

## Research Article

# *In vivo* matrix-guided human mesenchymal stem cells

J. Kramer<sup>a,b,\*</sup>, F. Böhrnsen<sup>a</sup>, U. Lindner<sup>c</sup>, P. Behrens<sup>d</sup>, P. Schlenke<sup>c</sup> and J. Rohwedel<sup>a</sup>

<sup>a</sup> Department of Medical Molecular Biology, University of Lübeck, 23538 Lübeck (Germany)

<sup>b</sup> Medical Department I, Division of Nephrology and Transplantation Unit, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck (Germany), Fax: +49 451 500 6579, e-mail: Jan\_Kramer@gmx.de

<sup>c</sup> Institute of Immunology and Transfusion Medicine, University of Lübeck, 23538 Lübeck (Germany)

<sup>d</sup> Department of Orthopaedic Surgery, University of Lübeck, 23538 Lübeck (Germany)

Received 3 November 2005; received after revision 15 December 2005; accepted 4 January 2006

Online First 16 February 2006

**Abstract.** Microfracture of subchondral bone results in intrinsic repair of cartilage defects. Stem or progenitor cells from bone marrow have been proposed to be involved in this regenerative process. Here, we demonstrate for the first time that mesenchymal stem (MS) cells can in fact be recovered from matrix material saturated with cells from bone marrow after microfracture. This also introduces a new technique for MS cell isolation during arthroscopic treatment. MS cells were phenotyped using specific cell surface antibodies. Differentiation of the MS cells into the adipogenic, chondrogenic and osteogenic lineage could be demonstrated by cultivation of MS cells

as a monolayer, as micromass bodies or mesenchymal microspheres. This study demonstrates that MS cells can be attracted to a cartilage defect by guidance of a collagenous matrix after perforating subchondral bone. Protocols for application of MS cells in restoration of cartilage tissue include an initial invasive biopsy to obtain the MS cells and time-wasting *in vitro* proliferation and possibly differentiation of the cells before implantation. The new technique already includes attraction of MS cells to sites of cartilage defects and therefore may overcome the necessity of *in vitro* proliferation and differentiation of MS cells prior to transplantation.

**Keywords.** Mesenchymal stem cells, cartilage, AMIC, extracellular matrix, chondrocytes, adipocytes, osteocytes.

Treatment of cartilage lesions by joint lavage and debridement of damaged cartilage [1, 2] as well as transplantation of allogenic and autologous cartilage [3–5] is not effective in restoring the original hyaline cartilage [for review, see ref. 6]. However, defects of articular cartilage, which perforate the subchondral plate, elicit an intrinsic repair response. Stem or progenitor cells from bone marrow have been proposed to be involved in this regenerative process. But arthroscopic techniques like microfracture [7–9] only yield a fibrocartilaginous repair tissue, which is a poor substitute for hyaline articular cartilage. Approaches such as transplantation of periosteum [10] or perichondrium [11, 12] bear the risk of calcification of the graft [13]. Therefore, lesions in joint cartilage

often end up being replaced by fibrous connective tissue or have a total lack of repair which can result in arthrosis. Several attempts have been undertaken to develop tissue-engineered grafts based on natural or synthetic biomaterials, but most of these attempts also failed to result in regeneration and restoration of complete functionality. The application of collagen I/III membranes in combination with autologous chondrocyte implantation at the side of the cartilage defect has been introduced to the clinic [14–17]. In comparison to this collagenous matrix, the use of periost includes the disadvantages of a second operation site combined with donor morbidity and the facultative occurrence of hypertrophic changes of the chondrocytes [18]. Moreover, all of these methods are based on the use of primary cultures of chondrocytes, which have been shown to dedifferentiate rapidly in culture and lose their

\* Corresponding author.

functional properties [19]. Isolation of autologous chondrocytes and periosteum involves the excision of healthy (cartilaginous) tissue from the joint for expansion [20]. In addition, adult chondrocytes show a restricted proliferation capacity in culture resulting in a limited number of cells, almost insufficient for regenerative strategies.

An alternative approach is the application of somatic stem cells [21–27]. Mesenchymal stem (MS) cells possess the ability to proliferate extensively in culture, and chondrocytes derived from stem cells have been observed to maintain a stable phenotype compared to chondrogenic cells derived from primary cultures. MS cells can be characterized by their cultivation behaviour, their differentiation potential at least into adipogenic, osteogenic and chondrogenic cell types, and by the expression of specific cell surface markers [26–31]. MS cells are most often derived from bone marrow by invasive iliac crest biopsy. Isolation and proliferation as well as chondrogenic differentiation of the MS cells *in vitro* are time intensive. In addition, optimal culture conditions to facilitate *in vitro* chondrogenesis of bone marrow-derived MS cells have not yet been established and standardized.

In this study, we hypothesize that MS cells can be sufficiently guided to a cartilage lesion by a collagen I/III matrix after microfracturing the subchondral bone. This procedure, called autologous matrix-induced chondrogenesis (AMIC [32]), leads to the accessibility of the intrinsic cartilage repair resources represented by MS cells in the bone marrow. Indeed, we were reproducibly able to detect the rapid appearance of human MS cells in the collagenous matrix and, therefore, suggest this simple method as sufficient for cartilage repair strategies. In the future this technique may make time-wasting *in vitro* proliferation and differentiation of MS cells after isolation and prior to transplantation unnecessary. A multicentre study comparing AMIC with microfracturing is currently underway at selected centres in Germany.

## Materials and methods

**Surgical transplantation technique.** Degenerative and detached cartilage was completely removed during an arthroscopy. A minimal invasive portal was used to gain access to the cartilage defect. Perforations into the subchondral bone were made with a sharp canula every 5 mm. The microfractured defect was covered with a porcine collagen I/III matrix (Geistlich Biomaterials, Wolhusen, Switzerland) for about 5 min, the matrix was removed after obvious bone marrow saturation and, finally, the matrix was trimmed to fit to the cartilage lesion by adaption to an appropriate template, which matches the size of the defect. Surplus collagenous matrix was directly transferred into phosphate-buffered saline (PBS) and immediately used for stem cell isolation to avoid

damage of the cells. The adapted matrix was placed back into the defect. Partial autologous fibrin glue (Baxter-Immuno, Heidelberg, Germany and autologous patient serum) was used regularly to fix the matrix. The knee joint was held in an extended position for 5 min before the joint was flexed ten times to test the stability and position of the matrix. The minimal invasive cut was closed in layers with standard techniques and a drainage without suction was applied. The knee was immobilized for 7 days in orthosis followed by continuous passive motion for 6 weeks and non-weight bearing for 6 weeks. The first preliminary results in the 1-year follow-up showed a functional improvement after the operation, and MR imaging showed no detachment of the matrix in 25 treated patients.

**MS cell culture and differentiation of MS cells.** The matrix was cut into small pieces (each sample with four particles with a diameter of around 3 cm) and cultured as described below. The medium marginally covered the matrices. Medium changes were performed every 4 days. Haematopoietic stem cells and non-adherent cells were removed with medium changes. Sequential microscopic observations were performed to detect adherent fibroblast colony-forming cells. The first colony-forming units-fibroblasts appeared after 3 weeks of cultivation. Trypsinization was performed if a culture confluence of 80% was observed, and the cells were split 1:2.

MS cells were cultured in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% sodium pyruvate, 1% L-glutamine, 1% enicillin/streptomycin and 10% fetal calf serum (FCS). Once adherent cells reached approximately 60–80% confluence, they were detached with trypsin-EDTA, washed twice with PBS, centrifuged at 1000 rpm for 5 min, and replated 1:2 under the same culture conditions or phenotyped using fluorescence-activated cell sorting (FACS). Initial cell density at the start of subculture was 7500 cells/cm<sup>2</sup>. Differentiation of MS cells was performed at a density of 1×10<sup>4</sup> cells/cm<sup>2</sup> onto 0.1% FCS-gelatine-coated two-well chamber slides (Falcon; BD Biosciences, Heidelberg, Germany) for indirect immunostaining and Sudan III staining as well as 6-well plates (TPP) for total RNA isolation. As a fast screen for chondrogenic differentiation, Alcian blue staining was performed using standard methods [33]. To detect adipocytic cells, lipid staining with Sudan III was performed. Cells plated onto chamber slides were washed with PBS followed by staining for 3 min with a 0.2% solution of Sudan III (Sigma, Taufkirchen, Germany) in 70% ethanol. Scion Image Software (Scion, Md.) was used to quantify the degree of adipogenic, osteogenic and chondrogenic differentiation. Areas containing differentiated cells were measured and calculated in relation to the entire area of cells.

**Adipogenic differentiation of MS cells.** To induce adipogenic differentiation, fifth- to seventh-passage cells were treated with adipogenic induction medium and adipogenic maintenance medium for 3 weeks. Induction was carried out using induction medium consisting of basal medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1  $\mu$ M dexamethasone, 200  $\mu$ M indomethacin and 2  $\mu$ M insulin. Following a 4-day induction period, the adipogenic induction medium was replaced with adipogenic maintenance medium consisting of basal medium supplemented with 2  $\mu$ M insulin for 3 days. The induction cycle was repeated three times followed by a 10-day period of adipogenic maintenance culture.

**Osteogenic differentiation of MS cells.** To induce osteogenic differentiation, fifth- to seventh-passage cells were treated with osteogenic medium for 3 weeks with medium changes twice weekly. Osteogenic medium consisted of basal medium supplemented with 0.1  $\mu$ M dexamethasone, 10 mM  $\beta$ -glycerolphosphate and 100  $\mu$ M ascorbic acid.

**Chondrogenic differentiation of MS cells.** To induce chondrogenic differentiation, fifth- to seventh-passage cells were transferred into 15-mL polypropylene tubes and centrifuged at 500 rpm for 5 min, to form a pellet, so-called micromass bodies, at the bottom of the tube (150,000–250,000 cells per body). The micromass cultures were then treated with chondrogenic medium for 3 weeks. Medium changes were carried out twice weekly. Chondrogenic medium consisted of basal medium supplemented with 0.1  $\mu$ M dexamethasone,  $3 \times 10^{-4}$  M ascorbic acid, 1% sodium pyruvate,  $10^{-3}$  M L-proline, 10 ng/ml transforming growth factor (TGF)- $\beta$ 3, and 1% ITS premix [Becton Dickinson, Heidelberg, Germany; 6.25  $\mu$ g/ml insulin, 6.25  $\mu$ g/ml transferrin, 6.25  $\mu$ g/ml selenious acid, 1.25 mg/ml bovine serum albumin (BSA) and 5.35 mg/ml linoleic acid].

**MS cell differentiation via mesenchymal microspheres.** To analyse differentiation of the MS cells into the adipogenic and osteogenic lineage in a three-dimensional environment, we performed MS cell culture via cellular aggregates, termed mesenchymal microspheres (MMSs). Undifferentiated MS cells, isolated as described above, were trypsinized, centrifuged and resuspended in basal medium. For differentiation protocols we used  $1 \times 10^3$  MS cells per MMS. Generation of MMSs was performed by using a primary dilution of  $5 \times 10^4$  cells/ml. Placing 20- $\mu$ l aliquots of the primary cell suspension onto the inner side of a 100-mm bacteriological petri dish, using a micropipettor with 100- $\mu$ l filter tips, resulted in around 25 hanging drops per lid. To avoid evaporation, a humidified environment was created by placing the bottom of a 60-

mm cell culture dish filled with PBS within the MMS cultivation chamber. Drops were cultured for 6 days until the formation of a spheroid was visible. On the 6th day of hanging-drop culture, the cellular aggregates were collected by turning the dish and submerging the MMS in basal medium. To avoid adherence to the plastic surface of the bacteriological petri dish, MMSs were immediately plated onto 0.1% FCS-gelatine-coated 6-well cell culture plates (for total RNA isolation) and chamber slides (for Sudan III, alkaline phosphatase and immunostaining). Plating was performed using a micropipettor with 100- $\mu$ l filter tips. MMSs attachment to the cell culture surface and basal medium change was performed every 3–4 days. When cells grew out from the spheroids around 3 weeks after plating, differentiation of MS cells was induced using the differentiation protocols described above.

**Detection of gene expression by RT-PCR analysis.** MS cells grown as micromass bodies or as a monolayer on 6-well plates were collected at different time points after plating, washed twice with PBS, and total RNA was isolated using an RNA Isolation Kit (Macherey & Nagel, Düren, Germany). The RNA concentrations were determined by measuring the absorbance at 260 nm. Samples of 500 ng RNA were reverse transcribed using oligo-dT primer and Superscript II reverse transcriptase following the manufacturer's recommendations. Aliquots of 1  $\mu$ l from the reverse transcriptase reactions were used for amplification of transcripts using primers specific for the analysed genes and Taq polymerase according to the manufacturer's instructions. Reverse transcriptase reactions were denatured for 2 min at 95 °C, followed by amplification for 34–45 cycles of 40-s denaturation at 95 °C, 40-s annealing at the primer-specific temperature (see Table 1) and 50-s elongation at 72 °C. Electrophoretic separation of PCR products was carried out on 2% agarose gels. Distilled water was always included as a negative control.

**Cryosections of MS cell-derived micromass bodies.** MS cell-derived micromass bodies were isolated, embedded in Tissue-Tek O. C.T. (Sakura Finetechnical, Tokyo, Japan) and frozen at  $-80$  °C. Cryosections (10  $\mu$ m) were prepared using a cryostat and placed onto slides. Indirect immunostaining was performed after fixation as described below.

**Indirect immunostaining.** MS cells cultured on chamber slides were rinsed three times with PBS, fixed for 5 min with precooled ( $-20$  °C) methanol-acetone at 4 °C, washed four times with PBS again and incubated at 37 °C for 30 min with 10% fetal bovine serum. Specimens were then incubated for 1 h with primary antibody in a humidified chamber at 37 °C. Antibodies specific for the following proteins were used (designation and dilution ratio

**Table 1.** Oligonucleotide sequences of primers used to study adipogenic, osteogenic and chondrogenic gene expression during MS cell differentiation *in vitro* by RT-PCR.

| Gene           | Forward primer (5'-3')<br>Reverse primer (5'-3') | Size<br>(bp) | Annealing<br>temperature (°C) | Reference |
|----------------|--|--------------|-------------------------------|-----------|
| aP2            | GCTTTGCCACCAGGAAAGTG<br>ATGACGCATTCCACCACCAG     | 279          | 60                            | 62        |
| PPAR $\gamma$  | AAACTCTGGGAGATTCTCCT<br>TCTTGTGAATGGAATGTCTT     | 247          | 56                            | 62        |
| C/EBP $\alpha$ | AGAAAGGGGTGGAAACATAGG<br>GAAAGCTGAGGGCAAAGG      | 685          | 58                            | 62        |
| Osteopontin    | ACTGATTTTCCCACGGACCT<br>CATTCAACTCCTCGCTTTCC     | 199          | 58                            | –         |
| Osteocalcin    | CTCACACTCCTCGCCTATT<br>CGCCTGGGTCTTCTCACTAC      | 143          | 58                            | –         |
| Collagen II    | ACGGCGAGAAGGGAGAAGTTG<br>GGGGGTCCAGGGTTGCCATTG   | 352          | 63                            | 31        |
| Aggrecan       | TCAGGAACTGAACTCAGTGG<br>GCCACTGAGTTCCACAGA       | 441          | 58                            | 31        |

The lengths of the amplified fragments, the annealing temperature and, if necessary, a reference declaring the creator of the primer pair are given. Primers used to detect expression of osteocalcin and osteopontin were designed by us.

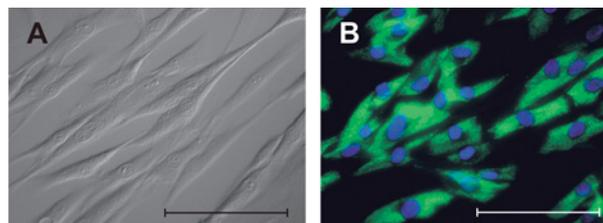
in PBS as well as a reference are given in parentheses): collagen II (II-II-6B3, 1:50 [34]), osteopontin (MPIIB101, 1:50 [35]), bone sialoprotein I + II (WVID1(9C5), 1:50 [35]) and STRO-1 (1:10 [36]). The antibodies were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. After rinsing four times with PBS, slides were incubated for 1 h at 37 °C with either FITC (1:200) or Cy3- (1:600) labelled anti-mouse IgG. Slides were washed four times in PBS and briefly washed in distilled water. Specimens were embedded in Vectashield mounting medium (Vector, Burlingame, Calif.) and analysed with the fluorescence-microscope Axioskop (Zeiss, Oberkochen, Germany).

**Flow cytometric analysis.** EDTA/trypsin- (0.25%) treated MS cells (second passage) were washed twice with FACS buffer (PBS, 1% BSA and 0.1 NaN<sub>3</sub>) and adjusted to approximately 0.5×10<sup>6</sup>/ml and subsequently stained. A 100- $\mu$ l cell suspension was incubated with either 20  $\mu$ l phycoerythrin(PE)-conjugated monoclonal antibodies (mAbs) or 10  $\mu$ l non-conjugated mAb and a secondary goat anti-mouse IgG1-PE at 4 °C for 20 min. All mAbs against the human antigens CD34, CD45, CD29, CD44, CD54, CD73, CD105, CD106, CD140b and CD166 were purchased from Becton Dickinson (Heidelberg, Germany). Prior to the flow cytometric analysis, all samples were filled up to a total volume of 500  $\mu$ l with FACS buffer. MS cells were analysed on an Epics XL flow cytometer using the System II software (Beckman Coulter, Krefeld, Germany). At least 10,000 events were acquired and analysed using a one-parametric protocol (FL2) and FSC/SSC dotplot diagram to exclude cell de-

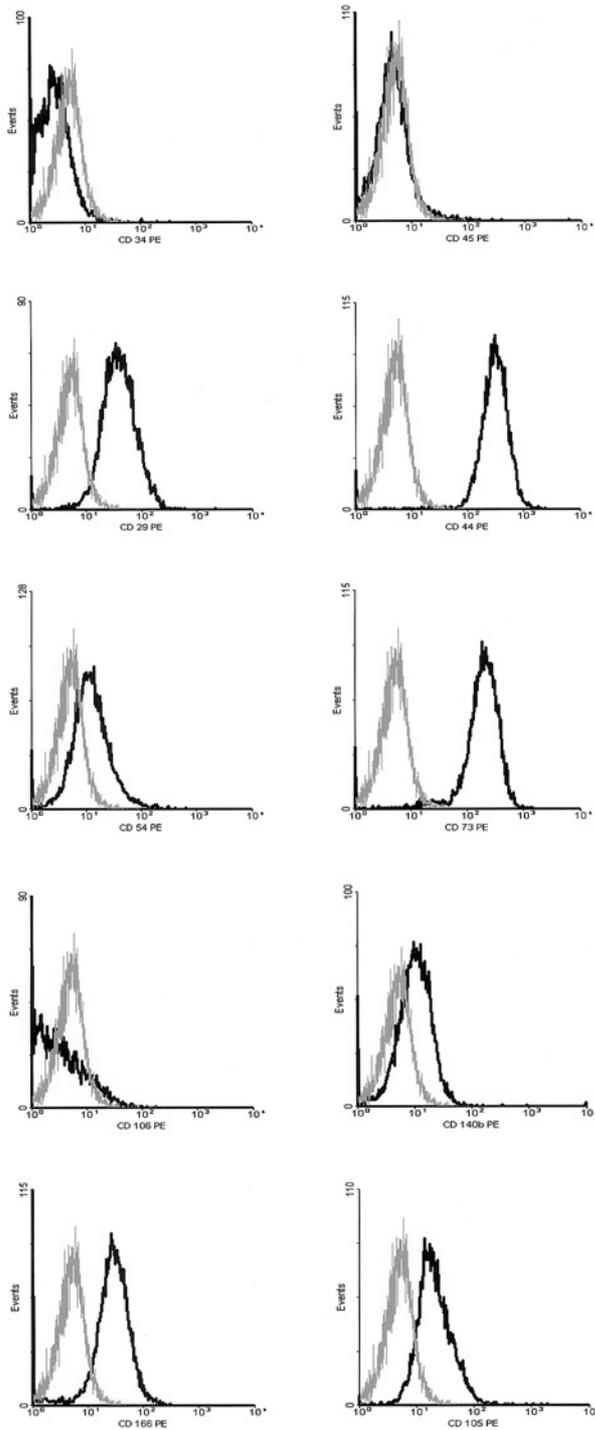
bris by gating. Non-specific isotype-matched controls were used to determine background fluorescence.

## Results

**Matrix-guided human cells show characteristic cultivation features of MS cells.** Using a collagen I/III matrix, cell material was sucked from microfractured cartilage lesions of five patients, 39–49 years old. The first colonies of fibroblast-like cells could be observed around 2–3 weeks after the onset of cultivation. Cultures were homogeneous and the morphology of the MS cells was similar among all cultures, showing a spindle-shaped phenotype (Fig. 1A). These cells expressed the MS cell marker STRO-1 [36, 37] as revealed by immunostaining (Fig. 1B). Later, cells lost their spindle-shaped morphology and the proportion of round-shaped cells increased.



**Figure 1.** Morphology of matrix-guided MS cells at passage 5. Phase contrast microscopy showing the typical spindle-shaped morphology of bone marrow-derived MS cells (A). These fibroblastoid cells express the MS cell marker STRO-1 as revealed by immunostaining (B). Blue, DAPI; green, STRO-1. Bar, 100  $\mu$ m.



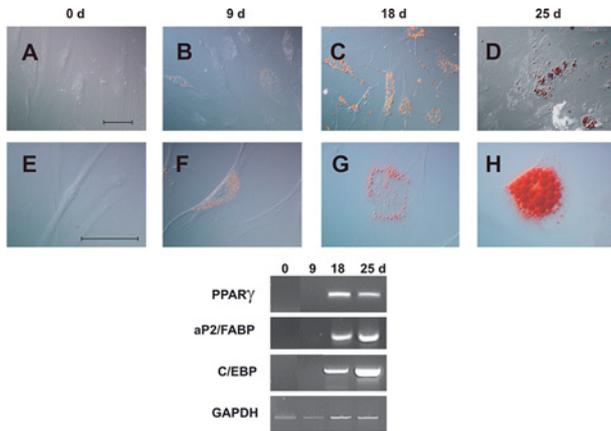
**Figure 2.** Flow cytometric analysis of matrix-guided MS cells at the second passage. Black lines indicate isotype-matched mouse IgG1 antibody control staining. Cells were detached with trypsin-EDTA, labelled with antibodies and analysed by a single-fluorescence (monoclonal antibodies conjugated to PE) flow cytometric protocol. Representative data of four independent experiments are shown.

An 80% confluence of the cells was observed after around 7 days of subculture. During later passages, the proliferation capacity of the MS cells decreased and was exhausted after passage 8. Haematopoietic cells were never

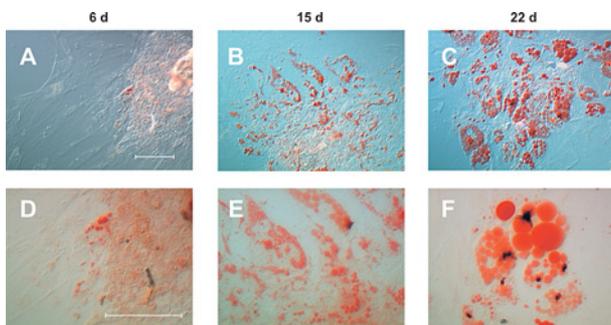
identified in the expanded cultures, and the cells did not differentiate spontaneously during culture expansion into any morphologically identifiable cell type.

**Immunophenotypic characterization of chondroglucid bone marrow-derived human MS cells.** Adherent fibroblast colony-forming cells were expanded, and characterization by flow cytometry was performed (Fig. 2). The cells were negative for CD34 (gp105-120) and CD45 (leukocyte common antigen), indicating that these cells were not of haematopoietic origin (Fig. 2A, B). The cells expressed CD29 ( $\beta$ 1 integrin) and showed weak expression of CD54 (intercellular adhesion molecule-1) and CD140b ( $\beta$  platelet-derived growth factor receptor). Analysis for the hyaluronate receptor (CD44) and ecto-5'-nucleotidase (CD73) revealed strong expression. The cells were negative for CD106 (vascular cell adhesion molecule-1). The adhesion molecule CD166 (activated leukocyte cell adhesion molecule) and the matrix receptor CD105 (endoglin, SH2) were found to be expressed. Taken together, the expression pattern was consistent with the pattern described for bone marrow MS cells [26, 31]. The isolated cultured mesenchymal cells comprised a distinct phenotypic population (>90% homogeneous in passage 2) by flow cytometric analysis.

**MS cells cultured in monolayer or via microspheres differentiate into adipogenic cell types.** Adipogenic differentiation was induced in the expanded MS cell monolayer cultures by treatment with IBMX, dexamethasone, insulin and indomethacin according to classical protocols. Morphologic changes in cells as well as the first formation of neutral lipid vacuoles were apparent as early as 1 week after induction and visualized by staining with Sudan III (Fig. 3) and oil red O (data not shown). These adipogenic cells started to express the nuclear receptor peroxisome proliferation-activated receptor  $\gamma$  (PPAR $\gamma$ ), the fatty acid-binding protein (FABP) aP2 and CCAAT enhancer-binding protein (C/EBP) – well known molecules controlling adipocyte differentiation and function – around 1 week later, as revealed by RT-PCR analysis (Fig. 3). Sudan III positivity could only be observed after application of adipogenic medium; initially, no staining was detectable in the MS cell cultures. Once observed, the lipid vacuoles continued to develop over time. Eighteen days after adipogenic induction, nearly all cells were positive for the lipid staining, and 25 days after induction, the adipocytes were eventually filled with the lipid vacuoles. To test whether human MS cells can also differentiate into adipogenic cell types, if they are cultured in a three-dimensional culture system, we performed MMS cultures. Hanging-drop cultivation (as described above) resulted in the formation of MMSs, which were plated onto gelatine-coated chamber slides and treated with the adipogenic induction medium. Comparable to the monolayer



**Figure 3.** Adipogenic differentiation of matrix-guided MS cells in monolayer culture. Sudan III stainings revealed that lipid vacuoles formed within the cells after induction with adipogenic medium demonstrated at low (*A–D*) and higher (*E–H*) magnification. Uninduced MS cells at 0 days showed no adipogenic phenotype (*A, E*). Later, small lipid vacuoles could be observed in the cells, indicating their adipogenic differentiation at 9 days after induction (*B, F*). The vacuoles increased in size during further cultivation as demonstrated by Sudan III staining at 18 days after induction (*C, G*). Finally, after 25 days of induction, the cells showed a round morphology and were almost filled with lipid vacuoles (*D, H*). Bars, 100  $\mu$ m. RT-PCR analysis showed that the adipocyte marker molecules PPAR $\gamma$ , aP2/FABP and C/EBP were expressed in the MS cell cultures after 18 and 25 days of induction with adipogenic medium. GAPDH served as an internal standard.



**Figure 4.** Matrix-guided bone marrow-derived MS cells cultured via MMSs differentiate into adipogenic cell types. Sudan III staining revealed that lipid vacuoles are formed within the cells after induction with adipogenic medium demonstrated at low (*A–C*) and higher (*D–F*) magnification, when MS cells were cultured as three-dimensional aggregates. Initially, after 6 days of induction, the adipogenic cells were predominantly detected in the centre of the spheroids (*A, D*). After 15 days of induction, adipogenic cells were also found in the outgrowing part of the mesenchymal microspheres (*B, E*). Finally, after 22 days of induction, large lipid vacuoles almost filled the adipogenic cells (*C, F*). Bars, 100  $\mu$ m.

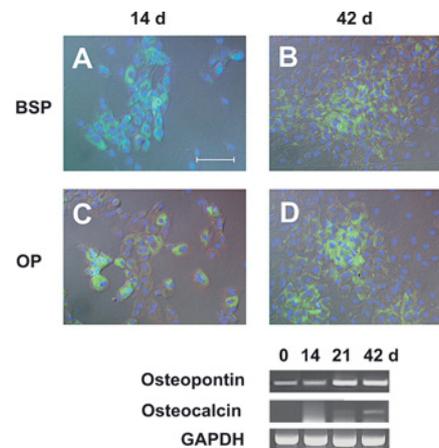
cultures, the first adipogenic cells were detectable by Sudan III staining around 1 week after onset of induction (Fig. 4). The lipid vacuoles were immediately larger compared to the monolayer cultures, and almost all Sudan III-positive cells were found near the centre of the microspheres. Already 2 weeks after induction, almost all cells

in the microsphere cultures showed large lipid vacuoles and, finally, around 3 weeks after induction, adipogenic differentiation of the MS cells throughout the microspheres was visible.

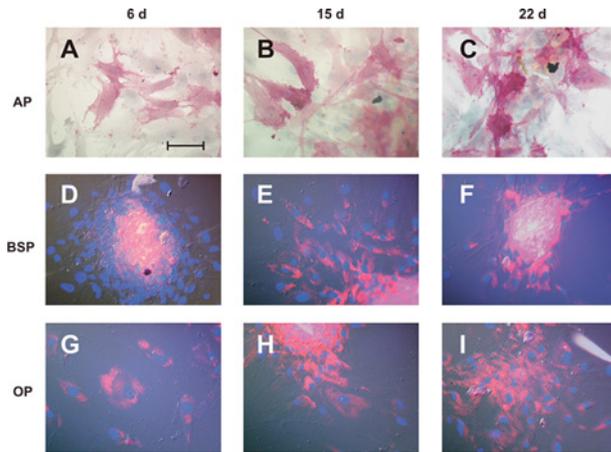
#### MS cells cultured in monolayer or via microspheres differentiate into osteogenic cell types.

Osteogenic differentiation of MS cells was induced by dexamethasone, glycerol phosphate and ascorbic acid in the presence of 10% FCS. After 14 days of induction, aggregates of cells expressing the osteogenic marker molecules bone sialoprotein (Fig. 5A, B) and osteopontin (Fig. 5C, D) were detectable in the MS cell monolayer cultures as revealed by immunostaining. During further culture, the number of positive cells increased, indicating osteogenic differentiation. These results were confirmed at the gene expression level by RT-PCR analysis (Fig. 5). Although osteopontin was already expressed at a basal level in undifferentiated MS cell cultures, as has been demonstrated previously [38], it was upregulated 3 weeks after treatment with osteogenic induction medium. In line with this, expression of osteocalcin was detected at day 21 and increased during further cultivation, indicating osteocyte formation of MS cells.

Cultivation of MS cells via three-dimensional microsphere cultures was performed and also resulted in osteogenic differentiation when treated with induction medium. Alkaline phosphatase-positive cells could be detected in the mesenchymal microspheres, indicating osteogenic differentiation of the MS cells (Fig. 6A–C). The alkaline phosphatase-positive cells were located in the centre as



**Figure 5.** Osteogenic differentiation of matrix-guided MS cells in monolayer culture. MS cells induced with osteogenic medium expressed bone sialoprotein (BSP; *A, B*) and osteopontin (OP; *C, D*) as revealed by immunostaining against these osteoblast marker molecules. The number of osteogenic cells subsequently increased during cultivation from 14 days of induction (*A, C*) up to 42 days of induction (*B, D*). Bar, 100  $\mu$ m. RT-PCR analysis demonstrated that expression of the osteogenic marker genes osteopontin and osteocalcin was upregulated during cultivation of MS cells in osteogenic medium. GAPDH was used as an internal standard.

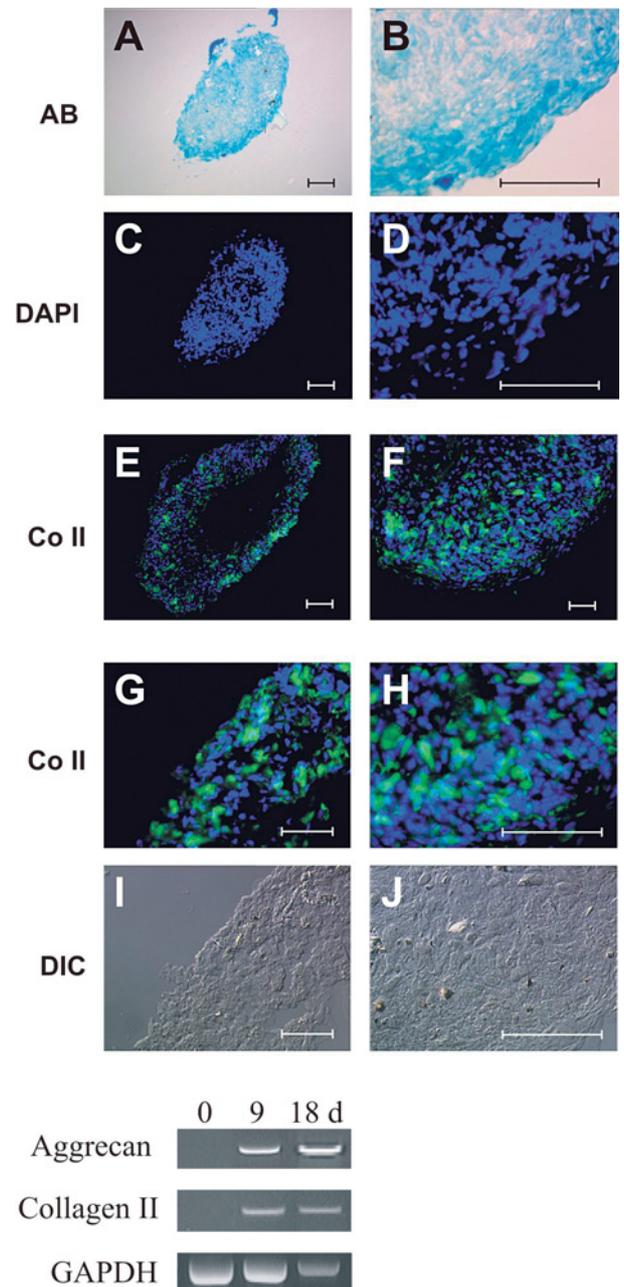


**Figure 6.** Matrix-guided bone marrow-derived MS cells cultured via MMSs differentiate into osteogenic cell types (A–I). MMSs were analysed 6 days (A, D, G), 15 days (B, E, H) and 22 days (C, F, I) after induction with osteogenic medium was started. Osteogenic differentiation of the cells within the three-dimensional cellular aggregates was indicated by staining for alkaline phosphatase (A–C). The number of alkaline phosphatase-positive cells increased during culture. Immunostaining against bone sialo-protein (D–F) and osteopontin (G–I) revealed that these osteogenic marker molecules were expressed in the centre (D, F, G) as well as in the outgrowing parts (E, H, I) of the cellular aggregates and that the number of positive cells subsequently increased during cultivation time.

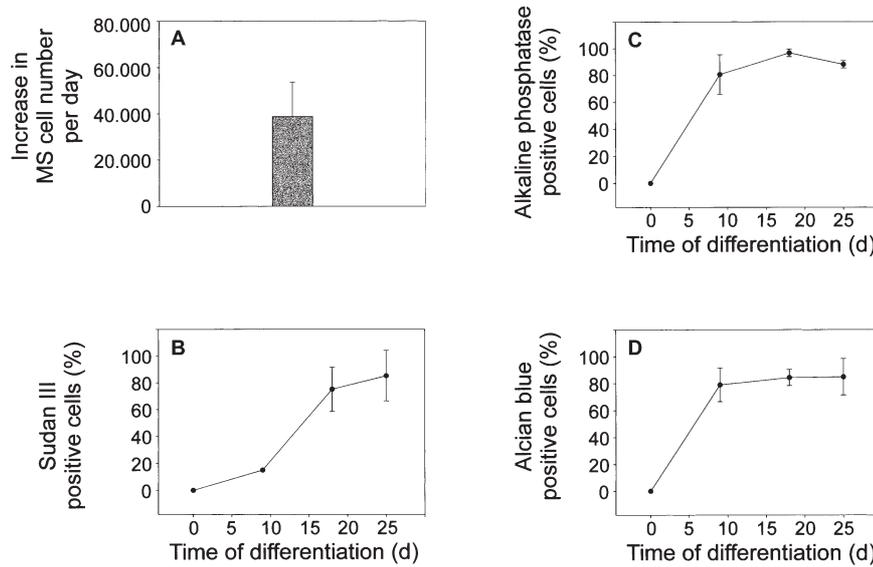
well as the outgrowing part of the spheroids and their number increased during further cultivation time. Immunostaining for bone sialo-protein (Fig. 6D–F) revealed that MS cell-derived cells express this marker molecule after induction of osteogenic differentiation and when cultured as three-dimensional aggregates. Expression of osteopontin (Fig. 6G–I) could also be observed by immunostaining in the MS cell-derived MMS cultures. Before application of the osteogenic medium, no protein expression of bone sialo-protein was observed in the undifferentiated MS cell cultures by immunostaining, whereas a basal expression could be detected for osteopontin (data not shown).

### MS cells cultured via micromass bodies differentiate into chondrogenic cell types.

To promote chondrogenic differentiation we gently centrifuged the isolated MS cells to form a pelleted micromass, and cultured the cells in a medium containing TGF- $\beta$ 3. The micromass bodies developed a multilayered morphology. Alcian blue (Fig. 7A, B) and safranin O (data not shown) staining revealed an increasing proteoglycan-rich extracellular matrix during culture. Collagen type II, the major component of hyaline cartilage tissue, was expressed by MS cell-derived chondrogenic cells, as revealed by immunostaining (Fig. 7E–H). RT-PCR analysis confirmed expression of collagen type II and demonstrated expression of aggrecan, the major proteoglycan of cartilage tissue (Fig. 7). Before application of chondrogenic medium, no



**Figure 7.** Chondrogenic differentiation of matrix-guided MS cells cultured via micromass bodies. Alcian blue (AB) staining of cryosectioned micromass bodies after 25 days of induction with chondrogenic medium revealed that cells within the three-dimensional aggregates produced proteoglycans typical for cartilage tissue (A, B). The cell nuclei were stained with DAPI (C, D). Immunostaining revealed that some cells in the micromass bodies expressed collagen type II (E–H), the main extracellular matrix protein of cartilage tissue, and showed the typical round-shaped morphology of chondrocytes (I, J). Due to the secondary antibody, conjugated with FITC, collagen type II (Co II) expression was indicated by green fluorescence. Bar, 100  $\mu$ m. RT-PCR analysis revealed that the chondrogenic marker genes aggrecan and collagen type II were expressed in MS cell-derived micromass cultures, 9 and 18 days after induction.



**Figure 8.** Efficient isolation and differentiation of human matrix-guided MS cells. MS cells were isolated from matrix material. The number of MS cells was counted during passaging. After 5 weeks of culture, around  $2\text{--}3 \times 10^5$  undifferentiated MS cells were obtained. The MS cell number increased during further passages with a rate of approximately  $4 \times 10^4$  cells per day (A). Differentiating MS cell cultures were regularly analysed by histochemical staining. The amount of differentiated adipogenic (B), osteogenic (C) and chondrogenic (D) cells increased up to nearly 100% after 3 weeks of culture in specific induction media.

protein expression of the analysed chondrogenic marker molecules could be found in the undifferentiated MS cell cultures by immunostaining.

**Quantification of MS cell isolation and differentiation.** When cultured as described (see Materials and methods), MS cells grew out from matrix material. The first colony-forming units-fibroblasts appeared after 3 weeks of culture. After 5 weeks of culture an initial MS cell number of approximately  $2\text{--}3 \times 10^5$  was obtained. During further passages, an increase in MS cell number of around  $4 \times 10^4$  per day was determined (Fig. 8A). Differentiating MS cell cultures were regularly analysed by histochemical staining for adipogenic, osteogenic and chondrogenic cell types. We found that the amount of differentiated cells increased up to almost 100% after 3 weeks of cultivation in induction medium (Fig. 8B–D).

## Discussion

Articular cartilage has a limited capacity for self repair. Partial-thickness defects that do not include the subchondral region show no regeneration. If the subchondral bone is penetrated by a full-thickness cartilage defect, a spontaneous fibrocartilaginous repair can be observed. But this repair cartilage shows degeneration and is a poor tissue replacement of native articular cartilage. However, many current operative procedures for cartilage lesions are based upon this mechanism of repair [for a review, see ref. 6]. Previously, participation of the stem cell pool of

the bone marrow has been hypothesized to lead to an optimized regenerative capacity, and perforation of the subchondral bone plate allows MS cells to access the defect [39]. In fact, our study demonstrates that MS cells from the bone marrow migrate to cartilage defects of the knee after microfracturing the subchondral bone. In general, isolation of MS cells is performed using human bone marrow after iliac crest biopsy. However human bone marrow obtained from long bones like the femur also contains MS cells [40]. Our study showed that these cells can be rapidly guided *in vivo* to a collagen I/III membrane at the site of a cartilage defect in the knee. The MS cells were characterized by their adherence to plastic culture dishes, their fibroblastoid morphology, their ability to proliferate in culture and by the expression of several MS cell marker molecules such as STRO-1 and a distinct set of cell surface markers. Finally, we have shown that these matrix-guided MS cells were capable of differentiating efficiently into adipogenic, osteogenic and chondrogenic cell types under specific culture conditions *in vitro*.

Human MS cells derived from bone marrow or other sources like adipogenic tissue [41] can differentiate at least into bone, cartilage and fat [for reviews see refs. 27, 29, 42, 43]. Moreover, there has been a serious discussion that bone marrow stem cells have a role in maintenance and repair of several other tissues [for a review, see ref. 44]. We detected the first plastic-adherent fibroblastoid cell colonies around 2 weeks after culture of the matrices *in vitro*. Previously fibroblast colonies have been suggested to arise from a single CFU-F [45]. This is also strongly supported by time-lapse observations [46]. But

adherence does not allow separation of CFU-F from monocytes [45]. However, we found a homogeneous cell population of spindle-shaped fibroblastoid cells, and phenotype analysis by FACS revealed no expression of CD45, but an expression pattern of typical MS cell surface molecules. Moreover, the cells expressed STRO-1, an established marker molecule of MS cells [36, 37] and showed a distinct proliferation capacity over several passages *in vitro*.

As mentioned, MS cells are widely regarded as the stem cells for osteoblasts, chondrogenic and adipogenic cells. *In vitro*, chondrogenesis by MS cells is typically carried out in micromass cultures in the presence of TGF [24, 25]. *In vitro* adipogenic differentiation of MS cells is classically induced by incubation of monolayers in medium supplemented with dexamethasone, IBMX and indomethacin [26], and *in vitro* differentiation of MS cells to osteoblasts has involved incubating a confluent monolayer of MS cells with ascorbic acid, beta-glycerophosphate and dexamethasone [47–50]. Regarding these protocols, we performed differentiation of matrix-guided MS cells and, indeed, demonstrated by RT-PCR, histochemistry and immunostainings that the fibroblastoid cells possess the ability to differentiate into adipogenic, osteogenic and chondrogenic cell types under classical culture conditions. Because three-dimensional cultivation leads to extensive cellular interactions influencing differentiation of MS cells [24, 25], we tested the differentiation behaviour of the MS cells into adipogenic and osteogenic cell types under three-dimensional conditions. We found that MS cells formed three-dimensional cellular aggregates, if cultured via hanging drops, a technique developed from embryonic stem cell culture techniques [for a review see refs. 33, 51]. The aggregates, we called mesenchymal spheroids, were plated and after induction with specific induction media, adipocytes and osteogenic cells could be detected. Further studies have to be performed to quantify the possible differences of differentiation that may occur using these culture techniques.

Microfracture has become one of the most frequently performed procedures in arthroscopic cartilage repair settings [7–9]. This technique resulted in a marked increase of tissue volume in cartilage defects compared to untreated osteochondral defects, and the tissue consisted of type II collagen. However, the quality of the repair cartilage was poor compared to native tissue [40, 52].

The extracellular matrix is a key regulator of stemness. Proliferation and differentiation of MS cells can be influenced by collagenous scaffolds [53–56]. Application of collagen I/III matrices in combination with autologous chondrocyte implantation at the side of the cartilage defect has already been introduced to the clinic [14–17]. Previously, an investigation established in a dog model revealed that fibrocartilage developed in the defect after collagen II matrix treatment combined with microfracture

[57]. However, the unseeded matrix was superior to the group containing individuals treated with collagen type II matrix seeded with autologous chondrocytes. But other groups have published the opposite findings; this may be due to another type of matrix used and to the time period from seeding chondrocytes to the matrix up to implantation [40]. For example, an animal study was recently performed in sheep to determine whether the results of microfracture can be improved when a bilayer composite of collagen I/III+II matrix is provided for bone marrow-derived MS cells [58]. A better repair was achieved in microfractured defects compared to untreated defects. But a poor repair in comparison to simple microfracture alone was described if the matrix was added additionally. Moreover, autologous chondrocytes must be present at the lesion to achieve a sufficient amount of defect fill and good repair quality. But in this study, the matrices were regularly sutured to the defect, which caused damage and degeneration of the adjacent tissue leading to a worse outcome.

Chondrogenic differentiation of MS cells is modulated by members of the TGF- $\beta$  family [25, 59, 60]. Therefore, a partial autologous fibrin (PAF) glue was used instead of regular suturing in this study. PAF glue is obtained by mixing a commercially available fibrin glue with autologous serum containing TGF- $\beta$ . This glue avoids suturing and may optimize MS cell migration to the matrix and differentiation into chondrogenic cells at the site of the cartilage defect. In addition, type I collagen matrices have been suggested to be superior to guide progenitor cells from the subchondral bone into the cartilage defect [61]. Therefore, a matrix composite with the deep layer of type I collagen next to the bone was used. Finally, currently running clinical trials may demonstrate that a collagen matrix in combination with bone marrow stimulation, but no further autologous cultured chondrocytes, might be sufficient to facilitate cartilage repair.

The AMIC technique [32] is easy to handle. No damage to healthy cartilage is carried out and this procedure can be done in one operation step. Moreover, *in vitro* cultivation and differentiation of cells can be avoided using AMIC. Therefore, this technique is less expensive, less time intensive, and risk factors like recurrent or prolonged anaesthesia and *in vitro* procedures are limited. In this study, we verified the hypothesis that perforation of the subchondral bone plate gives rise to the stem cell pool of the bone marrow. We have presented strong evidence that bone marrow cells can be guided directly to a cartilage defect by a collagenous matrix, and that MS cells can be isolated regularly from these matrices. Thus, time-intensive proliferation and differentiation processes of MS cell culture *in vitro* may no longer be necessary to receive MS cell-derived cartilage repair at the site of a defined defect. The goal of future studies is to optimize migration and stable differentiation of MS cells after matrix-assisted guidance to the cartilage defect.

*Acknowledgements.* The skilful technical assistance of A. Eirich and M. Dose is gratefully acknowledged. The work was funded by Intermed Service GmbH & CoKG (Geesthacht, Germany).

- 1 Jackson R. W., Marans H. J. and Silver R. S. (1988) Arthroscopic treatment of degenerative arthritis of the knee. *J. Bone Joint Surg.* **66**: 1040–1048
- 2 Baumgaertner M. R., Cannon W. D. Jr, Vittori J. M., Schmidt E. S. and Maurer R. C. (1990) Arthroscopic debridement of the arthritic knee. *Clin. Orthop.* **253**: 197–202
- 3 Beaver R. J., Mahomed M., Backstein D., Davis A., Zukor D. J. and Gross A. E. (1992) Fresh osteochondral allografts for post-traumatic defects in the knee: a survivorship analysis. *J. Bone Joint Surg. Br.* **74**: 105–110
- 4 Bobic V. (1996) Arthroscopic osteochondral autograft transplantation in anterior cruciate ligament reconstruction: a preliminary clinical study. *Knee. Surg. Sports Traumatol. Arthrosc.* **3**: 262–264
- 5 Convery F. R., Meyers M. H. and Akeson W. H. (1991) Fresh osteochondral allografting of the femoral condyle. *Clin. Orthop.* **273**: 139–145
- 6 Redman S. N., Oldfield S. F. and Archer C. W. (2005) Current strategies for articular cartilage repair. *Eur. Cell Mater.* **9**: 23–32
- 7 Steadman J. R., Rodkey W. G., Briggs K. K. and Rodrigo J. J. (1999) The microfracture technic in the management of complete cartilage defects in the knee joint (in German). *Orthopäde* **28**: 26–32
- 8 Steadman J. R., Briggs K. K., Rodrigo J. J., Kocher M. S., Gill T. J. and Rodkey W. G. (2003) Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up. *Arthroscopy* **19**: 477–484
- 9 Miller B. S., Steadman J. R., Briggs K. K., Rodrigo J. J. and Rodkey W. G. (2004) Patient satisfaction and outcome after microfracture of the degenerative knee. *J. Knee Surg.* **17**: 13–17
- 10 O'Driscoll S. W., Keeley F. W. and Salter R. B. (1986) The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion: an experimental investigation in the rabbit. *J. Bone Joint Surg. Am.* **68**: 1017–1035
- 11 Homminga G. N., Bulstra S. K., Bouwmeester P. S. and Linden A. J. van der (1990) Perichondral grafting for cartilage lesions of the knee. *J. Bone Joint Surg. Br.* **72**: 1003–1007
- 12 Bruns J., Behrens P. and Silbermann M. (1997) The principle of autogeneic rib perichondrial transplantation in the treatment of deep articular cartilage defects (in German). *Z. Orthop. Ihre Grenzgeb.* **135**: 138–144
- 13 Bab I., Sela J. and Stein H. (1982) Transplantation of free perichondrial grafts into rabbit articular cartilage is associated with matrix vesicle calcification. *Acta Anat.* **113**: 53–60
- 14 Behrens P., Ehlers E. M., Kochermann K. U., Rohwedel J., Rusli M. and Plötz W. (1999) New therapy procedure for localized cartilage defects: encouraging results with autologous chondrocyte implantation (in German). *MMW. Fortschr. Med.* **141**: 49–51
- 15 Bachmann G., Basad E., Lommel D. and Steinmeyer J. (2004) MRI in the follow-up of matrix-supported autologous chondrocyte transplantation (MACI) and microfracture (in German). *Radiologe* **44**: 773–782
- 16 Haddo O., Mahroof S., Higgs D., David L., Pringle J., Bayliss M. et al. (2004) The use of chondrocyte membrane in autologous chondrocyte implantation. *Knee* **11**: 51–55
- 17 Ronga M., Grassi F. A. and Bulgheroni P. (2004) Arthroscopic autologous chondrocyte implantation for the treatment of a chondral defect in the tibial plateau of the knee. *Arthroscopy* **20**: 79–84
- 18 O'Driscoll S. W. and Fitzsimmons J. S. (2001) The role of periosteum in cartilage repair. *Clin. Orthop. Relat Res.* **391**: S190–S207
- 19 Mark K. von der, Gauss V., Mark H. von der and Müller P. (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* **267**: 531–532
- 20 Brittberg M., Lindahl A., Nilsson A., Ohlsson C., Isaksson O. and Peterson L. (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl. J. Med.* **331**: 889–895
- 21 Caplan A. I. (1991) Mesenchymal stem cells. *J. Orthop. Res.* **9**: 641–650
- 22 Wakitani S., Goto T., Pineda S. J., Young R. G., Mansour J. M., Caplan A. I. et al. (1994) Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J. Bone Joint Surg. Am.* **76**: 579–592
- 23 Prockop D. J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**: 71–74
- 24 Mackay A. M., Beck S. C., Murphy J. M., Barry F. P., Chichester C. O. and Pittenger M. F. (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* **4**: 415–428
- 25 Yoo J. U., Barthel T. S., Nishimura K., Solchaga L., Caplan A. I., Goldberg V. M. et al. (1998) The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J. Bone Joint Surg. Am.* **80**: 1745–1757
- 26 Pittenger M. F., Mackay A. M., Beck S. C., Jaiswal R. K., Douglas R., Mosca J. D. et al. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147
- 27 Minguell J. J., Erices A. and Conget P. (2001) Mesenchymal stem cells. *Exp. Biol. Med. (Maywood)* **226**: 507–520
- 28 Majumdar M. K., Thiede M. A., Mosca J. D., Moorman M. and Gerson S. L. (1998) Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J. Cell Physiol.* **176**: 57–66
- 29 Short B., Brouard N., Occhiodoro-Scott T., Ramakrishnan A. and Simmons P. J. (2003) Mesenchymal stem cells. *Arch. Med. Res.* **34**: 565–571
- 30 Vogel W., Grunebach F., Messam C. A., Kanz L., Brugger W. and Buhning H. J. (2003) Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells. *Haematologica* **88**: 126–133
- 31 Lee O. K., Kuo T. K., Chen W. M., Lee K. D., Hsieh S. L. and Chen T. H. (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* **103**: 1669–1675
- 32 Behrens P. (2005) Matrix-coupled microfracture – a new concept for cartilage defect repair (in German). *Arthroscopie* **18**: 193–197
- 33 Kramer J., Hegert C. and Rohwedel J. (2003) *In vitro* differentiation of mouse ES cells: bone and cartilage. *Methods Enzymol.* **365**: 251–268
- 34 Linsenmayer T. F. and Hendrix M. J. (1980) Monoclonal antibodies to connective tissue macromolecules: type II collagen. *Biochem. Biophys. Res. Commun.* **92**: 440–446
- 35 Dorheim M. A., Sullivan M., Dandapani V., Wu X., Hudson J., Segarini P. R. et al. (1993) Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. *J. Cell Physiol.* **154**: 317–328
- 36 Simmons P. J. and Torok-Storb B. (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* **78**: 55–62
- 37 Gronthos S., Graves S. E., Ohta S. and Simmons P. J. (1994) The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood* **84**: 4164–4173
- 38 Kramer P. R., Nares S., Kramer S. F., Grogan D. and Kaiser M. (2004) Mesenchymal stem cells acquire characteristics of cells in the periodontal ligament *in vitro*. *J. Dent. Res.* **83**: 27–34

- 39 Shapiro F, Koide S. and Glimcher M. J. (1993) Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J. Bone Joint Surg. Am.* **75**: 532–553
- 40 Lee H. S., Huang G. T., Chiang H., Chiou L. L., Chen M. H., Hsieh C. H. et al. (2003) Multipotential mesenchymal stem cells from femoral bone marrow near the site of osteonecrosis. *Stem Cells* **21**: 190–199
- 41 Zuk P. A., Zhu M., Ashjian P., De Ugarte D. A., Huang J. I., Mizuno H. et al. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **13**: 4279–4295
- 42 Kassem M. (2004) Mesenchymal stem cells: biological characteristics and potential clinical applications. *Cloning Stem Cells* **6**: 369–374
- 43 Gregory C. A., Ylostalo J. and Prockop D. J. (2005) Adult bone marrow stem/progenitor cells (MSCs) are preconditioned by microenvironmental 'niches' in culture: a two-stage hypothesis for regulation of MSC fate. *Sci. STKE* **294**: pe37
- 44 Grove J. E., Bruscia E. and Krause D. S. (2004) Plasticity of bone marrow-derived stem cells. *Stem Cells* **22**: 487–500
- 45 Castro-Malaspina H., Gay R. E., Resnick G., Kapoor N., Meyers P., Chiarieri D. et al. (1980) Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* **56**: 289–301
- 46 Friedenstein A. J., Chailakhjan R. K. and Lalykina K. S. (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet.* **3**: 393–403
- 47 Pereira R. F., Halford K. W., O'Hara M. D., Leeper D. B., Sokolov H. P., Pollard M. D. et al. (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc. Natl. Acad. Sci. USA* **92**: 4857–4861
- 48 Colter D. C., Class R., DiGirolamo C. M. and Prockop D. J. (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA* **97**: 3213–3218
- 49 Jaiswal N., Haynesworth S. E., Caplan A. I. and Bruder S. P. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J. Cell Biochem.* **64**: 295–312
- 50 Phinney D. G., Kopen G., Isaacson R. L. and Prockop D. J. (1999) Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J. Cell Biochem.* **72**: 570–585
- 51 Kramer J., Hegert C., Hargus G. and Rohwedel J. (2003) Chondrocytes derived from mouse embryonic stem cells. *Cytotechnology* **41**: 177–187
- 52 Frisbie D. D., Oxford J. T., Southwood L., Trotter G. W., Rodkey W. G., Steadman J. R. et al. (2003) Early events in cartilage repair after subchondral bone microfracture. *Clin. Orthop. Relat. Res.* **407**: 215–227
- 53 Salasnyk R. M., Williams W. A., Boskey A., Batorsky A. and Plopper G. E. (2004) Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells. *J. Biomed. Biotechnol.* **2004**: 24–34
- 54 Batorsky A., Liao J., Lund A. W., Plopper G. E. and Stegemann J. P. (2005) Encapsulation of adult human mesenchymal stem cells within collagen-agarose microenvironments. *Biotechnol. Bioeng.* **92**: 492–500
- 55 Mauney J. R., Volloch V. and Kaplan D. L. (2005) Matrix-mediated retention of adipogenic differentiation potential by human adult bone marrow-derived mesenchymal stem cells during *ex vivo* expansion. *Biomaterials* **26**: 6167–6175
- 56 Chen C. W., Tsai Y. H., Deng W. P., Shih S. N., Fang C. L., Burch J. G. et al. (2005) Type I and II collagen regulation of chondrogenic differentiation by mesenchymal progenitor cells. *J. Orthop. Res.* **23**: 446–453
- 57 Breinan H. A., Martin S. D., Hsu H. P. and Spector M. (2000) Healing of canine articular cartilage defects treated with microfracture, a type-II collagen matrix, or cultured autologous chondrocytes. *J. Orthop. Res.* **18**: 781–789
- 58 Dorotka R., Bindreiter U., Macfelda K., Windberger U. and Nehrer S. (2005) Marrow stimulation and chondrocyte transplantation using a collagen matrix for cartilage repair. *Osteoarthritis Cartilage* **13**: 655–664
- 59 Huang Q., Goh J. C., Huttmacher D. W. and Lee E. H. (2002) *In vivo* mesenchymal cell recruitment by a scaffold loaded with transforming growth factor beta1 and the potential for *in situ* chondrogenesis. *Tissue Eng.* **8**: 469–482
- 60 Li W. J., Tuli R., Okafor C., Derfoul A., Danielson K. G., Hall D. J. et al. (2005) A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials* **26**: 599–609
- 61 Buma P., Pieper J. S., Tienen T. van, Susante J. L. van, Kraan P. M. van der, Veerkamp J. H. et al. (2003) Cross-linked type I and type II collagenous matrices for the repair of full-thickness articular cartilage defects – a study in rabbits. *Biomaterials* **24**: 3255–3263
- 62 Rodriguez A. M., Elabd C., Delteil F., Astier J., Vernochet C., Saint-Marc P. et al. (2004) Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem. Biophys. Res. Commun.* **315**: 255–263



To access this journal online:  
<http://www.birkhauser.ch>