Long-Term Survival and Bipotent Terminal Differentiation of Human Mesenchymal Stem Cells (hMSC) in Combination With a Commercially Available Three-Dimensional Collagen Scaffold

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Researchers working in the field of tissue engineering ideally combine autologous cells and biocompatible scaffolds to replace defect tissues/organs. Due to their differentiation capacity, mesenchymal-derived stem cells, such as human mesenchymal stem cells (hMSC), are a promising autologous cell source for the treatment of human diseases. As natural precursors for mesenchymal tissues, hMSC are particularly suitable for bone, cartilage, and adipose tissue replacement. In this study a detailed histological and ultrastructural analysis of long-term cultured and terminally differentiated hMSC on 3D collagen scaffolds was performed. Standardized 2D differentiation protocols for hMSC into adipocytes and osteoblasts were adapted for long-term 3D in vitro cultures in porous collagen matrices. After a 50-day culture period, large numbers of mature adipocytes and osteoblasts were clearly identifiable within the scaffolds. The adipocytes exhibited membrane-free lipid vacuoles. The osteoblasts were arranged in close association with hydroxyapatite crystals, which were deposited on the surrounding fibers. The collagen matrix was remodeled and adopted a contracted and curved form. Human MSC survive long-term culture within these scaffolds and could be terminally differentiated into adipocytes and osteoblasts. Thus, the combination of hMSC and this particular collagen scaffold is a possible candidate for bone and adipose tissue replacement strategies.

Key words: Mesenchymal stem cells; Autologous cells; Adipose tissue engineering; Bone tissue engineering; Collagen; Electron microscopy

INTRODUCTION

Tissue engineering in modern medicine aims to reconstruct damaged tissues or organs by transplanting cells, seeded on a biomaterial scaffold. Ideally, such biological hybrids would then be capable of fulfilling the original function of the damaged tissue or organ (8). However, suboptimal fulfillment of such demands results in continued research in this field. Novel findings in embryonic and adult stem cell research and the understanding of their biology both in vivo and in vitro offer new perspectives for their application in the field of reconstructive medicine. From the immunological point of view, autologous cells should be used to avoid transplant rejections. Adult stem cells from different organs, so called tissue-specific or somatic stem cells, provide an ideal cell source for tissue substitutes, because they can be differentiated into several cell types and can be isolated and retransplanted autologously (17). In the past decade extensive effort has been made to explore the differentiation capacity of various somatic stem cells. Human mesenchymal stem cells (hMSC) are adult stem cells that can be readily isolated from bone marrow (2). They can be expanded ex vivo and differentiated into several cell types, such as osteoblasts, adipocytes, chondrocytes, and smooth muscle cells (14).

The biomaterials used to create an artificial tissue substitute can be categorized into metals, ceramics, or polymers. Besides being biocompatible, such scaffolds should be biodegradable or permanent, depending on the intended use. Macromolecules of the extracellular matrix have been...
shown to be relevant in the simulation of a three-dimensional (3D) stem cell niche. Therefore, such biopolymers are preferred for the production of complex scaffolds.

Collagen, as a degradable biopolymer and a component of the extracellular matrix, is often used as a scaffold and is available in several 3D structures, such as gels, sponges, or foams. It can be easily colonized and remodeled by surrounding cells, which results in its integration into the natural microenvironment (10).

Whether seeded on a 3D scaffold or as monolayer culture on TCPS (tissue culture plastic), hMSC differentiation into adipocytes and osteoblasts is normally detected after a 21-day induction by staining with oil red O for the visualization of lipid vacuoles or alizarin red for calcium deposits. As other cell types like osteoblast and adipocyte precursor cells are also capable of producing positive staining signals, further definite characteristics have to be detected to identify terminally differentiated adipocytes and osteoblasts.

This study focused on detailed biochemical and ultrastructural morphological analyses of biohybrids designed by Suwelack Skin & Health Care AG using a hole punch. For cell culture experiments, collagen scaffolds were disinfected by incubating in 70% ethanol for 2 h, followed by rinsing twice in PBS before being placed in 24-well plates.

**Isolation and Expansion of hMSC**

Mesenchymal stem cells were isolated from human bone marrow aspirate according to protocols from Haynesworth (5) and Pittenger (14) and were characterized as previously described (13). In this study, aspirate was mixed with an equal amount of stem cell medium, vortexed, and spun down at 500 g for 10 min. After decanting the supernatant, the pellet was resuspended in 10 ml stem cell medium and seeded in a T75 cell culture flask. Cells were cultured at 37°C in a 20% O2, 5% CO2 humidified atmosphere. Nonadherent cells were removed by medium change 24 h after seeding. After reaching 90% confluence, cells were detached with stem cell trypsin (Cellsystems, St. Katherinen, Germany) and reseeded with a cell density of 5 × 10^4 cells/mm^2. The stem cell medium consisted of 60% DMEM low glucose (PAA, Colbe, Germany), 40% MCDB-201, 1% ITS plus, 1 nM dexamethasone, 100 µM ascorbic acid 2-phosphate, 10 ng/ml EGF (all from Sigma, Steinheim, Germany), and 2% fetal calf serum (FCS; Hyclone, Perbio Science, Erembodegem-Aalst, Belgium).

**hMSC Differentiation**

The differentiation protocols from Pittenger and coworkers (14) were followed to demonstrate the multipotency of the isolated cells. For adipogenic differentiation, hMSC were seeded with a density of 8 × 10^4 cells/mm^2. After 24 h, the stem cell medium was replaced...
with adipogenic induction medium. Thereafter, adipogenic induction medium was alternately changed with adipogenic maintenance medium every 3–4 days for up to 50 days. The adipogenic induction medium consisted of DMEM high glucose (PAA), 1 µM dexamethasone, 0.2 mM indomethacin, 0.01 mg/ml insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine, and 10% FCS (all from Sigma). The adipogenic maintenance medium consisted of DMEM high glucose (PAA), 0.01 mg/ml insulin, and 10% FCS (both from Sigma). To visualize lipid vacuole formation, cells were stained after 7, 14, 21, and 50 days with oil red O. Cells were fixed before staining with 50% ethanol for 30 min at 4°C, then incubated in an oil red O solution (0.2% oil red O in methanol was freshly diluted 1:3.5 in 1 M NaOH) for 10 min. Finally, cells were rinsed twice in 50% ethanol and then three times in aqua bidest.

For osteogenic induction, hMSC were seeded in a density of 3.1 × 10⁴ cells/mm². Twenty-four hours after seeding, the stem cell medium was replaced with osteogenic induction medium consisting of DMEM low glucose (PAA), 100 nM dexamethasone, 10 mM sodium β-glycerophosphate, 0.05 mM L-ascorbic acid 2-phosphate, and 10% FCS (all from Sigma). Medium was changed every 2–3 days. After 7, 14, 21, and 50 days of osteogenic differentiation, cells were stained with alizarin red to detect calcium deposits. Cells were fixed before staining with 70% ethanol for 1 h, washed three times with aqua bidest, and then stained with an alizarin red solution (40 mM, pH 4.1, Sigma) for 10 min. Finally, cells were washed three times with PBS.

**Immunohistochemistry and Laser Scan Microscopy**

hMSC/collagen biohybrids were fixed in formalin for 24 h, rinsed with PBS, followed by an antibody diluent (DAKO, Hamburg, Germany), and then incubated with an anti-vimentin antibody (1:10,000, mouse, monoclonal, DAKO) for 1 h at room temperature (RT) and at 4°C overnight. The cell/collagen hybrids were then washed in PBS overnight and, after removing the PBS, incubated with a biotinylated anti-mouse secondary antibody (DAKO) for 2 h at RT. The scaffolds were again rinsed in PBS overnight. For detection, the constructs were incubated in Cy3-conjugated streptavidin (1:100 in PBS, Jackson Immuno Research Laboratories, Soham, UK) for 1 h at RT. After further washing with PBS overnight, cell nuclei were stained with DAPI diluted in PBS (1 µg/50 ml) for 10 min at RT and rinsed three times in PBS. Samples were analyzed using a LSM-510-Meta laser scanning microscope (Zeiss, Jena, Germany). Stacks of 2-µm-thick optical sections were recorded, enabling the generation of 3D images.

**Scanning Electron Microscopy (SEM)**

hMSC/collagen biohybrids were fixed in 3% glutaraldehyde for at least 24 h, rinsed with sodium phosphate buffer (0.2 M, pH 7.39, Merck, Darmstadt, Germany), and dehydrated by incubating consecutively in 30%, 50%, 70%, and 90% acetone and then three times in 100% acetone for 10 min. Collagen scaffolds were then critical point dried in liquid CO₂, and then sputter-coated with a 30-nm gold layer. Samples were analyzed using an environmental scanning electron microscope (ESEM XL 30 FEG, FEI, Philips, Eindhoven, The Netherlands) in a high vacuum environment.

**Transmission Electron Microscopy (TEM)**

Scaffolds were fixed with 3% glutaraldehyde for 24 h, postfixed with 1% osmium tetroxide, washed with sodium phosphate buffer (0.2 M, pH 7.39, Merck), dehydrated in a series of increasing alcohol concentrations (30%, 50%, 70%, 90%, 3× pure ethanol), and then embedded in epoxy resin. Ultrathin sections were prepared with a Leica Ultracut microtome, stained with uranyl acetate and lead citrate, and analyzed using a transmission electron microscope (EM 400 T, Philips).

### RESULTS

**Biochemical and Morphological Characterization of Collagen Matrices**

The amino acid composition of the collagen scaffolds was comparable to pure collagen type I [according to standard values as determined by Harrington et al. in 1961 (4)] apart from the reduced amount of alanine and

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Collagen (Suwelack) (mol/100 mol)</th>
<th>Collagen Type I (mol/100 mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>7.57</td>
<td>11.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.86</td>
<td>5.0</td>
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<td>Aspartic acid</td>
<td>4.64</td>
<td>4.5</td>
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<tr>
<td>Glutamic acid</td>
<td>7.52</td>
<td>7.2</td>
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<tr>
<td>Glycine</td>
<td>27.03</td>
<td>32.0</td>
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<td>Histidine</td>
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<td>0.5</td>
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<tr>
<td>Hydroxyproline</td>
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<td>9.4</td>
</tr>
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<td>Isoleucine</td>
<td>2.62</td>
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<tr>
<td>Leucine</td>
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<td>Lysine</td>
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<td>Phenylalanine</td>
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<tr>
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<td>0.3</td>
</tr>
<tr>
<td>Valine</td>
<td>2.05</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Not detectable by ASA using acidic total hydrolysis.
Collagen as Scaffold for hMSC-Based Tissue Engineering

Human mesenchymal stem cells seeded on collagen matrices showed a long-term survival over a period of 50 days. FDA/PI staining (live/dead staining) showed a cell vitality of over 95% (data not shown). As shown in Figure 3, no spontaneous differentiation was detected (see controls). However, the lipid vacuole formation in adipocytes and calcium deposits of osteoblasts continuously increased over the time of induction, as visualized by oil red O and alizarin red staining, respectively.

hMSC displayed the ability to adhere to collagen fibers of the matrices. The 3D reconstruction of the cell/collagen hybrids showed strong Cy3-labeled vimentin-positive cells attached to the collagen fibers. These cells also demonstrated a spatial orientation within the filaments (Fig. 4A–C). After adipogenic differentiation, cells displayed a relatively round morphology (Fig. 4B), while hMSC-derived osteoblasts or undifferentiated cells appeared spindle shaped (Fig. 4A and C). On the matrix surface, hMSC were visibly connected to adjacent collagen fibers by bridging the pores (Fig. 4D–F). After induction of adipogenic and osteogenic differentiation, strong lipid vacuole formation and widespread calcium deposits were observed throughout the collagen matrices, respectively. The number of hMSC-derived adipocytes or osteoblasts continuously increased over a period of 50 days, as identified by oil red O and alizarin red...
staining, respectively (Fig. 3, Fig. 4G and I). Adipocytes built large intracytoplasmic lipid vacuoles (Fig. 4G and H), without a surrounding membrane, as verifiable by ultrastructural analysis (Fig. 6B and C). Collagen scaffolds containing emerging osteoblasts appeared to perform extracellular matrix remodeling, which resulted in a contracted and curved collagen matrix (Fig. 4I).

Compared to collagen scaffolds with undifferentiated hMSC or hMSC-derived adipocytes, the collagen area was reduced to 47.35 ± 3.65% (Fig. 5). Undifferentiated hMSC or hMSC-derived adipocytes did not influence the matrix structure at any of the studied time points (Fig. 4G). Light microscopical analysis of collagen constructs containing hMSC-derived osteoblasts showed a compact mesh of thickened and strongly calcified collagen fibers, as shown by alizarin red and toluidin blue staining (Fig. 4I and J). Only collagen fibers with adherent cells were calcified, while fibers without adherent cells on the surface appeared unchanged, suggesting a strict and highly specific cell-mediated calcification (Fig. 4J). On ultrastructural analysis, hMSC-derived osteoblasts were found to attach to each other by desmosomes (Fig. 6D, right inset) and to spread between collagen fibers via filopodia-like cytoplasmic connections (Fig. 6D, open arrow). Collagen fibers in contact with osteoblasts were entirely surrounded by electron-dense hydroxyapatite deposits, demonstrating the first step of specific, cell-derived and matrix-guided bone formation (Fig. 6D, arrows). The matrices were not degraded at any time of culture, indicating that the cells did not secrete collagenases.

**DISCUSSION**

In the present study detailed analyses of the in vitro interactions between hMSC and a large-scale commercially available 3D collagen matrix were performed. This

![Figure 3](image-url)

**Figure 3.** Differentiation of hMSC into adipocytes (top) and osteoblasts (bottom) over a time scale of 50 days on collagen scaffolds. Lipid vacuole formation and calcium depositions are visualized by oil red O and alizarin red staining, respectively. During inductions, the vacuole formation and calcium accumulation increased dramatically over time, while control constructs remained unstained. Scale bar: 100 µm.
Figure 4. Morphological analyses of hMSC, 50 days after seeding on collagen scaffolds. Laser scan microscopy (A–C), scanning electron microscopy (D–F), and light microscopy (G–J) images of cell/collagen hybrids. Three-dimensional reconstruction of stacked two-channel fluorescence confocal pictures and SEM view after adipogenic (B, E) and osteogenic (C, F) differentiation compared to undifferentiated hMSC (A, D). Both undifferentiated and differentiated cells showed a close association with the collagen fibers. Cells expressed vimentin (Cy3, red channel), which resulted in a yellow overlap with the green autofluorescing collagen (A–C). Cells connected collagen fibers on the scaffold surface (D–F). After adipogenic differentiation cells exhibited a relatively flat morphology (E). hMSC-derived adipocytes displayed large intracytoplasmatic vacuoles, as detected by oil red O staining (G, open arrow) and were clearly orientated along collagen fibers (G, arrows). These constructs did not show any macroscopical signs of contraction. Large lipid vacuoles displaced the nuclei of adipocytes to the edge of the cytoplasm (I, arrows, toluidine blue staining). In contrast, hMSC-derived osteoblasts built a compact mesh of calcified collagen fibers, visible by alizarin red staining (I), which resulted in a strong contraction of the constructs. Calcified collagen fibers (J, arrows) are located between bundles of uncalcified fibers (J, open arrows). Only collagen fibers in contact with osteoblasts were surrounded by a calcified matrix, while acellular regions remained unstained (J, toluidine blue staining). Scale bars: 50 µm (A–C), 25 µm (D–F), 10 µm (G, H), 100 µm (I), 500 µm (J).
study showed that long-term cell/matrix interactions can result in an appropriate hMSC-based construct for bone and adipose tissue engineering.

Collagen is one of the most important components of the extracellular matrices in many tissues and is indispensable for bone formation. Due to the mechanical properties (e.g., elasticity) of collagen, collagen-based scaffolds can be easily modified to adapt to the shape of damaged tissues. Moreover, due to its biochemical composition, the integration in vivo is facilitated and tissue rejection is unlikely. Different collagen types with varying tertiary structures can be isolated from several animal sources and used to produce biodegradable scaffolds (15,16). For our constructs, we chose a collagen sponge of purified collagen obtained from bovine skin (Fig. 1). These industrially produced matrices can be manufactured in different sizes and shapes, but with similar composition, fiber tightness, and matrix porosity.

Many natural and synthetic biomaterials are described as being suitable for stem cell-based tissue engineering. To date, mesenchymal stem cells have been seeded on a range of 3D scaffolds for various applications; for example, nanofibrous polycaprolactone scaffolds support the differentiation into adipocytes, chondrocytes, and osteoblasts (9). Honeycomb collagen scaffolds induce rat mesenchymal stem cells to produce pseudohydroxyapatite crystals, a sign of osteogenic differentiation (3). Gel–collagen sponge composites seeded with hMSC improve tendon repair compared to nonseeded scaffolds (7). Collagen I/III scaffolds are described as antimitotic, but osteoinductive (6), while poly-lactid-co-glycolic acid (PLGA) scaffolds are described as adipogenic (12).

Previous studies have reported the differentiation of mesenchymal stem cells into osteoblasts and adipocytes after 3D in vitro culture. In the majority of cases the differentiation state was analyzed using light microscopy and histochemical staining methods or by detection of adipocyte- or osteoblast-specific transcripts. However, these methods alone cannot allow the differentiation between a nonterminally differentiated and a terminally differentiated cell, and the detection of RNA transcripts is not sufficient to determine whether or not a cell is terminally differentiated. For example, Neubauer and coworkers detected lipid vacuoles and late markers (e.g., glycerol-3-phosphate dehydrogenase) for adipogenesis after 4 weeks of culture on PLGA scaffolds (12). Weinand and coworkers recently encapsulated mesenchymal stem cells into collagen I hydrogels and performed oscil-

**Figure 5.** Quantification of collagen areas after a 50-day cultivation with unstimulated hMSC, hMSC-derived adipocytes, and hMSC-derived osteoblasts, respectively. The area of the constructs with unstimulated hMSC and hMSC-derived adipocytes was $2.011 \pm 0.02 \text{ cm}^2$, while hMSC-derived osteoblasts reduced the collagen area to $1.267 \pm 0.02 \text{ cm}^2$. All values are given as mean $\pm$ SD. Below the graph, the corresponding macroscopic view of collagen samples is mapped.
Figure 6. Transmission electron microscopy (TEM) of hMSC after long-term culture (50 days) on collagen scaffolds without differentiation stimuli (A), and after differentiation into adipocytes (B, C) and osteoblasts (D). In all samples cells were in close contact with the collagen fibers (A–D, Col). After adipogenic differentiation emerged adipocytes produced large intracytoplasmatic lipid vacuoles without a surrounding membrane (C, arrows), and showed an eccentric nucleus, both morphological characteristics of adipocytes. Human MSC-derived osteoblasts formed filopodia-like cytoplasmic extensions attaching to collagen fibers, which were covered with a homogeneous layer of hydroxyapatite crystals (D, left inset, Ha). Strikingly, deposits of hydroxyapatite crystals were found both in regions with contact to cell membranes and in regions without adjacent cells. In cases where two osteoblasts were in physical contact, desmosomes were clearly detectable (D, right inset). Col, collagen; Cyt, cytoplasm; Ncl, nucleolus; Vac, vacuole; Ha, hydroxyapatite; Des, desmosome. Scale bars: 5 μm (A, B, D), 1 μm (A inset), 0.5 μm (C, D left inset), 0.2 μm (D right inset).
lating and rotating dynamic cell cultures for 6 weeks, which led to a bone-specific gene expression (18). However, detailed ultrastructural morphological features of terminal differentiated human mesenchymal stem cells after long-term culture were not shown.

To the best of our knowledge, this study is the first work to present detailed ultrastructural analyses of hMSC-derived osteoblasts and adipocytes after long-term culture in contact with a collagen scaffold. In this study, hMSC were seeded on industrially produced collagen matrices and were viable over a period of 50 days, whereby the differentiation capacity was neither spontaneously induced nor negatively influenced by the scaffold. Furthermore, the cells firmly adhered to the collagen fibers, adopted a 3D spatial orientation following desmosomes. In the presence of specific culture media, the matrix supported both adipogenic and osteogenic differentiation of hMSC. During adipogenic differentiation of hMSC, a large number of small intracellular lipid vacuoles emerged over a 21-day culture period with dramatically increasing size over time. After 21 days, ultrastructural morphological characteristics of adipocytes (e.g., membrane-free lipid vacuoles) were clearly detectable (Fig. 6B, C). The lack of a membrane is characteristic for lipid vacuoles of terminal differentiated adipocytes. In contrast, other cell types store lipids in peroxisomes or vesicles with a surrounding membrane.

The collagen scaffolds were also clearly integrated in the process of osteogenic differentiation of hMSC on the biohybrids. A time-dependent calcium deposit formation could be observed with conventional light microscopy during long-term induction (Fig. 3). After approximately 40 days, collagen remodeling and extracellular matrix formation, including deposits of hydroxyapatite, were detected. During this process, collagen constructs dramatically changed their shape (Fig. 5). Tensions in the collagen structure twisted and contracted the constructs, transforming them into curved scaffolds with thickened edges. At the ultrastructural level, deformed constructs revealed newly formed extracellular matrix, composed of electron-dense hydroxyapatite crystals covering the entire circumference of the collagen fibers. Strikingly, this matrix remodeling was highly restricted to collagen fibers, which were in close contact with hMSC-derived osteoblasts (Fig. 6D), demonstrating that this was not an unspecific phenomenon. These findings support the theory that cell–matrix interactions can play a crucial role in tissue engineering when developing biohybrids by combining stem cells with industrially manufactured matrices. Under physiological conditions, the remodeling process is additionally influenced by resident osteoclasts (1), thus enabling (hMSC-derived) osteoblasts to build new mineralized bone matrix. Our experiments clearly demonstrate that under specific cell culture conditions and in contact with a 3D collagen matrix, hMSC-derived osteoblasts are capable of producing their own extracellular matrix for bone formation without dependence on additional cell populations.

This study demonstrates that human adult mesenchymal stem cells can terminally differentiate into adipocytes and osteoblasts after long-term in vitro culture on collagen matrices fabricated by Dr. Suwelack Skin & Health Care AG. During osteogenic induction, a cell-mediated matrix remodeling occurred, which is a necessary step towards bone formation.

Further studies will focus on the physical and chemical manipulation of the constructs and their in vivo integration and functionality. These experiments will show whether these bioengineered, stem cell-based constructs can be considered to be used for bone and adipose tissue replacement.

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REFERENCES

10. Markowicz, M.; Koellensperger, E.; Neuss, S.; Koenigschulte, S.; Bindler, C.; Pallua, N. Human bone marrow mesenchymal stem cells seeded on modified collagen im-