCs and cartilage repair in a clinically relevant model.

In general, we agree with Dr Schmitt and his colleagues that it is useful to evaluate additional end points besides histologic testing for quality assessment of regenerated tissue in preclinical studies. However, our histologic analysis using the well-accepted cartilage grading scales (ICRS assessment scale[4] and the O'Driscoll score[7]) was performed in accordance with Good Clinical Practice by 3 investigators independently in a blinded fashion. The minimal differences between the observed scores strengthen the significant findings that were reported.

In a comparable study that we completed using the same model, we examined 12-month long-term results with end points including histologic evaluation and gross examination as well as micro-MRI analysis (MOCART [magnetic resonance observation of cartilage repair tissue] score) using a 7.1-T spectrometer at a pixel resolution of 62.5 μm.[5] The combined outcome of this long-term study confirms our short-term histological results presented in our herein-discussed AJSM article.[11]

Moreover, we are currently performing a confirmatory preclinical study with 32 animals under Good Laboratory Practice guidelines in which ultrasound biomicroscopy is used in addition to MRI.

Concerning the anchoring of the implants, we agree that restricted weightbearing would be optimal. However, as most do, we found this very difficult to accomplish. We are aware of different possible approaches including the use of external fixation, the use of a mat in combination with a rolling frame, or even performing surgery on just 1 hindlimb. However, these procedures are accompanied by problems for the sheep (eg, risk of infection, discomfort, and lack of sleep). This is exacerbated by the sheep's reduced ability to engage in natural flight from stressors during postoperative care. In addition, our suggested immobilization of the hindlimbs for 1 week was denied by our animal review board. Fixation using xenogenic glues like fibrin glue was avoided because increased infiltration of mononuclear cells has been observed with this method.[1] To evaluate possible dislocations of the implants, we performed preliminary testing on 2 additional animals using quantum dot–tagged MSCs, which indicated that implants remained in

situ. We believe that it is important to mention that because of the equivalent methods used in the different treatment groups, any dislocation of the implants would be distributed evenly among the groups. However, we cannot exclude the possibility that dislocations occurred, thereby resulting in possible distortions of the treatment effect.

While maintaining the experimental structure, the treatment method, observation and analysis of data as equal in both groups, we found that, based on our group size planning and also assuming nonparametric distribution, pre-MSC MACT remained significantly superior.

Contrary to the supposition made by Schmitt et al, the focal cartilage defects of 7 mm that we created in the medial femoral condyle of the sheep do indeed represent critical-size defects. This is supported by multiple preclinical studies on sheep that have confirmed that 4- to 7-mm–diameter defects are of a "critical size."[3,10] In addition, a comparative anatomic study by Osterhoff et al[8] has shown that the ovine stifle can be utilized as representative of a human knee scaled down by one-third. Thus, a 7-mm lesion in a sheep knee corresponds to the same in a human knee of more than 2 cm in diameter. In our study, we have created critical-size defects in all 4 test and reference groups.

Based on the definition of "critical size" defects as described by Schlichting et al,[10] in such lesions, erupted bone marrow cells did not regenerate either bone at the deep zone or cartilage at the surface. Thus, the reported "best result" for the untreated control group in our 6-month study does indeed show a nearly closed cartilaginous layer. However, the subchondral structure reveals no regeneration, including the presence of cysts. Moreover, another of our own previous studies focused on the development of our animal model. Specifically, it showed that 7-mm–diameter focal defects in Merino sheep did not tend to regenerate spontaneously.[2]

Concerning the doubts of Schmitt and colleagues on the origin of the "best case" toluidine blue staining of the pre-MSC gel, we can assure them that the histology has not missed the former implant location, as demonstrated by the added Figure 1, which shows the junction between native and regenerated cartilage. We were further able to confirm these superior results and especially the hyaline-like character of the regenerated tissue in some specimens after 12 months in our follow-up 12-month study.[5]

Regarding the possible interactions between the 2 different treatment modalities in the same joint cavity, we agree that this interaction cannot be ruled out. Therefore, that is why we stated in the discussion section of our article the following: "One limitation of this study is the use of 2 defect placements in 1 stifle joint. Although no difference between both defect locations in the condyle in terms of histological outcome was found in statistical analysis, a possible influence on the repair cannot be ruled out."

We would also like to note that our study was conducted only as an explorative proof of concept trial in which we decided for ethical reasons to reduce the number of animals rather than use a higher case number. Above all, we would like to express that we randomized the group and also the defect allocation. By doing so, the systematic bias was discarded. This is supported by the fact that in our study the interactions between the 2 distinct treatment groups in each joint cavity occurred in a fully randomized manner and when combined with the high number of the joints that were included (n = 18), we suggest that the terms of the interactions were balanced.

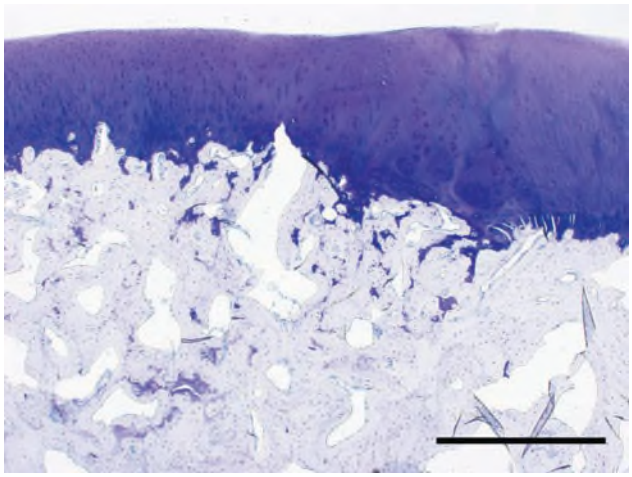

Figure 1. Border zone between native and regenerated cartilage. The figure shows a toluidine blue–stained section of the junction between the native articular cartilage on the right-hand side to the repair tissue on the left-hand side 6 months after treatment with predifferentiated mesenchymal stem cells (''best case'' in Figure 8 of the article discussed herein). It demonstrates clearly that the histologic slide has not missed the regenerate. Bar indicates 2 mm.

In their letter, Schmitt et al proposed that we should have randomized the joint cavity instead of the defect. However, this study design would not have allowed for the comparison of all 4 treatment modalities in a single animal. Consequently, this would have led to a bias of the results caused by the interindividual differences of the sheep and also no paired samples would have been present, thereby limiting the statistical validity of the study.

Despite our many respectful disagreements, we do fundamentally agree with Schmitt et al that the application of a single defect per joint cavity is optimal. For that reason, we are currently performing a confirmation study with only a single defect and a single treatment modality per joint cavity. This is to minimize the risk of secondary influences and interactions.

With respect to the comments of Schmitt et al regarding the depth of the defect, in our study we created full-thickness cartilage defects of 2-mm depth (as shown in Figure 1D).[11] We did this by the use of a drill with a mechanical stop. With this regimen, we reached the tidemark without opening the subchondral spongy bone and importantly caused no bleeding from the bone. This effectively prevented the migration of MSCs into the treated lesion site.

This is also consistent with the findings of Jubel et al[3] (see Figure 4), who studied similar lesions in the medial femoral condyle of sheep and described also that the bone layer was not opened.

However, we agree with Schmitt et al that our method may have caused a bone hypertrophy in some of the regenerated defects. Furthermore, we agree that this very likely may be attributable to local cell immigration from the subchondral bone. However, this remains a problem of all animal studies for cartilage repair, not only large animal models but especially in smaller animals such as rabbits. In sheep, for example, this is prevalent because of the relatively thin cartilage layer of sometimes only up to 2.0 mm.

Therefore, what is necessary is to eliminate this influence as much as possible. To reduce this influence, minimizing the risk of dislocation, and to accommodate the 2-mm–thick implant itself, it is critical to establish a certain defect depth for safe placement of the graft. But once again, this is a problem in every animal model used for cartilage repair. Furthermore, the influence of such local cells is equal for every treated defect in all 4 groups in our study.

Schmitt et al state that the terminal differentiation of MSCs is a difficult challenge to overcome in stem cell research. We agree. Indeed, the prevention of this process is daunting. However, their citation[9] referred to the ectopic implantation of MSCs in mice. This comparison is invalid because in our setting we applied an orthotopic implantation of MSCs into the hyaline articular cartilage of the sheep knee.

Moreover, despite Pelttari's outcome, we did not observe an ectopic calcification in any of the MSC groups. This is true for results observed at both 6 months and at 12 months.[5] In addition, these findings are in line with an actual investigation in humans, which compare the clinical outcomes of 36 patients treated with autologous chondrocyte implantation to 36 patients treated with autologous bone marrow–derived MSCs.[6] Histologic evaluation of biopsy specimens taken after 1 year showed no collagen type X in the MSC group. This indicates an absence of cell hypertrophy, the main marker for terminal differentiation of MSCs.

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