Influence of *In Vitro* Cultivation on the Integration of Cell-Matrix Constructs After Subcutaneous Implantation

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ABSTRACT

Dynamic cultivation of scaffolds loaded with undifferentiated stem cells can lead toward osteogenic differentiation *in vivo*. The aim of this study was to examine the influence of different *in vitro* cultivation setups on the integration of cell-matrix constructs after subcutaneous implantation. Human mesenchymal stem cells (hMSC) were inoculated on clinically approved scaffolds. These cell-matrix constructs were then cultured under static (12 hours or 14 days) or dynamic (14 days) conditions, followed by paravertebral subcutaneous implantation in athymic nude mice. After 2 weeks and 12 weeks the constructs and selected organs were harvested for histological evaluation, and qualitative and quantitative polymerase chain reaction (PCR). Histological analysis showed good integration of cell-matrix constructs independent of culture conditions and a differential effect of static and dynamic *in vitro* culture on fat cell formation *in vivo*. Human DNA (hDNA) was detected in explanted cell-matrix constructs at all time points with a significant decrease in human cells on the constructs compared to the initial amount of cells seeded. No hDNA was detected in the explanted organs. In conclusion, we could prove the survival of hMSC on scaffolds after *in vitro* cultivation and consecutive implantation *in vivo*. While the amount of adipose tissue increased after static cultivation, we could not achieve osteogenic differentiation.

INTRODUCTION

BONE DEFECTS CAUSED BY TRAUMA, tumors, or malformations still represent a major problem in reconstructive surgery. In the context of promising tissue engineering strategies, Vacanti *et al.* successfully reconstructed the distal phalanx of an amputated finger in a clinical experiment. Scaffolds were seeded with autologous cells that were cultivated *in vitro* prior to implantation.[1] However, the relatively poor clinical result paired with a time-consuming approach and therefore high operative stress for the patient do not justify wide clinical use of this approach.[2] More experimental research is needed to improve tissue engineering applications in this direction.

Graft materials are vitalized by cells *in vitro* prior to implantation into sites where formation of new tissue is needed. Much attention is paid to human mesenchymal stem cells (hMSC) because of their ability to progress from undifferentiated progenitors to biosynthetically mature cells and their self-renewing capacity.[3,4] Although ectopic as well as orthotopic bone formation has been observed in many preclinical animal models following implantation of 3D cell-matrix constructs, many issues, such as the recruitment and manipulation of an adequate cell population, choice of a suitable scaffold, and an effective initiation of osteogenesis still remain to be solved.[5–8]

To achieve new bone formation, implanted stem cells must differentiate toward osteoblastic cells, because these cells are

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crucial for the synthesis and deposition of new bone. To support this process, influence can be exerted *in vitro* to favor a certain lineage rather than allowing the cells to differentiate along all the lineages possible. Following expansion of cells, it is essential that they are seeded successfully onto suitable scaffolds. In this study we focus on the influence of the *in vitro* culture period on the *in vivo* integration of cell-matrix constructs after subcutaneous implantation. Some studies claim that cultivation of cell-matrix constructs in flow perfusion chambers enhances osteoblastic differentiation even without the addition of osteogenic supplements such as dexamethasone as opposed to static culture conditions *in vitro*.[9–11] Presumably, in the former case, shear forces promote undifferentiated stem cells to differentiate toward the osteogenic lineage. However, implantation of cell-matrix constructs in a calvarian defect model did not show differences in bone formation as a result of different culture conditions. The highest percentage of bone formation per implant was seen after static cultivation or flow perfusion for 1 day *in vitro*.[12]

Further, it is essential that implanted cells do not disseminate or exhibit unwanted cell growth afar the implantation site. Evidence exists that hMSC appear in neuronal tissue and other organs of the host following intravenous injection.[13] Also, cells injected intraperitoneally into inbred mouse embryos were detected in all harvested organs. To date no experimental study has examined the dissemination of hMSC in host organs following subcutaneous implantation.

Therefore, this study aimed to (i) examine the influence of the *in vitro* cultivation period on the *in vivo* performance of cell-matrix constructs after their subcutaneous implantation, and (ii) detect any migration of cells from the implantation site to organs of the host. To rule out the influence of osteoconduction, periosteal bone formation, and biomechanical features as seen in orthotopic implantation sites, we used a heterotopic model.

MATERIALS AND METHODS

Scaffold loading and cultivation of hMSC

The hMSC (Cambrex, Walkersville, USA) were cultivated in MSC basal medium (Cambrex). Fresh medium was supplied three times a week. When cell layers neared confluence, cells were detached using trypsin-EDTA. Cultures were maintained in a humidified atmosphere of 95% air with 5% carbon dioxide (CO_2) at 37°C.

Cells in the fifth passage were trypsinized, centrifuged, and resuspended to achieve a final concentration of 1.5×10^6 cells per mL. About 660 µL of this suspension (1.0×10^6 cells) was used for each scaffold (Tutobone®, Tutogen Medical, Neunkirchen, Germany) (Fig. 1A). The matrices were 9 mm in diameter and 3 mm in height. During the first 6 hours scaffolds were turned in regular intervals to ensure equal distribution of the cell suspension throughout. With this technique a seeding efficiency of 90% was reached.

For static cultivation, cell-matrix constructs were transferred to 48-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, containing 10% fetal bovine serum (FBS) and 40 IU/mL penicillin/streptomycin for either 12 hours or 14 days in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. Medium was changed every 2 days throughout the culture period.

Dynamic cultivation was performed in bioreactors (Gradient container, Minucells Minutissue, Weinheim, Germany) with cylindrical flow chambers (Fig. 1B, C). In this experimental setup Hepes buffer (4-(2-Hydroxyethyl)-1-piperazinethansulfonic acid, 25 mM) was added to the culture medium. Each chamber contained a cassette for one cell-matrix construct, sealed by two neoprene rings and held in place by two outside clamps. Bioreactors were connected by silicone tubes to both fresh medium and waste reservoirs. The high permeability of silicone for CO_2 and oxygen allowed for sufficient gas exchange. Flow through each chamber was driven by multichannel roller pumps (Ismatec, IPC, Switzerland), with each flow chamber on its own independent pumping system. In the bioreactor, media flowed through the scaffold from bottom to top to prevent air trapping (Fig. 1C).

Flow perfusion culture systems were assembled using sterile techniques in a laminar flow hood. Cell-matrix constructs were seeded, placed in the cassettes, and sealed. Systems were placed on a heating plate (37°C) and covered with a styrofoam box. Scaffolds were cultured at a medium flow rate of 18.1 µL/minute.

Experimental design and surgical procedure

A total of 24 (six per group) six- to eight-week-old athymic nude mice (nu/nu; Harlan Winkelmann, Borchen, Germany) were used in this study. The study was approved by the Government Committee of Upper Bavaria, and all animals were cared for following guidelines of the LMU Munich for the care and use of laboratory animals.

Following anesthesia using an intramuscular injection of a fentanyl-medetomidin-midazolam mixture, two pouches were prepared subcutaneously by blunt dissection. In one group, cell-matrix constructs that had been statically cultivated for 12 hours were implanted left and scaffolds without hMSC were implanted right paravertebrally. In the second group, matrices cultivated for 14 days under static or flow perfusion conditions, respectively, were used.

After 2 weeks and 12 weeks, scaffolds including the surrounding tissue and organs (brain, heart, lung, liver, kidney, spleen, and testis) were retrieved. One half of the harvested scaffolds were embedded in methylmethacrylate (MMA, Merck, Germany) for histological evaluation, and the other half and the organs were used for molecular analysis.

Histological analysis

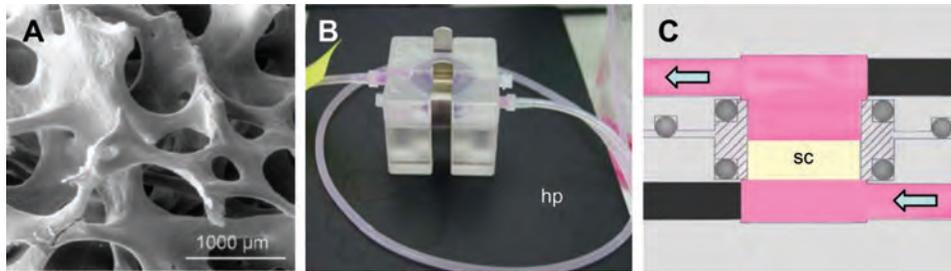Harvested implants were fixated in a formaldehyde/methanol solution containing 64% methanol, 35% formalde-

FIG. 1. (**A**) Microporous structure of subcutaneously implanted bovine spongious bone scaffolds (Tutobone; height 3 mm, diameter 9 mm, pore size 400–1200 μm). (**B, C**) Bioreactor setup used for dynamic cultivation: the bioreactor as well as tubes are located on a heating plate (hp). To keep constant temperature (37°C) a styrofoam box covers the setup (not shown). Nonrecirculating media flow (as indicated by arrows) is powered by a multichannel roller pump. The medium is forced through the seeded scaffolds (sc) at a continuous flow rate of 18.1 μL/minute. Color images available online at www.liebertpub.com/ten.

hyde, and 1% sucrose in phosphate buffered saline (PBS) for 48 hours, dehydrated in a graded series of ethanol, and embedded in MMA. Following polymerization, 4 μm thick sections were prepared of each implant using a microtome technique polycut (Jung, Heidelberg, Germany). Sections were stained with paragon and investigated using a light microscope (Axioskop, Zeiss, Jena, Germany). Digital images were obtained using a 3CCD color video camera (Sony) and processed on a personal computer with KS 400 (Zeiss). Sections were independently evaluated and graded by three observers judging the extent of inflammatory reaction, necrosis, granulation tissue, the occurrence of adipose tissue, neovascularization, and osteoclasts. The grading scale ranged from 1 to 5 according to van Gaalen *et al.*[14]

Molecular analysis

Extraction of DNA. The isolation of DNA from the harvested samples was performed with QiAmp blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. Briefly said, DNA contained in the lysed samples was absorbed onto a silica matrix, and cell debris was removed by washing with QIAamp washing buffer. Finally, the DNA was collected by elution in distilled water.

Conventional DNA PCR. Detection of human DNA (hDNA) within the scaffolds and organs of the mice was confirmed by polymerase chain reaction (PCR). We amplified an 850 bp fragment of the α-satellite region of the human chromosome 17 using primers corresponding to the primer pair 17a1 (5′ GGG ATA ATT TCA GGT GAC TAA ACa G 3′) and 17a2 (5′ TTC CGT TTA GTT AGG TGC AGT TAT C 3′) as previously described by Becker *et al.*[15]

Each reaction mixture with an end volume of 50 μL contained 250 ng of human genomic DNA, 200 μM of each nucleotide (GTP, ATP, TTP, and CTP), 250 μM of each primer, 2.5 units Taq polymerase, and Q-Solution (Qiagen) with a definite concentration of 15 mM $MgCl_2$.

An initial denaturation for 3 minutes at 94°C was followed by 40 cycles of 30 seconds each at 94°C. Then annealing was performed for 1 minute, followed by a polymerase reaction at 72°C for another minute. Finally, 10 minutes of polymerization and an interruption of the reaction by cooling down to 4°C completed the cycle. Amplified DNA fragments were transferred onto a 1.75% agarose gel and divided by electrophoresis. After staining with ethidium bromide, gels were analyzed using ultraviolet light.

Isolated DNA from hMSC serving as positive control and DNA extracted from murine fibroblasts serving as negative control ran with every PCR. To prove a reliable sensitivity, the genomic DNA of a cell mixture containing both murine fibroblasts and hMSC was determined with each PCR (1×10^6 murine fibroblasts + 10 hMSC and 1×10^6 murine fibroblasts + 1 hMSC).

Quantitative real-time PCR (qRT-PCR) on genomic DNA. The quantitative determination of hDNA in the samples was performed by qRT-PCR (LightCycler System, Roche, Mannheim, Germany) using SYBR Green I for detection. A specific human primer set from Search-LC (Heidelberg, Germany) was used (Sense Primer: 5′ AAC ATG GTG AAA CCC CGT CTC 3′; Anti-Sense Primer: 5′ TGC AGT GGC GCG ATC TTG 3′). The final volume of each PCR reaction was 20 μL, with 10 μL containing 5 ng genomic DNA in Aqua dest. and 10 μL of a PCR Mix (6 μL PCR grade water, 2 μL of primer pair, and 2 μL of LightCycler Fast Start DNA Master SYBR Green I; Roche).

Each PCR cycle consisted of a 10-second denaturation at 95°C followed by an annealing phase of 10 seconds initially at 68°C with a transition to 58°C in steps of 0.5°C per cycle. Elongation was performed at 72°C for 16 seconds, during which fluorescence intensity was measured in each cycle. The temperature transition rate of all steps was 20°C per second. Following PCR cycling, samples were denatured again at 95°C and cooled down to 58°C for 10 seconds. To confirm specificity, a melting curve analysis was performed by continuous measurements of the fluorescence intensity while heating the sample to 95°C with a temperature transition rate of 0.1°C per second. Serial dilution steps of human genomic DNA in 10 μL aqua dest. (60 ng, 6 ng, 0.6 ng,

0.06 ng, 0.006 ng, 0.0006 ng hDNA) were used to generate calibration curves. With every PCR, hDNA ran as a positive control, DNA of murine fibroblasts and aqua dest. served as negative controls.

Statistical analysis

Correlation analysis was performed using the nonparametric Wilcoxon signed rank test for histological results and the parametric t-test for results of the quantitative PCR. Statistical differences were considered significant when $p < 0.05$.

RESULTS

Histological findings

After an implantation period of 2 weeks an incipient ingrowth of fibrous tissue was seen histologically. In both treated and blank scaffolds many leukocytes indicating an inflammatory reaction as well as partial necrosis were to be seen (Figs. 2, and 3).

During the 12 weeks that followed implantation, further ingrowth of the surrounding tissue into the scaffolds occurred. In all seeded and unseeded scaffolds multinucleated giant cells were seen lying on the surface. The increase in osteoclasts compared to 2 weeks after implantation was statistically significant (Fig. 3). Most of them were seen aligned along the edge of the blank scaffolds. The initial inflammatory reaction, as seen in the 2-week groups, was steadily decreasing until 12 weeks. Instead, more and more scar tissue was viewable, particularly in the middle of the constructs, over time. Further, more adipose tissue was found within loose fibrous connective tissue in all scaffolds. Most of the adipose tissue was detectable within the cell-matrix constructs in the groups with static cultivation, either for 12 hours or 14 days. The increase in adipose tissue in all groups after 12 weeks was statistically significant compared to 2 weeks. In all groups, newly formed vessels could be detected throughout the whole scaffold after 12 weeks. All in all, apart from fat cell formation, no differences regarding tissue reaction as a result of varying culture conditions were seen (Figs. 2 and 3).

Semiquantitative PCR

As internal controls we could detect up to 10 hMSC in a pool of 10^6 murine cells. In all explanted composites hDNA was found at all time points equally. No hDNA could be detected in blank scaffolds (Fig. 4A). Moreover, we did not detect hDNA in any of the explanted organs at any time point (Fig. 4B). After 2 weeks of implantation hDNA was seen in the soft tissue surrounding the implanted cell-matrix constructs irrespective of the type and duration of *in vitro* cultivation.

Quantitative PCR (LightCycler)

With RT-PCR a significant decrease in human cells on cell-matrix constructs could be detected after 2 weeks of implantation compared to the amount of initially seeded cells. In the group with statically cultivated scaffolds over 14 days, there was a further significant decrease in human cells after 12 weeks. In the scaffolds statically cultured for 12 hours this decrease was not significant. In the dynamically cultivated group no further decrease in human cells was seen (Fig. 5).

DISCUSSION

In this study we evaluated the performance of hMSC seeded in bioresorbable and biocompatible scaffolds following subcutaneous implantation over a period of 12 weeks. There was an ingrowth of surrounding host tissue in all groups over time. Regardless of the culture conditions, multinucleated giant cells attached to the surface of the scaffold, which corresponds to the results of other studies.[16] Loose fibrous connective tissue could be detected mainly at the edge and granulation tissue at the center of the scaffolds. The appearance of adipocytes was especially prominent in the groups cultured under static conditions for either 12 hours or 14 days.

Although human cells are frequently used, one remaining problem is their detection in xenotransplantation. However, we could differentiate between implanted hMSC and ingrowing murine cells using RT-PCR. The hMSC were detectable until 12 weeks after implantation in the previously seeded scaffolds. Other studies failed to detect stem cells after 6 weeks by RT-PCR or *in situ* hybridization, although new bone formation was seen.[17,18] This might be explained by the missing sensitivity of the method or by the fact that human bone marrow stem cells (hBMSC) labelled with digoxigenin lose their specific properties needed for detection once they are differentiated into osteoblasts. In xenotransplanation it is important to detect implanted stem cells even at a very low level because unwanted cell growth must be ruled out. Although cell death occurred in our study, the PCR was sensitive enough to detect even 10 hMSC. The highly repetitive sequences of the α-satellite centromer region of human chromosome 17 are highly specific for human tissues and allow for detection of even smallest amounts of hDNA.[13] Dilution studies showed that the DNA amount of 10 hMSC was detectable in a pool of 10^6 murine fibroblasts. The quantitative PCR showed that the amount of cells significantly decreased 2 weeks after implantation compared to the initial amount of seeded cells. After 12 weeks there was a further significant decrease in cells in the group that had been statically cultured for 14 days, whereas in the dynamically cultured group the amount of cells remained stable. We presume that under dynamic culture conditions cells form an extracellular matrix (ECM), which prevents them
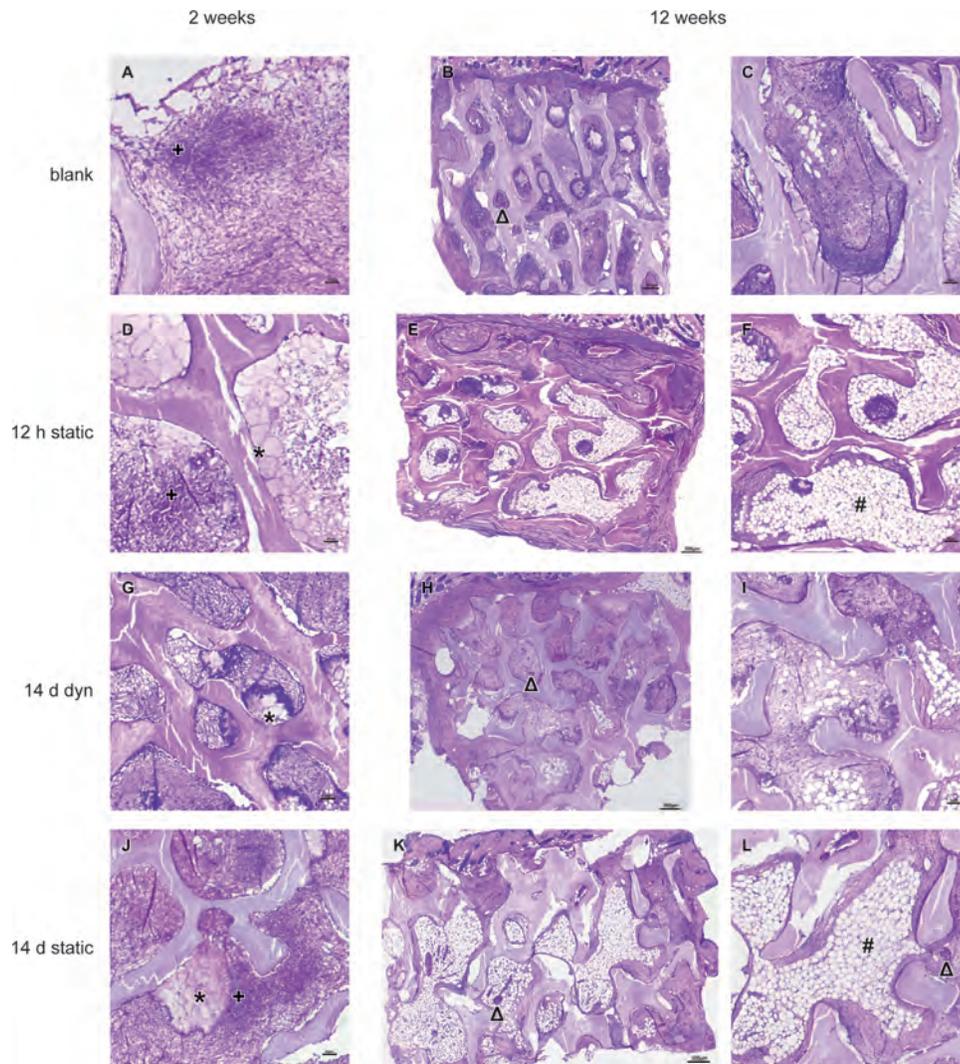
FIG. 2. Paragon-stained MMA-embedded sections 2 weeks (**A, D, G, J**) and 12 weeks (**B, C, E, F, H, I, K,** and **L**) after subcutaneous implantation. Independent of the kind of cultivation, 2 weeks after implantation, mainly inflammation (+), necrosis (*), and granulation tissue were detectable. Twelve weeks after implantation, there was a decrease in the initial inflammation reaction and an ingrowth of the surrounding tissue with neovascularization (Δ). Adipose tissue (#) was mainly detectable in statically cultured groups. Color images available online at www.liebertpub.com/ten.

from further cell death after 2 weeks. Holtorf *et al.* reported that rat MSC build a mineralized matrix and therefore differentiate toward the osteogenic lineage after dynamic cultivation even without the influence of additional osteogenic supplements. Further, cells are located throughout the whole scaffold, whereas under static culture conditions they are mainly localized in the perimeter of the scaffold.[19]

In contrast to other studies, we could not detect any differentiation toward the osteoblastic lineage.[12,20–22] Accordingly, no new bone or cartilage formation in the statically or dynamically cultivated groups could be noted. A higher density of cells seeded on the relatively large scaffolds might have had a positive influence on osteoblastic differentiation. Another reason for the difference might be the use of a dif-

ferent cell type in combination with a biological scaffold. Especially for MSC a comparison of experimental outcomes is rather difficult because of varied tissue sources and methodologies of cell preparations. Therefore, the International Society for Cellular Therapy proposed the adherence to plastic, the expression of specific surface antigen, and the multipotent differentiation potential as three minimal criteria to define MSC.[23] To be comparable with other results we used commercially available hMSC, which have quantitatively less progenitor cells than BMSC gained from bone marrow but fulfill the defined criteria.

Further, the osteogenically stimulating medium causes an *in vitro* differentiation toward the osteogenic lineage of hMSC. Also, dynamic compared to static cultivation of
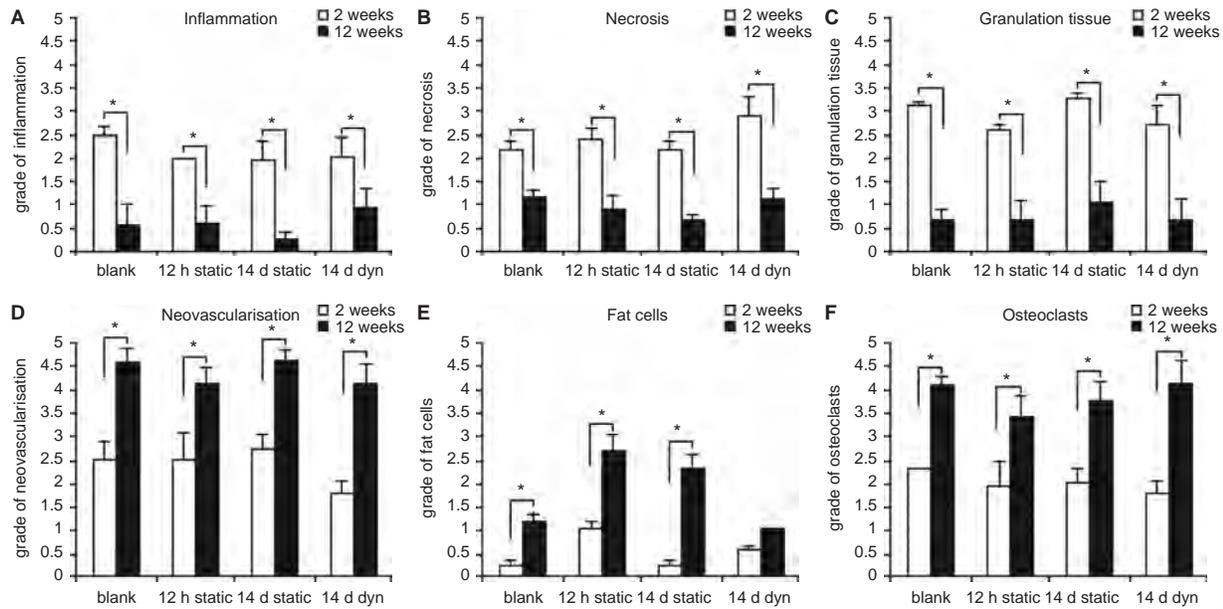
FIG. 3. Histological grade of inflammation, necrosis, granulation tissue, adipose tissue, neovascularization, and osteoclasts judged by three different examiners. A significant decrease in inflammatory reaction and granulation tissue was seen after 12 weeks. Further, there was a notable increase in appearance of adipose tissue after 12 weeks compared to 2 weeks in all groups, with significant increase in the statically cultivated groups. A significant increase in the grade of neovascularization and in the amount of osteoclasts was seen in all groups.
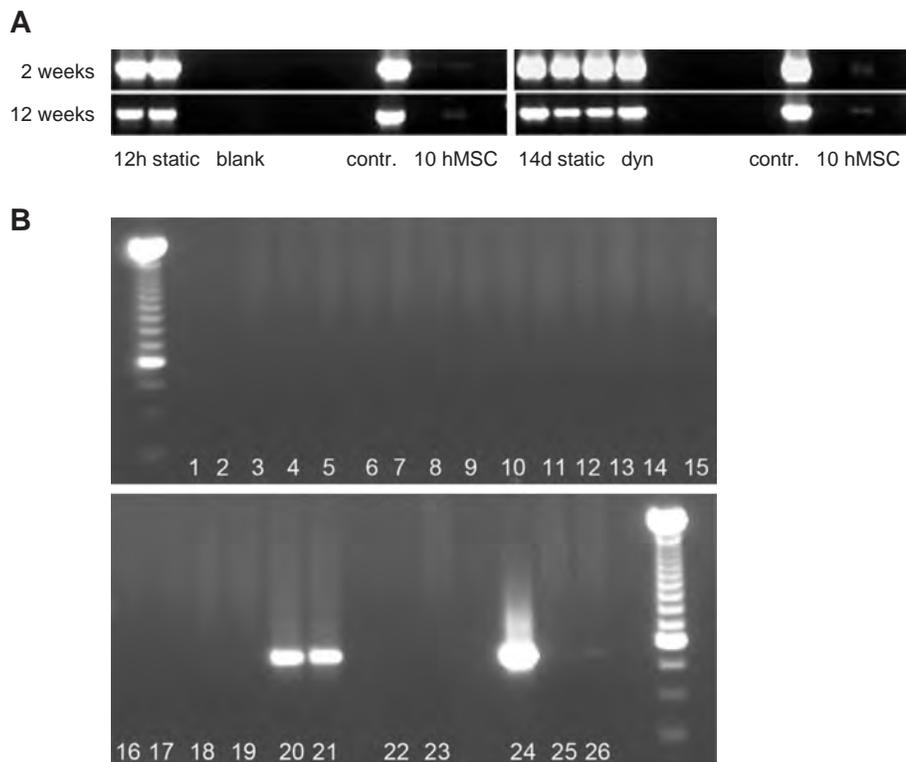

FIG. 4. (**A**) Semiquantitative PCR, 2 and 12 weeks after subcutaneous implantation. Shown are representative samples of detectable hDNA in cell-matrix constructs. The hDNA was detectable in all cell-matrix constructs except the blank scaffolds over 12 weeks. Internal controls show that DNA was verifiable up to 10 hMSC. Compared to controls (1×10^6 hMSC) intensity of the PCR signal of the probes in each group decreased over implantation time. (**B**) PCR of the organs, scaffolds, and controls, 12 weeks after subcutaneous implantation of cell-matrix constructs. No hDNA could be detected in any of the explanted organs at any time point. $1+2$: liver, $3+4$: spleen, $5+6$: kidney, $7+8$: lung, $9+10$: heart, $11+12$: brain, 13: testis, 14: muscle/skin left, 15: muscle/skin right, $16+17$: blood, $18+19$: blank scaffold, 20+21: 12 hours statically cultured scaffold, technical controls, 22: water, 23: murine fibroblasts, 24: hMSC (1×10^6 cells), 25: hMSC (1 cell in 1×10^6 murine fibroblasts), 26: hMSC (10 cells in 1×10^6 murine fibroblasts).
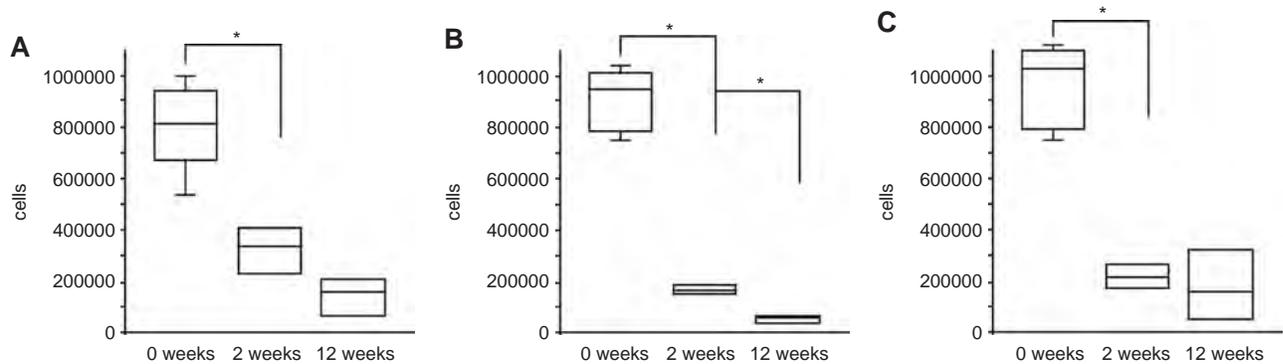
FIG. 5. Amount of cells detected in cell-matrix constructs before implantation, and after 2 weeks and 12 weeks of implantation with varying precultivation conditions: (**A**) static cultivation for 12 hours, (**B**) static cultivation for 14 days, and (**C**) dynamic cultivation for 14 days. The horizontal bar indicates the median, the lower and upper limits of the box indicate the 25th and 75th percentiles. The upper and lower whiskers mark the 90th and 10th percentiles, respectively. The decrease in number of cells after 2 weeks in all examined groups was statistically significant. Further decrease after 12 weeks was statistically significant only in group B. Following are the p values: (**A**) 0 vs. 2 weeks: $p = 0.0002$, 2 vs. 12 weeks: $p = 0.0673$; (**B**) 0 vs. 2 weeks: $p < 0.0001$, 2 vs. 12 weeks: $p = 0.0002$; (**C**) 0 vs. 2 weeks: $p < 0.0001$, 2 vs. 12 weeks: $p = 0.7592$.

osteogenic-stimulated cell-matrix constructs enhances the formation of an ECM *in vitro*, which facilitates more new bone formation *in vivo*.[24]

Although we tested the culture setup *in vitro* (unpublished data), we assume that the dynamic cultivation setup in flow chambers used in the present study is not sufficient to stimulate hMSC *in vitro* for differentiation processes in order to form new bone in a heterotopic site. While the bioreactor itself is comparable to the flow perfusion system introduced by Bancroft *et al.*,[11] we used nonrecirculated media supply without addition of osteogenic supplements. We also used a significantly lower flow rate. In comparison to the setup used by Wang *et al.*,[24] who also cultured without stimulating supplements, we used a different bioreactor system and lower flow rates.

Instead of osteogenic differentiation we observed adipose tissue in the seeded scaffolds, which was especially prominent in the groups cultured under static conditions. However, the origin of the adipocytes remains unclear: they may be either the differentiated hMSC or the differentiated precursor cells from the murine host. Our results suggest that static conditions *in vitro* may permit differentiation toward the adipogenic lineage of hMSC in larger extents than dynamic conditions. Further, it is possible that the implantation of a relatively small amount of differentiated precursor cells may enable considerable further fat cell formation by the host *in vivo* as has been described in recent fat engineering approaches.[25,26]

The size of the scaffold may be responsible for rapid cell death mainly during the first 2 weeks after implantation and for the lack of bone formation. Although many newly formed vessels were seen after an implantation period of 12 weeks, the results of the quantitative analysis by RT-PCR showed a noticeable decrease in hMSC 2 weeks after implantation. We believe that the death of hMSC mainly occurs during the first hours of implantation and can be attributed to the lack of vascular supply with insufficient nutrient supply by diffusion. These data also correlate with other studies where no new bone formation was seen in large unvascularized bony defects.[27] Although the critical size of ectopic implants did not exceed 3.5 mm,[6] the scaffolds used seemed to be oversized for the relatively poorly vascularized subcutaneous tissue.

Poor adhesion properties of the scaffold or missing differentiation possibilities can be ruled out, because we previously demonstrated new matrix formation after seeding scaffolds in dynamic culture systems for over 6 weeks *in vitro* (unpublished data). On the other hand, it cannot be ruled out that immunologic reactions, as seen histologically particularly after 2 weeks, lead to early cell death even though athymic nude mice were used. This thesis is supported by the studies of Mc Bride *et al.*, who showed a decrease in cells after intracerebral injection into athymic animals when using hMSC, while the initial amount of injected murine MSC remained unaltered.[28] Other studies have demonstrated that hMSC were detectable in bone marrow as well as in other organs such as lung and spleen after intravenous injection.[13,29,30] We showed that no unwanted cell migration from the implanted composites into the organs of the host occurred following ectopic implantation. The question if unwanted cell migration could occur over the vascular network present in bone marrow remains unanswered despite its relevance for orthotopic implantation.

In summary, we established an experimental model that allows for testing the influence of different cell populations and the onset of cell differentiation, as well as evaluate the performance of various scaffolds regarding their integration *in vivo* after subcutaneous implantation. The PCR described allows a sensitive and specific detection of human cells following xenotransplantation. The RT-PCR using Light-Cycler methodology allows a reliable quantification of

hMSC within the implanted scaffolds. In this study we were not able to detect a significant advantage of dynamic cultivation with respect to the formation of new bone following subcutaneous implantation. We believe that in addition to *in vitro* culture conditions, the surface properties of the used scaffold, the use of an adequate cell type, and the amount of implanted cells as well as their grade of differentiation are at least as important for new bone formation *in vivo*.

ACKNOWLEDGMENTS

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