Research Article

The In Vitro Human Fracture Hematoma Model - A Tool for Preclinical Drug Testing

Moritz Pfeiffenberger¹², Paula Hoff¹³, Christa Thöne-Reineke⁴, Frank Buttgereit¹², Annemarie Lang¹² and Timo Gaber¹²

¹Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Department of Rheumatology and Clinical Immunology, Berlin, Germany; ²German Rheumatism Research Centre (DRFZ) Berlin, a Leibniz Institute, Berlin, Germany; ³Endokrinologikum Berlin am Gendarmenmarkt, Berlin, Germany; ⁴Institute of Animal Welfare, Animal Behavior and Laboratory Animal Science, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Abstract

The aim of the study was to establish an in vitro fracture hematoma (FH) model, which mimics the in vivo situation of the human fracture gap in order to assess drug efficacy and effectiveness for the treatment of fracture healing disorders. Therefore, human peripheral blood and mesenchymal stromal cells (MSCs) were coagulated to produce in vitro FH models, incubated in osteogenic medium under normoxia/hypoxia, and analyzed for cell composition, gene expression and cytokine/chemokine secretion. To evaluate the model, we studied the impact of dexamethasone (impairing fracture healing) and deferoxamine (promoting fracture healing). Under hypoxic conditions, MSCs represented the predominant cell population, while the frequencies of leukocytes decreased. Marker gene expression of osteogenesis, angiogenesis, inflammation, migration and hypoxic adaptation increased significantly over time and compared to normoxia while cytokine/chemokine secretion remained unchanged. Finally, dexamethasone favored the frequency of immune cells compared to MSCs, suppressed osteogenic and pro-angiogenic gene expression and enhanced the secretion of inflammatory cytokines. Conversely, deferoxamine favored the frequency of MSCs over that of immune cells and enhanced the expression of the osteogenic marker RUNX2 and markers of the hypoxic adaptation. In summary, we demonstrate that hypoxia is an important factor for in vitro modeling the initial phase of fracture healing, that both fracture-healing disrupting and promoting substances can influence the in vitro model comparable to the in vivo situation. Therefore, we conclude that our model is able to mimic in part the human FH and to reduce the number of animal experiments in early preclinical studies.

1 Introduction

Approximately 10% of fractures lead to impaired fracture healing accompanied by pain and suffering of the affected patients and tremendous socio-economic costs (Gomez-Barrena et al., 2015; Gaston and Simpson, 2007). During the process of fracturing, the bone marrow canal is opened and adjacent blood vessels rupture. The cells emerging from the bone marrow (e.g. mesenchymal stromal cells – MSCs, hematopoietic progenitor cells and premature lymphocytes) mix with peripheral blood in the fracture gap coagulate and form the fracture hematoma. Furthermore, local inflammation appears to promote the migration and recruitment of MSCs, endothelial cells, immune cells and fibroblasts (Kolar et al., 2010). Particularly MSCs are considered to play a pivotal role in an adequate healing process, since they are able to differentiate in both chondrocytes and osteoblasts/osteocytes thereby facilitating bone healing (Knight and Hankenson, 2013). The transcription factor runt-related transcription factor RUNX2 drives MSCs towards the osteogenic lineage, while its secreted downstream target phosphoprotein 1 (SPP1) is a key marker for early osteogenesis and also induced by hypoxia-inducible factor 1 (HIF1α) (Gross et al., 2005; Li et al., 2004). Furthermore, matrix metalloproteinase 2 (MMP2) and MMP9, which are fundamental for appropriate bone healing (Henle et al., 2005) are induced via the HIF-1 pathway (Luo et al., 2006; O’Toole et al., 2008).

Additionally, MSCs are capable to attract other cells towards the fracture site that are crucial for the ongoing regeneration process such as T cells, granulocytes and macrophages. In brief, particularly MSCs in a pro-inflammatory microenvironment, distinctively secrete chemokine (C-X-C motif) ligand (CXCL)9, CXCL10, macrophage inflammatory protein (MIP)-1α, MIP-1β and Rantes, thereby enhancing lymphocyte recruitment. Additionally, through the enhanced secretion of...
granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin 6 (IL-6) and IL-8 granulocytes are recruited (Le Blanc and Davies, 2015). During the initial phase of fracture healing, which can be classified chronologically within the first 1-5 days after the fracture (Annamalai et al., 2018), immune cells play a pivotal role. Their inflammatory activity can be considered as a double-edged sword for the bone healing process. Although useful in initiating the bone healing process by recruiting and licensing MSCs and endothelial cells at the fracture site via cytokine release, immune cells also prolong the healing process by perpetuating the inflammatory response (El-Jawahri et al., 2016). More in detail, granulocytes but also monocytes/macrophages and natural killer (NK) cells are involved in the clearance of debris (Baht et al., 2018; Thomas and Pulero, 2011). Furthermore, NK-cells are capable of recruiting MSCs towards the fracture site, while monocytes/macrophages are able to conduct re-vascularization. Finally, T cells are assumed to orchestrate the inflammatory process during the initial phase of fracture healing by retaining but also ceasing the inflammation (El-Jawahri et al., 2016). However, the distinct roles of immune cell populations and their temporal and spatial distribution and composition within the FH still remain unclear.

Due to the disruption of blood supply, the established fracture hematoma is characterized by the severe reduction of nutrients and oxygen (Hoff et al., 2016b). Cellular adaption towards the hypoxic microenvironment of the fracture gap is mainly driven by the HIF-1. HIF-1 regulates the switch from oxidative phosphorylation towards glycolysis and also promotes the reestablishment of vascularization (Cramer et al., 2003). Thereby, HIF-1 induces genes of the glycolytic cascade such as phosphoglycerate kinase (PGK1) and lactate dehydrogenase (LDHA) and genes encoding pro-angiogenic factors such as vascular endothelial growth factor (VEGFA) and IL8 (Liu et al., 2012; Dengler et al., 2014). The inflammatory milieu in the FH is inter alia characterized by the presence of pro-inflammatory cytokines; e.g. IL-6 and IL-8 are also abundantly secreted during the initial phase leading to immune cell activation and the recruitment of further immune cells into the fracture site via e.g. CXC-motif chemokine receptor 4 (CXCR4) (Liu et al., 2012). Especially the initial phase of fracture healing – taking place within the FH - is susceptible to fracture healing disorders, which can lead to delayed or even incomplete healing of the affected bone (Claes et al., 2012; Schindeler et al., 2008).

The experimental removal of the FH in a rat femoral fracture model leads to a prolonged healing process (Grundnes and Reikeras, 1993), while the implantation of a FH leads to an improved bone healing (Mizuno et al., 1990; Tachibana et al., 1991). Briefly, Mizuno et al. transplanted the hematoma of the rat’s femur to subperiosteal sites, observing new bone formation indicating strong osteogenic potential (Mizuno et al., 1990). Tachibana et al. used fluid material of human fracture hematomas, which induced the osteoblast proliferation in the osteoblast-like cell line MC3T3E1 (Tachibana et al., 1991).

Until date, research concerning fracture healing is most often performed using animal – particularly rodent models (mice and rats). Not only the size of the used animals differs from the human patient (Perlman, 2016), also the similarity of inflammatory process between rodents and men have been controversially discussed (Mestas and Hughes, 2004; Seok et al., 2013; Takao and Miyakawa, 2015). Apart from the advantages of mice to study certain pathways and systemic effects (e.g. easy to handle, requirement of low space, gene editing), the variety of species relevant for fracture healing in order to reduce animal numbers in research and accelerate translation.

2 Methods

2.1 Blood sampling, bone marrow-derived MSC isolation and cultivation

Blood was collected in 6 mL EDTA Vacutainers (Becton Dickinson, Franklin Lakes, USA) from healthy donors while human mesenchymal stromal cells (hMSCs) were isolated from bone marrow of patients undergoing total hip replacement (provided by the Center for Musculoskeletal Surgery, Charité-Universitätsmedizin Berlin and distributed via the “Tissue Harvesting” core facility of the BCRT). Donor information and experimental usage of donor material is given in Table 1. All procedures were approved by the Charité-Universitätsmedizin Ethics Committee and were performed according to the Helsinki Declaration (ethical approval EA1/012/13). Bone marrow was subsequently transferred to a 175 cm² cell tissue flask (Greiner Bio-one, Kremsmünster, Austria) and incubated in DMEM + GlutaMAX™ (Gibco, Carlsbad, USA) supplemented with 10% FCS (Biovest, Riverside, USA), 1% Penicillin/Streptomycin (Gibco, Carlsbad, USA) and 20% StemMACS™ MSC Expansion Media Kit XF (Miltenyi Biotech, Bergisch Gladbach, Germany) under a humidified atmosphere (37 °C, 5% CO₂, 95% room air). After 2 days of cultivation, the supernatant was discarded, and the adherent cells were washed three times using PBS. Cell culture media was changed once a week; cells with a confluence of 80-90% were passaged using Trypsin-EDTA. For all FH models, bone marrow-derived non-differentiated hMSCs in passage 3-4 were used. In order to replace FCS during MSC propagation, we used xeno-free 20% of StemMACS™ MSC Expansion Media Kit XF (Miltenyi Biotech, Bergisch Gladbach, Germany) and 80% StemMACS™ MSC Expansion Media Kit XF (Miltenyi Biotech, Bergisch Gladbach, Germany). When trying to fully replace FCS by using pooled human platelet lysate according to the protocol of Schallmoser et al. (Schallmoser and Strunk, 2009), we observed a detachment of MSCs from the plastic surface of incubation chambers and additionally the absence of the typical characterization marker CD90 as determined by flow cytometry. Thus, we had to postpone the replacement of FCS, which will be in the focus of future studies.

2.2 Differentiation and characterization of bone marrow-derived MSCs

Bone marrow-derived MSCs were plated at a density of 1x10⁶ cells per well in 96-well plates (Greiner Bio-one, Kremsmünster, Austria) and subsequently cultivated in the respective differentiation media. For adipogenic and osteogenic differentiation, MSCs were incubated under medium exchange once a week in either StemMACS™ AdipoDiff (Miltenyi Biotech, Bergisch Gladbach, Germany) or StemMACS™ OsteoDiff (Miltenyi Biotech, Bergisch Gladbach, Germany), respectively (results are
given in Fig. S1A-D). After 3 weeks, samples of MSCs were fixed in 4% paraformaldehyde for 10 min at room temperature (RT). For evaluation of adipogenic differentiation, fixed MSCs were stained with a freshly prepared 60% Red Oil O working solution solved in ddH2O (Sigma-Aldrich Chemie Gmbh, Munich, Germany) -stock solution: 0.3% Red Oil O solved in 100% isopropanol- for 15 min at room temperature and washed again with 60% isopropanol. Lipid droplets were analyzed via microscopy. For evaluation of osteogenic differentiation, fixed MSCs were stained with 0.5% Alizarin Red (Sigma Aldrich, St. Louis, USA) dissolved in H2O4 for 15 min at RT to visualize calcium deposition for microscopy (Fig. S13). Immunophenotyping of bone marrow-derived MSCs via the expression profile of typical surface markers (CD73+, CD90+, CD105+; CD34-, CD45-, CD20-, CD14-, HLA-DR-) was conducted using the MSC Phenotyping Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers’ instructions (results are given in Fig. S21). Only cell cultures which fulfilled the minimal criteria for MSCs set by the “Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy” (Dominici et al., 2006) including differentiation towards adipogenic and osteogenic lineage and expression of the respective surface marker profile were used for the experiments (Fig. S1 and S22).

2.3 Generation of 3D in vitro FH models

To generate the in vitro FH models, we firstly isolated, expanded and characterized MSCs before using them in the subsequent allogeneic combination with whole blood which was immediately processed. To this end, 2.5 x 10^6 MSCs without any pre-differentiation per well were centrifuged at 300 g for 3 min at 4 °C in a 96-well plate (U-bottom, Greiner Bio one, Kremsmünster, Austria). After discarding the supernatant, the cell pellet was resuspended in 100 µL of 10 mM CaCl2 (solved in PBS). 100 µL of allogeneic EDTA-blood was added and gently mixed by pipetting. After 30 min of incubation at 37 °C, 5% CO2, the coagulated in vitro FH models were transferred into DMEM + GlutaMAX™ supplemented with 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin (Sigma Aldrich, St. Louis, USA), 10^8 M dexamethasone (Sigma Aldrich, St. Louis, USA) and 0.002% ascorbic acid (Sigma Aldrich, St. Louis, USA), within this study further referred as osteogenic medium (OM). For the treatment studies, either 10^7 dexamethasone (DEX, impairing fracture healing) or 250 µmol deferoxamine (DFO, promoting fracture healing) was added to the medium. Afterwards, the generated in vitro FHs were incubated under either hypoxia (37 °C, 5% CO2 and 1% O2) or normoxia (37 °C, 5% CO2 and 18% O2) in a humidified atmosphere for up to 48 h. Donor information for the material used in this study is given in Tab. 1. Hypoxic conditions were achieved using an incubator (Binder, Tuttinglen, Germany) and flushed with nitrogen. Normoxic and hypoxic conditions were constantly monitored using incubators equipped with CO2-sensors and O2-sensors (Binder, Tuttinglen, Germany).

For the flow cytometry analysis, the mRNA expression analysis and the Bioplex assays in the initial experiments, the blood of the blood donors B1-B4 was mixed with either MSC1 or MSC2 (n=6). For the treatment studies with either 10^7 DEX or 250 µmol DFO blood donors B4-B6 were mixed with MSC3 or MSC4 respectively (n = 6).

Tab. 1: Blood donors and MSC donors for the generation of the in vitro fracture hematomas

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cell type</th>
<th>Age</th>
<th>Sex</th>
<th>FH model</th>
<th>Effect of hypoxia</th>
<th>Effect of DFO/DEX</th>
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<tr>
<td>B1</td>
<td>blood</td>
<td>44</td>
<td>m</td>
<td>MSC1, MSC2</td>
<td>FC, GE, CCS</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>blood</td>
<td>23</td>
<td>m</td>
<td>MSC1, MSC2</td>
<td>FC, GE, CCS</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>blood</td>
<td>27</td>
<td>m</td>
<td>MSC1, MSC2</td>
<td>FC, GE, CCS</td>
<td>-</td>
</tr>
<tr>
<td>MSC1</td>
<td>bmMSCs</td>
<td>77</td>
<td>m</td>
<td>B1, B2, B3</td>
<td>FC, GE, CCS</td>
<td>-</td>
</tr>
<tr>
<td>MSC2</td>
<td>bmMSCs</td>
<td>75</td>
<td>m</td>
<td>B1, B2, B3</td>
<td>FC, GE, CCS</td>
<td>-</td>
</tr>
<tr>
<td>B4</td>
<td>blood</td>
<td>37</td>
<td>m</td>
<td>MSC3, MSC4</td>
<td>-</td>
<td>FC, GE, CCS</td>
</tr>
<tr>
<td>B5</td>
<td>blood</td>
<td>26</td>
<td>m</td>
<td>MSC3, MSC4</td>
<td>-</td>
<td>FC, GE, CCS</td>
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<tr>
<td>B6</td>
<td>blood</td>
<td>38</td>
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<td>MSC3, MSC4</td>
<td>-</td>
<td>FC, GE, CCS</td>
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<tr>
<td>MSC3</td>
<td>bmMSCs</td>
<td>72</td>
<td>m</td>
<td>B4, B5, B6</td>
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<td>FC, GE, CCS</td>
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<tr>
<td>MSC4</td>
<td>bmMSCs</td>
<td>62</td>
<td>m</td>
<td>B4, B5, B6</td>
<td>-</td>
<td>FC, GE, CCS</td>
</tr>
</tbody>
</table>

FC: flow cytometry analysis, GE: gene expression analysis, CCS: cytokine/chemokine secretion analysis

2.4 Preparation of in vitro FH models for flow cytometry and gene expression analysis

Immediately after coagulation (0 h = control) or after cultivation for 6, 12, 24 or 48 h in OM, the in vitro FH models were washed twice in PBS. Cells were separated using a cell strainer (70 µm, Corning, New York, USA). Subsequently, erythrocyte lysis was conducted twice at 4 °C for 6 min by osmotic shock using erythrocyte lysis buffer (0.01 M KHCO3, 0.155 M NaCl, 0.1 mM EDTA, and pH 7.5). Cells were washed in 0.5% BSA in PBS (PBS/BSA).

2.5 Flow cytometry analysis

After blocking the unspecific binding of Fc-receptor a solution containing 5 mg/ml human IgG (IgG1 66.6%, IgG2 28.5%, IgG3 2.7%, IgG4 2.2%; Flebogamma, Grifols, Frankfurt, Germany), cells were washed in PBS/BSA and antibody staining was performed for 15 min on ice, using anti-human (ab) antibodies and dilutions as depicted in Tab. 2. Cells were washed (PBS/BSA) and centrifuged at 300 g for 3 min in a U-bottom 96-well-plate, supernatants were discarded, and the pellets were resuspended in 0.05% NaN3 in PBS/BSA (PBS/BSA/Azide). Shortly before analyzing, the cells were incubated with 1:25 diluted 7-AAD (BioLegend®, San Diego, USA) for 2 minutes at room temperature. Cells were assessed using a MACS Quant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany) and evaluated using FlowJo software (Tree Star, USA). The gating strategy is depicted in Fig. S31.

1 doi:10.14573/altex.1910211s
Tab. 2: Dilution for the antibodies used for the characterization of immune cells and MSCs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marker for</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
<th>Species of origin</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal anti-hCD3</td>
<td>T cells</td>
<td>Miltenyi Biotech</td>
<td>130-113-139</td>
<td>REAfinity™</td>
<td>1:100</td>
</tr>
<tr>
<td>Monoclonal anti-hCD4</td>
<td>T helper cells</td>
<td>Miltenyi Biotech</td>
<td>130-113-223</td>
<td>REAfinity™</td>
<td>1:100</td>
</tr>
<tr>
<td>Monoclonal anti-hCD8</td>
<td>Cytotoxic T cells</td>
<td>Miltenyi Biotech</td>
<td>130-110-684</td>
<td>REAfinity™</td>
<td>1:100</td>
</tr>
<tr>
<td>Monoclonal anti-hCD14</td>
<td>Monocytes</td>
<td>Miltenyi Biotech</td>
<td>130-110-521</td>
<td>REAfinity™</td>
<td>1:100</td>
</tr>
<tr>
<td>Monoclonal anti-hCD19</td>
<td>B cells</td>
<td>Miltenyi Biotech</td>
<td>130-113-649</td>
<td>REAfinity™</td>
<td>1:100</td>
</tr>
<tr>
<td>Monoclonal anti-hCD45</td>
<td>Pan-Leukocytes</td>
<td>Miltenyi Biotech</td>
<td>130-110-633</td>
<td>REAfinity™</td>
<td>1:100</td>
</tr>
<tr>
<td>Monoclonal anti-hCD73</td>
<td>MSCs</td>
<td>BioLegend®</td>
<td>3444004</td>
<td>Mouse</td>
<td>1:20</td>
</tr>
<tr>
<td>Monoclonal anti-hCD90</td>
<td>MSCs</td>
<td>BioLegend®</td>
<td>328114</td>
<td>Mouse</td>
<td>1:20</td>
</tr>
</tbody>
</table>

2.6 Gene expression analysis

Total RNA was extracted using the Arcturus™ PicoPure™ RNA Isolation Kit (Applied Biosystems, Foster City, USA), according to the manufacturer’s instructions. cDNAs were synthesized by reverse transcription using the SensiScript® Reverse Transcriptase Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. cDNAs were stored at -20 °C until further processing. Quantification of gene expression was performed by qPCR using the DyNamo Flash SYBR Green qPCR Kit (Thermo Fisher, Waltham, USA) according to the manufacturer’s instructions and assessed in the Stratagene Mx3000P (Agilent Technologies, California, USA) using the following program: initial denaturation: 7 min at 95 °C, amplification: 45 cycles with 5 s at 95 °C, 7 s at 60 °C and 9 s at 72 °C, melting curve analysis: stepwise increasing the temperature from 50 °C to 95 °C every 30 s. Data were normalized to the expression of elongation-factor 1-a (EF1A), using the ΔCt-method. Here, we used EF1A because of its stable expression in MSCs under conditions of inflammation (involving immune cell activation and morphological changes), hypoxia and different cell types (peripheral immune cells) under different drug treatments (Curits et al., 2010). Therefore, we had to exclude several typical house-keeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin (ACTB) well-known to be regulated under hypoxic conditions and after immune cell activation, respectively (Foldager et al., 2009). All primers were purchased from TIB Molbiol (Berlin, Germany) and are listed in Tab. 3.

Tab. 3: Primers used

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP1</td>
<td>Secreted phosphoprotein 1</td>
<td>GCGGAGGTGATAGTGTGTT</td>
<td>TGAGGTGATGTCCCTCGTGCTGCTG</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
<td>AGCCTTGCCTGCTGCTCTTA</td>
<td>GTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
<td>TTACTTACACCCCCGACGT</td>
<td>TATGGAGTGCCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>EF1A</td>
<td>Elongation factor 1-alpha</td>
<td>GTTGATAATGGTTCCCTGCGAAC</td>
<td>TTGCCCCGCTCCAGACGACCT</td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix metalloproteinase-2</td>
<td>GATACCCCTTGGACGTTAGGA</td>
<td>CCTTCCAAAGTGCCATAGC</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
<td>GACGCCCAAGGAAAACCTG</td>
<td>CAAACCTACAGAACAGACACAC</td>
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<tr>
<td>IL6</td>
<td>Interleukin 6</td>
<td>TACCGCCAGGAAAAGATCC</td>
<td>TTCTGCAGGTGCGCTTTC</td>
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<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase 1</td>
<td>ATGGATGAGGTGTTGAAAG</td>
<td>CAATGGCGATGCATGCTGACT</td>
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<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase 1</td>
<td>ACCCAAGTTCCACCATGATT</td>
<td>CCAAAATGCAAGGAACACT</td>
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<tr>
<td>MMP9</td>
<td>Matrix metalloproteinase-9</td>
<td>CCTGAGAGCTGAGAAACACTC</td>
<td>CACCCGCCATGACATGACGTA</td>
</tr>
<tr>
<td>HIF1A</td>
<td>Hypoxia-inducible factor 1-alpha</td>
<td>CCATTAGAAAGCAGTTCCGC</td>
<td>TGGTAGGAGATGCGAGATGC</td>
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</table>

2.7 Cytokine and chemokine quantification

Supernatants of the in vitro FHs were immediately frozen after 48 h and stored at -80 °C. The concentration [pg/mL] of cytokines and chemokines was determined using multiplex suspension assay (Bio-Rad Laboratories, München, Germany) according to the manufacturers’ description. Following cytokines and chemokines (lower detection limit) were measured: IL-1β (7.55 pg/mL), IL-2 (18.99 pg/mL), IL-4 (4.13 pg/mL), IL-5 (20.29 pg/mL), IL-6 (25.94 pg/mL), IL-7 (16.05 pg/mL), IL-8 (37.9 pg/mL), IL-10 (37.9 pg/mL), IL-12 (7.21 pg/mL), IL-17 (24.44 pg/mL), interferon-γ (IFNγ, 56.32 pg/mL), tumor necrosis factor-alpha (TNFα, 59.53 pg/mL), monocyte chemotactic protein-1 (MCP-1, 27.02 pg/mL), macrophage inflammatory protein MIP-1β (6.27 pg/mL), granulocyte colony-stimulating factor (G-CSF, 50.98 pg/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF, 11.82 pg/mL) and macrophage migration inhibitory factor (MIF, 57.78 pg/mL).

For the dexamethasone and DFO treatment experiments, we set the values of cytokines/chemokines which were not detectable in the samples to the values of the corresponding detection limit.
2.8 Statistical analysis
Statistical analysis was conducted using Graphpad Prism (Version 7, La Jolla, USA). The gene expression and Bioplex data are depicted as mean ± SEM. Flow cytometry data are depicted as median ± range. All data sets were tested for normal distribution. Since the data sets were not normally distributed, non-parametric tests were performed. Differences between the time points were compared using the Mann Whitney U test. Differences between normoxia and hypoxia or hypoxia vs. dexamethasone/DFO treatment were compared using the Wilcoxon signed rank test (*p<0.05, **p<0.01, ***p<0.005, ****p<0.0001).

3 Results
3.1 Temporal cellular composition of the in vitro FH models is dominated by long-term survival of MSCs
In order to simulate the first phase of fracture healing in vitro, we generated in vitro FH models consisting of human MSCs and peripheral blood cells. Here and in the following experiments, we only used cells meeting the minimal criteria for MSCs set by the “Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy” (Dominici et al., 2006), provided in Fig. S1 and Fig S2, which guarantees the consistent quality of the MSCs in the various tests performed. In consideration of mimicking the fracture gap microenvironment characterized by hypoxia and to evaluate its impact on temporal distribution and cell composition, we incubated the in vitro FH models for 6, 12, 24 and 48 h under hypoxic conditions (1% O₂, which is the average oxygen level measured in the incubator at 5% CO₂ and high humidity flushed with nitrogen) as compared to normoxia (18% O₂, which is the average oxygen level measured in the incubator at 5% CO₂ and high humidity flushed with room air) (Fig. 1). 18% oxygen Using flow cytometry, we observed a more severe but not significant continuous decline in the frequency of living cells after an incubation period of 48 h under hypoxia (45 ± 4%) as compared to normoxia (55 ± 3%; Fig. 1A). More in detail, frequency of innate and adaptive immune cells constantly decreased under hypoxic and normoxic conditions (Fig. 1B). Interestingly, the frequencies of cells representing the adaptive immune response, namely T helper cells (CD45+/CD3+/CD4+), cytotoxic T cells (CD45+/CD3+/CD8+), and B cells (CD45+/CD19+) significantly decreased at 48 h under hypoxia as compared to normoxic incubation. In contrast, cells from the innate compartment of immune response such as granulocytes (CD45+/SSChigh), which were the most abundant cell population until 24 h (25 ± 4%), and monocytes (CD45+/SSCintermediate/CD14high) did not differ in their frequencies with regard to oxygen availability.

Moreover, the frequency of MSCs (CD45-/CD90+/CD73+) within the in vitro FH models constantly increased over time, turning out to be the most abundant cell population with approximately 20% after 48 h of incubation. Interestingly, this effect is even more pronounced under hypoxic conditions, particularly after 48 h, where the frequency of MSCs within the in vitro FH models is significantly higher under hypoxic compared to normoxic conditions (23 ± 4% vs. 13 ± 4%). Thus, hypoxic incubation reduced the frequencies of the adaptive immune cell compartment and increased the frequencies of MSCs, the precursors of chondrocytes and osteoblast/osteocytes important for bone healing.

Fig. 1: Hypoxia favors survival of MSCs while T- and B-cell fractions significantly decreased
(A) Frequency of total cells negative for 7-AAD in the FH cultured in osteogenic differentiation medium under normoxic (NOX; 18% O₂) or hypoxic conditions (HOX; 1% O₂) for 6, 12, 24 and 48 h (median ± range, n = 6). (B) Frequency of immune cell populations (granulocytes, CD14+ monocytes, CD4+ T cells, CD8+ T cells, CD19+ B cells) and MSCs (CD73+, CD90+, CD45-, CD14-) negative for 7-AAD in the in vitro FHs cultured in osteogenic differentiation medium under normoxic or hypoxic conditions for 6, 12, 24 and 48 h (median ± range, n = 6). Statistical analysis was conducted, using the Wilcoxon signed ranked test (*p<0.05).
Fig. 2: Within the in vitro FHs, osteogenic, angiogenic, inflammatory, migration and metabolic markers were significantly upregulated after 48 h of incubation, more pronounced under hypoxia.

Depicted is the relative RNA expression of the osteogenic markers RUNX2 and SPP1 (A), the angiogenic/inflammatory markers VEGFA, IL8, IL6 (B), the migration markers CXCR4, MMP2 and MMP9 (C) and the metabolic markers HIF1A, LDHA and PGK1 within the in vitro FHs after cultivation in OM for 48 h. The expression is depicted at 0 h (white bars) and either under normoxia (NOX; light grey bars) or hypoxia (HOX; dark grey bars). All values are normalized to the “housekeeping gene” EF1A (mean ± SEM, n=12). Statistical analysis was conducted using the Mann Whitney U test comparing the values to 0 h and the Wilcoxon signed rank test comparing normoxia and hypoxia (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
3.2 RNA-expression profile revealed the upregulation of fracture healing-relevant markers in the in vitro FH models

With the intention to analyze the impact of hypoxia – most prominent at 48 h - on a selected gene expression pattern, we investigated the expression of marker genes for osteogenesis (RUNX2, SPP1), angiogenesis (VEGFA, IL6, MMP2, MMP9), inflammation (SPP1, IL6, IL8), migration (CXCR4, MMP2, MMP9) and hypoxic adaptation (HIF1A, LDHA, PGK1) of whole in vitro FH models incubated in OM under hypoxic and normoxic conditions at 48 h (Fig. 2). Although, we also investigated the gene expression for the time points 0, 6, 12 and 24 h (Fig. S4), we here focus on the effects after 48 h of incubation. Almost all marker gene expressions analyzed demonstrated a significant hypoxia-mediated induction at 48 h as compared to 0 h except IL6, HIF1A, and PGK1. Moreover, as compared to normoxic incubation only CXCR4 and IL8 were not significantly induced by hypoxic incubation at this time point.

More in detail, the osteogenic markers namely the transcription factor RUNX2 as well as SPP1 were significantly up-regulated after 48 h incubation under hypoxic conditions as compared to 0 h (pRUNX2 = 0.006, pSPP1 < 0.0001) (Fig. 2A). Only SPP1 was significantly induced after 48 h incubation under normoxic conditions (pSPP1 < 0.0001) but not RUNX2. Both osteogenic marker genes demonstrate a significant increase in gene expression under reduced oxygen availability (pRUNX2 = 0.023, pSPP1 = 0.009).

The angiogenic marker VEGFA was significantly up-regulated after 48 h incubation under hypoxia (pVEGFA = 0.0003) but not under normoxia as compared to 0 h (Fig. 2B). Furthermore, VEGFA was significantly up-regulated after 48 h as compared to incubation under normoxic conditions (pVEGFA = 0.0049). The pro-inflammatory and pro-angiogenic IL6 was significantly up-regulated under hypoxia when compared to normoxia (pIL6 = 0.0017), with no significant alterations in expression after 48 h incubation under normoxic conditions when compared to 0 h (Fig. 2B). In contrast, IL6 gene expression, which is well-known to be induced under inflammatory conditions was significantly lowered after 48 h incubation under normoxic (pIL6 = 0.0138), but remained unaltered after 48 h incubation under hypoxic conditions, resulting in a significant higher expression under hypoxia as compared to normoxic conditions after 48 h of incubation (pIL6 = 0.03; Fig. 2B).

The CXCR4 transcript encodes the receptor for stromal cell derived factor 1 (SDF-1), which is well-known to facilitate migration of a variety of cell types, including the recruitment of MSCs towards the fracture site. The expression of CXCR4 was significantly and to a similar extent induced after 48 h as compared to 0 h irrespective of oxygen availability (pSDF1a = 0.005; pHIF1a = 0.002; Fig. 2C). Moreover, gene expression of MMP2 and MMP9 - encoding for matrix metalloproteinases and important for vascularization and migration - were significantly up-regulated after 48 h as compared to 0 h under hypoxic but not under normoxic conditions (pMMP2 =0.0197, pMMP9 = 0.002; Fig. 2C). Both genes were significantly higher expressed when comparing normoxia vs. hypoxia (pMMP2 = 0.0244, pMMP9 = 0.0137).

Typical markers for cellular adaptation towards hypoxia (LDHA, PGK1, and HIF1A) were significantly higher expressed after 48 h of incubation under hypoxic when compared to normoxic conditions (pLDHA =0.008, pPGK1 = 0.031, pHIF1A = 0.027; Fig. 2D). While LDHA was also significantly up-regulated after 48 h of incubation under hypoxia but not normoxia as compared to 0 h (p = 0.0002), PGK1 demonstrated a trend for an increased expression (p = 0.086) while HIF1A showed decrease for both incubation conditions normoxia and hypoxia.

Taken together, almost all gene expressions of markers important for proper fracture healing demonstrated a significant hypoxia-mediated induction after 48 h.

Fig. 3: The secretion of angiogenic as well as pro-inflammatory cytokines/chemokines was induced

In vitro FHs were incubated under normoxia (NOX) or hypoxia (HOX) for 48 h in osteogenic medium. Depicted is the concentration (median ± range, n=6) of secreted protein [pg/mL] in the supernatant. Statistical analysis via Wilcoxon signed rank test. p values indicate statistical trends (p<0.1).
Fig. 4: Dexamethasone favored the survival of immune cells over MSCs, suppressed osteogenesis and enhanced the secretion of inflammatory cytokines.

*In vitro* FHs were incubated under hypoxia (HOX; grey bars) for 48 h in osteogenic medium with the supplementation of $10^{-7}$ M dexamethasone (HOX+Dex; dark grey bars). Depicted is the frequency of total cells and single cell populations negative for 7-AAD in the FH (A), the relative RNA-expression of relevant genes normalized to EF1A (B) and the concentration of secreted protein [pg/mL] in the supernatant (C). Depicted is the median ± range, ($n=6$) for the cell composition analysis and the mean ± SEM ($n=6$) for the RNA-expression and the secreted proteins. Statistical analysis was conducted using the Wilcoxon signed rank test (*p<0.05*).
3.3 Hypoxia did not significantly impact cytokine/chemokine levels in the supernatant of the in vitro FH models

In order to confirm the observed gene expression pattern for pro-angiogenic and pro-inflammatory markers after 48 h incubation and to analyze further secretion cytokines and chemokines, we analyzed the respective supernatants for the presence of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IFNγ, TNFa, MCP-1, MIP-1β, G-CSF, and GM-CSF (listed in section 2.7). While IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, IL-17, TNFa and IFNγ were not detectable in the supernatants of the in vitro FH models, certain levels of the pro-inflammatory IL-6, pro-inflammatory and pro-angiogenic MIF and the pro-inflammatory and pro-angiogenic IL-8 as well as the chemoattractant protein MCP-1 and the pro-inflammatory granulocyte/macrophage stimulating factors G-CSF and GM-CSF were observable (Fig. 3). The pro-inflammatory IL-6 and the pro-inflammatory/angiogenic IL-8 were detectable in the medium after 48 h incubation under both normoxia and hypoxia with only a trend to a higher level in the latter one (pₐₛ = 0.063), while MIF, MCP-1, G-CSF and GM-CSF levels were comparable irrespective of oxygen availability.

3.4 Dexamethasone favors the survival of immune cells over MSCs, suppressed osteogenesis and enhanced the secretion of inflammatory cytokines

In the interest if the established in vitro FH model is suitable to reflect the glucocorticoid-mediated disturbance of the initial healing phase, we analyzed the impact of the glucocorticoid dexamethasone (DEX) at a clinically relevant dose of 10⁻⁷ M under physiological conditions (under hypoxia and in a pro-osteogenic environment) after 48 h of incubation as compared to the untreated control. Surprisingly, DEX treatment diminished the frequency of living cells within the in vitro FH models to a lesser extent than the corresponding control (Fig. 4A). In detail, DEX significantly reduced the frequency of MSCs while increasing the proportions of all immune cell populations analyzed leading to an enrichment of the granulocyte population (25 ± 4%) within the DEX treated in vitro FH models.

Moreover, gene expression analysis demonstrated that the expression of osteogenic marker-genes (RUNX2, SPP1) was significantly diminished in the FH models after treatment with DEX as compared to the untreated control (Fig. 4B). Of note, DEX abolished osteogenic differentiation while inducing lipid droplet formation under normoxic cultivation conditions only at a high concentration of 10⁻³ M DEX but not at clinically relevant dose of 10⁻⁷ M (Fig. S7). Similarly, the gene expression of pro-inflammatory IL6 and CXCR4 was significantly reduced in the DEX treated FH models. However, DEX treatment did neither influence the expression of pro-angiogenic genes (VEGFA, IL8) nor genes involved in the adaptation towards hypoxia (PGK1, LDHA, and HIF1A). Focusing on the release of cytokines and chemokines, we detected IL-4, IL-6, IL-8, IL-17, IFNγ, TNFa, G-CSF, GM-CSF, MIF and MCP-1 in considerable amounts in the supernatant of in vitro FH models (Fig. 4C). Interestingly, all factors detected increased in an abundant in the supernatant of in vitro FH models treated with DEX but remained low in the untreated controls.

3.5 Chemical induction of hypoxia using DFO did not completely mimic hypoxic conditions observed by incubation in a hypoxia-incubator

DFO has been widely reported to increase fracture-healing properties by supporting HIF-mediated angiogenesis and osteogenesis independent of the species, model and evaluation methods (Donneys et al., 2013b,c, 2015, 2016; Drager et al., 2016, 2017; Farberg et al., 2012; Guzey et al., 2016; Matsumoto and Sato, 2015; Shen et al., 2009; Stewart et al., 2011; Yao et al., 2016; Zhang et al., 2012; Kang et al., 2016; Kusumbe et al., 2014; Li et al., 2015; Liu et al., 2014; Wang et al., 2017b). To simulate the clinical application of DFO to overcome an inadequate hypoxic response in patients that are prone to delayed healing, we treated the in vitro FH models with 250 µM DFO under normoxic condition (Fig. 5) or left them untreated (treatment under hypoxic conditions are shown in Fig. S5).

Focusing on the frequencies of all cells alive (Fig. 5A), we did not observe any differences after treatment with DFO as compared to the normoxic untreated control in vitro FH models. Interestingly, the frequency of the single cell populations displayed a significantly reduced frequency of T cells after treatment using DFO supplementation. Both, the frequency of T helper cells as well as the frequency of cytotoxic T cells significantly declined upon DFO treatment in the in vitro FH models (10 ± 3% to 7 ± 2% and 5.5 ± 1% to 4 ± 1%; p = 0.03). Conversely, the proportion of granulocytes was significantly higher in the DFO-treated group (28 ± 3%) when compared to the normoxic control group (18 ± 2%; p = 0.03). No other cell population analyzed demonstrated any considerable differences between both groups.

Focusing on gene expression pattern (Fig. 5B), we observed a higher expression of the early osteogenic transcription factor RUNX2 (p = 0.06) and the inflammatory markers IL6 (0.1) as well as IL8 (p = 0.06) after treatment with DFO as compared to the control group. Additionally, genes for the adaption towards hypoxic conditions were higher expressed in the group treated with DFO as compared to the corresponding normoxic controls (p_DFGRK = 0.04, p_DFIF1A = 0.03). Generally, the treatment with DFO did not lead to a significant higher cytokine/chemokine release as compared to normoxia (Fig. 5C).

4 Discussion

In order to provide a pre-clinical model of the initial healing phase, we established an in vitro FH model-based on human cells which can be used (i) to investigate cellular mechanisms and adaptive processes during the initial phase of fracture healing. (ii) to identify new potential therapeutic targets and (iii) to determine the efficacy and effectiveness of therapeutics for the treatment of fracture healing disorders (iv) effectively reducing the number of animal experiments.

Mimicking the in vivo situation of the fracture gap by applying a hypoxic microenvironment, we observed a predominant reduction in the frequencies of the adaptive immune cell compartment and an increase in the frequency of MSCs (Fig. 1). Of note, the trauma-induced rupture of blood vessels opens bone channel and forms a hematoma of clotted bone marrow/blood in majority consisting of immune cells, their precursors and endothelial and stromal cells which initiates the healing cascade of bone regeneration by further recruitment of immune cells and MSCs (Knight and Hankenson, 2013). While
Fig. 5: DFO significantly increased the frequency of granulocytes, decreased the frequency of T cells and enhanced the expression of hypoxia-related and inflammatory markers.

In vitro FH models were incubated under normoxia or normoxia with the supplementation of 250 µM DFO (dark grey bars) for 48 h in osteogenic medium with. Depicted is the frequency of total cells and single cell populations negative for 7-AAD in the FH (A), the relative RNA-expression of relevant genes normalized to EF1A (B) and the concentration of secreted protein [pg/mL] in the supernatant (C). Depicted is the median ± range, (n = 6) for the cell composition analysis and the mean ± SEM (n=6) for the RNA-expression and the secreted proteins. Statistical analysis was conducted using the Wilcoxon signed rank test (*p<0.05).
the importance of MSCs as precursors of chondrocytes and bone cells (osteoblasts and osteocytes) (Knight and Hankenson, 2013; Kolar et al., 2011b; Hoff et al., 2016a) and as terminators of the inflammatory phase resembling the in vivo situation (Kolar et al., 2011b) is undisputed, the role of immune cells such as granulocytes, T and B cells remains controversial (Kovtun et al., 2016; Grogaard et al., 1990; El Khassawna et al., 2017; Konnecke et al., 2014; Reinke et al., 2013; Toben et al., 2011). Generally, cells owing lots of mitochondria often have an intrinsic need to be sufficiently supplied by oxygen and barely able to cope with hypoxic conditions (e.g. neuronal cells), the surviving capacity and activation status of immune cells (T cells, B cells, monocytes, neutrophils) is enhanced under hypoxic condition mainly due to the activation/stabilization of the HIF-signaling pathway in an oxygen-restricted environment (Krzywinska and Stockmann, 2018). In terms of MSCs, hypoxia seems to favor MSC survival and their differentiation towards chondrogenic and osteogenic lineage once differentiation is induced while adipogenesis is reduced (Wagegg et al., 2012; Lee et al., 2016). However, whether inflammatory cells and MSCs differ in their tolerance of oxygen is still a matter of research.

Using RAG1-deficient mice, which lack an adaptive immune system, Toben et al. (2011) demonstrated an enhanced fracture healing due to accelerated endochondral ossification and a shift from pro-inflammatory to anti-inflammatory cytokines (Toben et al., 2011). Moreover, depletion of only CD8+ T cells also enhanced/accelerated fracture healing in a mouse osteotomy model indicating a possible contribution of adaptive immune cells to a delayed or disturbed fracture healing (Reinke et al., 2013; Toben et al., 2011). Nevertheless, T and B cells evidently contribute to a higher bone quality in the later stages of fracture healing by facilitating the collagen organization process (El Khassawna et al., 2017; Konnecke et al., 2014). However, granulocytes, which are responsible for the removal of dead cells and cell debris in the very initial phase after trauma (Soehnlein et al., 2009), and which are the most abundant cell fraction in the early fracture hematoma (Kovtun et al., 2016), provide an “emergency extracellular matrix” for infiltrating stromal cells (Bastian et al., 2016) thereby essentially contributing to proper fracture healing (Kovtun et al., 2016). Although, Grogaard et al. (1990) did not observe any impact of neutrophilia on callus formation in rats (Grogaard et al., 1990), Kovtun et al. (2016) observed diminished bone content, impaired mechanical properties and bone healing after 21 days in fractured mice using an anti-Ly-6G-antibody to reduce neutrophil numbers (Kovtun et al., 2016). Using patient-derived isolated ex vivo fracture hematomas we previously reported a decrease of granulocytes already after 24 h of incubation, which was even more pronounced under hypoxia (Hoff et al., 2013).

In our FH model, we observed an increase in the frequency of MSCs, key players in the initial phase of fracture healing, which generate the precursors of chondrocytes and osteoblasts/osteocytes being essential for proper bone healing (Knight and Hankenson, 2013; Kolar et al., 2011b; Hoff et al., 2016a) and are capable of terminating the inflammatory phase (Kolar et al., 2011b) by inhibition of immune cell proliferation (Le Blanc et al., 2003; Madrigal et al., 2014; Gieseke et al., 2007; Bocelli-Tyndall et al., 2007; Potian et al., 2003; Kovach et al., 2015), thereby modulating the immune response and conveying immune tolerance (Nauta and Fibbe, 2007).

The shift of the cellular distribution to a pattern dominated by MSCs also influences the gene expression pattern in our FH model demonstrating a significant hypoxia-mediated induction after 48 h especially for hypoxia-response and osteogenic marker gene expression analyzed (Fig. 2), while not significantly altering but enhancing inflammatory cytokine and chemokine levels in the supernatant (Fig. 3). Both, an induction of hypoxia-response and osteogenic marker gene expression as well as no further induction of inflammatory cytokine and chemokine levels, have been reported to be important for proper fracture healing (Hoff et al., 2011).

Focusing on gene expression pattern and using hypoxic incubation conditions, we could successfully mimic the situation in the fracture gap by confirming the findings observed in hematomas derived from 40 patients obtained between 48 and 72 h after surgery as summarized in Table 4 (Kolar et al., 2011a). Additionally, within this study we confirmed that hypoxia promotes osteogenesis of MSCs as reported previously (Haque et al., 2013; Wagegg et al., 2012; Lennon et al., 2001). Moreover, we observed secretion of pro-angiogenic (IL-8) and pro-inflammatory cytokines/chemokines (IL-6, G-CSF, GM-CSF, and MCP) after incubation for 48 h under both normoxia and hypoxia (Fig. 3) confirming the findings of our previous study using hematomas derived from 40 patients (Hoff et al., 2016a); factors well-known to be responsible for the recruitment and activation of leukocytes in an inflammatory milieu. Unexpectedly, we could not observe a certain amount of the early inflammatory markers TNFα and IL-1β. However, Granero-Molto et al. also reported a decreased secretion of TNFα and IL-1β after transplanting MSCs into the fracture gap using a stabilized tibia fracture mouse model (Granero-Molto et al., 2009).

Although our FH model has some limitations with regard to the completeness of cell types involved, we could demonstrate a decrease in the frequency of lymphocytes and an increase in the frequency of MSCs after 48 h of incubation under hypoxic conditions, suggesting the capacity of MSCs to restrict the initial inflammatory phase of fracture healing while initiating osteochondral differentiation.

For technical reasons, we combined peripheral blood and MSCs from different donors (allogenic combination) based on the consideration that MSCs are immune-privileged, immune evasive (Ankrun et al., 2014) and/or in some conditions immunosuppressive (e.g. in case of T cell proliferation). However, the activation of immune cells via allogenic MSCs is still controversially discussed as previously reviewed in detail (Hare et al., 2012; Rozier et al., 2018a; Zhang et al., 2015). Here, we observed that T cells in combination with allogenic MSCs remained quiescent with regard to the expression of the early activation marker CD69 and late activation marker CD25 (Fig S6). Nevertheless, we here like to mention that the observed restriction of inflammation may underline the well-known therapeutic benefit of allogenic MSC transplants without any obvious disadvantages as compared to autologous MSC transplantation (Rozier et al., 2018b).

Finally, we characterized and stratified the established in vitro FH model to ex vivo data from primary human fracture hematomas obtained between 48 and 72 h after trauma and found profound similarities as demonstrated by flow cytometry, gene expression and cytokine secretion (Tab. 4 and 5).
We and others have also reported that DEX has been shown to even reverse the anti-proliferation and immunosuppressive effects of hypoxia (Chen et al., 2014). Moreover, DEX belongs to a wide class of glucocorticoids which are master regulators of the adaptation towards a hypoxic microenvironment in a wide variety of cell types (Semenza, 1992; Gaber et al., 2005).

In order to further validate the applicability of the in vitro FH model for drug testing, we treated the in vitro FHs with either DEX (impairs fracture healing) or DFO (supports fracture healing), in order to mimic either impaired or supported bone healing processes during the initial phase of fracture healing (Fig. 4 and 5). DEX belongs to wide class of glucocorticoids which significantly improve the quality of life of many patients suffering from diseases caused by a dysregulated immune system based on their strong immunosuppressive, anti-inflammatory and anti-allergic effects on immune cells, tissues and organs (Strehl et al., 2019). However, glucocorticoids are also known to influence bone metabolism by inhibiting bone formation, enhancing bone resorption and impairing adequate bone healing (Canalis, 2003; Frenkel et al., 2015; Sato et al., 1986; Sawin et al., 2001; Waters et al., 2000).

Here, we incubated the in vitro FH models with a therapeutic dose of $10^{-7}$ M DEX for 48 h under pathophysiologic hypoxia. Upon treatment with DEX, we observed a significantly lower frequency of MSCs and significantly higher frequencies of immune cells, suppressed osteogenic gene expression (RUNX2, SPP1) and an enhanced secretion of inflammatory cytokines. In vivo, DEX is known to induce apoptosis particularly in T cells and suppresses T cell activation (Xing et al., 2015), during co-incubation of MSCs and immune cells (e.g. PBMCs) DEX has been reported to enhance immune cell proliferation and reversed the immunosuppressive effect of MSCs (Chen et al., 2014; Buron et al., 2009). In the latter of that findings, DEX has been shown to even reverse the anti-inflammatory effect of transplanted MSCs as shown by Chen et al. in a mouse model of liver cirrhosis (Chen et al., 2014). Furthermore, in vitro osteoblastic differentiation is delayed by the treatment with DEX (Canalis, 1999) as indicated also in the in vitro FH model by a reduced osteoinductive capacity (RUNX2, SPP1). We and other have also reported that DEX suppresses the hypoxia-induced HIF-target gene expression (Wu et al., 2014; Gaber et al., 2011).

### Tab. 4: Gene expression data from our in vitro FH model (n=12, 48 h incubation under hypoxia) and from an ex vivo study using primary human fracture hematomas obtained between 48 and 72 h after trauma (n=40) (Kolar et al., 2011a)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Importance in the fracture healing process</th>
<th>In vitro FH model (hypoxia for 48 h)</th>
<th>ex vivo primary human FHs (&lt; 72 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX2</td>
<td>key regulator that directs mesenchymal stromal cells towards the osteoblastic lineage (Vimalraj et al., 2015; Komori, 2010)</td>
<td>↑***</td>
<td>↑</td>
</tr>
<tr>
<td>SPP1</td>
<td>coding gene for osteopontin (OPN) is a differentiation marker for osteoblastic cells induced by hypoxia (Denhardt and Noda, 1998; Sila-Asna et al., 2007; Gross et al., 2005),</td>
<td>↑****</td>
<td>↑*</td>
</tr>
<tr>
<td>VEGFA</td>
<td>most important pro-angiogenic factor (Schipani et al., 2009; Martin et al., 2009; Beamer et al., 2010) essential for the reestablishment of oxygen supply and promotes osteogenesis (Hoff et al., 2016a; Grosso et al., 2017),</td>
<td>↑***</td>
<td>↑</td>
</tr>
<tr>
<td>IL8</td>
<td>responsible for the activation and differentiation of leukocytes in an inflammatory environment (Herman et al., 2008),</td>
<td>↑**</td>
<td>↑***</td>
</tr>
<tr>
<td>IL6</td>
<td>key cytokines in the initial phase of fracture healing (Cassuto et al., 2018),</td>
<td>↑</td>
<td>↑***</td>
</tr>
<tr>
<td>CXCR4</td>
<td>responsible for the homing of MSCs, promotes bone repair (Liu et al., 2013; Yellowley, 2013) and reflects the migratory capacity of immune and stem cells (Campbell et al., 2003; Kunkel and Butcher, 2002),</td>
<td>↑**</td>
<td>↑</td>
</tr>
<tr>
<td>LDHA</td>
<td>(hypoxia-mediated) marker of induced glycolysis, contributes to acidic pH, HIF-target gene (Semenza, 1998; Gaber et al., 2005),</td>
<td>↑***</td>
<td>↑**</td>
</tr>
<tr>
<td>PGK1</td>
<td>(hypoxia-mediated) marker of induced glycolysis (Semenza, 1998; Gaber et al., 2005),</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>HIF1A</td>
<td>master regulator of the adaptation towards a hypoxic microenvironment in a large quantity of different cell types (Semenza, 1998; Gaber et al., 2005) regulated on protein-, but not on mRNA-level (Semenza, 1998; Gaber et al., 2005),</td>
<td>↑</td>
<td>n.a.</td>
</tr>
<tr>
<td>MMP2</td>
<td>remodeling of extracellular matrix, crucial for the survival of bone cells and vasculo genesis / angiogenesis and fracture healing (Stamenkovic, 2003; Paiva and Granjeiro, 2017; Varghese, 2006; Cui et al., 2017; Liel et al., 2011),</td>
<td>↑**</td>
<td>n.a.</td>
</tr>
<tr>
<td>MMP9</td>
<td>remodeling of extracellular matrix, crucial for the survival of bone cells and vasculo genesis/angiogenesis and fracture healing (Stamenkovic, 2003; Paiva and Granjeiro, 2017; Varghese, 2006; Cui et al., 2017; Colnot et al., 2005),</td>
<td>↑***</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

**up- † or down- † regulation of the genes. *p<0.05, **p<0.01, ***p<0.001, n.a. = not analyzed

### Tab. 5: Cell frequencies from our in vitro FH model (n=6, 48 h incubation under hypoxia) and from an ex vivo study using primary human fracture hematomas obtained between 48 and 72 h after trauma (n=40) (Kolar et al., 2011a).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CD surface marker</th>
<th>In vitro FH model (hypoxia for 48 h)</th>
<th>ex vivo primary human FHs (&lt; 72 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>CD45+CD3+</td>
<td>3.7% (2.4-10.6%)</td>
<td>8.2% (1.1-39.4%)</td>
</tr>
<tr>
<td>T helper</td>
<td>CD45+CD3+CD4+</td>
<td>1.9% (1.1-6%)</td>
<td>3.1% (1.4-15.4%)</td>
</tr>
<tr>
<td>Cytotoxic T cells</td>
<td>CD45+CD3+CD8+</td>
<td>1.3% (0.9-2.5%)</td>
<td>1.9% (0.2-12.6%)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Scatter and CD45+CD14+</td>
<td>0.6% (0.3-1.7%)</td>
<td>4.9% (0.1-38.5%)</td>
</tr>
<tr>
<td>B cells</td>
<td>CD45+CD19+</td>
<td>0.26% (0.2-0.4%)</td>
<td>0.9% (0.1-5.8%)</td>
</tr>
<tr>
<td>MSCs</td>
<td>CD45-CD73+CD90+</td>
<td>23.4% (16.6-24.5%)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>Scatter and CD45+ vs. CD16+</td>
<td>16.1% (5.6-24.7%)</td>
<td>64.2% (0.8-94%)</td>
</tr>
<tr>
<td>Not assigned cells</td>
<td>n. a.</td>
<td>37.1% (27.6-45.8%)</td>
<td>12.2% (1.2-85.6%)</td>
</tr>
</tbody>
</table>

Frequency of cells (min-max in %). Granulocytes in vitro gated via CD45+/ FSC-, SSC-gate, ex vivo via CD16, n.a. = not analyzed

Interestingly, in an equine in vitro FH model we found similar trends on behalf of the cell composition, RNA-expression pattern and the effects of hypoxia after 48 h of incubation (Pfeiffenberger et al., 2019). In order to further validate the applicability of the in vitro FH model for drug testing, we treated the in vitro FHs with either DEX (impairs fracture healing) or DFO (supports fracture healing), in order to mimic either impaired or supported bone healing processes during the initial phase of fracture healing (Fig. 4 and 5).
et al., 2011) which could be confirmed in the in vitro FH model by a reduced gene expression of VEGFA, LDHA, and CXCR4. Although, DEX is well-known to suppress inflammation (Strehl et al., 2019), we observed an enhanced secretion of inflammatory cytokines which may lead to delayed or disturbed bone healing as suggested from ex vivo patient derived data (Hoff et al., 2011). Indeed, Liu et al. (2018) demonstrated that glucocorticoids delayed fracture healing and impaired bone biomechanical properties in mice (Liu et al., 2018). Taken together, the effects of DEX within our model may resemble the processes of delayed or impaired fracture healing.

In contrast to DEX, DFO has been reported to support angiogenesis (Farberg et al., 2014), to enhance the vascular response to fractures (Donneys et al., 2012) and to augment the restoration and mineralization of the callus (Donneys et al., 2013a), making it an attractive off-label therapeutic target with regard to fracture healing. Additionally, DFO stabilizes HIF-1α by suppressing the oxygen-sensitive proxil hydroxylases (PHDs), which are responsible for the tagging of HIF-1α for proteasomal degradation, thereby mimicking hypoxic conditions and markedly improving osteogenesis (Qu et al., 2008) and bone regeneration (Wan et al., 2008).

To simulate the clinical application of DFO to overcome an inadequate hypoxic response in patients that are prone to delayed healing as demonstrated previously (Kolar et al., 2011b), we treated the in vitro FH models with 250 µM DFO under normoxic (Fig. 5) or left them untreated (treatment under hypoxic conditions are shown in Fig. S5). Here, we used DFO as a hypoxia mimicking agent to overcome a delay or failure of the cells to adapt to hypoxic conditions which we simulated using normoxic incubation conditions. As a result, we observed in our established in vitro FH model that the frequency of T helper cells as well as of cytotoxic T cells (Fig. 5) is diminished when treated with DFO compared to normoxia, while the frequency of granulocytes is significantly higher. This finding can be explained by an anti-proliferative effect of DFO on activated T lymphocytes, however, with barely any effect on granulocytes as demonstrated by Hileti et al. (1995). Similarly, DFO has been demonstrated to diminish proliferation and survival of MSCs (Zeng et al., 2011; Wang et al., 2017a), which may explain the decline in the frequency of MSCs as compared to normoxia in our model. However, as expected DFO-treatment as well as hypoxia up-regulated HIF1A, PGK1 and LDHA as compared to their corresponding controls while only RUNX2 which is a very early marker for osteogenic processes but not SPP1 the downstream marker of osteogenesis is induced by DFO-treatment. With regard to the secretion of cytokines, IL-6 and IL-8 are both similarly secreted, whereas DFO-treatment resulted in scarcely any enhanced secretion of G-CSF, GM-CSF, MCP-1 and MIP-1β compared to normoxia and therefore does not contribute to an enhanced secretion of IL-6 as observed for DEX treatment. Although, the treatment with DFO could not thoroughly reflect the situation obtained by the incubation under hypoxic conditions DFO enhanced the expression of hypoxia-adaptation relevant genes and pro-osteogenic factors (RUNX2), thus promoting the cellular adaptation to hypoxic conditions and the pro-osteogenic phenotype of MSCs such as found in the fracture gap.

Although we were able to mimic key features of the initial phase of fracture healing in vitro, the model still has room for improvement. The in vitro FH model presented here is under development and still requires optimization and qualification. More in detail, to optimize the model we will have to overcome technical challenges that urged us to use an allogenic approach. The optimal model should be derived from autologous and xeno-free material (autologous serum, MSCs and peripheral blood). Furthermore, we will have to include further cell types present in the bone marrow and in the fracture gap including hematopoietic stem cells and cells of their subsequent progenitor lineages, endothelial progenitor cells and perivascular cells (Braham et al., 2019). Finally, we have to implement the model into a perfusion system to provide nutrients diffusion and waste removal which we plan to achieve by using a bioreactor platform. This will provide us then with the opportunity to extend the life-time of our model in order to study later phases of bone regeneration.

Prospectively, the established and optimized model will provide the opportunity to (1) study cellular and humoral processes of bone regeneration, (2) investigate the underlying mechanisms of how hypoxic conditions modulate cell survival, proliferation, communication and differentiation during the initial phase of fracture healing, (3) screen for new potential therapeutics and their efficacy to support fracture healing and to treat fracture healing disorders and (4) determine side effects of pharmacological substances.

5 Conclusion

In the study presented here, we developed an in vitro human-based FH model using human MSCs and human peripheral blood as a tool for preclinical drug testing. We in depth characterized key mechanisms important for proper fracture healing. We demonstrated that hypoxia preferred the survival of MSCs to immune cells. Fracture-healing relevant genes/factors were considerably upregulated after 48 h of incubation, most often significantly enhanced by hypoxia. Additionally, cytokines/chemokines that are crucial during the initial phase of fracture healing were secreted. These findings resemble previous results from our group from an ex vivo study using patient-derived fracture hematomas. We could highlight significant similarities to human in vitro and ex vivo and animal-based in vivo data. To proof the suitability of our 3D in vitro FH model for drug testing, we treated the developed system with DEX and DFO thereby confirming the responsiveness to commonly used drugs and newly developed therapeutics. In summary, we were able to show that both fracture-healing disrupting and fracture-healing promoting substances can influence the in vitro FH model in a similar way as it was observed in the in vivo situation. Therefore, we conclude from our data, that our model is able to correctly mimic human fracture hematoma and reduce the number of animal experiments in early preclinical studies.

References


Yao, Q., Liu, Y., Tao, J. et al. (2016). Hypoxia-mimicking nanofibrous scaffolds promote endogenous bone regeneration. ACS Appl Mater Interfaces 8, 32450-32459. doi:10.1021/acsami.6b10538


Competing financial interest statement
The authors declare that they have no conflict of interest.

Authors’ contributions
Study design: MP, AL, TG; Data collection and analysis: MP, AL, TG; Drafting manuscript: MP, AL, TG; Data discussion and interpretation: MP, PH, CTR, FB, AL, TG; Revising manuscript: MP, PH, CTR, FB, AL, TG.

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