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DIFFERENTIAL CARTILAGINOUS TISSUE FORMATION BY HUMAN SYNOVIAL MEMBRANE, FAT PAD, MENISCUS CELLS AND ARTICULAR CHONDROCYTES

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ABSTRACT

Objective. To identify an appropriate cell source for the generation of meniscus substitutes, among those which would be available by arthroscopy of injured knee joints.

Methods. Human inner meniscus cells, fat pad cells, synovial membrane cells and articular chondrocytes were expanded with or without specific growth factors (Transforming Growth Factor-beta1, TGF-β1, Fibroblast Growth Factor-2, FGF-2 and Platelet-Derived Growth Factor bb, PDGF-bb, TFP) and then induced to form 3D cartilaginous tissues in pellet cultures, or using a hyaluronan-based scaffold (Hyaff®-11), in culture or in nude mice. Human native menisci were assessed as reference.

Results. Cell expansion with TFP enhanced glycosaminoglycan (GAG) deposition by all cell types (up to 4.1-fold) and mRNA expression of collagen type II by fat pad and synovial membrane cells (up to 472-fold) following pellet culture. In all models, tissues generated by articular chondrocytes contained the highest fractions of GAG (up to 1.9% of wet weight) and were positively stained for collagen type II (specific of the inner avascular region of meniscus), type IV (mainly present in the outer vascularized region of meniscus) and types I, III and VI (common to both meniscus regions). Instead, inner meniscus, fat pad and synovial membrane cells developed tissues containing negligible GAG and no detectable collagen type II protein. Tissues generated by articular chondrocytes remained biochemically and phenotypically stable upon ectopic implantation.

Conclusions. Under our experimental conditions, only articular chondrocytes generated tissues containing relevant amounts of GAG and with cell phenotypes compatible with those of the inner and outer meniscus regions. Instead, the other investigated cell sources formed tissues resembling only the outer region of meniscus. It remains to be determined whether grafts based on articular chondrocytes will have the ability to reach the complex spatial organization typical of meniscus tissue.

Key Indexing Terms: Chondrogenesis, meniscus, fibrocartilage, tissue engineering

Running headline: Comparison of chondrogenic cells

Meniscus is a complex fibrocartilaginous tissue, which is essential in the knee joint for shock absorption, load distribution, maintenance of stability and protection of articular cartilage (1-3). Injuries to the meniscus are often treated by partial or total meniscectomy, which is known to be associated with detrimental changes in joint function, ultimately increasing the risk of early degenerative joint diseases (4-6). Surgical approaches currently in use to substitute the damaged meniscus (e.g., the use of allografts or of a collagen-based material) can initially restore a stable and pain-free joint, but long-term clinical results, especially related to the protection of the articular surface, are still uncertain (7; 8). Recently, tissue engineering strategies have been proposed for the generation of meniscus substitutes, based on the loading and culture of suitable cells into appropriate biodegradable porous scaffolds (9-12). The promising approach has been pre-clinically validated by several studies (11; 13), indicating the potential of cell-based meniscus substitutes to improve healing of meniscus tissue.

Several cell sources have been used for meniscus repair, including meniscus fibrochondrocytes (11; 12; 14; 15), chondrocytes (13; 16; 17) or bone marrow-derived mesenchymal progenitor cells (18-20), but to the best of our knowledge a direct comparison of the different cell types has not yet been established. With the ultimate goal to identify an appropriate cell source for the generation of meniscus substitutes, the aim of our work was to compare the growth and post-expansion chondrogenic capacity of different cell types that would be readily available during meniscectomy or arthroscopic examination of an injured knee joint. In particular, we investigated the following human cell types: (i) inner meniscus cells (hereafter referred to as “meniscus cells”), which constitute the native target tissue and can be expanded even following meniscus injury (15); (ii) fat pad cells, known to include multipotent mesenchymal progenitor cells (21); (iii) synovial membrane cells, reported to have a progenitor nature (22; 23) and to be involved in healing of cartilage lesions (24; 25); and (iv) articular chondrocytes, currently in clinical use for articular cartilage repair and capable of differentiation into a variety of mesenchymal tissues (26-28). All cells were expanded with or without growth factors previously reported to enhance chondrogenesis of different

chondrocyte types (29; 30), and then induced to form 3D cartilaginous tissues in a scaffold-free model system (i.e., pellet culture), or using a hyaluronan-based scaffold, following *in vitro* culture or *in vivo* ectopic implantation. Each model included a culture phase in the presence of chondrogenic supplements, typically used to induce the expression of GAG and collagens (31; 32). Tissues were assessed for the content of GAG, and the expression pattern of different collagen types was compared to that in the different regions of native human menisci.

Materials and Methods

Human material

Meniscus, fat pad and synovial membrane tissues were obtained from a total of 15 donors (26-68 years of age, mean 45), after informed consent. Biopsies were harvested during partial meniscectomy or anterior cruciate ligament reconstruction, no longer than 2 months after trauma. At the time of surgery, knee joints were not overtly inflamed or osteoarthritic, although a few cases displayed minor inflammation (4/15 donors), ICRS Grade 1 cartilage damage (2/15 donors), or moderate osteoarthrosis (3/15 donors). Meniscus (n=15), fat pad (n=7) and synovial membrane (n=12) biopsies were all relatively small in size, with a weight range of respectively 10-500 mg, 10-100 mg, and 20-400 mg. Meniscus biopsies were only from the inner avascular region, where injured tissue is typically resected. Synovial membrane was from the suprapatellar recess and adipose tissue from the infrapatellar fat-pad. For ethical reasons, articular cartilage could not be harvested from the same donors. Cartilage tissues were thus collected from the medial femural condyle of 6 cadavers (22-55 years of age, mean 42), with no known clinical history of joint disorders, within 24 hours after death. Based on a preliminary study, the growth and differentiation ability of chondrocytes isolated from macroscopically normal cartilage shortly after knee joint trauma was within the range of chondrocytes isolated from cadaver joints. The weight range for the articular cartilage specimens was 500-1000 mg.

Complete human meniscus specimens were also obtained with ethical permission and consent from 32 individuals made anonymous (18-86 years of age, mean 56), undergoing above knee amputation or partial meniscectomy for traumatic or degenerative tears. Complete meniscus specimens were processed for immunohistochemical analysis as described below and used to define the pattern of collagen types expressed in the deep inner avascular and deep outer vascularized regions of the native meniscus tissue. Analysis did not include characterization of the superficial ~~fibroblastic~~ layer of the meniscus.

Cell Isolation and Culture

Inner meniscus and articular cartilage biopsies were finely minced and digested by incubation for 22 hours at 37°C in 0.15% type II collagenase (Worthington Biochemical Corporation, Lakewood, NJ) (1ml solution per 100mg tissue) (33). Meniscus cells and articular chondrocytes were resuspended in Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g/L glucose with nonessential amino acids), containing 10% fetal bovine serum, 4.5 mg/mL glucose, 0.1mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.29 mg/mL L-glutamine (*control medium*).

Fat pad and synovial membrane biopsies were grossly cut and digested by incubation respectively for 7 hours and for 22 hours in 0.4% type II collagenase (1ml solution per 40mg tissue). For the isolation of fat pad cells, floating adipocytes were removed by centrifugation at 300g for 5 minutes (34). Collagenase concentration and incubation times were selected by adapting a previously published protocol (22), following preliminary tests.

Meniscus, fat pad, synovial membrane cells and articular chondrocytes were plated in tissue culture flasks, at a density of 10^4 cells/cm^2, and cultured in a humidified incubator at 37°C and 5% CO_2. Throughout the phase of expansion in monolayer, all cell sources were cultured in control medium, without or with further supplementation of 1 ng/mL Transforming growth factor-β1 (TGF-β1), 5 ng/mL Fibroblast growth factor-2 (FGF-2) and 10 ng/mL Platelet-derived growth factor-bb (PDGF-bb) (*TFP medium*). The growth factors (all from R&D Systems, Minneapolis, MN) were selected

based on their previously reported capacity to enhance the growth and post-expansion chondrogenic ability of different human chondrocyte types (29; 30).

Differentiation assays

Upon reaching subconfluence, meniscus, fat pad, synovial membrane cells and articular chondrocytes were detached using 0.05% trypsin/ 0.53mM EDTA (GIBCO-BRL, CH) and replated at a density of $5x10^3$ cells/cm^2. Following an additional passage of expansion, cells were used in the three different models described below.

Model I: chondrogenic differentiation in 3D pellet culture

Meniscus, fat pad, synovial membrane cells and articular chondrocytes from all donors, expanded with or without TFP, were suspended in a defined serum free medium, consisting of DMEM supplemented with ITS^{+1} (10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 0.5 µg/mL bovine serum albumin, 4.7 ng/mL linoleic acid, from Sigma Chemical, St Louis, MO), 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.29 mg/mL L-glutamine, 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/mL human serum albumin, 10 ng/mL TGF-β1 and 10^{-7} M dexamethasone. The serum free medium was initially developed for the chondrocytic differentiation of human mesenchymal progenitor cells (35) and is typically used for the redifferentiation of human articular chondrocytes (29). Aliquots of 5 x 10^5 cells in 0.5mL of serum free medium were centrifuged in 1.5 mL conical polypropylene tubes (Sarstedt, Nümbrecht, D) at 1300 rpm for 4 min to form spherical pellets. These were placed on a 3D orbital shaker (Bioblock Scientific, Frenkendorf, CH) at 30 rpm in a humidified incubator at 37°C and 5% CO_2.

Pellets were cultured for 2 weeks, with medium changes twice per week, and subsequently processed for histological, immunohistochemical, biochemical or mRNA analysis as described below. Each analysis was performed independently in at least two entire pellets for each primary culture and expansion condition.

Model II: chondrogenic differentiation in 3D scaffold based cultures

Meniscus, fat pad, synovial membrane cells and articular chondrocytes from 2 donors, expanded with TFP, were seeded into non-woven meshes (5 mm diameter, 2 mm thick disks), made of esterified hyaluronan (Hyaff®-11, Fab, Abano Terme, IT) at the density 4 x 10^6 cells/scaffold (7 x 10^7 cells/cm^3). The scaffold was selected based on previous reports indicating that it supports redifferentiation of human articular chondrocytes (36) and since it is already in clinical use for articular cartilage repair (37). Cells were resuspended in 28µL of *control medium* and slowly dispersed over the top surface of the dry meshes with a micropipette. The seeded scaffolds were transferred to a 37°C incubator to allow for initial cell attachment. After 45 minutes, 100 µL of medium were carefully added to the base of each well. After a further 1.5 hours of incubation, 2 mL of medium were slowly added along the side of each well to cover the scaffold. Cell-scaffold constructs were cultured in *control medium* supplemented with 1ng/ml of TGFβ-3, 0.1 mM ascorbic acid 2-phosphate and 10µg/ml of human insulin, with medium changes twice a week. After 2, 4 and 6 weeks, the resulting tissues were processed for histological, immunohistochemical, biochemical or mRNA analysis as described below. Each analysis was performed independently in at least three engineered tissues for each primary culture and cell source.

Model III: ectopic implantation in nude mice

Meniscus, fat pad, synovial membrane cells and articular chondrocytes from 1 donor, expanded with TFP, were loaded and cultured for 2 weeks into Hyaff®-11 meshes as described for Model II and implanted subcutaneously in nude mice (CD-1 nude/nude, Charles River, Germany). After 6 weeks, mice were euthanised and explants processed for histological, immunohistochemical, biochemical or mRNA analysis as described below. Each analysis was performed independently in two engineered tissues for each cell source.

Analytical methods

Proliferation rate during expansion

The number of doublings of meniscus, fat pad, synovial membrane cells and articular chondrocytes during the second passage of expansion was determined as the logarithm in base 2 of the fold increase in the number of cells during expansion. The proliferation rate was defined as the number of doublings during the second passage of expansion divided by the time required for expansion and was expressed as doublings/day.

Real time RT-PCR (global amplification)

Total RNA was prepared from pellet cultures using Tri-Reagent (Sigma, UK). Pellet cultures were ground up in the Tri-Reagent using Molecular Grinding Resin (Geno Technology Inc, St Louis, USA). cDNA was synthesised from 10-100ng of total RNA by global amplification (38). Globally amplified cDNA was diluted 1:1000 and 1µl aliquots of the diluted cDNA were amplified by real time RT-PCR in a 25µl reaction volume on a MJ Research Opticon using a SYBR Green Core Kit (Eurogentec, Seraing, Belgium), with gene specific primers designed using ABI Primer Express software. Relative expression levels were normalised using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated using the $2^{-\Delta Ct}$ method (39). All primers were from Invitrogen, Paisley, UK. Primer sequences were for (i) GAPDH: Forward 5'-3' CACTCAGACCCCCACCACAC, and Reverse 5'-3' GATACATGACAAGGTGCGGCT; (ii) collagen type I: Forward 5'-3' TTGCCCAAAGTTGTCCTCTTCT, and Reverse 5'-3' AGCTTCTGTGGAACCATGGAA; (iii) collagen type II: Forward 5'-3' CTGCAAAATAAAATCTCGGTGTTCT, and Reverse 5'-3'GGGCATTTGACTCACACCAGT; (iv) SOX9: Forward 5'-3' CTTTGGTTTGTGTTCGTGTTTTG, and Reverse 5'-3' AGAGAAAGAAAAAGGGAAAGGTAAGTTT. The expression levels of collagen types I, II and SOX-9 were investigated to determine the relative extent of cell differentiation towards a fibrocartilaginous or hyaline-like phenotype.

Biochemistry

Tissues generated by meniscus, fat pad, synovial membrane cells and articular chondrocytes were digested with protease K (1mg/mL protease K in 50 mM Tris with 1 mM EDTA, 1 mM

iodoacetamide, and 10 mg/mL pepstatin- A; 0.5 mL solution for cell pellets and 1mL for Hyaff®-11 based constructs) for 15 h at 56°C (34). The glycosaminoglycan (GAG) content was measured spectrophotometrically using dimethylmethylene blue (40), with chondroitin sulfate as a standard, and normalized to the DNA amount, measured using the CyQUANT cell proliferation assay kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard.

Histology

Tissues were rinsed in PBS, fixed in 4% buffered formalin for 24 h at 4°C, embedded in paraffin, and sectioned to a thickness of 5 μm for cell pellets and of 7 μm for Hyaff®-11 based constructs. Sections were stained with Safranin-O for sulfated GAG.

Immunohistochemistry

Cryostat sections (3 to 5 μm thick) were cut from tissues and pellets stored frozen at -70°C. Sections, on Superfrost slides (BDH, Poole, UK), were fixed for 5 minutes in ice cold acetone and then stored at -20°C until required for immunostaining by standard ABC immunoperoxidase method with a panel of antibodies against collagen type I (COL-1 Mouse, Sigma, Gillingham, UK), collagen type II (CIIC1, Developmental Studies Hybridoma Bank, Iowa City, USA), collagen type III (FH-7A, Sigma, Gillingham, UK), collagen type IV (CIV22 Dako, Ely, UK) and collagen type VI (poly, Dako, Ely, UK). Sections immunostained with anti-type I and type II collagen antibodies were pre-incubated with 1mg/ml hyaluronidase (Sigma type 4 hyaluronidase from bovine testes) for 30 minutes at 37°C. Negative control sections with appropriate IgG or non-immune animal serum were run in parallel with the experimental sections to confirm the specificity of any positive staining.

The slides were blindly examined by light microscopy and the results were scored based on the percentage of positive tissue as: no matrix positive (-); <50% of matrix positive (+/-); >50% of matrix positive (+).

Statistical Analysis

Values are presented as mean ± standard deviation. Differences between the experimental groups were evaluated by Mann Whitney U tests and considered statistically significant with $p < 0.05$.

Results

Characterization of native human meniscus

The inner and outer regions of human native meniscus displayed a uniformly positive immunohistochemical staining for collagen types I, III and VI. Instead, staining for collagen type II, the major fibril collagen in articular cartilage, was weakly positive only in the inner meniscus region. Staining for collagen type IV, usually absent from articular cartilage, was positive, predominantly in a dendritic pattern, in the outer vascular zone of the meniscus and also, but to a lower extent, in the inner meniscus (Table I, section A), as previously described for ovine meniscus (41).

Cell proliferation during monolayer expansion

Meniscus, fat pad, synovial membrane cells and articular chondrocytes cultured in *control medium* proliferated at similar rates (Fig. 1). However, probably due to the limited biopsy size (i.e., if lower than 20 mg), some cultures of meniscus (three primaries), fat pad (four primaries) and synovial membrane cells (one primary) could not be expanded.

TFP supplementation during expansion significantly increased the proliferation rate of all four cell types, and allowed cell expansion even in cases of limited biopsy size. In the presence of TFP, articular chondrocytes proliferated significantly faster than all other cell sources.

Model I: 3D pellet cultures

After two weeks of culture, meniscus, synovial membrane cells and articular chondrocytes expanded in control medium formed spherical pellets, in contrast to fat pad cells, which did not generate regular tissue structures (Fig. 2). Following expansion in *control medium*, only articular chondrocytes generated tissues positively stained for GAG. Medium supplementation with TFP during expansion of all cell sources resulted in pellets more intensely stained for GAG. A

chondrocytic cell morphology and overtly positive staining for GAG was observed in pellets generated by articular chondrocytes and in restricted regions of pellets generated by fat pad and synovial membrane cells.

Pellets generated by all cell sources, expanded with or without TFP, contained similar amounts of DNA (data not shown). Following expansion in *control medium*, articular chondrocytes generated pellets with significantly higher GAG/DNA fractions than the other cell sources (Fig. 3A). Medium supplementation with TFP during expansion induced a significant increase in the GAG/DNA content of pellets generated by all cell sources (1.4, 4.1, 1.9 and 3.1 fold, respectively for meniscus, fat pad, synovial membrane cells and articular chondrocytes). Following TFP expansion, tissues formed by articular chondrocytes contained the largest fractions of GAG/DNA.

Following expansion in *control medium* and differentiation in pellets, articular chondrocytes expressed the highest mRNA levels of collagen types I, II and SOX-9 (Fig. 3B, C, D). Medium supplementation with TFP during expansion induced a significant increase in the mRNA expression of collagen type II by fat pad and synovial membrane cells (respectively 66- and 472-fold). Following TFP expansion and differentiation in pellets, articular chondrocytes expressed mRNA levels of SOX-9 higher than all other cell sources and of collagen type II higher than meniscus and synovial membrane cells.

Pellets generated by all cell sources displayed positive immunohistochemical staining for collagen types I, III, IV and VI, with generally increased levels of staining following TFP expansion (Table I, section B). Only articular chondrocytes, expanded with or without TFP, formed tissues positively stained for collagen type II, although only in discrete areas.

Model II: culture into Hyaff®-11 meshes

All cell sources, expanded in medium supplemented with TFP and loaded into Hyaff®-11 scaffolds, generated tissues which developed with *in vitro* culture time. In particular, DNA fractions of wet weight decreased with time, reaching after 6 weeks similar levels in all constructs (between 0.062% and 0.071% of the wet weight). The GAG content of all constructs, expressed as percentage of wet

weight, increased with culture time, and after 6 weeks' culture were the highest in tissues generated by articular chondrocytes (1.90 ± 0.09% of the wet weight, respectively 2.6-, 2.9- and 3.0-fold higher than in tissues formed by meniscus, fat pad and synovial membrane cells), approaching the levels measured in the inner region of native menisci (approximately 2%, calculated based on AufderHeide et al. (42)). Histological staining of tissue cross-sections indicated that only articular chondrocytes were able after 6 weeks to engineer cartilaginous tissues positively stained for GAG (Fig. 4).

The pattern of collagen types deposited in cell-Hyaff®-11 constructs was similar to that described in cell pellets (Table I, section C). In particular, tissues generated by articular chondrocytes were positively stained for all assessed collagen types, including collagen type II, thus indicating a hybrid fibro-hyaline cartilaginous nature (Fig. 5). Instead, tissues formed by meniscus, fat pad and synovial membrane cells were negatively stained for collagen type II.

Model III: ectopic implantation following culture into Hyaff®-11 meshes

In order to assess the intrinsic capacity of engineered cartilage tissues to further develop upon implantation in an environment supportive, but not inductive, of chondrogenesis (43), constructs generated by all cell sources, following two weeks of pre-culture *in vitro* (44), were implanted in subcutaneous pockets in nude mice for 6 weeks. Following *in vivo* implantation, the DNA content decreased and reached similar levels in all constructs (between 0.030% and 0.047% of the wet weight). In parallel, the GAG content increased in all constructs, and reached the highest levels in tissues generated by articular chondrocytes (1.4 ± 0.3% of the wet weight, respectively 2.6-, 1.9- and 3.5-fold higher than in tissues formed by meniscus, fat pad and synovial membrane cells). Histologically, tissues formed by articular chondrocytes were qualitatively similar to those maintained in culture for a total of 6 weeks (Model II), with a mixture of fibroblastic and chondrocytic cell phenotypes, large intercellular spaces positively stained for GAG, and no evidence of vascularization (Fig. 6). Instead, tissues formed by meniscus, fat pad and synovial membrane cells contained mostly fibroblastic cells and negligible extracellular matrix, with no

detectable positive stain for GAG. As compared to articular chondrocytes-based constructs, the lower amount of extracellular matrix in meniscus-, fat pad- and synovial membrane cells-based tissues appeared to be associated with a more advanced degradation of the esterified hyaluronan fibers, which were markedly more swollen, likely due to increased absorption of water (Fig. 6). Immunohistological staining of explant cross-sections indicated that tissues generated by articular chondrocytes were positively stained for all assessed collagen types, including type II (Table I, section D). Instead, the amount of extracellular matrix in meniscus-, fat pad- and synovial membrane cells-based tissues was too limited to allow for reliable immunohistochemical characterization.

Discussion

In this study, with the ultimate goal of identifying a suitable cell source for engineering autologous meniscus grafts, we compared the growth and post-expansion chondrogenic capacity of human meniscus, fat pad, synovial membrane cells and articular chondrocytes using three different model systems. Our results indicate that (i) articular chondrocytes reached the highest proliferation rates and (ii) only tissues formed by expanded articular chondrocytes contained molecules typically found in the inner avascular region of the meniscus (i.e., GAG and collagen type II), in the outer vascularized region of the meniscus (i.e., collagen type IV), and common to both regions (i.e., collagen types I, III and VI). Instead, under our specific experimental setup, tissues formed by meniscus, fat pad, and synovial membrane cells contained negligible GAG amounts and no detectable collagen type II.

We initially studied the response of the different cell sources to a specific growth factor combination (i.e., TFP) supplemented during cell expansion. Medium supplementation with TFP induced a significant increase in the proliferation rate during expansion and in the deposition of GAG in pellet cultures by all cell sources. In addition, it induced an upregulation of the mRNA levels of collagen type II in pellet cultures by fat pad and synovial membrane cells. We previously

reported that in addition to articular chondrocytes (29), also chondrocytes from ear and nasal septum increased their growth and post-expansion ability to generate cartilaginous tissues in response to TFP (30). The fact that different chondroprogenitor cells and mature chondrocytes from different cartilage sources similarly responded to the TFP growth factor combination during expansion suggests that these different cell types share certain pathways controlling chondrogenic commitment; in this context, it would be interesting to assess which common specific gene sets are up- or down-regulated in the different cell sources when exposed to TFP.

Expanded meniscus, fat pad and synovial membrane cells displayed evidences of differentiation towards the chondrogenic lineage, as assessed by the detection of type II collagen and Sox-9 mRNA expression. However, these cells developed merely fibrocartilaginous tissues, as indicated by the low amounts of deposited GAG and by the immunohistochemical detection of all the investigated collagen types, with the exception of type II collagen. Previous studies reported the production of type II collagen by meniscus cells (45), fat pad cells (21) and synovial membrane cells (22). The apparent discrepancy might be explained by the use of different detection methods and/or by the fact that biopsies taken with different methods or from healthy vs injuried knees may result in a different quality of the isolated cells. Indeed, one main limit of our study is related to the use of likely heterogeneous cells, without definition of typical phenotypes by assessment of surface markers. Our *in vivo* results are anyway consistent with the work by De Bari et al. (46), reporting that no stable cartilage could be formed ectopically by synovial membrane cells, and with that by Park et al., indicating that chondrogenesis by synovium-derived cells was not complete, and anyway inferior – with respect to type II collagen and Sox 9 mRNA expression – to that by articular chondrocytes (47). In terms of mRNA expression of chondrogenic genes and GAG deposition, our findings are also in line with other recent studies, reporting the chondrogenic differentiation of human synovial membrane cells in the presence of high concentrations (500 ng/ml) of bone morphogenetic protein (BMP)-2 (23,48). However, since these studies did not include assessment

of type II collagen at the protein level, a direct comparison with our data, potentially addressing the role of BMP-2 in the system, cannot be established.

In all investigated models, cartilaginous tissues generated by articular chondrocytes contained all the investigated collagen types in addition to abundant GAG and thus displayed a mixed hyaline/fibrous phenotype, consistently with several previous studies (33; 49-51). The quality of engineered cartilage based on articular chondrocytes seeding into Hyaff®-11 meshes improved during time in culture or during the time of subcutaneous implantation, as assessed by the increased content of GAG and decreased cellularity. Moreover, cells remained phenotypically stable and tissues implanted *in vivo* were not vascularized, also consistent with a previous report (44). The fact that the GAG content of articular chondrocytes-based constructs was in the range of that measured in the inner region of the native meniscus would be important to guarantee initial resistance of the graft upon implantation (42; 52) and biological responsiveness to compressive loads (53). Moreover, the expression and deposition by articular chondrocytes of all collagen types identified in the native meniscus indicates a compatibility of their phenotype with meniscus cells. Last but not least, the relatively higher proliferation rate of articular chondrocytes, especially in the presence of the growth factor combination TFP, would allow to generate a sufficient number of cells in a shorter time.

Although we cannot exclude that different experimental conditions (e.g., use of different sets of growth factors) might result in a different trend, data generated using the described model systems indicate that articular chondrocytes represent a more appropriate cell source than meniscus, fat pad or synovial membrane cells for the generation of a meniscus substitute. Recent studies have already explored the possibility to heal lesions in the avascular region of the meniscus using lamb (16) or swine articular chondrocytes (17), with promising results. Clearly, tissues engineered by articular chondrocytes only remotely mimic the complex structure of native meniscus; moreover, in our study we have not investigated the spatial organization of aggrecan or of collagen fibers, which is known to be highly specific in meniscus cartilage (54; 55). In this regard, it should be pointed out

that differential localization of specific matrix molecules in articular chondrocytes-based tissues may be induced by appropriate physical conditioning *in vitro*, e.g. by application of hydrodynamic forces (Marsano et al., Biorehology, In Press). Moreover, it is likely that the composition and mechanical properties of the graft will correctly develop by adapting to local mechanical forces upon implantation, as it is described during the process of embryonic joint development (56-58). In this context, studies are ongoing in a sheep model to assess the efficacy of autologous articular chondrocytes loaded into appropriately shaped scaffolds as engineered substitutes for total meniscectomies.

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Figure Legends

Figure 1. Proliferation index for inner meniscus cells (IMC), fat pad cells (FPC), synovial membrane cells (SMC) and articular chondrocytes (AC) expanded without (CTR) or with growth factors (TFP). * = significantly different from same cells expanded without TFP. ° = significantly different from IMC, FPC and SMC.

Figure 2. Representative sections of pellets generated by meniscus (A,B), fat pad (C,D), synovial membrane cells (E,F) and articular chondrocytes (G,H) expanded with (B, D, F, H) or without (A,C, E, G) the growth factor combination TFP and stained by Safranin O. Bar = 100 μm.

Figure 3. Fractions of GAG (A) and mRNA expression levels of collagen types I (B), II (C) and Sox9 (D) in pellets generated by inner meniscus cells (IMC), fat pad cells (FPC), synovial membrane cells (SMC) and articular chondrocytes (AC), expanded without (CTR) or with growth factors (TFP). * = significantly different from same cell source expanded without TFP. ° = significantly different from all the other cell sources, expanded in the same condition. § = significantly different from AC, expanded in the same condition.

Figure 4. Representative Safranin-O stained sections of tissues generated by culture of inner meniscus cells (A), fat pad cells (B), synovial membrane cells (C) and articular chondrocytes (D) into Hyaff®-11 for 6 weeks. Bar = 300 μm.

Figure 5. Representative immunohistochemical stain for collagen types I (A,B), II (C,D), III (E,F), IV (G,H) and VI (I,J) of cartilaginous tissues generated by culture of articular chondrocytes (A, C, E, G, I) and synovial membrane (B, D, F, H, J) into Hyaff®-11 for 4 weeks. Immunohistochemical stains were similar in tissues generated by inner meniscus, fat pad and synovial membrane cells. Bar = 100 μm.

Figure 6. Representative Safranin O-stained sections of tissues generated by culture of meniscus (A), fat pad (B), synovial membrane cells (C) and articular chondrocytes (D) into Hyaff®-11 for 2 weeks, followed by 6 weeks of ectopic implantation. Bar = 300 μm.

Table 1. Immunophenotypic characterization of native and engineered tissues.

The expression of Collagen types I, II, III, IV and VI was assessed in the inner avascular (IN) and outer vascular (OUT) regions of native human meniscus (section A), in pellets generated by inner meniscus cells (IMC), fat pad cells (FPC), synovial membrane (SMC) cells and articular chondrocytes (AC) expanded without (CTR) or with TFP, after 2 weeks (section B), in tissue constructs generated by the four different cell sources after 6 weeks of culture into Hyaff®-11 (section C), and in tissue constructs generated by AC cultured for 2 weeks into Hyaff®-11 and implantated ectopically for 6 weeks (section D). Scores are based on the percentage of positive tissue as: no matrix positive (-); <50% of matrix positive (+/-); >50% of matrix positive (+).

	A		**B**		**Model I**						**C**		**Model II**		**D**	**Model III**
	Meniscus		**IMC**		**FPC**		**SMC**		**AC**		**IMC**	**FCP**	**SMC**	**AC**	**AC**	
	IN	**OUT**	**CTR**	**TFP**	**CTR**	**TFP**	**CTR**	**TFP**	**CTR**	**TFP**						
Col I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Col II	+/-	-	-	-	-	-	-	-	+/-	+/-	-	-	-	+	+	
Col III	+	+	+/-	+	+/-	+	+/-	+	+	+	+	+	+	+/-	+	
Col IV	+/-	+	+	+	+/-	+	+	+	+/-	+	+	+/-	+	+	+	
Col VI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

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