Discussion

We have previously demonstrated the feasibility of the MSC-derived TEC approach for cartilage repair and showed the equivalent overall morphology and macro-scale compressive properties of TEC-mediated repair cartilage compared to normal cartilage (Ando *et al.*, 2007; Katakai *et al.*, 2009). However, we also pointed out the morphological abnormality in the superficial area of the TEC-mediated repair cartilage, with the predominance of fibro-cartilaginous tissue. The introduction of micro-scale biomechanical analysis along with detailed ultrastructural observations has enabled a more precise evaluation of zone-specific structure and function between normal and TEC-mediated repair cartilage. The present study specifically focused on the superficial zone of the repair cartilage and elucidated a number of unique characteristics of the tissue, morphologically and biomechanically. Grad *et al.* recently investigated the surface micro-scaled biomechanical properties of *in vitro* engineered cartilaginous tissues (Grad *et al.*, 2012). However, to our knowledge, the present study is the first demonstration of these unique functional abnormalities in the superficial zone of repair cartilage following implantation in a large animal model.

Morphologically, the combination of SEM with picrosirius red staining under polarised microscopy clearly demonstrated the difference in the surface structure of uninjured cartilage and the TEC-mediated repair tissue. SEM detected the dense paralleled fibrous bundle with the thickness of approximately 20 µm at the most superficial layer of uninjured articular cartilage. Interestingly, picrosirius red staining of the corresponding area showed the presence of a bright orange band, with approximately 100-150 µm thickness, aligned parallel with the surface of uninjured articular cartilage. Previous studies suggested that the thickness of the superficial tangential zone of articular cartilage has 10-20 % of the thickness of the whole cartilage (Mow *et al.* 1990) and the average thickness of porcine femoral articular cartilage is reported to be about 1 mm at 11 months of age, which corresponds to the period at the tissue sampling in the present study (10-11 months old) (Rieppo *et al.*. 2009). Thus, the thickness of the superficial tangential zone could be expected to be approximately 100-200 µm. The similarity in the thickness suggests that the surface orange band detected by picrosirius red staining may correspond to the superficial tangential zone and that the dense tangential fibrous band detected by SEM, which has less than 20 % of the thickness of the orange band, might be the superficial specialised structure involved in the superficial tangential zone. Notably, a bright red thin band with the thickness of 3 µm was observed at the very surface of uninjured cartilage. MacConaill first reported a thin bright line at the articular surface which consisted of parallel aligned fibres and termed it the lamina splendens (MacConaill, 1951). The width of the lamina splendens was reported to be 3.2-5.2 µm (Dunham, *et al.*, 1988) and 4-8 µm (Teshima *et al.*, 1995) in canine tibial plateau and human femoral head, respectively. Based on the similarity in the thickness, the red bright band presumably corresponds to the lamina splendens. On the other hand, SEM showed a thin fibre with the thickness of 3 µm at the surface of the TEC-treated cartilaginous tissues. However, picrosirius red staining did not show such a bright red or orange band at the tissue surface. Therefore, it is likely that the lamina splendens, or the structure of the superficial tangential zone, was not restored in the TEC-treated cartilaginous repair tissue. Significantly, inferior O'Driscoll scores in the TEC-mediated repair cartilaginous tissue at the superficial zone level may also be a reflection of the loss of the superficial structures. Thus, the lack of the lamina splendens coupled with the altered superficial tangential structure at the surface of the TEC-mediated repair cartilaginous tissue could be the most significant morphological finding in the present study.

The lamina splendens, together with the superficial tangential zone, is regarded as playing various important roles in maintaining the mechanical response of articular cartilage to load (Thambyah *et al.*, 2007, Hollander *et al.*, 2010), joint physiology – such as withstanding extrinsic compression and intrinsic swelling pressure – and facilitating joint lubrication (Teshima *et al.*, 1995). Therefore, the lack of these superficial structures could lead to a functional insufficiency at the superficial zone of the repaired cartilage in many properties, such as surface roughness, stiffness, lubrication and water retaining potential to control intrinsic swelling pressure. This may compromise the long term functionality of the repair tissue unless it continues to improve with time post-implantation.

In order to assess the potential functional insufficiency of the superficial zone of repair–cartilage, the atomic force microscopy (AFM) approach was employed. AFM has been reported to allow evaluation of the structure-mechanical property relationships at the surface of articular cartilage at the µm and nm scales (Stolz *et al.*, 2004; Hsieh *et al.*, 2008) and thus, to examine surface roughness and stiffness of the repair cartilage. There were no significant differences in the surface roughness between the TEC-mediated repair–cartilage and uninjured cartilage, while significantly lower micro-scale stiffness was detected in the TEC-mediated repair cartilage than in the uninjured cartilage. This result was in contrast with those of previous macro-scale compression assessments, where no significant differences between TEC-mediated repair cartilage and the uninjured cartilage were detected. Such findings indicate the lack of sensitivity of the macro approach and the importance of micro-scaled mechanical testing.

The proteoglycan 4 (PRG4) (lubricin) is a mucinous glycoprotein expressed in cartilage and one which is believed to play an important role in the boundary lubrication of articular cartilage (Swann *et al.*, 1977; Jay *et al.*, 1992). Therefore, expression and localisation of this molecule may estimate the lubrication properties of the TEC-mediated repair cartilage. In the present study, we demonstrated that PRG4 was similarly localised to the superficial zone of the TEC-mediated repair cartilage and in normal uninjured cartilage. In accordance with the similar localisation pattern of PRG4, there were no significant differences in the coefficient of friction between the TEC-mediated repair cartilage and the uninjured cartilage. We previously reported that the coefficient of friction of

the TEC-mediated repair cartilage was not significantly different from that of normal cartilage (Ando *et al.*, 2007). However, the results were obtained immediately after loading of the specimens, when the lubrication mode was thought to be a combination of hydrodynamic lubrication and boundary lubrication. Therefore, in the previous study, there might have been no clear effect of loading duration on the friction in which the boundary lubrication becomes dominant (Pickard *et al.*, 1998). In the present study, in order to eliminate that potential problem, we started the examination after 60 s of loading, and again obtained similar results. Taken together, the structural abnormality in the superficial zone of repair cartilage did not likely affect the surface localisation of PRG4 or the boundary lubrication properties of the repair cartilage.

Finally, we investigated the permeability (inverse relation to water retention capacity) of the repair cartilage. This capacity is considered essential to influence the crucial function of articular cartilage to control intrinsic swelling pressure (Mansour *et al.*, 1976). Interestingly, the TEC-mediated repair cartilage exhibited permeability similar to the uninjured cartilage at the level of the middle and deep zones, while higher permeability was detected in the repair cartilage at the surface/superficial zone. The permeability of normal cartilage has been reported to increase as one assesses deeper zones (Muir *et al.*, 1970), while the tendency of the TEC-mediated cartilaginous repair tissue was shown to be the opposite in the present studies. The present results clearly indicated that the superficial zone of the TEC-mediated repair cartilage exhibited significantly lower water retaining capacity. This is the first clear demonstration of a novel functional insufficiency involved in the superficial zone of the TEC-mediated repair cartilage, an insufficiency which could be closely related to the superficial structural abnormalities also detected in the present studies. A future detailed analysis in the structure and composition of the superficial tangential zone will be required to identify the key molecule or structure which is responsible for the water retaining capacity of normal uninjured cartilage and why the TEC-mediated repair tissue is compromised in this regard.

Despite the differences in the structure and permeability between the superficial zone of the uninjured and the TEC-mediated repair cartilage, no statistically significant differences were observed in the coefficient of friction. The coefficient of friction is known to strongly depend on interstitial fluid pressurisation (Ateshian *et al.*, 2009). Given the higher permeability of the superficial zone, a diminished ability to maintain fluid load support in the TEC group was expected, translating into an increase in the measured coefficient of friction. This, however, was not observed in the present study. There are likely two points contributing to such discrepancy. First, the coefficient of friction was measured 60 s after the application of normal load, when the boundary lubrication became dominant. At that moment, the boundary film on the cartilage surface, such as lipid and protein, likely played a major role in lubrication and thus, the effect of interstitial fluid pressure may have been diminished. Secondly, even if the effect of interstitial fluid pressure remained, the modulus of the repair cartilage became softer than normal cartilage while the permeability became greater at the surface.

One of the limitations of this study was that it followed the results up to only 6 months after surgery and it is not clear whether the findings observed in the present study would continue over the long-term, or improve with time post-implantation. Specifically, there may be the possibility for the further improvement of the structure of the superficial tangential zone and of the corresponding mechanical properties of the TEC-mediated repair tissue. Longer follow up studies will be required. The use of immature pigs might be another concern. However, we had previously confirmed that skeletal maturity does not affect the quality of the TEC-mediated repair cartilage, using the same surgical model (Shimomura *et al.*, 2010), and thus the use of immature animals has been somewhat validated. Another limitation was using uninjured specimens from the same condyle which had undergone surgery. Even "uninjured" articular surfaces may have been influenced by inflammatory responses following implantation, as well as the mechanical effect of incongruence created by those chondral defects. Therefore, these specimens might not be the most perfect "normal" control. However, extensive morphological analyses showed that the uninjured cartilage showed exactly the features as normal cartilage. Finally, we failed to directly prove the structure-function relationship in the articular surface due to the missing sample to sample correspondence between the histological scoring and biomechanical testing, and, therefore, direct statistical correlations could not be calculated. However, the significant inferiority in histological scores of the superficial zone of the TEC-mediated repair cartilage compared to the uninjured cartilage, as well as the significantly inferior biomechanical properties – including micro-scale stiffness and permeability in the superficial zone of the TEC-treated tissue to the uninjured cartilage – strongly suggest correlations between the microscopic structural features, with the altered micro-biomechanical properties in the articular surface, do exist.

This study revealed unique structural abnormalities in the superficial zone of the TEC-mediated cartilaginous repair tissue. Although not discussed widely, a critical review of publications focused on cell-based cartilage repair suggests that the predominance of fibro-cartilaginous tissue at the superficial zone of the repair cartilage was likely not peculiar to the present study, but was rather commonly observed in cell-based cartilage repair using autologous chondrocytes and MSCs (Knutsen *et al.*, 2004; Saris *et al.*, 2008; Gooding *et al.*, 2006; Nejadnik *et al.*, 2010). It is our speculation that it is not likely to develop functional articular surface structure by the implantation of a biomechanically immature implant without any follow-up treatment. In this regard, the restoration of the lamina splendens and the superficial tangential zone, which is the specialised membrane-like structure composed of densely packed collagen fibrils, should be an important target to focus on for the improvement of cartilage repair quality as the field moves towards tissue regeneration.

Conclusions

Cartilage defects, repaired by implantation of a scaffold-free tissue engineered construct (TEC) derived from synovial mesenchymal stem cells, are cartilaginous tissue which exhibited macro-scale compressive properties similar to uninjured cartilage. However, the TEC-mediated repair cartilage lacks the lamina splendens, as well as the superficial tangential zone, and exhibits inferior micro-scaled mechanical properties such as surface stiffness and water retaining capacity. Further improvement of these surface structures will be required to optimise cartilage regeneration.

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Discussion with Reviewers

Reviewer I: The presence and function of a distinct lamina splendens layer has been a somewhat controversial topic within the field of cartilage research. Here you suggest that this layer could be important for establishing the permeability and, thereby, the viscoelastic properties of the repair tissue. Does the literature suggest what sort of starting material might be placed in the defect to reconstitute the lamina splendens? Alternatively, do you think it would be feasible to reproduce this thin, acellular material *in vitro* and "coat" it onto a freshly-filled defect as a means to improve the immediate functionality of the repair?

Authors: As far as we can ascertain, there are unfortunately no reports in the available literature which address the reconstruction of the lamina splendens itself. Hollander *et al.* (2010; text reference) also emphasised the importance of regeneration of the lamina splendens at the joint surface. However, it may be a significant challenge to restore the integrity of the lamina splendens and future innovative studies are certainly needed to solve this problem. The suggestion of reproducing a thin, acellular material *in vitro* and "coating" it onto a freshly-filled defect is very interesting and worth trying for the immediate functional repair.

Reviewer II: This study follows on from previous studies conducted by this group, namely; Ando *et al.* (2007), Ando *et al.* (2008), Katakai *et al.* (2009) and Shimomura *et al.* (2010) (text references). These studies have explored the various properties of TEC derived from synovial mesenchymal stem cells in relation to biomechanics, tissue maturity, cartilage repair and chondrogenicity. Figures 1,

3 and 4 have appeared in subtly different forms in earlier manuscripts and essentially duplicate the results seen in these earlier manuscripts. The results of the macro-scale tangent modules (Fig. 3d) between Normal and TEC cartilage were reported in Fig. 5 of Ando *et al.* (2007), and the results of frictional coefficient also reported in the same Figure in the same manuscript for essentially the same experiment with different parameters. Please comment.
Reviewer V: Outcomes of a similar experiment were already reported in Ando *et al.* (2007), and are hence not real "novel" data. Similar data as presented in Fig. 3 was reported previously, as well (is Fig. 3D not based on the same data as Fig. 6B in Katakai *et al.*, 2009?). Please comment.
Authors: We used the same immature animals as in the previous studies of Ando *et al.* (2007) and Katakai *et al.* (2009) (text references). However, we have performed more detailed and different analysis, namely histological and micro-scaled biomechanical analysis of the surface structure of the repair cartilage. Although there are some similar data observed, analogous to the previous studies, such data have been used as the introduction of the new investigations conducted in the present study. Furthermore, even with regard to the somewhat similar data, the detailed experimental conditions of the mechanical analysis were quite different from the previous studies. The histological figures also have been obtained from different areas of the samples, something not done previously. Therefore, all data in this paper are not the simple reuse of those from the previous studies. We believe the inclusion of these data helps the readers to clearly understand the whole story of the present study (and why we focused on the analysis of details of the superficial structure).

Reviewer II: As PRG4 is a secreted protein most of antibody labelling is extracellular, therefore we are not able to observe if cells in the TEC are producing this protein. Following on from the latter point, it is well known that PRG4 from the synovium or surrounding cartilage that is secreted into the synovial fluid can be deposited onto surrounding (TEC) cartilage. Therefore, the presence of antibody labelling in TEC cartilage does not reveal anything conclusive about cellular production of PRG4 by differentiated MSCs in TEC cartilage. The authors' analysis of these observations is insufficient. Please comment.
Authors: Since the localisation of PRG4 (Lubricin) has been considered to be related to its tissue lubrication property, we investigated the localisation of PRG4 by immunohistochemical analysis as a potential indicator of tissue surface lubrication integrity. PRG4 was localised at the surface layer of normal cartilage, as well as the TEC-mediated repair cartilage. On the other hand, the purpose of PRG4 staining was not to detect the cellular source of the PRG4 but to assess the localisation of the PRG4 molecule at the superficial zone of repair tissue, which might be related to lubrication at the tissue surface. Therefore, we have decided not to add further analysis regarding the localisation of the cellular production of PRG4 (e.g., by *in situ* hybridisation) in the present study.

Reviewer III: The permeability of cartilage is known to be strain-dependant. The authors compressed their samples by 30 % prior to permeability testing. Please clarify the motivation for this.
Authors: To avoid leaking water, it was required to tighten the water pipe to the cartilage surface in the experiment. Therefore, the permeability test was performed at the compressive strain of 30 %. Using the equation for Darcy's law indicated in the text, it is possible to calculate the permeability at any strain.

Reviewer III: The micro-indentation testing revealed differences in the surface properties of normal and engineering tissues. This was not extended to testing deeper regions of the tissue. Would this be possible? Such analysis may reveal more subtitle difference in the mechanics of other regions of the repair tissue.
Authors: Unfortunately, we did not perform such an AFM measurement on the tissue although it should be possible to do in the future. In the future, we would like to perform the AFM measurement on each layer of uninjured cartilage and the repair tissue.

Reviewer IV: The authors describe positive staining for PRG4 to be in the surface (Fig. 7a). There appears to be some staining in/of the cells lower in the cartilage also, at least in the image from the untreated group. Do the authors have any comment on this?
Authors: The positive staining of PRG4 was observed in the image of the untreated defect group. It is well known that synovial cells synthesise PRG4 (Schumacher *et al.*, 1999, additional reference). We speculated that this fibrous tissue in the untreated group might have been developed by cells from the surrounding synovial tissues and thus express the PRG4. With this speculation, there may have been some positive staining detected in the deeper area due to infiltration of the defect by such cells.

Reviewer IV: How relevant do the authors think that an injury model in a 4 month old pig is to the reparative capacity of mature and ageing humans with degenerative musculoskeletal conditions?
Authors: We have previously confirmed the feasibility of the TEC to facilitate cartilage repair regardless of skeletal maturity (Shimomura *et al.*, 2010, text reference). Therefore, the factor of aging (maturity) could be of negligible influence on the outcome of treatment. In other words, the use of immature animal has been validated to have clinical relevance. We have currently no evidence regarding the effect of potential degenerative conditions on repair using a TEC approach. Clearly osteoarthritis is an inflammatory condition, so the point is relevant but answers must await results of future investigations.

Reviewer V: Can the shape and size of the TEC be controlled? This might be a necessary requirement for future application.
Authors: The size of the TECs can be controlled by changing the size of the culture dishes to be used and

cell number to be inoculated. Thus, we chose the most suitable size of culture dish for the present studies, as well as prepared the required number of cells based on the estimated volume of the cartilage lesions/defects which is measured macroscopically, arthroscopically, or by MRI before implantation surgery. Details can be found in Ando *et al.* (2007) and Ando *et al.* (2008) (text references).

Reviewer V: Why were the TECs prepared as allografts without chondrogenic stimulation? If the allografts were stimulated chondrogenically, would there still be such a difference in the biomechanical stability of the superficial layer?
Authors: We are currently investigating the implantation of the TEC with stimulation by relevant growth factors. We are interested in the potential improvement of the superficial zone by any biological manipulation of the TEC prior to or concurrent at the time of implantation. Hopefully, we will be able to report the results of such studies in the not so distant future.

Reviewer V: It would actually be quite interesting to also test the edges of the specimens, rather than only the centre for biomechanical stability, especially as this can be hugely important in preventing graft failure.
Authors: We have recently completed measurement of the integration strength of the implants, and the results are in preparation for a future submission. Interestingly, we have found that the integration strength also depends on the depth (deep lesion shows the highest strength and superficial shows the lowest value). However, some details remain to be confirmed, but we hope to be able to report the results in the near future.

Reviewer V: The introduction of depth dependent differences may well be very important for the successful long term performance of regenerative implants. Other cell sources, such as chondrocytes or bone marrow derived stem cells may, based on recent findings in the literature, be (more) suitable. Have the authors any experience with these in their model and/or could they hypothesise how these cells would be have in their approach?
Authors: The reason for the use of synovial MSCs is based on the reported characteristics of synovial MSCs such as the retention of multipotent differentiation potential over many passages of culture (De Bari *et al.*, 2001, text reference) and superior chondrogenic potential among MSCs derived from many tissues (Sakaguchi *et al.*, 2005, text reference). We have not had any experience with MSCs derived from other tissues; however, based on the cellular characteristics, we believe that the synovium could be one of the most suitable cell sources for cartilage regeneration therapies.

Reviewer VI: Can you explain the large degree of variability in the quasi-static compression testing, as reflected by the high coefficients of variance (= SD/mean)? Is such inter-sample variability typical for this sort of test?
Authors: Since the edge of the AFM probe is nano-scale and the indentation depth is less than micro-scale, the results of the indentation test are site-specific. Therefore, it is likely that the variability in the surface condition of tissue samples could potentially have led to the somewhat high inter-sample variability in the test. Perhaps with further time post-implantation some of this variability may disappear, but currently it is the "state of the art".

Additional Reference

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Treatment of Partial Growth Arrest Using an In Vitro-generated Scaffold-free Tissue-engineered Construct Derived From Rabbit Synovial Mesenchymal Stem Cells

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Background: Injuries to the epiphyseal plate sometimes result in partial growth arrest, which can lead to the development of angular deformities and limb length discrepancies in growing children. The aim of this study was to develop a new treatment for partial growth arrest of the physis. For this purpose, we investigated the feasibility of an in vitro-generated scaffold-free tissue-engineered construct (TEC) derived from synovial mesenchymal stem cells (MSCs) in a rabbit growth arrest model.

Methods: An experimental model for growth arrest was created by excising the medial half of the proximal growth plate of tibias from 6-week-old New Zealand White rabbits. Three experimental groups were set to evaluate TEC implantation: group 1, no implantation as controls; group 2, implantation of bone wax as additional controls; and group 3, implantation of TEC in the lesion.

Results: In group 1, all damaged growth plates were arrested and angular deformities appeared 4 weeks later. In groups 2 and 3, angular deformities were less than in the control group. Histologic images showed bone bridges developed at the damaged growth plate in group 1. Regeneration of growth plates was recognized in groups 2 and 3. Histologic examination showed greater regeneration of the growth plate in group 3 than in group 2. In addition, MSCs in the TEC differentiated into proliferative and prehypertrophic chondrocyte-like cells.

Conclusions: A scaffold-free 3D TEC made using cultured synovium-derived MSCs differentiated into proliferative and prehypertrophic chondrocyte-like cells.

Clinical Relevance: The results of this experimental study suggest that scaffold-free 3D TEC made using cultured synovium-derived MSCs can be a new approach for the repair of epiphyseal injury. Clinical effectiveness of a scaffold-free 3D TEC for growth arrest remains to be determined.

Key Words: epiphyseal plate, physeal growth arrest, mesenchymal stem cell, tissue-engineered construct (TEC)

(*J Pediatr Orthop* 2012;32:314–321)

Injuries to the epiphyseal plate sometimes result in partial growth arrest, which can lead to the development of angular deformities and limb length discrepancies in growing children. Langenskiold[1,2] first demonstrated the prevention of growth arrest of the epiphyseal plate by physeal bridge resection and subsequent free transplant of fat in 1967. Since those reports, implantation of soft tissues including fat,[2–5] silastic,[6,7] and bone wax[3] have been investigated, and certain positive effects have been confirmed. However, those procedures may show limitations in terms of the control of bone growth and of the long-term safety of implant materials in vivo.

Stem cell therapies have focused on facilitating regenerative tissue repair of various tissues and organs. Among several cell sources, mesenchymal stem cells (MSCs) have the capability to differentiate into a variety of connective tissue cells, including bone, cartilage, tendon, and muscle.[8] These cells may be isolated from various tissues, such as bone marrow, skeletal muscle, and synovial membrane.[8–11] In addition to the selection of a cell source, finding an appropriate scaffold that provides a favorable 3-dimensional environment for cell proliferation and differentiation is important. For this purpose, many studies have placed priority on the development of scaffolds and various scaffolds have been approved for clinical use.[12] However, several issues associated with long-term safety remain. To minimize the risks involved, scaffold-free cell delivery systems may offer an excellent alternative.

Meanwhile, the unique matrix organization of articular cartilage results in antiadhesive properties that present an obstacle to the integration of tissues implanted adjacent to the cartilage matrix in the treatment of chondral injuries.[13] To overcome this problem, most implantation procedures into chondral lesions have required enzymatic treatment of the cartilage matrix surface,[14] or reinforcement of the initial fixation by sutures[15,16] or absorbable pins.[17] However, an animal study revealed

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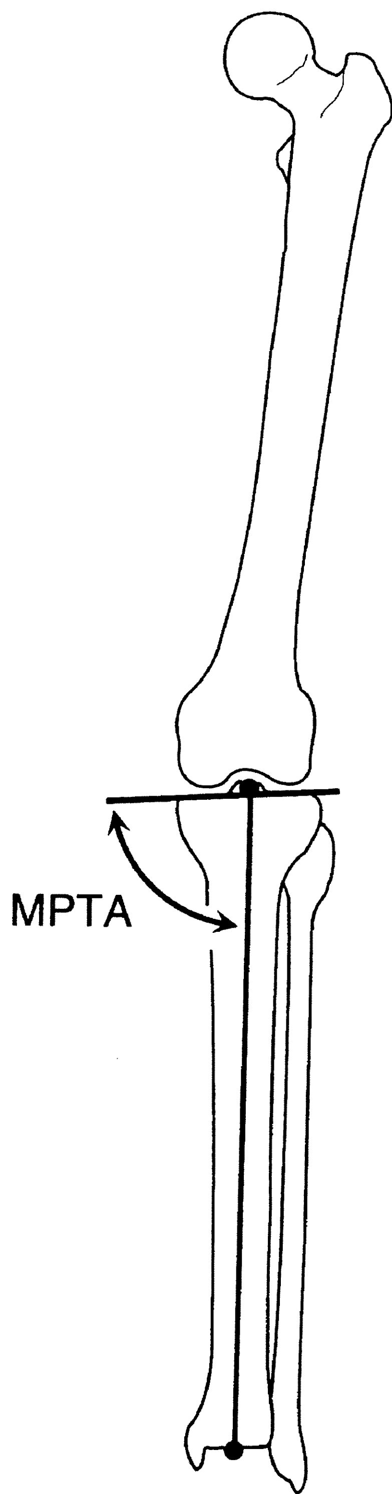

FIGURE 1. Medial proximal tibial angle (MPTA).

that suture tracks in the surrounding articular cartilage remain unhealed, representing a defect that could potentially act as a trigger site for subsequent matrix degradation around the margin between the implant and the adjacent cartilage tissue.[16] Implantable tissues that possess highly adhesive properties to cartilage tissue are thus needed for secure tissue integration.

TABLE 1. Histologic Grading Scale

Grade	Description
Cell morphology	
2	Columnar cartilage cells
1	Disordered cartilage cells
0	Noncartilage only
Staining (safranin O)	
3	
2	Slightly reduced
1	Significantly reduced
1	No staining
Thickness of epiphyseal plate*	
4	> 100%
3	75%-100%
2	50%-74%
1	25%-49%
0	< 25%

*Mean thickness of the repaired epiphyseal plate compared with that of the unoperated side.

We recently described the development of a scaffold-free 3-dimensional (3D) tissue-engineered construct (TEC) comprising MSCs derived from synovium and extracellular matrix synthesized by the cells.[18] Development of a 3D construct without the use of any artificial materials could be a key advantage for TECs. Implantation of a TEC into chondral defects in porcine knee joints initiated repair with a chondrogenic-like tissue, showing secure tissue integration with adjacent cartilage tissue.[18,19] Implantation of the TEC could thus be feasible to repair a damaged epiphyseal plate, based on the capacity for chondrogenic and osteogenic differentiation and high tissue adhesiveness.

The aim of the present study was thus to investigate the feasibility of using a TEC to repair damaged epiphyseal plates in a rabbit model.

METHODS

All procedures in this study followed the principles of the Declaration of Helsinki.

Harvest of Synovial Tissue and Isolation of MSCs

Synovial tissue was harvested from the knee joints of New Zealand White rabbits. Cells were isolated according to a previously reported protocol for the isolation of human synovial-derived MSC. Briefly, synovial membrane specimens were rinsed with phosphate-buffered saline (PBS), minced meticulously, and digested with 0.1% collagenase IV (Sigma, St Louis, MO) for 2 hours at 37°C. After neutralization of the collagenase with growth medium comprising high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Gibco BRL, Life Technologies), cells were collected by centrifugation, washed with PBS, resuspended in the growth medium, and plated in culture dishes.

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TABLE 2. MPTA at 4 Weeks After Surgery

Group 1	Group 2 (Bone Wax)	Group 3 (TEC)
62	70	74
64	76	76
64	80	79
65	83	80
75	90	84
—	—	85
—	—	88
66.0	79.8	80.9

MPTA indicates medial proximal tibial angle; TEC, tissue-engineered construct.

For expansion, cells were cultured in the growth medium at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced once a week. After 15 to 28 days of primary culture, when cells reached confluence, they were washed twice with PBS, harvested by treatment with trypsin-EDTA (0.25% trypsin and 1 mM EDTA; Gibco BRL, Life Technologies), and replaced at a 1:3 dilution when cultures reached near confluence. Cells from passages 4 to 7 were used in the present study.

Development of Scaffold-free 3D TECs

TECs were developed using the previously reported method.[18–20] Synovial MSCs were plated onto culture dishes at a density of $4.0 \times 10^5/cm^2$ in the growth medium containing 0.2 mM ascorbate 2-phosphate. After 7 to 14 days in culture, a complex of cultured cells and the extracellular matrix synthesized by the cells was detached from the substratum by application of shear stress using gentle pipetting. The detached complex was left in suspension to form a 3D structure by active tissue contraction. This tissue represented a basic scaffold-free 3D TEC.

Creation of Partial Growth Arrest and Implantation of the TEC

Male 6-week-old New Zealand White rabbits were selected because these animals would have open physes for 4 to 6 months. Rabbits were operated under general anesthesia using intramuscular injection of ketamine

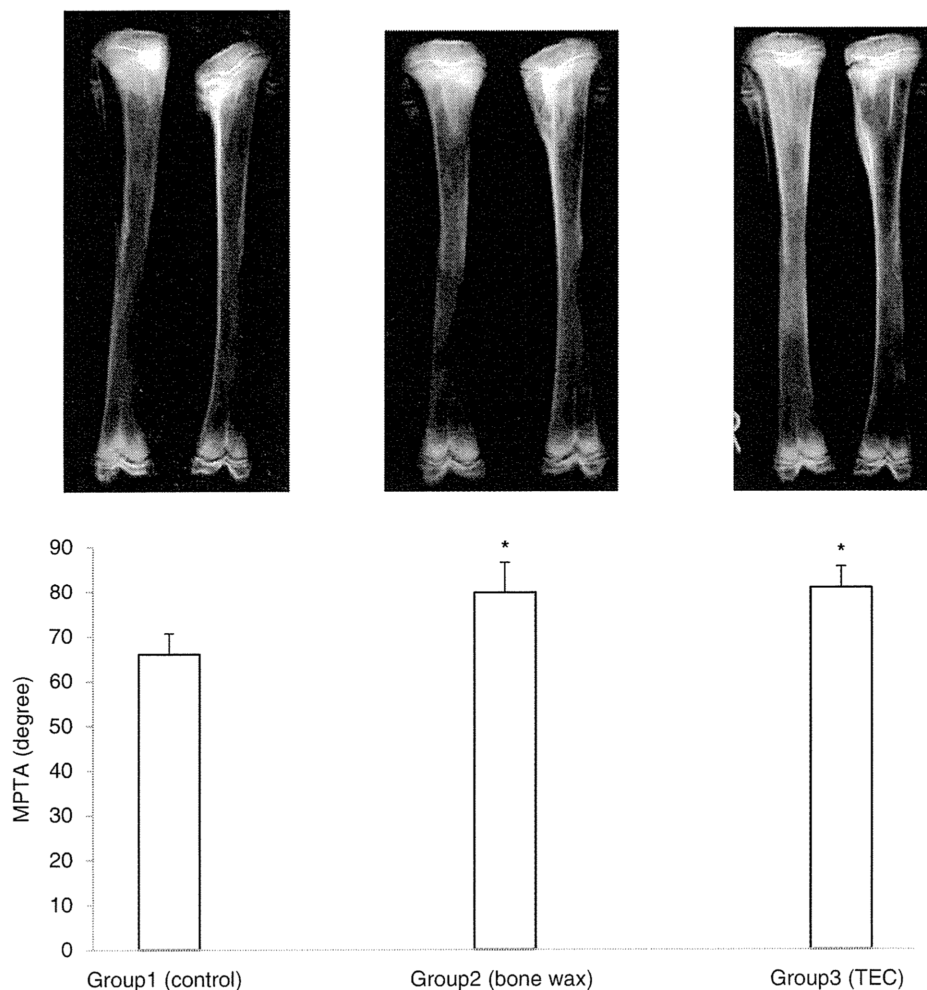

FIGURE 2. Medial proximal tibial angle (MPTA) at 4 weeks after surgery. Group 1 (control) showed varus deformity. Varus deformities were significantly better in groups 2 (bone wax) and 3 (TEC) than in group 1. $*P<0.05$.

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TABLE 3. MPTA at 8 Weeks After Surgery

Group 1	Group 2 (Bone Wax)	Group 3 (TEC)
37	56	56
41	58	59
45	65	61
47	69	65
65	73	80
—	—	88
47.0	64.2	68.2

MPTA indicates medial proximal tibial angle; TEC, tissue-engineered construct.

hydrochloride (30 mg/kg of body weight) and intravenous injection of propofol (80 mg/kg). The proximal part of the left tibia was exposed through an anteromedial incision. A window in the medial side of the growth plate was first made using a high-speed dental burr (diameter, 2 mm). The drill was then introduced and passed centrally into the epiphyseal growth plate region. Physeal defects of 3 mm diameter and 5 mm depth were created. The periosteum, subcutaneous tissue, and skin were then closed in layers. Rabbits were allowed free, weight-bearing movement with no immobilization of the legs. Operated rabbits were randomly divided into 3 groups as follows:

Group 1: nothing embedded into the physeal defect (n = 5).
Group 2: bone wax embedded into the physeal defect (n = 5).
Group 3: TECs were implanted into the physeal defect (n = 7).

Evaluation of TEC Effectiveness

Rabbits in all groups were killed at 4 or 8 weeks postoperatively. For radiographic evaluation, radiographs of the right (unoperated) and the left (operated) tibias were taken, and the medial proximal tibial angle (MPTA) was measured (Fig. 1).

For histologic evaluation, sections through the proximal tibia were made and stained with hematoxylin and eosin or safranin O. Histologic findings were scored according to a histologic grading scale (Table 1), with

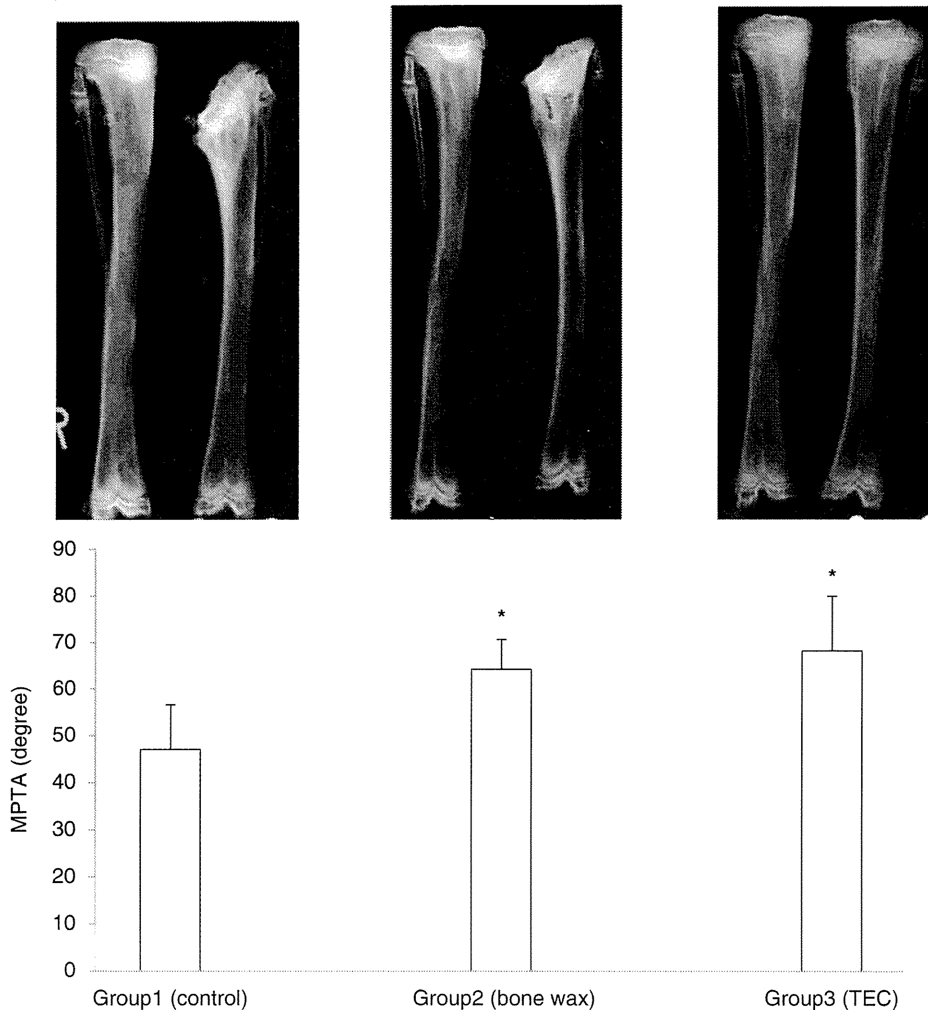

FIGURE 3. Medial proximal tibial angle (MPTA) at 8 weeks after surgery. Varus deformities were more severe in all groups than at 4 weeks. Varus deformities were significantly better in groups 2 (bone wax) and 3 (TEC) than in group 1. *$P<0.05$.

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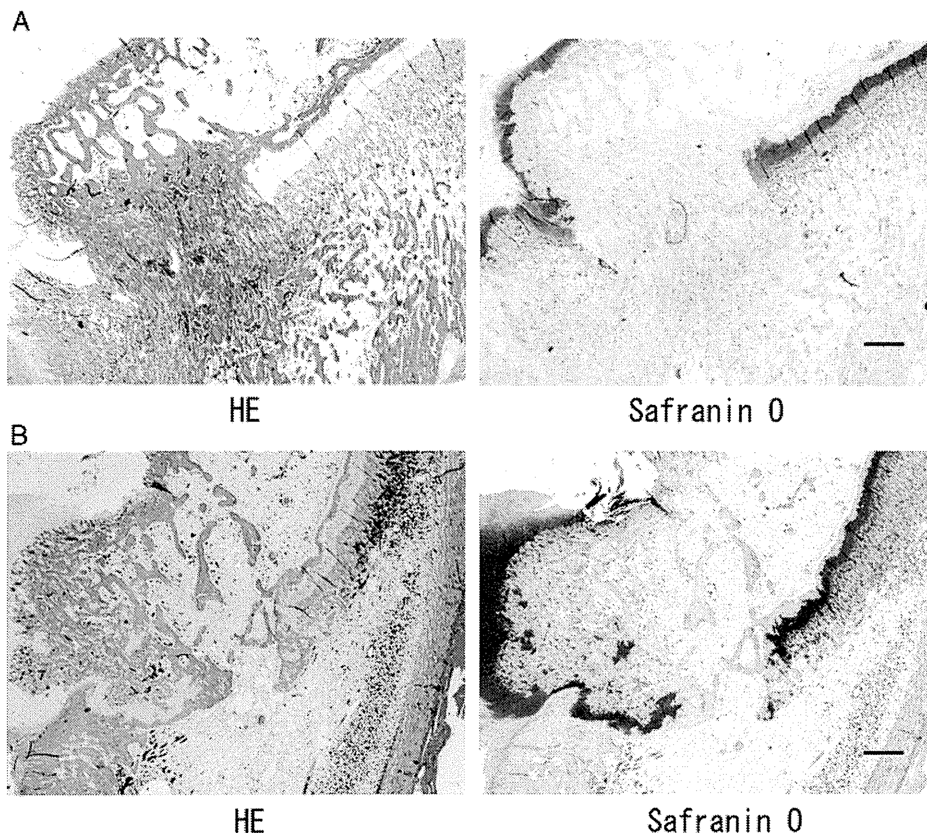

FIGURE 4. Histology in group 1. A, Histology at 4 weeks after surgery, showing bony bridge formation. B, Histology at 8 weeks. Bony bridge formation is increased and the epiphysis shows a wide connection with the metaphysis. Bar, 500 μm. HE indicates hematoxylin and eosin.

higher scores indicating higher quality of the growth plate repair.

Statistical Analysis

All data from at least duplicate samples are expressed as means ± SD, and a minimum of 3 independent experiments were performed. Unpaired Student *t* test or analysis of variance for multiple comparisons were used for statistical analysis. Differences between experimental groups were considered significant for values of $P < 0.05$.

RESULTS

Radiographic Results

Group 1 demonstrated angular deformity of the left tibia, with a mean MPTA of 66.0 degrees (range, 62 to 75 degrees) at 4 weeks after surgery (Table 2 and Fig. 2) and 47.5 degrees (range, 37 to 65 degrees) at 8 weeks after surgery (Table 3 and Fig. 3). Radiography in this group showed partial growth arrest of the proximal medial tibias (Figs. 2, 3). The mean MPTA of the right tibia was 88.0 degrees.

The mean MPTA in group 2 was 79.8 degrees (range, 70 to 90 degrees) at 4 weeks after surgery (Table 2 and Fig. 2) and 64.2 degrees (range, 56 to 73 degrees) at 8 weeks after surgery (Table 3 and Fig. 3). Varus deformity was milder than in group 1 at 4 weeks, postoperatively (Fig. 2). At 8 weeks after surgery, all cases showed varus deformity (Table 3 and Fig. 3). MPTA was significantly better than in group 1 at both 4 and 8 weeks after surgery (Figs. 2, 3).

The mean MPTA in group 3 was 80.9 degrees (range, 74 to 88 degrees) at 4 weeks after surgery (Table 2 and Fig. 2) and 68.2 degrees (range, 56 to 88 degrees) at 8 weeks after surgery (Table 3 and Fig. 3). Varus deformity was much milder than in group 1 at 4 weeks after surgery (Fig. 2), and MPTA in 3 of the 6 operated tibias was almost the same as in the unoperated tibias (Table 2). At 8 weeks postoperatively, 1 of the 6 operated tibias showed normal MPTA, and another case showed very little varus deformity. However, 4 of the 6 operated tibias showed varus deformity (Table 3 and Fig. 3). Compared with group 1, MPTA was significantly improved at both 4 and 8 weeks after surgery (Figs. 2, 3).

Histologic Findings

In group 1, a bony bridge formation replaced the epiphyseal growth plate at the operated site, with the epiphysis showing a wide connection with the metaphysis at 4 weeks after surgery (Fig. 4A) and increased connection at 8 weeks (Fig. 4B).

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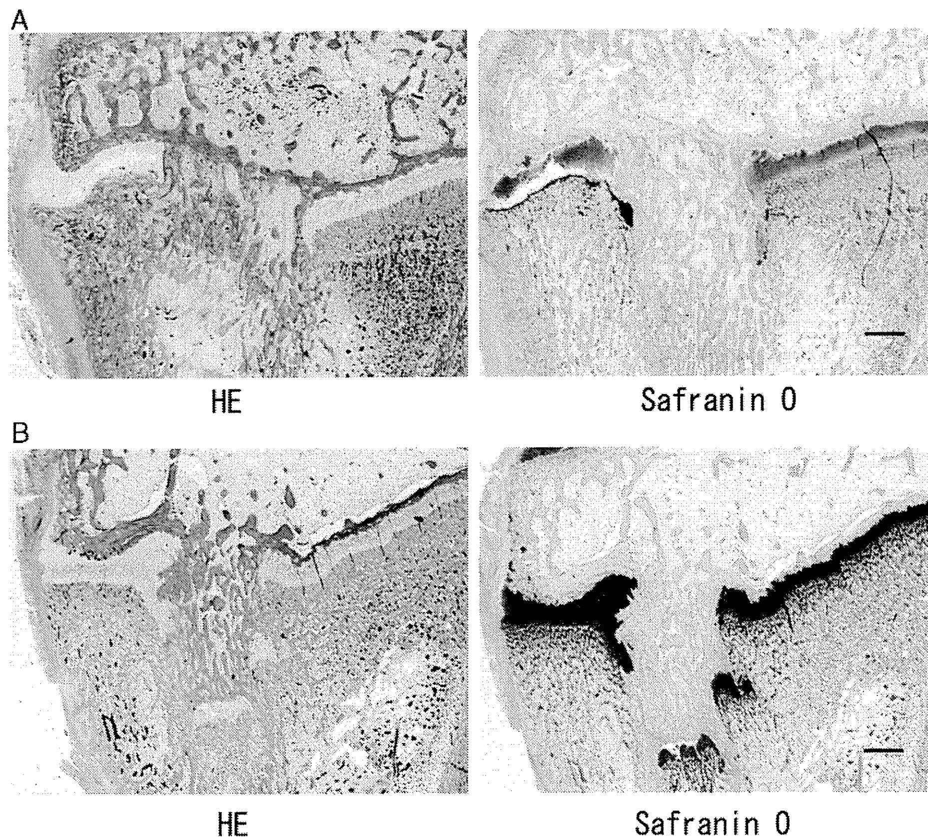

FIGURE 5. Histology in group 2 (bone wax). A, Histology at 4 weeks after surgery, showing partial bony bridge formation. B, Histology at 8 weeks, showing partial growth arrest over a wider area than at 4 weeks. Bar, 500 μm. HE indicates hematoxylin and eosin.

In group 2, all cases showed partial bony bridge formation. The transplanted bone wax remained at the operated site and showed no staining with hematoxylin and eosin or safranin O. However, some cases showed disappearance of transplanted bone wax and replacement with a bony bridge and growth plate-like tissue (Fig. 5). At 8 weeks after surgery, many cases showed a wider bony bridge area than at 4 weeks.

In group 3, all cases showed partial bony bridge formation. However, proliferative and prehypertrophic chondrocyte-like cells were seen at the operated site 4 weeks postoperatively (Fig. 6A). The cells took on a columnar arrangement like a normal physis and the physeal area was thickened (Fig. 6B). The cartilage matrix showed staining with safranin O, indicating the chondrogenic potential of MSCs in the TEC. Regeneration of the growth plate was recognized in the tibias without deformity (Fig. 6D).

Histologic Scores

Histologic findings at 4 and 8 weeks after surgery were evaluated and rated using a histologic grading scale (Table 1). At 4 weeks after surgery, histologic scores for the repaired growth plate were higher in groups 2 and 3 than in group 1 (Table 4). At 8 weeks after surgery, histologic scores were lower in group 2 compared with scores at 4 weeks (Table 5). Scores were significantly higher in group 3 than in group 2. At 8 weeks after surgery, group 2 showed a wider area of bony bridge formation, resulting in lower scores. Group 3 showed a thickened physeal area, resulting in higher scores.

DISCUSSION

The physis has limited ability to repair itself. Injury and damage may thus lead to deformities and shortening of long bones by growth arrest with bone bridge formation. Various treatments such as gradual correction and bone bridge resection have been used with varying rates of success. In treatment by bone bridge resection, several types of interposition material have been used in clinical practice. Langenskiold[21] reported 38 cases of physeal bridge resection using autogenous body fat, suggesting that 82% of cases benefited from the procedure. Bright reported a series of 100 patients treated using silastic as the interposition material,[22] with 81% of patients demonstrating some growth after bridge resection, and 70% achieving good to excellent results. Klassen and Peterson[23] reviewed the Mayo Clinic experience with 50 cases of physeal bridge resection using cranioplast (methylmethacrylate) as the interposition material, reporting early closure of the operated physis. These findings

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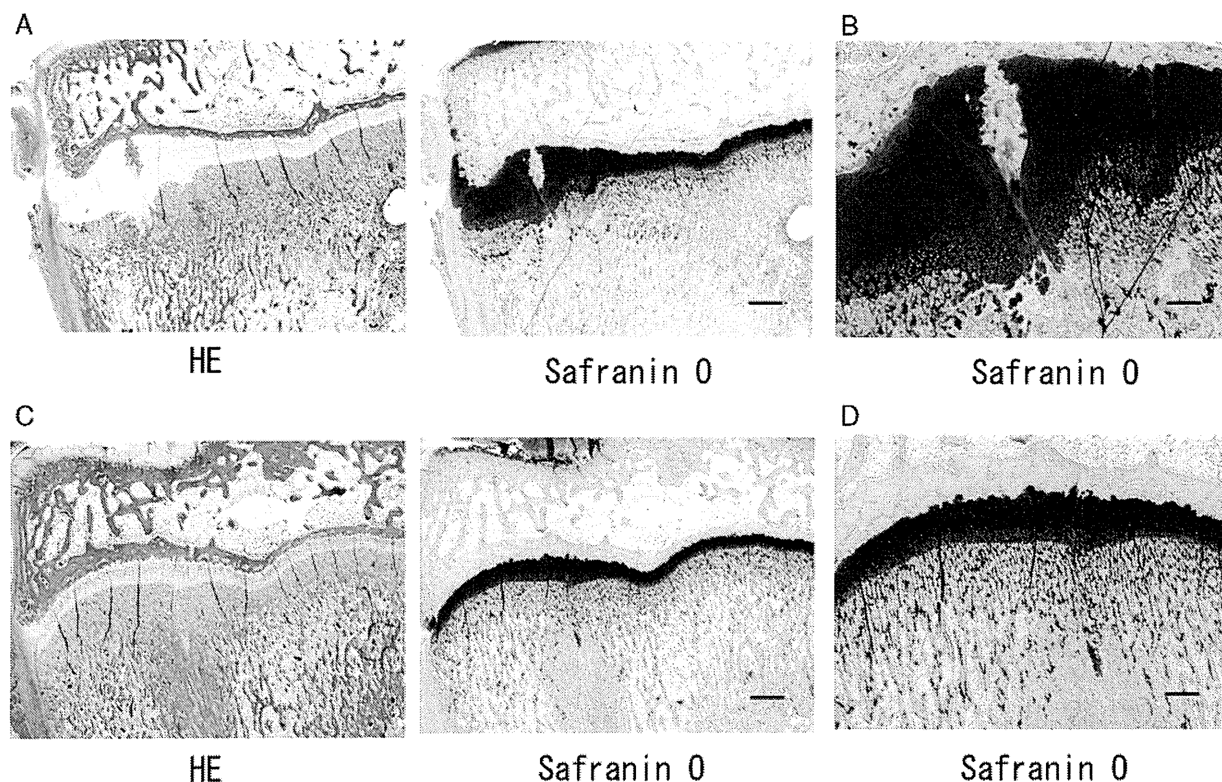

FIGURE 6. Histology in group 3 (TEC). A, Histology at 4 weeks after surgery, showing little bony bridge formation and repaired epiphyseal plate on the operated site. B, High-power section (×2) of the new physis at 4 weeks after surgery. Proliferative and prehypertrophic chondrocyte-like cells are seen on the operated site, and the physeal area is thickened. Bar, 500 μm. C, Histology at 8 weeks after surgery, showing repaired epiphyseal plate. D, High-power section (×2) at 8 weeks after surgery, showing the thickened physeal area on the operated site. Bar, 500 μm. HE indicates hematoxylin and eosin.

suggest that the interposition of fat, silastic, and other artificial materials might be associated with limitations to bone growth and several issues associated with long-term safety. We used bone wax as the interposition material in group 2. Many orthopaedic surgeons perform fat grafting in human epiphyseal plate injury. However , the bone wax is more difficult to be absorbed than the fat, and so, we used bone wax as the interposition material in this study.

The present study has demonstrated the feasibility of using a unique scaffold-free TEC from synovium-derived MSCs to repair the injured epiphyseal plate. The use of this unique TEC has the following benefits.

First, TECs can be autogeneously developed without any need for an exogenous scaffold. Implantation of TECs thus has minimal risk of potential side effects induced by biological and artificial materials in the scaffold. Some papers have reported transfer of MSCs using scaffolds for the treatment of growth arrest.[24,25] No similar reports in the literature have mentioned the use of cultured MSCs without scaffolds to repair epiphyseal plate injury.

Second, TECs have the ability to allow chondrogenesis. We implanted pluripotent MSCs into the epiphyseal plate area, because reconstructing the columnar structure of the growth plate by MSCs in vitro is difficult.

TABLE 4. Histologic Scores 4 Weeks After Surgery for the Repaired Growth Plate

Rabbit Number	Group 1	Group 2 (Bone Wax)	Group 3 (TEC)
1	0.0	1.0	3.0
2	0.5	2.5	3.0
3	0.5	3.0	3.5
4	4.0	6.0	3.5
5	5.5	8.5	5
6	—	—	6.5
7	—	—	7.0
Mean ± SD	2.1 ± 2.4	4.2 ± 3.0	4.5 ± 1.6

TEC indicates tissue-engineered construct.

TABLE 5. Histologic Scores 8 Weeks After Surgery for the Repaired Growth Plate

Rabbit Number	Group 1	Group 2 (Bone Wax)	Group 3 (TEC)
1	1.0	1.5	2.0
2	1.0	1.5	3.0
3	3.0	1.5	5
4	3.0	1.5	6.0
5	5.0	2.0	7.0
6	—	—	8.0
Mean ± SD	2.6 ± 1.6	1.6 ± 0.2	5.2 ± 2.5*

*Significantly different from groups 1 and 2 ($P < 0.05$).
TEC indicates tissue-engineered construct.

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The local in vivo environment might have stimulated differentiation of the TEC. For example, multiple cytokines such as parathyroid hormone-related protein and Indian hedgehog exist at various concentration gradients in the growth plate. We hypothesized that these cytokines could help with regeneration of the injured physis. Our results showed that using MSCs without scaffolds resulted in less bony bridge formation and contributed to longitudinal bone growth. In the control group, bony bridge trabeculae replaced the epiphyseal plate. In the TEC group, proliferative and prehypertrophic chondrocyte-like cells were seen on the operated site. These cells took on a slightly disordered columnar arrangement like a normal physis, and the physeal area was thickened (Fig. 6). The implanted stem cells may thus show differentiation responses in the epiphyseal plate. In groups 2 and 3, the radiographic results were similar. However, the histologic scores of group 3 were significantly better than that of group 2. This is because the implanted stem cells could help the regeneration of the injured physis. We are planning to perform a longer duration of examination in the next study.

Further studies are needed to clarify the function of the new physis from the TEC with regard to bone growth and to examine applications in other large animals, including humans. This study used a small number of animals and only continued observations for 8 weeks. A larger number of subjects and a longer duration of examination are thus warranted. To attain more extensive chondrogenic differentiation responses, biological manipulation of the TEC may be required before implantation. This study used synovium-derived cells, which reportedly exhibit the most enhanced chondrogenic potential among mesenchymal tissue-derived cells.[11] If TECs are to be used for repair of epiphyseal injury in large animals such as humans, large quantities of cells will be needed. To overcome this problem, we are planning to use TECs cultured from induced pluripotent stem cells after confirming the quality of TECs developed using induced Pluripotent Stem cells in the next study.

CONCLUSIONS

A scaffold-free 3D TEC made using cultured synovium-derived MSCs differentiated into proliferative and prehypertrophic chondrocyte-like cells. We have demonstrated the feasibility of a scaffold-free 3D TEC as a new approach for the repair of epiphyseal injury.

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Inflammation Research

ORIGINAL RESEARCH PAPER

Ovine synovial membrane-derived mesenchymal progenitor cells retain the phenotype of the original tissue that was exposed to in-vivo inflammation: evidence for a suppressed chondrogenic differentiation potential of the cells

Wataru Ando · Bryan J. Heard · May Chung · Norimasa Nakamura · Cyril B. Frank · David A. Hart

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Abstract
Background The purpose of this study was to characterize the effect of post-surgery joint inflammation on the chondrogenic differentiation capacity of mesenchymal progenitor cells (MPCs) derived from the synovial membrane (SM).
Methods Six Suffolk-cross sheep were subjected to experimental anterior cruciate ligament (ACL) core surgeries. After they were killed 2 weeks after surgery, the volume of synovial fluid in the knees was measured and SM was collected for mRNA extraction and cell isolation. Cells were propagated and used for lineage-specific differentiation assays using cell pellet cultures and mRNA extraction. Chondrogenic differentiation assays in the presence of exogenous interleukin-1β (IL-1β) were also performed.
Results The volume of synovial fluid from the operated knees was significantly greater than from the contralateral knees. Quantitative RT-PCR revealed that mRNA levels for IL-1β and matrix metalloproteinases-3 and -13 in SM from the operated knees were significantly higher than those from the contralateral knees. The size of MPC pellets from operated knees (opMPC) cultured in chondrogenic medium were significantly smaller than the corresponding pellets generated with MPCs from contralateral knees (conMPC). Addition of 1–100 ng/ml IL-1β significantly suppressed the resultant size of chondrogenic cell pellets from normal ovine SM-MPC.
Discussion From these results, we conclude that cells from SM exposed to post-surgical inflammation are compromised by the inflammatory environment and that IL-1β can inhibit the latent chondrogenic potential of normal MPCs. This suggests that if MPCs from injured joints do contribute to cartilage repair, their endogenous repair potential may become compromised by such post-injury joint inflammation.

Keywords Chondrogenesis · Inflammation · Interleukin (IL) · Matrix metalloproteinases (MMP) · Mesenchymal progenitor cells (MPCs)

Abbreviations

ACL	Anterior cruciate ligament
BM	Bone marrow
GAG	Glycosaminoglycan
IL-1β	Interleukin-1β
opMPC	MPC from operated knee
conMPC	MPC from contralateral knees
MPCs	Mesenchymal progenitor cells
MMPs	Metalloproteinases
OA	Osteoarthritis
SF	Synovial fluid
SM	Synovial membrane
TNFα	Tumor necrosis factor-α

Introduction

Mesenchymal stem/progenitor cells (MSCs/MPCs) have the capacity to differentiate to a number of connective

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tissue lineages such as osteogenic, chondrogenic, and adipogenic [1]. These cells may be isolated from various tissues such as bone marrow (BM), skeletal muscle, synovial membrane (SM), adipose tissue, umbilical cord blood, and placenta [1–6]. Among cells from a number of tissue sources, SM-MSCs/MPCs exhibit the most chondrogenic potential [7]. These cells are proposed to play an important role in the intraarticular environment to enhance endogenous repair and to be an appropriate cell source for cartilage regeneration therapy [8].

The surface 'intima' and the underlying 'subintima' are the two distinct anatomical layers forming the synovial tissue. The former layer is avascular and loosely organized, as it is not supported by a basement membrane, whereas the subintima consists of a network of connective tissue intermingled with cell types and blood vessels. The cell types predominantly present in this tissue are macrophage- and fibroblast-like synoviocytes [9] which are involved in the production of specialized matrix constituents such as hyaluronan, collagens, and fibronectin for the intimal interstitium and synovial fluid [10]. Although MSCs have been obtained from both synovium and synovial fluid, no specific location has been proposed where they may be present. In culture, cell surface markers, especially $CD44^+$, are used to isolate these cells from a mixed population of cells of synovium or synovial fluid [11]. However, for the sheep model there is a shortage of reagents available.

Intraarticular injuries can lead to the onset and progression of osteoarthritis (OA). The mechanism of cartilage degradation is believed to be multi-factorial, and biological factors are believed to play important roles in the pathophysiology of OA [9, 12]. Such biological factors can aggravate cartilage degeneration by contributing to an unstable balance between catabolic and anabolic influences on cells. Inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) are known to be increased in the synovial fluid (SF) volume from patients with anterior cruciate ligament (ACL) injury [13–16], and these cytokines could potentially stimulate the synthesis of matrix degrading metalloproteinases (MMPs) [17, 18]. Such MMPs are believed to be partially responsible for the degradation of extracellular matrix in cartilage, contributing to the progression of OA.

The effect of intraarticular inflammation on the differentiation capacity of MSCs is controversial. It has been reported that advanced OA is correlated with reductions in the differentiation activity of human BM-MPCs [19], while normal cartilage contains $CD105^+$/$CD166^+$ cells that have the phenotype of MPCs and the frequency of these cells has been reported to be increased in OA cartilage [20]. On the other hand, some studies have reported that BM-MSCs from patients with arthritis possess chondrogenic potential similar to BM-MSCs isolated from healthy donors [21]. Furthermore, the chondrogenic potential of human adult BM-MSCs has been reported to be independent of OA etiology [22]. The controversy is based, in part, on the fact that the differentiation capacities of BM-MPCs, and not SM-MPCs that have more chondrogenic potential than BM-cells [7], were compared in all of these previous studies.

Cells and tissues are exposed to the surgery-associated inflammation after surgery, and it is unclear how such inflammation affects both the endogenous tissues and cells in such environments in either the short term or long term. In spite of ACL reconstruction surgery, current ligament reconstruction procedures may not eliminate the progression of OA [23, 24], possibly related to inflammation in the joint. The purpose of this study was to characterize the effect of post-surgery inflammation on cells in the SM and the mesenchymal differentiation capacity of MPCs derived from SM exposed to short-term post-surgery inflammation in an established ovine model.

Materials and methods

Tissue collection, isolation and expansion of the cells

This study was performed in accordance with a protocol approved by the institutional animal ethics committee. Six skeletally mature 3–4-year-old female Suffolk-cross sheep were subjected to anatomically positioned reconstructive ACL autograft surgeries which were accomplished via an arthrotomy to the right stifle joint. The surgical procedure has been described in detail in Heard et al. [25]. Briefly, each surgical animal received an injection of Liquamycin (Pfizer Canada, Inc., Kirkland, QC, Canada) 24 h pre- and post-surgery, Atro-Sa (Rafter Products, Calgary, AB, Canada), Acevet (Vetoquinal, Inc., Lavaltrie, QC, Canada), and Temgesic (Schering-Plough, Hertfordshire, UK) which were all administered as a pre-anesthetic cocktail. Under anesthesia, the patella was dislocated medially to expose the ACL. The proximal head of the lateral femoral condyle was the entry point for a guide pin that was inserted to mark the femoral insertion of the ACL. A dry pneumatic nitrogen drill with a hollow bore coring device was fitted over the guide pin and was used to core out the ACL from its femoral insertion and the ligament was later replaced at the same anatomical position. All animals were observed to be healthy and were killed 2 weeks after surgery.

The legs were severed from the hip joint within 30 min of death and incised for joint capsule exposure. Next, the synovial fluid was aspirated with a 10 ml syringe as much as possible, prior to collection of all other tissues of the joint capsule. Following collection, the total volume of the synovial fluid was measured. SMs were obtained

aseptically from both knees of each sheep. Half of each SM was used for cell isolation and the remainder was snap-frozen in liquid nitrogen and later used for mRNA extraction. The cell isolation protocol from SM was according to that described previously [8]. Briefly, the tissues were rinsed with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA), and then minced meticulously. The minced tissue was then digested with 0.2% collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) for 2 h at 37°C. After neutralization of the collagenase with basic culture medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, lot #439145) and 1% penicillin/streptomycin (Invitrogen), the cells were collected by centrifugation, resuspended in basic culture medium, and plated in T-25 cell culture flasks. For expansion, cells were cultured in the basic culture medium at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced twice every week. After 7–10 days of primary culture, the cells were washed with PBS, harvested by brief treatment with 0.25% trypsin and 1 mM EDTA (Invitrogen), and replated in two T-75 flasks for the first subculture. Cells at passage 2 were used for the subsequent differentiation assays and mRNA analyses.

In-vitro chondrogenic differentiation assays

For cell pellet culture analysis, 2×10^5 cells were transferred into 15 ml polypropylene tubes, centrifuged at 500*g* for 5 min to form a pelleted micromass at the bottom of the tube, and these pellets were then treated with chondrogenic medium for 14 days with medium changes twice weekly. The chondrogenic medium consisted of DMEM supplemented with 1% insulin–transferrin–selenium supplement (Invitrogen), 100 nM dexamethasone (Sigma-Aldrich), 0.2 mM ascorbic acid 2-phosphate (Asc-2p; Sigma-Aldrich) and 500 ng/ml recombinant human BMP2 (PeproTech Inc., Rocky Hill, NJ, USA) [26, 27]. After 14 days of culture, macroscopic analysis was performed by stereomicroscopic procedures [27]. The images were analyzed by Scion image (Scion Corporation, Frederick, MD, USA), and then the cross-sectional areas of the resultant cell pellets were calculated as a parameter of pellet size. Glycosaminoglycan (GAG) levels in the pellet cultures were measured as previously described [28]. Briefly, cell pellets were digested with a 0.25 mg/ml papain solution (Sigma), and then the eluates were assessed using the 1,9-dimethylmethylene blue binding assay. Absorbance of the extracted product was measured using a spectrophotometer (Benchmark Plus; Bio-Rad, Hercules, CA, USA) at 510 nm. The total amount of protein was also measured by the bicinchoninic acid protein assay method (Sigma) for normalization. Each of the cell pellet samples was assessed in duplicate.

For mRNA extraction, cells were seeded in 12-well plates at a density of 1×10^4 cells/cm^2 in the basic culture medium. The medium was then changed to the chondrogenic induction medium at 24 h and day 3. Samples were collected at day 7 and mRNA extracted using the TRIspin method [29].

In-vitro osteogenic differentiation assays

Cells were seeded in 24-well plates at a density of 1×10^4 cells/cm^2 in the basic culture medium. After the medium was changed to an osteogenic medium at 24 h, cells were continuously cultured with medium changed twice weekly. Osteogenic medium consisted of basic culture medium, 100 nM dexamethasone, 10 mM α-glycerophosphate, and 0.2 mM Asc-2P (all from Sigma-Aldrich) [3]. At day 10, some cells were fixed with 10% neutral buffered formalin and then stained with Alizarin Red S solution (Sigma-Aldrich) for 3 min to stain for calcium deposits. For measurement of calcium deposition, other cell preparations were lysed in 10% formic acid and calcium content in solutions was measured using a colorimetric kit (Arsenazo III; Diagnostic Chemicals Limited, Charlottetown, PE, Canada). Each of the samples was assessed in duplicate.

In-vitro adipogenic differentiation assays

Cells were seeded in 24-well plates at a density of 1×10^4 cells/cm^2 in basic culture medium for 2 days. The medium was then shifted to adipogenic induction medium consisted of basic culture medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM hydrocortisone, 0.1 mM indomethacin and 5 mM octanoate. After 2 days, the medium was replaced with basic culture medium supplemented with 5 μg/ml insulin and 5 mM octanoate (all from Sigma-Aldrich) [1, 30]. Cells were cultured for an additional 6 days before staining with Oil Red O (Sigma-Aldrich). After fixation in 10% neutral buffered formalin, the cells were immersed in a 60% Oil Red O solution (Sigma) for 30 min. The cultures were then washed thoroughly with distilled water to remove background Oil Red O staining. The associated Oil Red O was eluted with 100 μl of 100% isopropanol, and the optical density of 70 μl of the eluates was quantified using a spectrophotometer at 510 nm [31]. The absorbance of the cells cultured in the basic culture medium was used as the background value to compare each sample. Each of the eluates was tested in duplicate.

Chondrogenic differentiation assay in the presence of exogenous IL-1β

To examine the effect of IL-1β on chondrogenesis of MPCs, normal SM were obtained from normal sheep not involved in the ACL core surgery aims of the study. Cells were isolated and expanded using the same procedure as described above. Pelleted micromasses from these cells were cultured in the chondrogenic medium containing human 0–100 nM IL-1β (PeproTech) starting on day 1. At day 14, the cross-sectional areas of the resultant cell pellets were calculated as described above.

Semi-quantitative real-time polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR (RT-qPCR) analysis

Total RNA was isolated from both SM tissue and the cells isolated from these tissues using the TRIspin method [29]. One microgram of total RNA from each sample was initially reverse-transcribed with random RT primers using an Omniscript RT kit (Qiagen, Hilden, Germany). Type II collagen expression was assessed for determining the extent of chondrogenesis by semi-quantitative RT-PCR using specific PCR primers as described in Table 1. All amplicons were sequenced to confirm specificity for sheep molecules (data not shown). The protocol described previously was used throughout [32]. The optional cycle number for both col2a1 and 18S was 24. Amplicons were separated on a 2% agarose gel followed by staining with ethidium bromide and band detection using a GelDoc system (Bio-Rad). RT-qPCR was performed using Bio-Rad iQ SYBR Green Supermix (Bio-Rad) as previously described [33]. PCR primers and the annealing temperatures are listed in Table 1. Amplification and detection were performed using an iCycler Thermal Cycler (Bio-Rad). To normalize for input load of cDNA between the samples, 18S was used as an endogenous standard for normalization. Preliminary studies indicated that 18S mRNA levels did not change during the response to injury (data not shown). The quantification of the relative fold change between samples was analyzed using iCycler iQ Opticak System Software, ver. 3.0a (Bio-Rad). Each of the cDNA preparations was tested in duplicate. The standard curve method was used to quantify the relative fold change between samples. The correlation coefficient of each standard curve was $\sim$0.99 [34].

Statistical analysis

The results are presented as mean ± SD. Measurement of synovial fluid volume, mRNA levels, and adipogenesis parameters were analyzed by the Mann–Whitney *U* test. Measurements of chondrogenesis and osteogenesis parameters were analyzed by ANOVA with Bonferroni's multiple comparison *t* test. STATVIEW version 5.5 software performed statistical calculations (SAS Institute, Cary, NC, USA) and significance was set at $p < 0.05$.

Table 1 Primer sequences and conditions for PCR analysis of sheep mRNA

Gene	Gene ID	Primer sequence	T_m (°C)	Size (bp)
18S (human)	X03205	F: TGGTCGCTCGCTCCTCTCC R: CGCCTGCTGCCTTCCTTGG	65	360
Col2a1 (bovine)	X02420	F: GAGCAGCAAGAGCAAGGACAAG R: GTAGGTGATGTTCTGAGAGCCCTC	53	163
IL-1β	NM_001009465	F: CGAACATGTCTTCCGTGATG R: TCTCTGTCCTGGAGTTTGCAT	57	144
IL-6	NM_001009392	F: ACAGCAAGGAGACACTGGCA R: GCCGCAGCTACTTCATCCGA	57	396
MMP-2	AF267159	F: CTACCACCTCCAACTACGAT R: AGAATGTGGCTACTAGCAG	57	412
MMP-3 (bovine)	AF135232	F: TTAGAGAACATGGGGACTTTTTG R: CGGGTTCGGGAGGCACAG	65	360
MMP-13	AY091604	F: GGTCTGTTGGCTCACGCTTTCC R: GAGTGCTCCTGGGTCCTTGG	65	171

18S 18S ribosomal RNA gene, *Col2a1* collagen type 2a1, *IL-1β* interleukin-1β, *IL-6* interleukin-6, *MMP2* matrix metalloproteinase-2, *MMP3* matrix metalloproteinase-3, *MMP13* matrix metalloproteinase-13

Results

Synovial fluid volumes are increased and mRNA levels for IL-1β and matrix metalloproteinase (MMP)-3 and -13 are upregulated in SM after surgery

At 2 weeks after surgery, the knees were swollen with significantly increased SF volumes in operated knees (9.21 $\pm$ 4.00 ml) compared to the contralateral knees (0.583 $\pm$ 0.342 ml, $p = 0.003$). Thus, this time point was chosen to assess the acute response to injury/surgery. To further investigate the differences in SM between operated knees and contralateral knees, mRNA levels for catabolic biomarkers were assessed. RT-qPCR revealed that mRNA levels for IL-1β, MMP-3, and MMP-13 normalized to 18S in SM from the operated knees were significantly higher than those from contralateral knees (Fig. 1a–c). Regarding mRNA levels for IL-6 and MMP-2, significant differences were not uniformly detected (Fig. 1d, e). These results suggested that surgical invasion of the joint leads to induction of selective inflammatory mediators in the joint, and this is reflected in elevated mRNA levels for a subset of catabolic biomarkers in the SM tissue.

Cells from SM exposed to post-surgery inflammation exhibit diminished chondrogenic potential compared to those from non-operated contralateral knees

Cells were isolated from the operated (opMPC) and contralateral (conMPC) knees. To evaluate the chondrogenic potential of both opMPCs and conMPCs, cell pellets were cultured in chondrogenic medium. Pellets from cells cultured in chondrogenic medium were significantly larger than those for the corresponding cells cultured in control

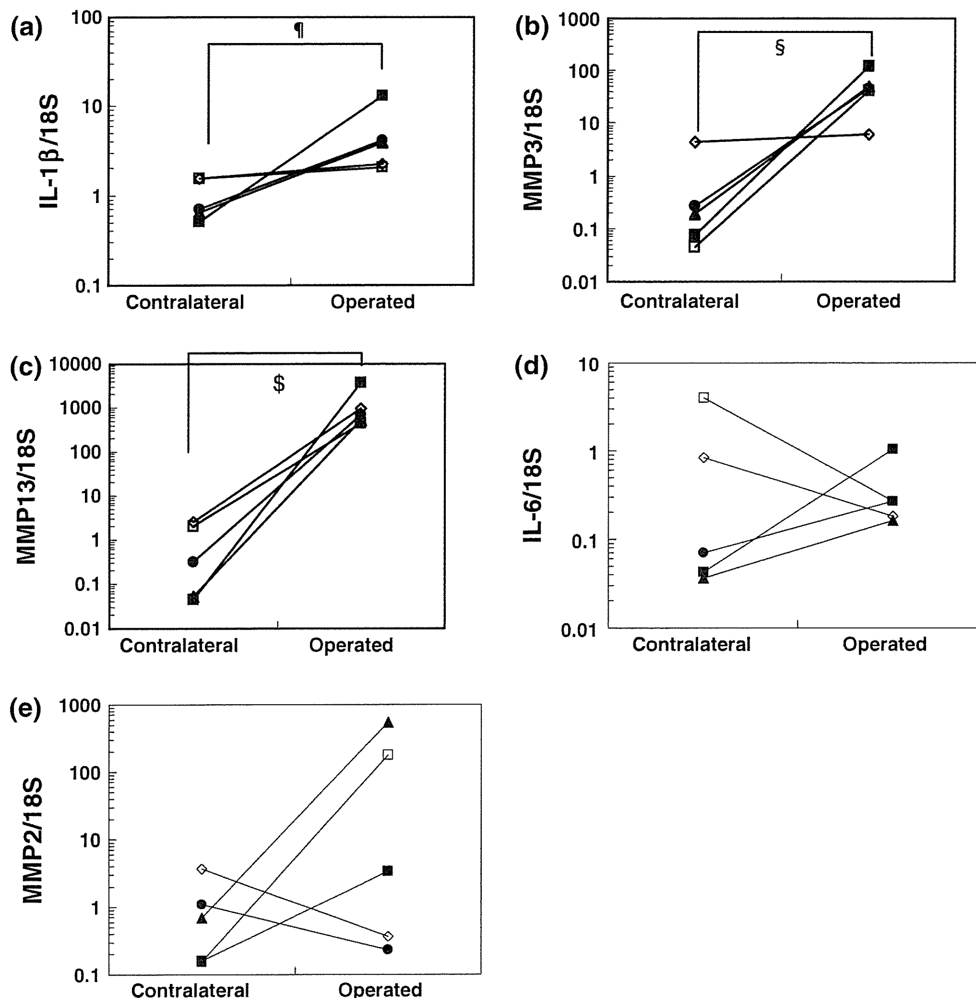

Fig. 1 mRNA levels for inflammatory mediators and MMPs in the SM after surgery. Quantification of the results of RT-qPCR for inflammatory mediators and MMPs including IL-1β (**a**), MMP-3 (**b**), MMP-13 (**c**), IL-6 (**d**), and MMP-2 (**e**) normalized to 18S in SM from the operated and contralateral knees of the sheep at 2 weeks after surgery. mRNA extraction failed from one sheep under ACL reconstruction surgery. ¶$p = 0.006$, §$p = 0.009$, \$$p = 0.006$. *Filled triangle, filled square, filled circle, open diamond, open square*: sheep 1–5

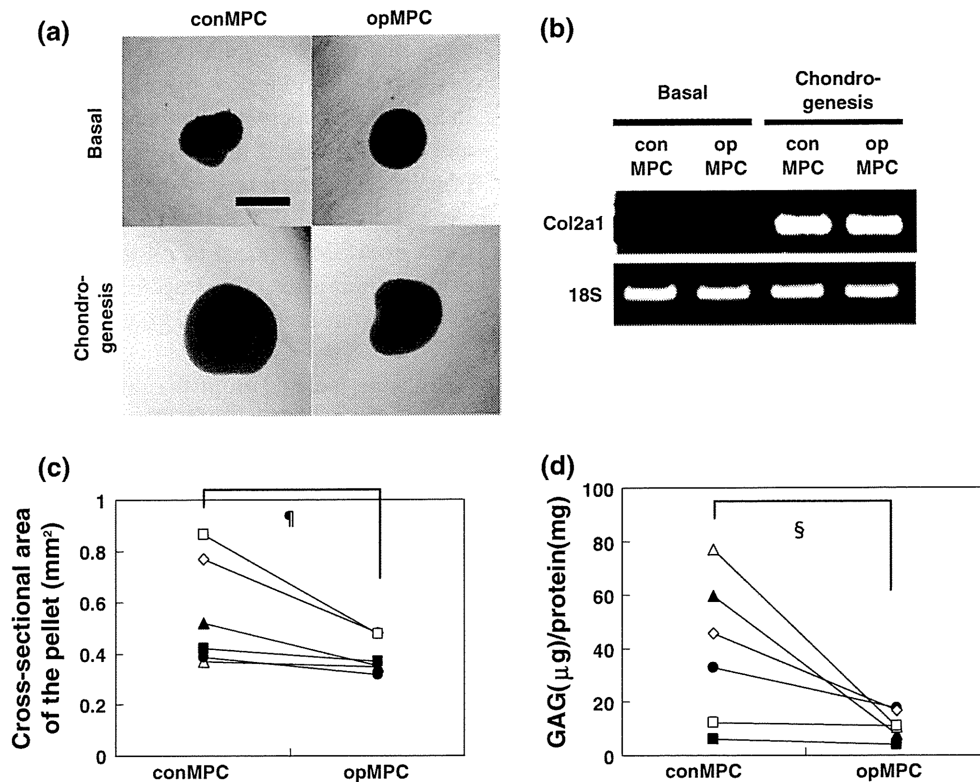

Fig. 2 Comparison of the chondrogenic potential between opMPC and conMPC. **a** A photograph of cell pellets from opMPC and conMPC cultured in basic culture medium (control) or chondrogenic medium (chondrogenesis). *Bar* 500 μm. **b** mRNA analysis of conMPC and opMPC for chondrogenic marker gene, type II collagen (*Col2a1*), and 18S. **c** The cross-sectional area from a representative 2D image of cell pellets. $^{¶}p = 0.02$. **d** The measurement of GAG by cell pellets normalized to protein level. $^{§}p = 0.004$. *Filled triangle, filled square, filled circle, open diamond, open square, open triangle*: sheep 1–6

medium for both opMPC and conMPC (Fig. 2a). RT-PCR analysis confirmed elevated mRNA expression for type II collagen for both cell sources cultured in chondrogenic medium (Fig. 2b). Measurement of the cross-sectional area of the opMPC pellets (0.390 ± 0.0690 mm^2) revealed they were significantly smaller than the corresponding conMPC pellets (0.554 ± 0.210 mm^2, $p = 0.02$) (Fig. 2c). The glycosaminoglycan content of the conMPC pellets cultured in the chondrogenic medium (39.0 ± 27.4 GAG/protein) was significantly higher than those of the opMPC pellets (11.4 ± 5.22 GAG/protein, $p = 0.004$) (Fig. 2d). These data suggest that surgery-associated inflammation appeared to have a deleterious influence on the chondrogenic potential of MPCs derived from the SM.

Cells from SM exposed to post-surgery inflammation exhibit osteogenic and adipogenic potential similar to cells from the non-operated contralateral knees

Both opMPCs and conMPCs cultured in osteogenic medium were stained with Alizarin Red; however, cells cultured in basal medium did not stain (Fig. 3a). Calcium deposition by opMPCs cultured in osteogenic medium (6.84 ± 2.56 μg/ml) was similar to that of corresponding conMPCs (6.47 ± 2.90 μg/ml) (Fig. 3b). Furthermore, the adipogenic potential of both cell populations was also examined. Oil Red O staining showed that cells cultured in control medium did not stain with Oil Red O to any detectable extent, while cells from both knees of the two groups cultured in adipogenic medium did stain in a detectable manner (Fig. 3c). The eluates of associated Oil Red O revealed no significant differences in lipid accumulation between opMPCs (0.100 ± 0.095 OD) and conMPCs (0.118 ± 0.102 OD) (Fig. 3d).

Cells from SM exposed to post-surgery inflammation continue to over-express mRNA for IL-1β and MMP-3 and -13

Cells isolated from the operated (opMPC) and contralateral (conMPC) knees retained the mesenchymal multi-lineage differentiation potencies, as shown in Figs. 2, 3. These cells were therefore defined as mesenchymal progenitor cells (MPCs). To investigate the effect of an altered intraarticular environment on cells isolated from the tissue exposed to inflammation, mRNA levels for catabolic