Aus der Klinik mit Schwerpunkt
Rheumatologie und Klinische Immunologie
der Medizinischen Fakultät Charité - Universitätsmedizin Berlin

eingereicht über das
Institut für Tierschutz, Tierverhalten und Versuchstierkunde
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Mimicking the initial phase of fracture healing *in vitro*

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
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Moritz Pfeiffenberger
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“Where the flowers they bloom, well it’s there I’ll be found” – Flogging Molly

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Abbreviations

µCT	Micro-computed tomography
ALP	Alkaline phosphatase
AOVET	Veterinary specialty group of the AO Foundation
ARI	AO Research Institute
BMP	Bone morphogenetic protein
BTE	Bone tissue engineering
CD	Cluster of Differentiation
COL1	Collagen 1
COX-2	Cyclooxygenase 2
CTSK	Cathepsin K
CXCL	Chemokine (C-X-C motif) ligand
CXCR4	CXC-motif chemokine receptor 4
DEX	Dexamethasone
DFO	Deferoxamine
DLX5	Distal less homeobox protein X5
ECM	Extracellular matrix
EU	European Union
FCS	Fetal calf serum
FH	Fracture hematoma
FGF	Fibroblast growth factor
FGM	Fracture gap model
GM-CSF	Granulocyte macrophage colony-stimulating factor
HA	Hydroxyapatite
HIF	Hypoxia-inducible factor
HOX	Hypoxia
HRE	Hypoxia-responsive elements
HSCs	Hematopoietic stem cells
HUVECs	Human umbilical vein endothelial cells
IL	Interleukin
LDHA	Lactate dehydrogenase A
MIP	Macrophage inflammatory protein
MIF	Macrophage migration inhibitory factor

MMP	Matrix metalloprotease
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stromal cells
NK cells	Natural killer cells
NOX	Normoxia
NSAIDs	Non-steroidal anti-inflammatory drugs
OC	Osteocalcin
OECD	Organization for Economic Co-operation and Development
OPN	Osteopontin
PCL	Poly(ε-caprolactone)
PDE	Partial differential equation
PET	Positron emission tomography
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PHD	Prolylhydroxyloase
PLGA	Poly(lactid-co-glycolid)
pVHL	Von-Hippel-Lindau-Tumorsuppressor
PGK1	Phosphoglycerate kinase
PPARγ	Peroxisome proliferator activated receptor gamma
RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
REACH	Registration, Evaluation, Authorization of Chemicals
RUNX2	Runt-related transcription factor 2
SFBC	Scaffold-free bone-like construct
SLC2A	Glucose transporter
SOX9	SRY-box transcription factor 9
SPECT	Single photon emission computed tomography
SPI1	Spi-1 Proto-Oncogene
SPP1	Secreted phosphoprotein 1
TCP	Tricalcium phosphate
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
VEGF	Vascular endothelial growth factor

1 Introduction

Fractures are one of the most frequent traumatic injuries worldwide (Mathew and Hanson 2009) and related therapies are often accompanied by incommodities and pain for the patients. Approximately 10% of fractures are accompanied by fracture healing disorders (Tzioupis and Giannoudis 2007), resulting in pain, decreased mobility and a significant loss of quality of life. Moreover, their treatment leads to a financial burden on society. Certain risk groups, such as elder people, people suffering from osteoporosis or significant malnutrition, post-menopausal women as well as patients with a disrupted blood supply, are particularly prone to developing fracture healing disorders (Giannotti, Bottai et al. 2013). The initial phase of fracture healing is most susceptible to healing disorders. Right after fracture, the bone is shattered, blood vessels are ruptured, and the bone marrow channel is opened. Leaking cells from the bone marrow, such as mesenchymal stromal cells (MSCs), progenitor cells and immune cells, are mixed with immune cells from the peripheral blood and coagulate in the fracture gap to form the so called fracture hematoma (FH) (Kolar, Schmidt-Bleek et al. 2010). FH formation is the starting point for several processes that are essential to appropriate fracture healing, including endochondral ossification, osteogenesis and angiogenesis. Furthermore, it acts as a temporary scaffold for the effective invasion of supplementary immune cells and MSCs (Claes, Recknagel et al. 2012). It is mainly characterized by a hypoxic microenvironment, inflammatory processes and the activation and migration of immune cells and MSCs (Kolar, Schmidt-Bleek et al. 2010). To date, most fracture healing research has been conducted with small animal models, using mice or rats (Histing, Garcia et al. 2011), which may bear significant translational limitations regarding their utility to humans. More recent approaches focus on enhancing transferability of experimental findings to human patients, by closely linking human cell systems to human diseases and disease pathways. Hence, preclinical *ex vivo* and *in vitro* models, whether in 2D or 3D, using human material are of increasing importance. In order to generate an alternative model, with the ultimate objective of creating a platform to test new fracture-healing-relevant therapeutics, the overall goal of this thesis was to establish a 3D *in vitro* model, capable of mimicking the initial phase of fracture healing. The final model consists of two main components, the bone component (mesenchymal condensation of MSCs) and the immune component (coagulated mixture of peripheral blood and MSCs), which were ultimately combined to obtain a fracture gap model. Since fractures are also of great relevance to equine patients, often ending fatal or in subsequent euthanasia (Riggs 2002), and research on the initial phase of fracture healing in horses is sparse, the FH model was also set up in an equine FH model, providing the opportunity to contextualize both the human and equine model with existing literature.

2 Literature review

2.1 Bone physiology

The main physiological functions of bone within the musculoskeletal system are i) structural support of the body, ii) locomotion support due to its mechanical nature, iii) protection of inner organs from infringement, iv) allocation of a suitable environment for hematopoietic cell development and v) provision of a permanent reservoir for phosphate and calcium within the body (Grabowski 2009). Generally, four categories of bones exist (long bones, short bones, flat bones and irregular bones). Long bones, such as the humerus, femur and tibia, are formed by endochondral and membranous bone formation, while flat bones, like the skull, sternum and ribs are formed by membranous bone formation. Finally, short bones incorporate the tarsal and carpal bones, whereas the sacrum is an example of an irregular bone (Clarke 2008). Healthy bone is dynamically remodeled by the permanent degradation of old, fatigued bone and formation of new bone (Rucci 2008).

Typically, bone consists of an inorganic and an organic part. The inorganic part mainly consists of extracellular matrix (ECM), predominantly hydroxyapatite (HA, composed of calcium and phosphate), while the organic matrix consists of collagens, most of which is type 1 collagen (COL1). HA crystals provide mechanical stiffness as well as load-bearing assets, whereas the organic matrix is capable of providing flexibility and elasticity to bone (Scheinpflug, Pfeiffenberger et al. 2018). Due to the manifold functional demands on bones, the matrix composition of bone consists of an outer shell of cortical bone characterized by a compact, dense and ordered structure, and cancellous, trabecular bone in the inner cavity. Hereby, the cortical bone provides stiffness and resistance to rotation forces and bending, while cancellous bone supports mechanical elasticity. Generally, long bones are composed of the diaphysis, a hollow shaft with a medullar cavity containing the bone marrow, a cone-shaped, flat metaphysis below and a rounded epiphysis above the growth plate. The diaphysis is mainly comprised of cortical bone, whereas the epiphysis and metaphysis are primarily composed of trabecular bone, embedded in a thin layer of cortical bone (Clarke 2008).

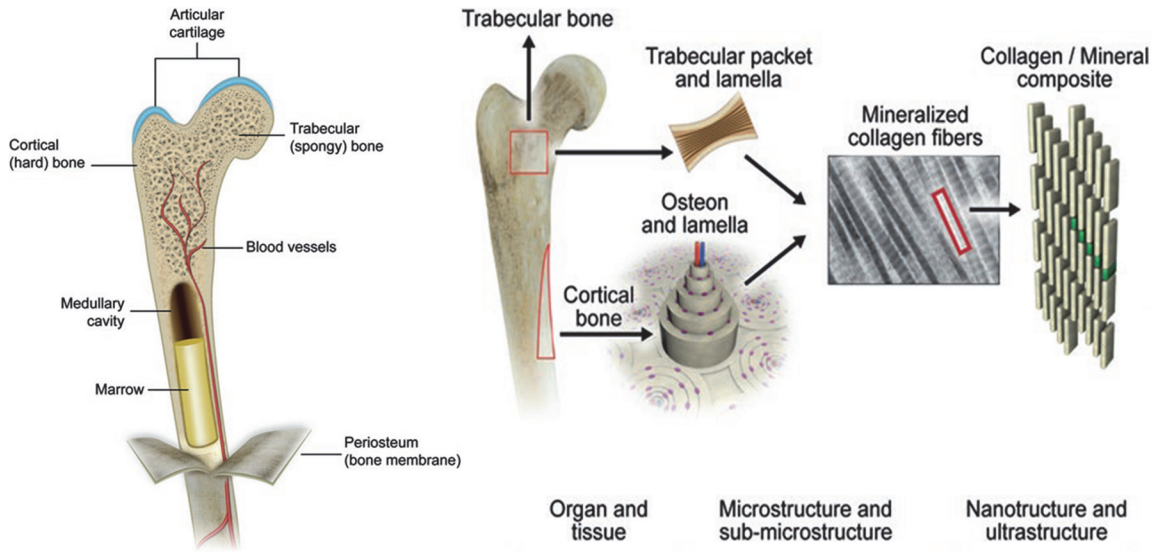

Figure 1: *Hierarchical organization of bone. Macroscopically, long bones are composed of compact cortical bone, spongy trabecular bone and the medullary cavity containing the bone marrow, enclosed by the highly vascularized and innervated periosteum. Microscopically, cortical bone is characterized by the functional main unit, the osteon, consisting of a central canal with a blood vessel and nerves surrounded by concentric lamellae. Trabecular bone consists of lamellar structures parallel to the trabecular surface. Cortical and trabecular bone are composed of collagen fibers with respective intermittent minerals. Modified from Gasser and Kneissel 2017 (Gasser and Kneissel 2017)*

In detail, cortical bone contains the osteon as the basic functional unit of the cortex. The osteon is comprised of an inner channel (Haversian canal) carrying a blood vessel, which supplies the bone with oxygen and nutrients, nerves and lymphatics, surrounded by concentrically arranged lamellae, consisting of multiple layers of osteoblasts and osteocytes. Cortical bone is externally covered by the periosteum, while the inner surface is covered by the endosteum, which serves as a functional bridging area between cancellous and cortical bone. Trabeculae are the main habitual feature of cancellous bone, consisting of lamellae placed parallel to the trabecular surface (Fig. 1). Cancellous bone cavities contain bone marrow, where the hematopoietic stem cell (HSC)-niche is habituated. This niche gives rise to immune and blood cells (Grabowski 2009, Gasser and Kneissel 2017, Scheinpflug, Pfeiffenberger et al. 2018) and is the primary functional site of HSC maintenance and hematopoiesis (Morrison and Scadden 2014). It is noteworthy that osteo-progenitor cells such as MSCs and osteoblasts are also part of the HSC niche and considered to provide the basis for its physical structures (Shiozawa, Havens et al. 2008).

Osteoblasts, osteocytes and osteoclasts are the most prominent cells in mature bone (Mohamed 2008). Osteoblasts, building the soft, not yet mineralized matrix (osteoid), are capable of assembling bone tissue and establishing the basis for new bone material by secreting COL1, calcium phosphates and calcium carbonates into the interstitial space. Additionally, COL1 and osteopontin (OPN), which play decisive roles in the calcification process during bone development and bone regeneration, are secreted from osteoblastic cells. Fur-

thermore, osteoblasts, which are part of the non-collagen bone matrix, are susceptible to secreting osteoblast-specific proteins that are substantial markers for ossification processes, like osteocalcin (OC) and alkaline phosphatase (ALP) (Einhorn 1998). Osteoblast-derived osteocytes are unable to divide, show a typical star-like morphology and are important for the maintenance of the bone matrix and calcium homeostasis (Cullinane 2002). Furthermore, osteocytes coordinate the skeletal response to mechanical loading by sensing mechanical strain, thereby orchestrating the formation and resorption of bone (Bonewald and Johnson 2008).

In contrast to osteoblasts and osteocytes that are derived from the mesenchymal lineage, osteoclasts are derived from the hematopoietic stem cell-lineage, though belonging to the monocyte-macrophage lineage. Osteoclastogenesis is mainly triggered by the induction of the transcription factor PU.1 (gene: *SPI1*), which enhances the differentiation from osteoclast-progenitor cells (derived from macrophages) to osteoclasts (Crotti, Sharma et al. 2008). Additionally, the receptor activator of NF-κB (RANK) / receptor activator of NF-κB ligand (RANKL) system plays an important role in the mediation of osteoclastogenesis. RANKL is expressed on the surface of osteoblasts and interacts with RANK, which is expressed by pre-osteoclasts (Mohamed 2008). The interplay of osteoblasts and osteoclastic precursors through the RANK/RANKL system induces the enhanced expression of osteoclast genes, which leads to the maturation of osteoclasts and to the enhanced secretion of typical osteoclastic marker proteins such as Cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP) (Wilson, Peters et al. 2009). Therefore the cell-cell interaction between osteoblasts and osteoclast precursors is crucial for the process of osteoclastogenesis (Rucci 2008). Osteoclasts are capable of resorbing bone and therefore crucial for bone remodeling. CTSK, a catalytic enzyme expressed predominantly in osteoclasts, functions as a protease and is involved in the degradation of the protein matrix during bone resorption, though being jointly involved in bone remodeling (Bossard, Tomaszek et al. 1996). TRAP, a glycosylated monomeric metalloprotein enzyme secreted by active osteoclasts, is a basic marker for activated osteoclasts and plays a critical role in bone remodeling, since it degrades skeletal proteins such as osteopontin (Habermann, Eberhardt et al. 2007, Hayman 2008).

Bone development can be divided into two different mechanisms: intramembranous and endochondral ossification (Shapiro 2008). In intramembranous ossification, bone is developed directly from initially primitive mesenchymal tissue without cartilage involvement. In contrast, endochondral ossification is characterized by the replacement of hypertrophic chondrocytes, derived from MSCs, with bone via mesenchymal condensation processes, which is followed by a process of controlled bone growth and remodeling processes, while forming and shaping the skeleton (Olsen, Reginato et al. 2000, Mackie, Ahmed et al. 2008, Teti 2011). Several

transcription factors are involved in mediating the endochondral ossification process. Thus, the *SRY-box transcription factor* 9 (SOX9) plays the most important role in impelling MSC towards the chondrogenic lineage (Hardingham, Oldershaw et al. 2006, Hino, Saito et al. 2014); *runt-related transcription factor 2* (RUNX2) is responsible for osteoblast development (Bruderer, Richards et al. 2014), while *peroxisome proliferator activated receptor gamma* (PPARγ), more accurately PPARγ2, thrives MSCs into the adipogenic lineage and inhibits osteogenesis (Aprile, Ambrosio et al. 2014). *Distal less homeobox protein X5* (Dlx5), a protein of the homeobox family and activator of *RUNX2,* functions as a mediator in the development of osteoblasts from osteo-progenitor cells (Samee, Geoffroy et al. 2008, Heo, Lee et al. 2017).

2.2 Fracture healing

Bone is one of the few tissues in the human body that can heal without forming a visible, fibrous scar (Marsell and Einhorn 2011). The process of bone regeneration after fracture is thought to postnatally recapitulate ontological events taking place during the embryological development of the skeleton. Therefore, many genes are highly expressed in embryonic stem cells as well as cells of the fractured callus and skeletal repair tissues (Morgan, De Giacomo et al. 2014). Hence, these recapitulation processes are considered to facilitate the pre-injury structure of bone as well as their biomechanical function in the body. Thus the well-orchestrated interplay between different cell types (endothelial, immune and skeletal cells) as well as the temporal and spatial distribution of these cells are crucial for the complete restoration of traumatized bones (Morgan, De Giacomo et al. 2014).

2.2.1 Consecutive phases of fracture healing

Fracture healing can be roughly divided into four different phases. The inflammatory phase comes first and includes the typical formation of a fracture hematoma, which occurs right after fracture and lasts 1-5 days. It is followed by the second phase, which consists of fibrocartilaginous callus formation and is subsequently followed by the third phase, where the fibrocartilaginous callus is substituted by a bony callus. Finally, the fourth and last phase follows, where the bone is fully remodeled and compact bone is added (Tian, Tang et al. 2019), Fig. 2.

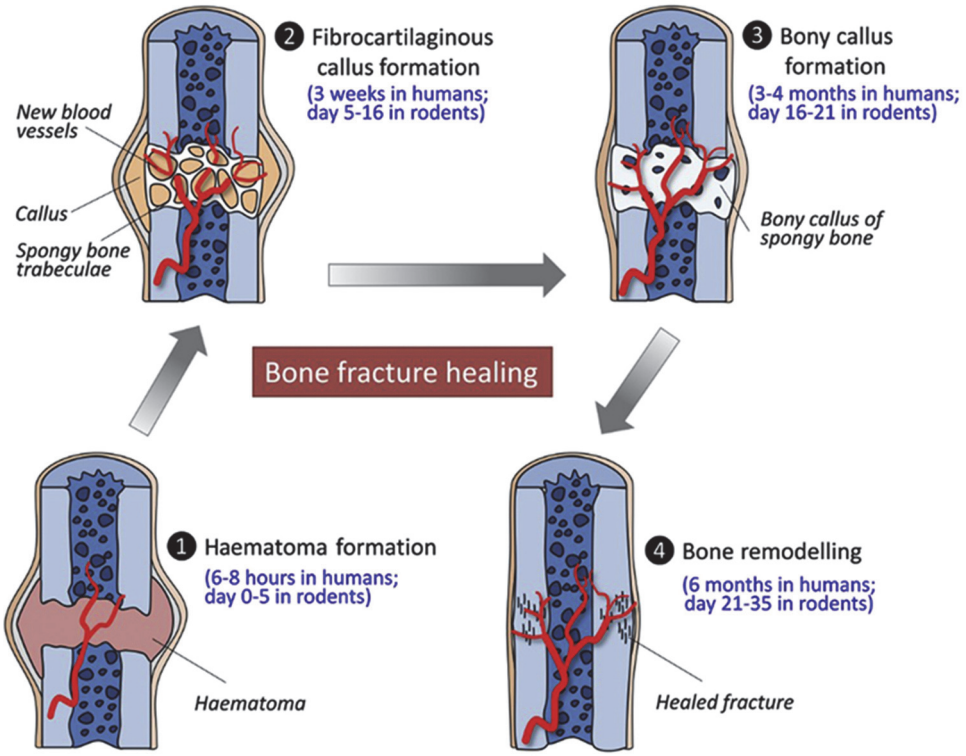

Figure 2: *Schematic description of the four phases of fracture healing. After fracture, fracture hematoma formation occurs during the first phase, followed by the formation of a fibrocartilaginous and later bony callus, until bone remodeling is completed in the last phase. Modified from Tian et al. 2018 (Tian, Tang et al. 2019).*

As a consequence of the trauma that fractured the bone, the bone marrow channel and blood vessels that supply both bone and periosteum with nutrients and oxygen are ruptured. Cells leaking from the bone marrow (e.g. MSCs and immune cells) are mixed in the fracture gap with blood cells (e.g. lymphocytes, granulocytes, monocytes, macrophages and erythrocytes) and subsequently coagulate, thus forming the fracture hematoma (Kolar, Gaber et al. 2011, Sheen and Garla 2020). In the second phase, primarily caused by the release of pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8), angiogenic processes are triggered. Furthermore, fibrin-rich granulation tissue is formed within the fracture hematoma and more MSCs are recruited towards the fracture site, where they differentiate into chondroblasts. These chondroblasts start chondrogenesis by building a fibrocartilaginous network, thereby spanning the bony ends of the fracture. Afterwards, osteoprogenitor cells produce an initial layer of woven bone (Fazzalari 2011, Sheen and Garla 2020). In contrast to lamellar bone, woven bone is initially formed under conditions where rapid deposition of minerals is needed in order to respond to fractures or distracted osteogenesis. This is characterized by an arbitrary organization of collagen fibers, high cellularity and low mineral density (McKenzie and Silva 2011). Additionally, newly formed vessels integrate the fracture site, supporting the regenerative process by supplying oxygen and nutrients. These first two phases are considered to be in great parts anabolic/regenerative by

means of building new tissue to restore the bone after fracture (Einhorn and Gerstenfeld 2015), Fig. 3.

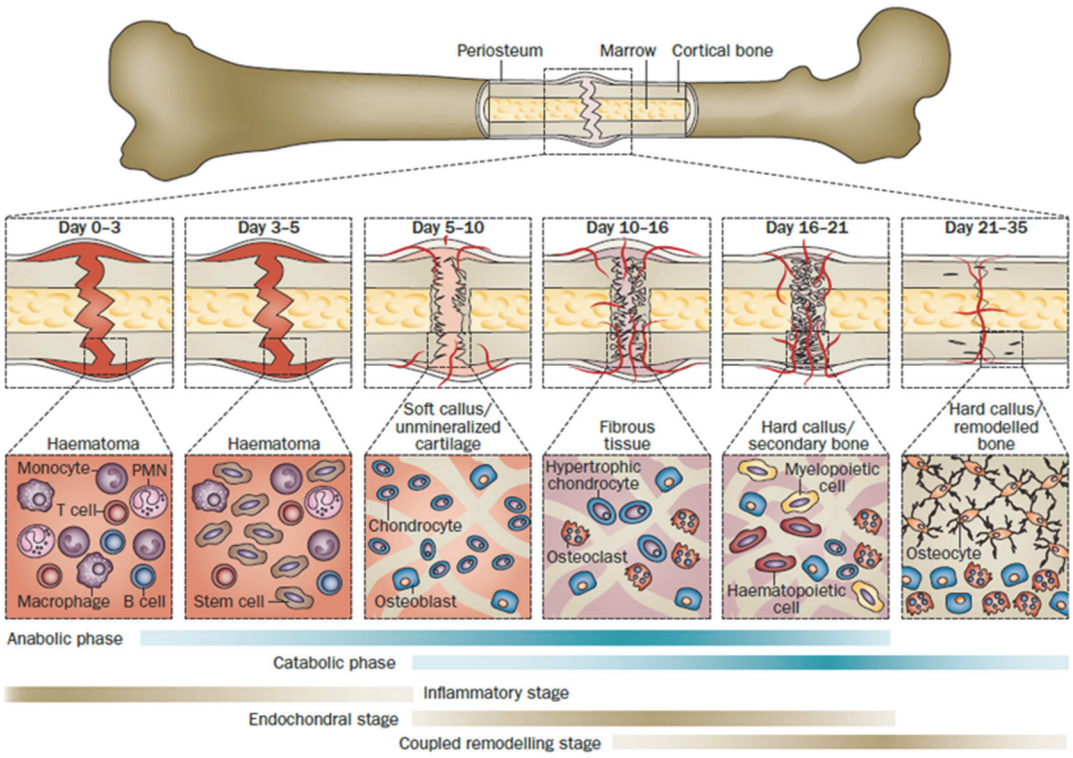

Figure 3: *Schematic description of the catabolic and anabolic phases during the process of fracture healing. While the first phases are mainly characterized as anabolic stages, building up new tissue, particularly the phase of remodeling is also characterized by catabolic processes due to the osteoclastic resorption of bone. Additionally, the main cell populations involved during the different phases are depicted (Einhorn and Gerstenfeld 2015).*

In the subsequent phase, the cartilaginous soft callus is resorbed and calcified. Osteoblasts derived from recruited MSCs deposit calcium and the soft and later hard callus is formed through endochondral ossification. Furthermore, blood vessel formation and sprouting continuously occur, which allows further migration of MSCs towards the immature callus area. Enhanced expression of RANKL on the surface of osteoblasts, osteocytes and progenitor cells induces osteoclastogenesis in RANK-expressing cells from the monocyte-macrophage lineage (Boyce and Xing 2007, Xiong and O'Brien 2012), inducing the transition towards the last phase - the remodeling, where anabolic and catabolic processes are in equilibrium (Sheen and Garla 2020). In this phase, osteoblasts and osteoclasts invade and the hard callus undergoes repeated remodeling. In a highly orchestrated manner, osteoclasts resorb and osteoblasts form new bone, thereby completely replacing the center region of the corticalis with compact bone, while the callus ends are replaced with lamellar bone. Accompanying vasculature is also remodeled, ultimately leading to the complete regeneration of the functional bone structure (Fazzalari 2011, Einhorn and Gerstenfeld 2015, Ghiasi, Chen et al. 2017, Sheen and Garla 2020).

2.2.2 Key events in the initial phase of fracture healing

Immediately after trauma, the bone marrow channel is shattered and attached blood vessels are ruptured. The most characteristic features of the initial phase of fracture healing are

- the formation of the fracture hematoma,
- a hypoxic microenvironment,
- a restricted nutrient supply and
- a local inflammation process,

resulting in cell activation and migration. The duration of the initial phase in humans ranges between 1 and 5 days (Annamalai, Turner et al. 2018).

➢ **Formation of the fracture hematoma**

Leaking cells from the bone marrow channel, such as premature immune cells, hematopoietic precursor cells, endothelial cells, pericytes, HSCs and MSCs, are released in the fracture gap and mixed with immune cells (lymphocytes, granulocytes, monocytes and macrophages) and erythrocytes from the ruptured blood vessels. The mixture immediately coagulates and forms the so called FH, which is essential for the appropriate healing cascade following the fracture (Kolar, Schmidt-Bleek et al. 2010). Thus, cell populations detected in the fracture hematoma include immune cells, hematopoietic stem cells (Schmidt-Bleek, Schell et al. 2009, Kolar, Gaber et al. 2011, Hoff, Gaber et al. 2016) as well as progenitor cells with multilineage capacity, such as MSCs (Oe, Miwa et al. 2007). Notably, the fundamental role of the FH was revealed in several studies, which are explained in the following. Experimental removal of the FH leads to an elongated healing process (Grundnes and Reikeras 1993), while the implantation of a FH leads to an improved bone healing (Mizuno, Mineo et al. 1990, Tachibana, Matsubara et al. 1991). The formation of the FH is the starting point for several processes essential for appropriate fracture healing, including angiogenesis, endochondral ossification and osteogenesis, and acts as a temporary scaffold for the effective invasion of supplementary immune cells (Claes, Recknagel et al. 2012).

➢ **Hypoxic microenvironment**

The cellular adaptation toward hypoxic conditions in the body is essential to reduce tissue injury in response to ischemia (Bernhardt, Warnecke et al. 2007), to be neuroprotective with regard to brain injuries (Sharp, Bergeron et al. 2001, Sirén, Fratelli et al. 2001), protective for the myocardium (Jurgensen, Rosenberger et al. 2004), nephroprotective (Rosenberger, Griethe et al. 2003) and important for fracture healing (Steinbrech, Mehrara et al. 1999, Kolar, Gaber et al. 2011).

Due to the rupture of blood vessels, nutrient and oxygen supply of the fracture site is severely limited. Facing limited oxygen concentrations and the demand of oxygen for cellular activity, cells adapt towards the hypoxic microenvironment through radical changes in their metabolism, driven by the hypoxia inducible factors (HIFs), mainly HIF1. HIF1 is a heterodimeric protein consisting of two subunits, namely HIF1α and HIF1β. HIF1β is constitutively expressed, whereas HIF1α is highly sensitive to oxygen. Although HIF1α is constitutively transcribed and translated under normoxic conditions and hydroxylated by oxygen-sensitive prolyl hydroxylase domain proteins (PHD), ubiquitinated by von Hippel-Lindau tumor suppressor protein (pVHL), it is rapidly degraded through proteasomes. When challenged with hypoxic conditions, HIF1α is stabilized via post-transnational modifications such as hydroxylation, acetylation or phosphorylation, while PHD and pVHL are suppressed (Masoud and Li 2015). As a consequence, HIF1α is translocated to the nucleus, dimerizes with HIF1β, co-activated by the transcriptional coactivator CBP and binds to the regulatory units (hypoxia-response elements, HRE), thereby inducing the expression of HIF target genes (Gaber, Dziurla et al. 2005, Ziello, Jovin et al. 2007).

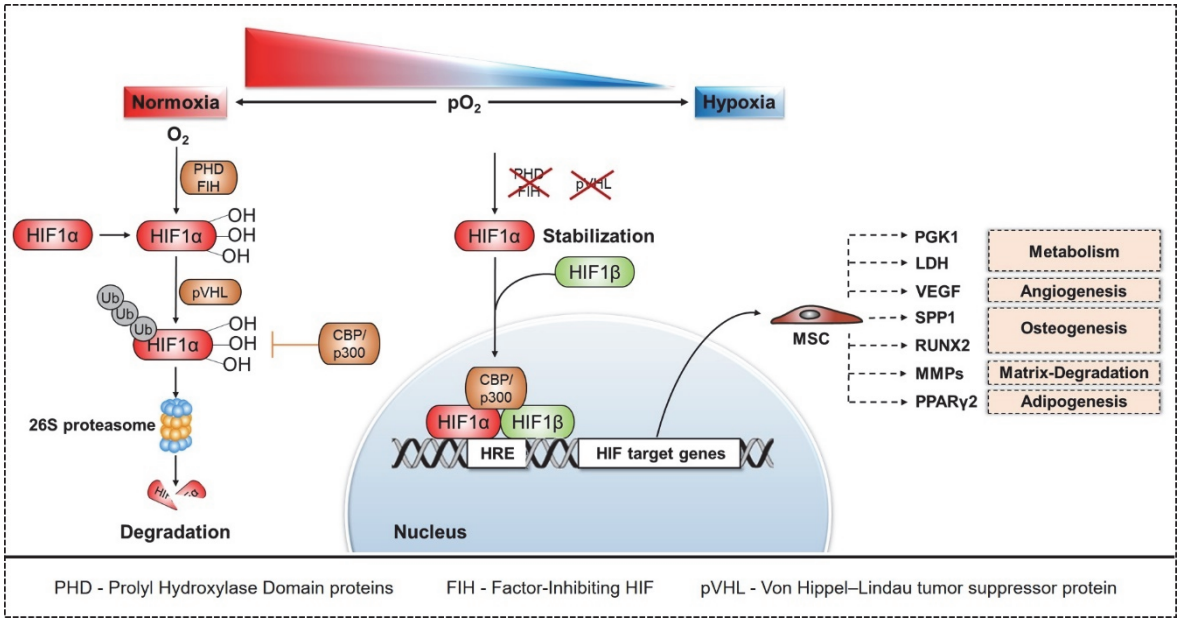

Figure 4: *Schematic description of the HIF-pathway under either normoxic or hypoxic conditions. Under normoxia, HIF1α is hydroxylated through PHD and ubiquitinated by pVHL, and is thereby labeled for constant degradation by proteasomes. Under hypoxic conditions the oxygen-sensitive PHD is suppressed. Thus HIF1α is stabilized, translocated to the nucleus, dimerizes with HIF1β and subsequently binds to the HRE, which allows the transcription of HIF target genes downstream.*

Through this mechanism, the transcription factor *HIF1*α synchronizes the switch from oxidative phosphorylation towards O_2-independent glycolysis. In the following, HIF1α additionally induces the enhanced expression of genes coding for enzymes of the glycolytic cascade, such as *lactate dehydrogenase A* (LDHA), *phosphoglycerate kinase* (*PGK1*), glucose transporter genes (*SLC2A*) and *6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3* (coding

gene: *PFKFB3*) to metabolically adapt to hypoxia, and genes encoding pro-angiogenic factors like *IL8* and *VEGF* to ensure re-vascularization (Liu, Shen et al. 2012, Dengler, Galbraith et al. 2014). These adaptive processes play a pivotal role in the cellular adaptation, the survival under hypoxia and the following process of angiogenesis. In terms of osteogenic processes, HIF1α is additionally involved in the enhanced expression and secretion of *phosphoprotein 1* (*SPP1,* gene coding for OPN), a key marker for early osteogenesis (Li, Oparil et al. 2004, Gross, King et al. 2005). Under hypoxic conditions the surviving capacity and activation status of immune cells (T cells, B cells, monocytes, neutrophils) is mainly enhanced due to the activation/stabilization of the HIF1α-signaling pathway in an oxygen-restricted microenvironment (Krzywinska and Stockmann 2018). Regarding MSCs, hypoxia seems to favor MSC survival and their differentiation towards chondrogenic and osteogenic lineage once differentiation is induced, while adipogenesis is reduced (Wagegg, Gaber et al. 2012, Lee, Byeon et al. 2016). Furthermore, the HIF1 pathway induces the enhanced expression of *matrix metalloproteinase 9* (MMP9) and MMP2 (Luo, He et al. 2006, O'Toole, van Koningsveld et al. 2008), both fundamental proteins for appropriate bone healing (Henle, Zimmermann et al. 2005).

- **Inflammation and cell activation**

Interestingly, an evident intensity of inflammation is essential to a proper fracture healing process. Moreover, the treatment with anti-inflammatory drugs such as cyclooxygenase-2 (COX-2) inhibitors, are known to impair fracture healing (Cottrell and O'Connor 2010). However, since non-steroidal anti-inflammatory drugs (NSAIDs) are very effective at alleviating pain, they are commonly used after surgery. Waeli et al. showed that the treatment with NSAIDs is very effective during the active phase of the circadian rhythm and causes severe bone healing disorders when applied during the resting phase (Al-Waeli, Nicolau et al. 2020) The inflammatory milieu in the FH is characterized by the enhanced release of pro-inflammatory cytokines such as IL-6 and IL-8 (Kidd, Stephens et al. 2010, Hoff, Gaber et al. 2017). Through this release and the enhanced secretion of granulocyte macrophage colony-stimulating factor (GM-CSF), granulocytes are recruited into the fracture site. Additionally, further lymphocytes are attracted via apparent secretion of chemokine (C-X-C motif) ligand (CXCL)9, CXCL10, macrophage inflammatory protein (MIP)-1α, MIP-1β and Rantes by MSCs, among others (Le Blanc and Davies 2015). Furthermore, additional lymphocytes and MSCs are activated and migrate towards the fracture site through the axis of CXC-motif chemokine receptor 4 (CXCR4) and CXCL12 (Liu, Shen et al. 2012). Immune cells, in particular, play an essential role during the initial phase of fracture healing. The inflammatory potential of immune cells can be best described as a double-edged sword for the fracture healing process. They are useful in initiating the bone healing process by recruiting and li-

censing MSCs and endothelial cells at the fracture site via cytokine release and prolonging the healing process by propagating the inflammatory response (El-Jawhari, Jones et al. 2016). In detail, granulocytes as well as monocytes/macrophages and natural killer (NK) cells are involved in the elimination of cell debris (Thomas and Puleo 2011, Baht, Vi et al. 2018). Moreover, NK-cells in particular are capable of recruiting MSCs for the fracture site, while monocytes/macrophages are capable of initiating re-vascularization processes. Furthermore, T cells are expected to orchestrate the inflammatory process during the initial phase of fracture healing by preserving as well as ceasing the inflammation (El-Jawhari, Jones et al. 2016). Finally, our group observed both the migration of monocytes and HSCs as well as the maturation of granulocytes within the FH. Additionally, we could show that T helper as well as cytotoxic T cells proliferate in and migrate towards the fracture site, thereby retaining their capacity to survive, mature, migrate and remain functional even under bioenergetically restricted conditions predominant in the FH (Hoff, Gaber et al. 2016). However, the distinct roles of immune cell populations and their temporal and spatial distribution and composition within the FH remain unclear.

2.2.3 Fracture epidemiology and fracture healing disorders

Fractures are one of the most common traumatic injuries worldwide (Mathew and Hanson 2009). An exact estimation of worldwide cases is almost impossible, mainly due to the huge inter-country differences of reported or treated fractures. Thus, concrete, country- or region-based data within the framework of existing risk groups (e.g. age, osteoporosis) are summarized in Table 1 (Foundation 2019).

Table 1: *Estimated number of fractures (in thousands) in men and women over 50 years in 2000. Modified from (Foundation 2019).*

Region	Hip	Spine	Forearm	Humerus	Other
Africa	8	12	16	6	33
Americas	311	214	248	111	521
Southeast Asia	221	253	306	121	660
Europe	620	490	574	250	119
Eastern Mediterranean	35	43	52	21	109
Western Pacific	432	405	464	197	1039
Total	**1627**	**1416**	**1660**	**706**	**3550**

Generally, the incidence of fractures in western industrial countries is increasing (Amin, Achenbach et al. 2014, Hemmann, Ziegler et al. 2020). In an ageing society, the trend is expected to remain ascending in the future. This correlates with increased morbidity and an upcoming immense economic burden for society (Giannotti, Bottai et al. 2013). Therapeutic interventions after fracture range from conservative procedures like plaster casts to cost-intensive and incisive interventions like hip replacements. These therapies on their own are often accompanied by incommodities and pain for the patients. In addition, fracture healing disorders occur in approximately 10% of fractures (Tzioupis and Giannoudis 2007), resulting in pain, decreased mobility and significant loss of quality of life, while the treatment leads to an intensified financial burden on society. Certain risk groups, such as elderly people, people suffering from osteoporosis or significant malnutrition, post-menopausal women and patients with disrupted blood supply, are prone to developing fracture healing disorders (Giannotti, Bottai et al. 2013). It is worthy of note that the size of the fracture gap is an essential factor for the development of fracture healing disorders, since larger gaps, multi-traumatic fractures and splinter fractures are more likely to show delayed healing or fail to heal (Jahagirdar and Scammell 2009, Giannotti, Bottai et al. 2013). Furthermore, patients suffering from diabetes are known to be prone to fracture healing disorders, due to an enhanced expression of inflammatory cytokines, particularly in chondrocytes (Alblowi, Tian et al. 2013), and the pronounced secretion of tumor necrosis factor α (TNFα) (Alblowi, Kayal et al. 2009). The initial phase is particularly susceptible to fracture healing disorders, since the microenvironment is restrictive during this phase (Schindeler, McDonald et al. 2008, Claes, Recknagel et al. 2012). Fracture healing disorders are commonly subdivided into delayed unions and atrophic or hypertrophic non-unions. While the fracture passes through the normal stages of fracture healing (clinically and radiologically) during delayed unions, albeit slower than expected, non-unions show no progression towards a union and the callus ends remain visible within the first 6 months after fracture (Freigang, Gschrei et al. 2019). However, the timeframe of delayed healing and the concept itself remain controversial (Marsh 1998, Bhandari, Fong et al. 2012). Hypertrophic non-unions, often caused by inadequate stability of the fracture, are characterized by viable yet sclerotic bone ends and a fracture gap mainly filled with cartilaginous or fibrous tissue, while non-unions typically show no signs of attempted healing processes (Jahagirdar and Scammell 2009). These severe cases need rigid fixation, oftentimes combined with supplementary bone grafting and other additional treatment, such as the supply of bone morphogenetic proteins (BMPs), in order to fully restore the functional callus properties (Jahagirdar and Scammell 2009, Giannotti, Bottai et al. 2013). However, the use of BMPs is controversial. Undoubtedly, the efficacy of BMPs as potent stimulators of bone healing has been verified in various models and clinical trials. Nevertheless, their potential in a clinical setting still lacks proof (Lissenberg-Thunnissen, de Gorter et al. 2011). Additionally,

a broad range of side effects, ultimately even life-threatening ones, have been unraveled in recent years (James, LaChaud et al. 2016). The gold standard for the treatment of severe fractures is an autogenous bone graft derived from the iliac crest, as it exhibits the best osteogenic potential for fracture healing (Baldwin, Li et al. 2019, Sohn and Oh 2019). However, this technique entails causing a new bone defect, which can lead to complications of its own, especially in older patients. Furthermore, therapeutic interventions with the focus on cell-based strategies are providing very encouraging results. The transplantation of MSCs has shown enhanced healing properties in cardiomyopathy (Hare, Fishman et al. 2012) and kidney transplantations (Pool, Leuvenink et al. 2019). Moreover, the transplantation of MSCs (Noel, Djouad et al. 2002, Granero-Molto, Weis et al. 2009, Cohn Yakubovich, Sheyn et al. 2017) or pooled cells from bone marrow (Jäger, Hernigou et al. 2010) show promising results regarding appropriate fracture healing. Nevertheless, processes involved in the initial phase of fracture healing as well as the treatment of fractures and fracture healing disorders have to be evaluated, analyzed and improved in further studies.

2.2.4 Status quo on preclinical models to study fracture healing

Various research approaches have aimed to gain new insights into fracture healing processes and address the clinical need for new therapeutic strategies to fight fracture healing disorders. Nowadays, most of the research regarding fracture healing is conducted on animal models. In recent years, there has been a shift from large animal models and rats towards mouse models (Histing, Garcia et al. 2011). However, preclinical *ex vivo* and *in vitro* models using human material, both 2D and 3D, are of increasing importance. They result in enhanced transferability of experimental findings to human patients, by closely linking human cell systems to human diseases and disease pathways. Since inter-species differences in the composition, density, and quality of bone exist (Aerssens, Boonen et al. 1998), the model selection must be done with regard to the adequate scientific question, in order to obtain results that can be interpolated to the human clinical situation. However, no animal species satisfies all requirements of an ideal model (Pearce, Richards et al. 2007).

- **Small animal models**

Due to the considerable advantages, which include low costs, easy and safe handling, small size, therefore space-saving housing, profound genetic and pathophysiological similarities to humans, short breeding cycles and the availability of genetically modified strains, a large number of small animal models has been established and is preferably used to study the mechanisms of bone healing (Elefteriou and Yang 2011, Histing, Garcia et al. 2011, Haffner-Luntzer, Kovtun et al. 2016). As reviewed by Brommage and Ohlsson, 249 mouse models capable of adequately mimicking human skeletal disorders in mice have been identified

(Brommage and Ohlsson 2020). While most fracture-related research, until the 2000s, was done in rats, most notably because of the easier application of certain fixation techniques, most of the research in recent years was performed in mouse models. This change is a consequence of the development of mice-specific antibodies as well transgenic or knockout mouse strains, which enabled researchers to study molecular patterns in detail (Elefteriou and Yang 2011, Histing, Garcia et al. 2011, Haffner-Luntzer, Kovtun et al. 2016). Particularly monoclonal antibodies that allow the detection of a large quantity of antigens and therefore target individual molecules involved in fracture, provide a sophisticated analytic tool for *in vivo* imaging approaches (Haffner-Luntzer, Kovtun et al. 2016). Another huge milestone was the complete sequencing of the mouse genome in 2002, which provided a key tool for analysis and interpretation of the contents of and correlations with the human genome as well as an experimental device for state-of-the-art biomedical research (Chinwalla, Cook et al. 2002). Furthermore, long-established *in vivo* imaging approaches like radiography and micro-computed tomography (µCT), together with recently developed sophisticated methods such as high resolution magnetic resonance imaging (MRI) (Haffner-Luntzer, Müller-Graf et al. 2017), single photon emission computed tomography (SPECT) and positron emission tomography (PET) (Fragogeorgi, Rouchota et al. 2019) allow specialized analyses in mouse models. To a great extent, research is conducted using fracture models of the tibia (Hiltunen, Vuorio et al. 1993) or femur (Histing, Garcia et al. 2010), since these long bones are relatively easy to access and proportionally big. Most modern surgical and fixation techniques are based on the studies of Bonnarens and Einhorn, who fractured the diaphysis of the rats´ femur with a blunt guillotine and fixed the femur with an intramedullary pin (Bonnarens and Einhorn 1984). Following this, most studies introduced fractures in the diaphyseal part of the bone (Adili, Bhandari et al. 2002, Garcia, Speidel et al. 2011), mostly owed to the fact that the stabilization is easier than in the epiphysis (Histing, Garcia et al. 2011). However, fractures in the epiphyseal part have also been studied (Kolios, Hoerster et al. 2010). Generally, fracture models in small animals can be divided into two different groups. In the first approach (closed model), the fracture is set using a 3-point fracture device and fixation with an intramedullary pin (Manigrasso and O'Connor 2004, Claes, Maurer-Klein et al. 2006). In the second approach (open model), the bone is osteotomized, most commonly using a Gigli wire, and stabilized with an external fixator (Cheung, Kaluarachi et al. 2003, Connolly, Li et al. 2003, Histing, Garcia et al. 2011). Within recent years, several new implants for the stabilization of the fracture have been developed and established (Histing, Garcia et al. 2011). These new devices include modified intramedullary pins (Krischak, Augat et al. 2007), locking nails (Holstein, Menger et al. 2007), pin-clip devices (Matthys and Perren 2009), interlocking nails (Garcia, Herwerth et al. 2011) and plates (Histing, Garcia et al. 2010).

- **Large animal model**

In the very early years of fracture research, a broad variety of large animal models, predominantly using rabbits, dogs and sheep, were established (Histing, Garcia et al. 2011). In addition, a few studies were performed on pigs, goats, cows and horses (Reinwald and Burr 2008, Decker, Reifenrath et al. 2014). These species were selected due to the comparability of their bone healing process, equivalent composition, similar load-bearing properties of the bone and their bone size, which made surgery and fixation easier (Reinwald and Burr 2008). With regard to dogs, the focus is to study the effects of an ovariohysterectomy (mainly using beagles), for better understanding of the development of osteoporosis (Faugere, Friedler et al. 1990, Shen, Dempster et al. 1992). Foremost, dog models are attractive because of the very similar composition of bone compared to humans (Aerssens, Boonen et al. 1998) and the analogous cancellous remodeling system. Furthermore, dogs have a Haversian system equivalent to that in humans (Martin, Albright et al. 1981). Sheep are also frequently used in orthopedic research, since they are very amendable, trainable animals and less expensive compared to dogs or pigs. Fracture healing models involving experiments with sheep are mainly used to study the implantation of prosthetic implants, because the size of these devices is similar to those used in human surgery (Rocca, Fini et al. 2002). Additionally, sheep models are extensively used in the study of alveolar and mandibular bone loss (Johnson, Gilbert et al. 2002). Hence, the initial phase of fracture healing, more specifically the cellular composition of the fracture hematoma, was also examined in sheep (Schmidt-Bleek, Schell et al. 2009). Mainly because of the striking similarities to humans and the augmented availability of genetically modified strains, pigs are a commonly used model in biomedical research for a variety of human diseases (Almond 1996). In fracture healing-related research, pig models are frequently used to determine the effects of biomaterials after transplantation (Schlegel, Kloss et al. 2003, Schaller, Matthias Burkhard et al. 2018), but also in *in situ* bone tissue engineering approaches (Bez, Sheyn et al. 2017) or basic research questions (Swiontkowski, Tepic et al. 1993). The preferred large animal model depending on the scientific question is summarized in Table 2.

Table 2: *Summary of key similarities of human and animal bone, ranging from +least similar to +++most similar. Modified from (Pearce, Richards et al. 2007).*

	Pig	Sheep	Dog
Macrostructure	++	+++	++
Microstructure	++	+	++
Composition of the bone	+++	++	+++
Bone remodeling	+++	++	++

- **Equine models**

Unlike mice, rats or pigs, the treatment of fractures in horses is of great interest to their owners. Therefore, horses are mostly used for research regarding treatment for equine patients, e.g. implementing new surgical techniques (Barnes, Tucker et al. 1995, Rossignol, Brandenberger et al. 2016). Fractures in horses are often life-ending injuries and the treatment of the horse as a patient is of great interest (Riggs 2002). Most equine fractures are stress fractures and occur in the lower limb area (Mackey, Trout et al. 1987), with a high rate of post-operative complications (Crawford and Fretz 1985). However, there are hardly any studies about the exact course of the horse's initial healing process. The initial phase of fracture healing in horses is predominantly a phase of cleansing (removal of dead cells and tissue) (Auer and Grainger 2015). Due to the usually insufficient and slowed healing of fractures in horses, the initial phase is considered as the starting point of immunological processes that initiate bone regeneration and is altered/delayed in comparison to humans (Auer and Grainger 2015).

- **3D *in vitro* models**

In recent years, conventional simplified 2D cell culture settings, focusing on MSCs, osteoblasts and osteocytes, have been more and more replaced by complex 3D *in vitro* methods. Since 3D tissue models are able to capture cell-cell as well as cell-matrix interactions, these models offer enormous advantages compared to monolayer cultures. The cellular microenvironment established in these 3D models allows a closer approximation of the *in vivo* situation with respect to bone regeneration and cellular responses to external influences. Furthermore, a 3D environment approximates the normal morphology of the tissue as well as proliferation, migration and differentiation of the included cells. Consequently, 3D models are a good alternative to monolayer experiments and have higher translational potential (Elliott and Yuan 2011).

Currently, most of the *in vitro* approaches focusing on bone regeneration rely on the bone tissue engineering (BTE) strategy. This strategy is based on the use of 3D scaffolds seeded with cells or cells in combination with bioactive molecules, in order to design an osteogenic substitute for bone defects (Amini, Laurencin et al. 2012). Thus, a lot of new scaffolds have been developed, which are able to closely mimic mechanical and structural properties of bone and are in combination with cells, capable of inducing tissue formation (Curry, Pensa et al. 2016). BTE is generally comprised of four key features: (i) mimicking the bone ECM niche by using biocompatible scaffolds, (ii) implementing osteogenic or osteogenic precursor cells capable of producing bone tissue matrices, (iii) inducing the differentiation of the implemented cell types towards the desired phenotype by morphogenetic signals and (iv) supporting

the growing tissue with nutrients and oxygen by restoring vasculature (Amini, Laurencin et al. 2012). With regard to scaffolds, a large variety of materials have been used. Usually, scaffolds from HA-composites or tricalcium phosphate (TCP) are applied, which allow cells to adhere to and migrate into the scaffold, due to their macro- and microporous structure. In addition, hydrogel-based and naturally or chemically derived polymers are used as scaffolds. Furthermore, scaffold-free tissue matrices like organoids or spheroids have been employed extensively (Scheinpflug, Pfeiffenberger et al. 2018). These scaffolds are commonly seeded with MSCs, osteoblasts or osteocytes and sometimes in combination. Additionally, differentiation and proliferation is enhanced by the addition of BMPs, fibroblast growth factors (FGFs) or VEGFs (Kent Leach, Kaigler et al. 2006, Thompson, Epari et al. 2010, De Witte, Fratila-Apachitei et al. 2018, Yin, Qiu et al. 2018). To include the vasculature component, endothelial cells, e.g. human umbilical vein endothelial cells (HUVECs), have been added, inter alia resulting in enhanced proliferation of osteoblastic cells (Leszczynska, Zyzynska-Granica et al. 2013). However, fully functional bone tissue with a perfusable vasculature is still missing and therefore a matter of further research, although recent approaches tried to implement vasculature in *in vitro* studies (Klotz, Lim et al. 2018, Chiesa, De Maria et al. 2020). The implementation of bioreactors was a big step towards approaching the *in vivo* situation, as it provides the opportunity to (i) supply the tissue models with nutrients and oxygen, (ii) remove metabolites and debris as well as (iii) prevent the formation of necrotic regions, as reviewed by Scheinpflug et al. (Scheinpflug, Pfeiffenberger et al. 2018).

2.3 Aims and Objectives of the Thesis

Fractures are one of the most common injuries worldwide and, due to ageing society, the prevalence continues to increase, particularly in western industrialized countries. Approximately 10% of fractures show disbalances in an appropriate healing process, leading to pain, immobility, comorbidities and high treatment expenses. However, the pathogenesis of fracture healing disorders is yet to be fully understood, mainly due to the complex interplay of bone metabolism and the immune component. To understand the underlying mechanisms, most findings have been derived from animal models (mostly mice and rats). Even though bone of humans and mice share several similar properties, fundamental differences exist. Mice lack the Haversian canal system and have different load-bearing properties, particularly due to the different way of locomotion. Furthermore, the sheer size of the bones complicates the experimental design of conducted approaches. In contrast, large animal models such as horses show closer similarities to human bone and can be considered as patients themselves, therefore potentially sharing benefits from experimental findings. However, the translation from experimental findings in animals to humans remains at least questionable.

The overall goal of this thesis was to establish a fully characterized 3D *in vitro* model, mimicking the initial phase of fracture healing.

Ultimately, the established 3D *in vitro* model will provide the opportunity to (i) study cellular and humoral processes of fracture healing, (ii) investigate the underlying mechanisms of how hypoxia alters cell survival, cell proliferation, interplay and differentiation during the initial phase of fracture healing, (iii) screen for new promising therapeutics and their efficacy to support fracture healing as well as to treat fracture healing disorders and (iv) determine side effects of pharmacological substances.

For that purpose, (i) an equine and a human *in vitro* fracture hematoma model was established in order to provide the opportunity to compare findings between different species. Additionally, (ii) scaffold-free bone-like constructs (SFBC), solely consisting of human MSCs were established and characterized which was finally combined with a human FH model to generate a (iii) fracture gap model.

3 Results

3.1 Hypoxia and mesenchymal stromal cells as key drivers of initial fracture healing in an equine *in vitro* fracture hematoma model

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RESEARCH ARTICLE

Hypoxia and mesenchymal stromal cells as key drivers of initial fracture healing in an equine *in vitro* fracture hematoma model

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Abstract

Fractures in horses–whether simple fractures with just one clean break, or incomplete greenstick with stress fractures, or complications such as shattered bones can all be either minimal or even catastrophic. Thus, improvement in fracture healing is a hallmark in equine orthopedics. The fracture healing process implements a complex sequence of events including the initial inflammatory phase removing damaged tissue, re-establishment of vessels and mesenchymal stromal cells, a soft and hard callus phase closing the fracture gap as well as the remodeling phase shaping the bone to a scar-free tissue. Detailed knowledge on processes in equine fracture healing in general and on the initial phase in particular is apparently very limited. Therefore, we generated equine *in vitro* fracture hematoma models (FH models) to study time-dependent changes in cell composition and RNA-expression for the most prominent cells in the FH model (immune cells, mesenchymal stromal cells) under conditions most closely adapted to the *in vivo* situation (hypoxia) by using flow cytometry and qPCR. In order to analyze the impact of mesenchymal stromal cells in greater detail, we also incubated blood clots without the addition of mesenchymal stromal cells under the same conditions as a control. We observed a superior survival capacity of mesenchymal stromal cells over immune cells within our FH model maintained under hypoxia. Furthermore, we demonstrate an upregulation of relevant angiogenic, osteogenic and hypoxia-induced markers within 48 h, a time well-known to be crucial for proper fracture healing.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Fractures in horses are often fatal, extremely expensive to treat, and in certain cases an injury leading to euthanasia [1, 2]. Additionally, equine fractures and their subsequent treatment are of great economic interest [3], especially for horses deployed in racing. Various studies indicated an incidence of fractures in races between 1–2% per race start [2, 4, 5], with pelvic and tibial stress fractures identified as the most common cases of fracture [5]. Furthermore, the number of horses used in leisure sports is still increasing and includes a trend towards free-range husbandries in groups leading potentially to injuries and contortions especially in the fetlock area [6, 7]. Similar to treatment in humans, current strategies towards long bone fractures in horses focus on stabilization of the fracture site by means of screws or nails [8, 9]. Nowadays more elicit methods of treatment utilizing internal fixators [1], locking compression plates [10], external fixators [11] or hydrogels [12] are being applied in clinics. Moreover, cell therapy with either mesenchymal stromal cells [13, 14] or osteoprogenitor cells [15] is of upcoming interest especially for fractures that cannot be stabilized due to the location (fetlock, coffin bone). Nevertheless, the biggest challenge still remains the appropriate stabilization that remains perpetuated during the recovery time after surgery when approximately 400–600 kg of body weight are loaded onto the bones. As to the processes of fracture healing and particularly those during the initial phase of fracture healing, only little is known. In horses, bone healing is generally considered to be delayed [16] and contradictory to phylogenetically lower developed animals, the bone quality is diminished after trauma [17].

Generally, fracture healing can be divided into four different phases: (i) initial/inflammatory phase, (ii) soft callus formation, (iii) hard callus formation, and (iv) remodelling phase. During fracture, the bone marrow channel is shattered and evading cells such as mesenchymal stromal cells (MSCs), hematopoietic stem cells (HSC), immune cells and their precursor cells are mixed with cells from ruptured blood vessels (immune cells) within the fracture gap. These cells coagulate and form the so-called fracture hematoma (FH), which initiates the ongoing inflammatory phase within a hypoxic milieu [18]. Main research progress focusing on the initial phase has been conducted in sheep or rodents [19, 20], facing the problem of translation towards the human situation and/or the horse as a patient. Mice for instance lack the Haversian canal system [21], which is typical for human and equine bone physiology and remain in general an arguable model for disease patterns with ongoing inflammation processes [22]. In contrast, large animal models show considerably more similarity to human bone physiology concomitant processes when it comes to the pathophysiology of fracture healing [23]. In a human *ex vivo* study in 2011, Hoff et al. could show that besides myeloid cells of the innate immune system (monocytes, granulocytes) and cells of the adaptive immune system (T and B cells), also hematopoietic stem cells and MSCs are prominent cells in the FH [18].

Based on the general assumption that within the initial phase of bone healing immediately following the trauma, a hematoma is generated which accumulates cells from both peripheral and intramedullary blood, as well as bone marrow cells including mesenchymal stromal cells (MSCs) [24]. The initial phase is known to involve an acute inflammatory response including the production and the release of several important molecules, such as IL6, IL8 and MIF [25], and the recruitment of MSCs in order to generate a primary cartilaginous callus [24]. Thus, we generated a blood clot with MSCs to simulate the shift from the initial hematoma to the soft callus phase and to determine the importance of MSCs in the equine fracture healing process. In brief, the rationale of our study was to study the influence of MSCs, which are considered to be the main driver of tissue regeneration during the initial phase of fracture healing, since MSCs are the progenitor cells both for cartilage (endochondral ossification) and bone cells (intramembranous ossification). We also implemented microenvironmental conditions found

at the fracture site in vivo (hypoxia). For simulating the hypoxic conditions, we incubated our FH models under hypoxia (1% O_2), with normoxia serving as a control. To underline the impact of MSCs, we also incubated in parallel blood clots without the supplementation of MSCs as a second control. This way we had the opportunity to (i) contextualize our data to existing *in vivo* and *ex vivo* data and (ii) to use this system to eventually analyze the impact of fracture healing-relevant drugs or therapies in subsequent studies. To this end, we used our *in vitro* FH model in order to study the influence of hypoxia and mesenchymal stromal cells on the initial phase of fracture healing.

Materials and methods

Blood samples

EDTA blood samples (residual material from diagnostic blood drawings) were obtained from the equine clinic at the Department of Veterinary Medicine, Freie Universität Berlin (clinic's own horses). The horses showed no indication of illness, systemic inflammation or infection. For the *in vitro* FH models we used the blood of three different horses and mixed them with 2.2 x 10^5 MSCs. Correspondingly, we used the blood of three different horses for the coagulation of the blood clots. Age and gender of the respective donors can be found in Table B in S1 Appendix. Blood collection was approved by the local legal representative animal rights protection authorities (Landesamt für Gesundheit und Soziales Berlin: O 344/13)

Bone marrow-derived MSC isolation and incubation

Bone marrow was obtained from the sternum shortly after euthanasia from horses which were euthanatized for other ethical justifiable reasons (cadavers) at the equine clinic Seeburg (Dallgow-Döberitz, Germany). Horses showed no indices of illness, systemic inflammation or infection. Bone marrow was transported aseptically in phosphate-buffered saline (PBS) and at RT. Collected bone marrow was transferred into 175 cm^2 cell culture flasks (Greiner Bio-one, Kremsmünster, Austria) and flushed with 25 ml of DMEM plus GlutaMAX (Thermo Fisher Scientific, Waltham, USA) supplemented with 20% (v/v) StemMACS MSC Expansion Media Kit XF (Miltenyi Biotech, Bergisch Gladbach, Germany), 10% (v/v) FCS (Thermo Fisher, Waltham, USA), 100 units/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher, Waltham, USA), further referred to here as MSC culture medium. Incubation was carried out at 37˚ C in humidified atmosphere containing 5% CO_2. The MSC culture medium was completely replaced after two days of incubation in order to remove remaining bone marrow, blood and non-adherent cells. Hereafter the medium was replaced weekly.

Differentiation and characterization of bone marrow-derived MSCs

To ascertain the differentiation capability, cells were plated at 1x10^4 cells/well in 96-well plates (Greiner Bio-one, Kremsmünster, Austria) and incubated in the appropriate differentiation medium for 3 weeks.

Osteogenesis: MSCs were differentiated in StemMACS OsteoDiff (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were fixated with a 4% (w/v) paraformaldehyde solution (Carl Roth, Karlsruhe, Germany) for 10 min at RT and stained with 2% (w/v) Alizarin Red (in H_2O_{dd}, pH 4.1; Sigma Aldrich, St. Louis, USA) for 10 min at RT.

Adipogenesis: MSCs were incubated in α-MEM (Sigma Aldrich, St. Louis, USA) supplemented with 10% (v/v) human serum AB (EUROCLONE, Via Figino, Italian), 100 units/ml penicillin, 100 mg/ml streptomycin, 12 mM L-glutamine (GE Healthcare, Little Chalfont, England), 5 μg/ml insulin (Lilly, Bad Homburg, Germany), 50 μM indomethacin (Sigma

Aldrich), 1 μM dexamethasone (Sigma Aldrich) and 0.5 μM isobutylmethylxanthine (Sigma Aldrich, St. Louis, USA). Cells were fixed with 4% (w/v) paraformaldehyde for 10 min at RT and stained with 0.3% (v/v) Oil Red O (Sigma Aldrich, St. Louis, USA) in 60% (v/v) isopropanol (Merck, Darmstadt, Germany) for 15 min.

Further phenotypic characterization was carried out by the expression analysis of three surface MSC markers. Antibodies against equine CD29 and CD105 were used as positive markers and an antibody against equine CD14 as negative marker. Further procedure is described below ("Flow cytometric analysis").

Establishment of 3D fracture hematoma model and control hematomas

For the production of one hematoma model, 100 μl of blood (collected in vacutainer tubes with EDTA) were mixed with 2.2 x 10^5 MSCs and 100 μl of a 10 mM $CaCl_2$ solution in a 96-well-plate (round bottom, Greiner Bio-one, Kremsmünster, Austria). Control hematomas (blood clots) were produced analogously without any supply of MSCs. After 30 min incubation at 37˚C the blood clots (n = 4) and the FH models (n = 4) were transferred into DMEM + GlutaMAX supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 mg/ml streptomycin, 0.2% (w/v) β-glycerophosphate (Sigma Aldrich, St. Louis, USA), 10 nM dexamethasone (Sigma Aldrich, St. Louis, USA) and 0.002% (w/v) l-ascorbic acid (Sigma Aldrich, St. Louis, USA), further referred as osteogenic differentiation medium. Hematomas/blood clots were incubated for 6, 12, 24, 48, and 72 h under hypoxia at 5% CO_2 and 1% O_2, balanced with N_2. Normoxic controls were incubated at 37˚C under 5% CO_2 balanced with room-air in a humidified atmosphere (resulting in 18% O_2) for 6, 12, 24, 48, and 72 h as well.

RNA isolation

After incubation, coagulated hematoma models were washed with PBS and cells were separated via a cell strainer (70 μm, Corning, New York, USA). Erythrocyte lysis was performed (erythrocyte lysis buffer: 0.01 M $KHCO_3$, 0.155 M NH_4Cl, 0.1 mM EDTA, pH 7.5) for 6 min at 4˚C three times, and cells were washed with 0.5% (w/v) BSA in PBS (PBS/BSA). Total RNA was extracted using Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, USA), according to the manufacturer´s instructions and the RNA concentration was determined using Nanodrop ND-1000 (Peqlab Biotechnologie, Erlangen, Germany). RNA was stored at -80˚C until further processing.

Quantitative PCR (qPCR)

The cDNA was synthesized by reverse transcription using TaqMan Reverse Transcription Reagents (Applied Biosystems) for RNA concentrations >10 ng/μl or Sensiscript Reverse Transcription Kit (QIAGEN GmbH, Hilden, Germany) for RNA concentrations ≤ 10 ng/μl. cDNA was stored at -20˚C until further processing. qPCR was performed using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher, Waltham, USA) and the Stratagene Mx3000P (Agilent Technologies, California, USA). Initial denaturation was for 7 min at 98˚C followed by 45 cycles with 5 s at 98˚C, 7 s at 58˚C and 9 s at 72˚C. Finally, the melting curve was analyzed by a stepwise increase of the temperature from 50 to 98˚C every 30 s.

All primers were purchased from TIB Molbiol (Berlin, Germany; gene symbol: forward primer, reverse primer):

B2M: CCCCTGATAGTTAAGTGGGATCG, AGTACAGCTTCCTGATTTATGTGC;
MIF: GCAAGCCAGCCCAGTACATC, GCTGTAGGAGCGGTTCTGTG;
VEGFA: TTGCTGCTCTACCTCCACCAT, ATCAGGGGCACACAGGAT;
RUNX2: TGTCATGGCGGGTAACGAT, TCCGGCCCACAAATCTCA;

SLC2A1: GAAACCTCACCCCACATCCT, TTCGCCTTCCGTAGTTCTCA;
LDHA: GCCGTCTTAATTTGGTCCAG, TGGATTGGAAACAACAAGCA;
PFKFB3: GATTTAGCACAAAGCACGTTT, CTCCAAGGGCATCTTCACAG;
PGK1: GAACACGGAGGATAAAGTCAGC, AGGAACCAAAAGGCAGGAAA;
SPP1: CCAGTGAGCATTCCGATGTG, TCTCCCACCCCGCTATTATTT;
PPARG: GGGTGTCAGTTTCGCTCAGT, GGGCTCCATAAAGTCACCAA.

Data were normalized to the expression of *Beta-2-Microglobulin* (B2M) and to the time point 0 h, using the ΔΔCt-method. Focusing on the influence of hypoxia and the effect of MSCs on a model of a fracture hematoma using equine samples under a sterile inflammatory situation, we had to exclude commonly used housekeeping genes that are known to be regulated by hypoxia or inflammation such as *GAPDH* or *ACTB*. We have ultimately chosen *B2M* as a housekeeping gene which has been reported to be a stable housekeeping gene in horse and under hypoxic conditions at least in human MSCs [26, 27]. Furthermore, using qPCR based on the same template concentrations we observed neglectable deviations of the Ct-values of *B2M* with regard to incubation duration for different time points (0 h, 12 h, 48 h) data not shown.

Flow cytometric analysis

After erythrocyte lysis, the isolated cells were washed with PBS/BSA. After Fc-receptor blocking with Flebogamma the cells were washed with PBS/BSA and antibody staining was performed for 15 min on ice. Table A in S1 Appendix shows all antibodies with their specificity, dilution used and the corresponding isotype controls. All isotype controls were obtained from Miltenyi Biotech GmbH (Bergisch Gladbach, Germany). The cells were washed with PBS/BSA and incubated with 1:25-diluted 7-AAD (BioLegend, San Diego, USA) for 15 min at RT. After a further washing step with PBS/BSA, the cells were resuspended in 0.05% (w/v) NaN_3 in PBS/BSA (PBS/BSA/Azide). The cells were recorded using flow cytometry with a MACS Quant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany) and analyzed with FlowJo software (Tree Star, USA). The antibodies and gating strategy utilized are given in the supplementary files (Table A and Fig A in S1 Appendix).

Embedding, cryosections and DAPI stain

For immunofluorescence, FH models (0 h, 24 h and 48 h) were embedded as follows: FH models were transferred into 4% paraformaldehyde, then into a 10%, 20% and finally 30% glucose solution, each for 24 h. Storage was at 4˚C. Cryo-embedding was followed by cryosections as described previously [28]. Slides were air dried and subsequently stained with DAPI. The DAPI staining solution was 0.1% (v/v) DAPI (Sigma Aldrich, St. Louis, USA); 0.1% (v/v) Tween 20 (Carl Roth, Karlsruhe, Germany); 5% (v/v) FCS (Thermo Fisher, Waltham, USA) in PBS. The whole procedure was performed at RT. Sections were first incubated in PBS with 0.1% (v/v) Tween 20 for 10 min. After 10 min incubation in DAPI staining solution, the sections were washed three times in PBS with 0.1% (v/v) Tween 20. Stained sections were put on a slide and then mounted (Fluoromount™ Aqueous Mounting Medium, Sigma Aldrich, St. Louis, USA) under a cover slip. Examination of the sections was performed and photos were taken, using a KEYENCE BZ-X700 fluorescence microscope and depicted in pseudo-colors.

Statistical analysis

Statistical tests were performed using Graph Pad Prism Software (La Jolla, USA). Differences were compared using the Mann–Whitney U-test. Probability values of $p<0.05$ were

considered to be statistically significant, and values of $p<0.1$ were considered to have a statistical trend ($^{*}p<0.05$; $^{+}p<0.1$).

Results

MSCs play a fundamental role in the initial phase of fracture healing. Therefore, MSCs represent an important cell fraction within our hematoma models. For the use of MSCs, we established well-defined minimal criteria based on their potential to adhere to plastics, to differentiate into osteoblasts and adipocytes, and to express typical surface markers. Only MSCs that fulfill these criteria were utilized to establish the equine *in vitro* FH model, consisting of peripheral blood and MSCs.After cultivation for three passages, the MSCs adhered to the plastic surface and showed their typical fibroblastoid morphology (Fig 1A). They also could be differentiated into the osteogenic lineage as the Alizarin Red S staining showed

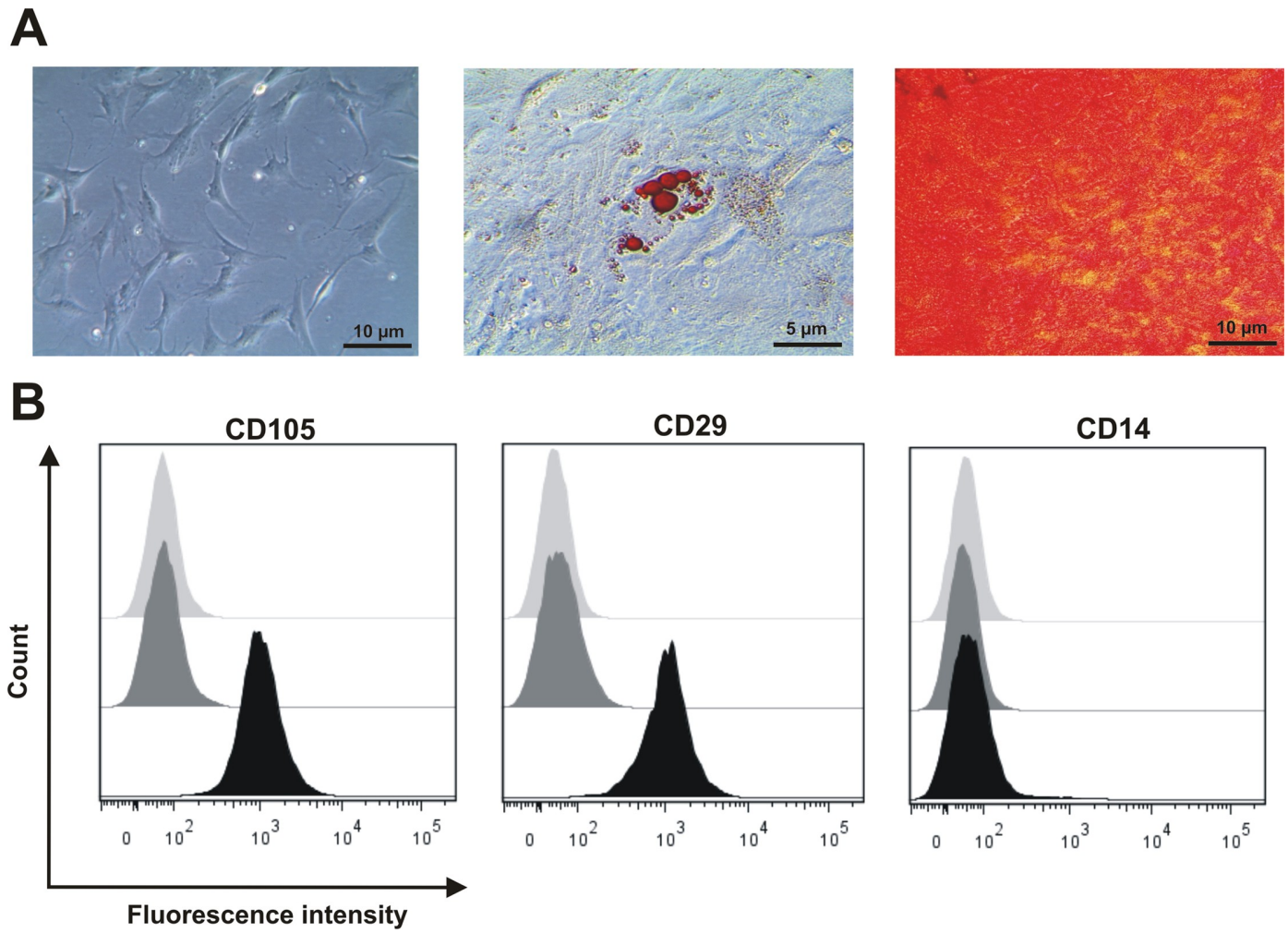

Fig 1. Bone marrow-derived cells obtained from sternum-biopsies are characterized as MSCs. Characterization of equine bone marrow-derived MSCs obtained from sternum-biopsies with regard to their potential to (A) adhere to plastic surfaces and typical morphology, their differentiation potential towards adipogenic and osteogenic lineage and (B) express surface markers CD29, CD105, and CD14 (unstained fractions, isotype control stainings and antigen-specific stainings are depicted in light grey, dark grey and black, respectively).

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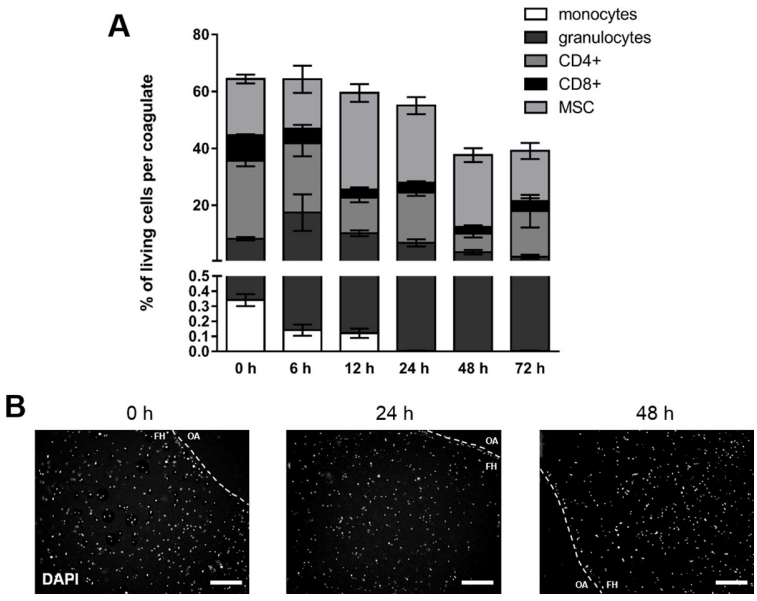

Fig 2. Immune cell vitality in the FH model decreases over time irrespective of subpopulation and spatial distribution after incubation under normoxic conditions. (A) Frequency of immune cell populations (granulocytes, CD14+ monocytes, CD4+ T cells, CD8+ T cells) and MSCs (CD29+, CD 105+, CD14-) negative for 7-AAD present in the *in vitro* FH model as incubated in osteogenic differentiation medium under normoxic conditions (37˚C, 5% CO_2, 18% O_2) for 6, 12, 24, 48, and 72 h (mean ± SEM, n = 3). Depicted is the frequency of total cells and the corresponding frequencies of the cell populations. (B) Spatial distribution of cells within the *in vitro* FH model as determined by DAPI-staining and depicted as representative staining for incubation periods of 0, 24, and 48 h (The dotted line indicates the border of the *in vitro* hematoma; FH = area of the fracture hematoma model and OA = outer area).

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calcium-complexes stained in red colour and the adipogenic lineage as the cells secrete lipid droplets which are stained red via Red oil staining (Fig 1B). Additionally, the typical surface markers CD29 and CD105 were expressed with no expression of the exclusion marker CD14 (Fig 1C; Table A in S1 Appendix).

Cell composition of *in vitro* FH models under normoxic conditions

To mimic the initial phase of fracture healing *in vitro*, we generated *in vitro* FH models by mixing and coagulating MSCs and blood cells. After incubation of the *in vitro* FH models for 6, 12, 24, 48, and 72 h under normoxic conditions (37˚C, 5% CO_2, 18% O_2), we observed a continuous decline in the frequency of cells alive which resulted in 45 ± 3% of cells alive after an incubation period of 72 h (Fig 2A). Within the FH model, the frequency of immune cells decreased over time, while the frequency of MSCs increased within the first 12 h. As a result, the MSC population became the major cell population in the *in vitro* FH model, although it decreased between 12 and 72 h (Fig 2A). With regard to the proportion of immune cells, we observed a continuous decrease in the frequency of granulocytes over time. The frequency of monocytes was negligible, with almost no cells detectable after 6 h of cultivation, while the frequency of CD8+ cells also decreased perpetually. Interestingly, the most prominent population at 0 h–namely CD4+ T cells–remains the most stable cell population within the incubation period analyzed (Fig 2A). In contrast, with regard to the blood clots (Fig B in S1 Appendix) the frequency of granulocytes as well as T cells was very stable. As to the spatial distribution, we observed no clustering of cells but an even distribution within the FH model using DAPI-staining (Fig 2B).

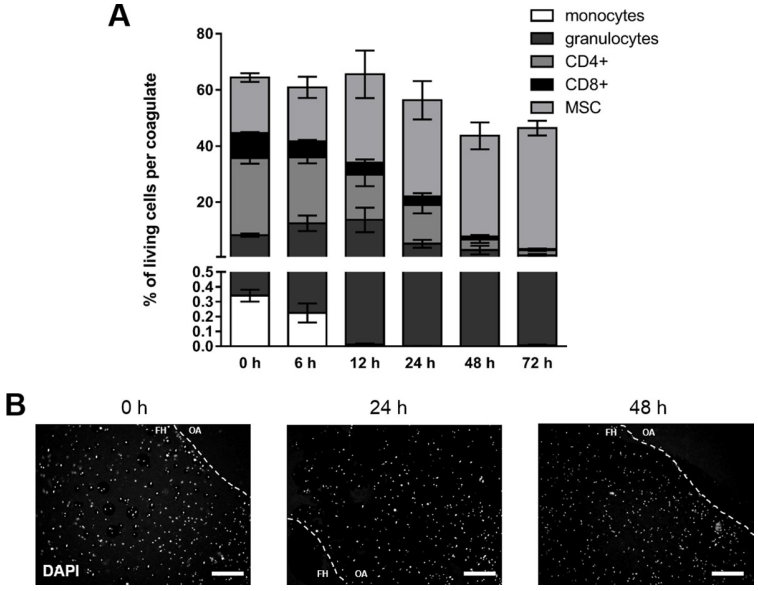

Fig 3. Hypoxia favors survival of MSCs while decreasing immune cell fractions. (A) Frequency of immune cell populations (granulocytes, CD14+ monocytes, CD4+ T cells, CD8+ T cells) and MSCs (CD29+, CD 105+, CD14-) negative for 7-AAD in the FH models cultured in osteogenic differentiation medium under hypoxic conditions (37˚C, 5% CO_2, 1% O_2) for 6, 12, 24, 48, and 72 h (mean ± SEM, n = 3). Depicted is the frequency of total cells and the corresponding frequencies of the cell populations. (B) Spatial distribution of cells within the FH model as determined by DAPI-staining and depicted as representative staining for 0, 24, and 48 h of incubation (The dotted line indicates the border of the *in vitro* hematoma; FH = area of the fracture hematoma model and OA = outer area).

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Cell composition of *in vitro* FH models under hypoxic conditions

To mimic the restricted microenvironment in the initial phase of fracture healing more adequately, we generated *in vitro* FH models and incubated them under hypoxic conditions (37˚C, 5% CO_2, 1% O_2) for 6, 12, 24, 48, and 72 h. We observed a perpetual decline of the cells alive with a final frequency of 43 ± 2% after 72 h of incubation (Fig 3A). With regard to the immune cell populations, the frequency of CD4+ T cells and CD8+ T cells again constantly decreased over time, whereas the frequency of granulocytes increased from zero to 12 h before massively decreasing. The frequency of monocytes alive was barely detectable at any time point analyzed. Finally, none of the analyzed immune cell populations survived the FH model after 72 h of cultivation under hypoxic conditions. In contrast, the proportion of MSCs within the FH model permanently increased from a ratio of 20% ± 1% at 0 h to 42 ± 2% at 72 h after cultivation (Fig 3A). With regard to the blood clots (Fig B in S1 Appendix), we observed a stable frequency of granulocytes and T cells. Regarding the spatial distribution, we again observed no clustering of cells but an even distribution within the FH model using DAPI-staining (Fig 3B).

Impact of oxygen availability on cellular vitality and composition in the *in vitro* FH models

When comparing cellular vitality and composition of the FH models incubated under normoxia with those incubated under hypoxia, we observed no differences in respect to overall cell survival, either after 72 h, or throughout the whole decline in time. However, as far as the cellular composition is concerned, the frequencies of all immune cell populations in the FH model declined under hypoxia, whereas under normoxia, survival of CD4+ and CD8+ T cells

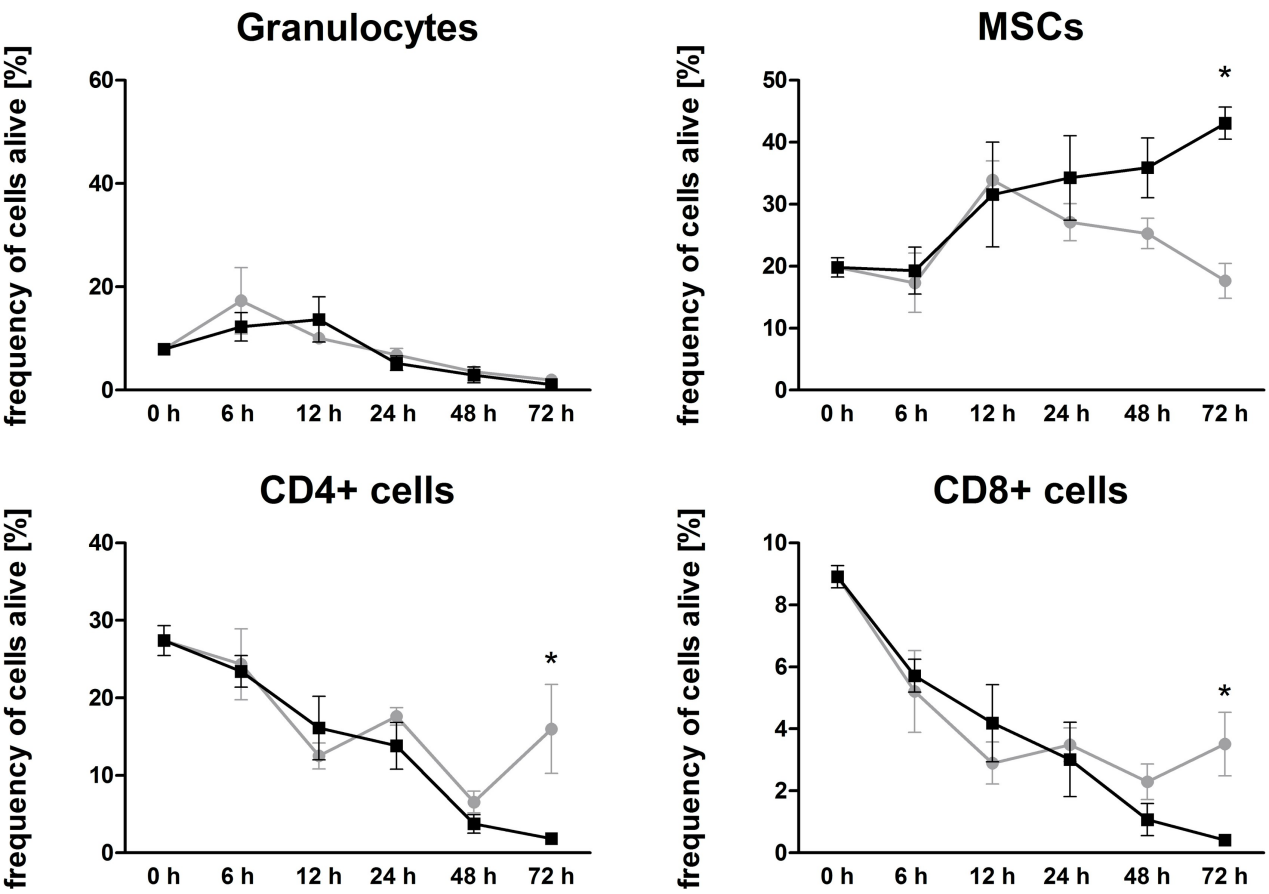

Fig 4. Hypoxia favors survival of MSCs while decreasing granulocytes, CD4+ and CD8+ immune cells. Frequency of granulocytes, CD4+ and CD8 + immune cells negative for 7-AAD in the FH model cultured in osteogenic differentiation medium under either normoxic (grey) or hypoxic (black) conditions (n = 3). Statistical analysis was conducted using the Mann-Whitney U-test, *p<0.05.

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after 72 h of incubation was significantly higher than that under hypoxic incubation (Fig 4). In contrast, the frequency of granulocytes remains unaffected by the incubation conditions, and for monocytes the case was likewise negligible. Hence, the frequency of MSCs was significantly higher under hypoxic conditions after 72 h of cultivation when compared to that of the corresponding control (Fig 4).

MSCs along with their time-dependent increase were by far the most abundant cells, a most striking situation seen from 12 to 72 h. Compared to normoxic conditions, MSCs survived better under hypoxic conditions, whereas immune cells seem to have a diminished survival rate (Fig 4).

Time-dependent RNA-expression of fracture-healing-relevant markers in the *in vitro* FH models

To analyze the impact of hypoxia on all cells in the FH model, we focused on markers for osteogenesis, glycolytic adaptation towards hypoxia and angiogenesis on the transcriptional level (Fig 5). Therefore we analyzed the RNA-expression of fracture-relevant markers. We cultivated the *in vitro* hematomas for 6, 12 and 48 h. Within the hematomas, osteogenic (*RUNX2*, *SPP1*), angiogenic (*VEGFA*, *MIF*) as well as hypoxia-induced (*LDHA*, *PGK1*, *PFKFB3*,

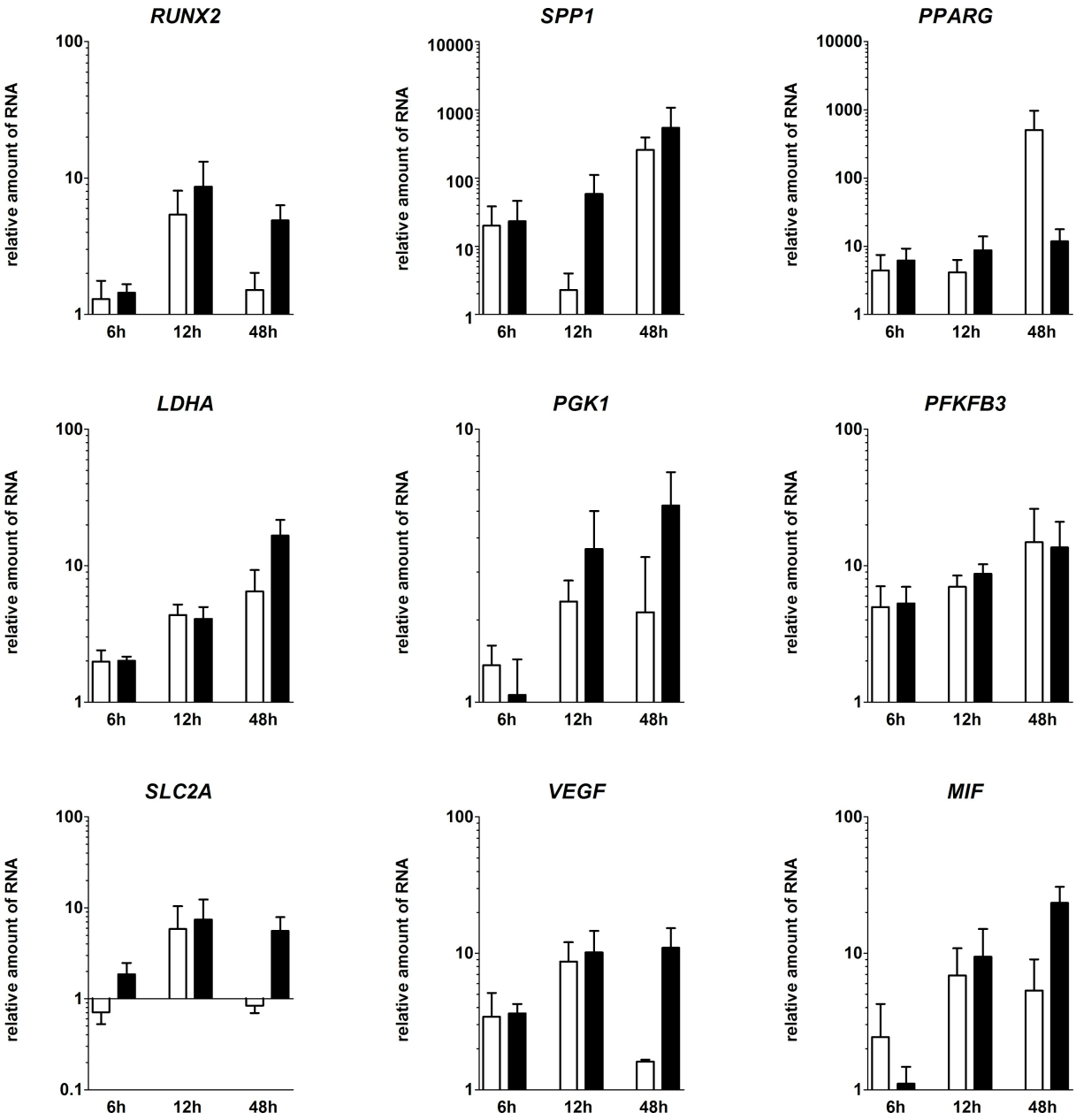

Fig 5. Within the FH models, osteogenic, hypoxia-induced and angiogenic markers were upregulated over time until 48 h of incubation. Depicted is the relative RNA-expression of the osteogenic markers *RUNX2*, *SPP1*, the adipogenic marker *PPARG*, the hypoxia induced genes *LDHA*, *PGK1*, *PFKFB3*, *SLC2A1*, and the angiogenic genes *VEGFA*, and *MIF* within the *in vitro* FH models after cultivation in osteogenic differentiation medium for 6, 12, and 48 h under either normoxia (white bars) or hypoxia (black bars). All values are normalized to the "housekeeping gene" *B2M* and 0 h (Mean ± SEM, n = 3). Statistical analysis was conducted using Mann-Whitney U-test.

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SLC2A1) markers were higher expressed after 48 h. Concerning the osteogenic markers, we demonstrated a time-dependent upregulation of *RUNX2* and *SPP1*. While *RUNX2* was upregulated after 12 h of cultivation, the highest upregulation of *SPP1* was observed at 48 h. Additionally, this effect was even stronger under hypoxic conditions, where *RUNX2* was upregulated to a higher extent. In contrast, the adipogenic transcription factor *PPARG* was less

strongly induced in FH models incubated under hypoxic conditions. Drawing the focus towards angiogenic markers, we could show the upregulation of typical markers (peaks: *VEGFA* after 12 and *MIF* after 48 h). Interestingly, both *VEGFA* and *MIF* showed higher levels of expression in FH models incubated under hypoxic conditions. *LDHA*, *PGK1*, *PFKFB3* and *SLC2A1* were also upregulated at least after 12 h. After 48 h, the majority of genes analyzed were expressed to a higher extent under hypoxic conditions except for *PFKFB3* and *PPARG* (Fig 5).

Differences of the mRNA-expression of *in vitro* hematomas versus blood coagulates after 48 h of cultivation under normoxic or hypoxic conditions

To analyze if the observed changes in gene expression patterns are due to the different survival rates of the analyzed immune cell populations and MSCs, we compared the data gained from *in vitro* hematomas to blood coagulates without MSCs. Focusing on the differences between the mRNA-expression of blood coagulates and *in vitro* hematomas, we could show that except for *SPP1* the expression of relevant genes was higher in the *in vitro* hematomas (significant for *PFKFB3* and *VEGFA*). In the *in vitro* FH model, osteogenic-relevant genes (*RUNX2*, *SPP1*) were upregulated, even more pronounced under hypoxic conditions, whereas the adipogenic marker *PPARG* was upregulated, but about 100 times less under hypoxic conditions. Hypoxia-induced genes (*PGK1*, *PFKFB3* and *SLC2A1*) were also upregulated under both cultivation methods, except for *SLC2A1* which was only upregulated under hypoxic conditions. *PGK1* and *SLC2A1* showed a higher expression under hypoxic conditions. For the angiogenic markers *VEGF*A and *MIF* as well as for the pH-regulating marker *LDHA*, we could show an upregulated expression which was even more evident under the influence of hypoxia (Fig 6).

Discussion

To study the underlying cellular mechanisms of the initial inflammatory phase of fracture healing in horses, we aimed to establish an equine *in vitro* FH model. To this end, we first created protocols for the isolation and characterization of major cell types involved in the generation of the FH model, namely immune cells–released from the ruptured vessels and from the bone marrow–and mesenchymal stromal cells (MSCs) from bone and bone marrow. The limited availability of suitable antibodies for immunological characterization but also the limited knowledge of lineage markers for equine cells especially for equine MSCs belong to the challenging issues we wanted to address in the study presented here.

To characterize MSCs, standardized criteria for human material have already been established and defined more than ten years ago [29]. In brief, human MSCs have to fulfill minimal requirements including attachment to plastic surfaces, the capacity to differentiate into multiple lineage [30], as verified by differentiation towards osteogenic, chondrogenic and adipogenic lineage, and finally the presence or absence of typical surface markers (CD73+, CD90+, CD105+; CD45-, CD34-, CD14-, CD19- and HLA-DR-) [29]. Focusing on equine MSCs, it is disparately more difficult to characterize these cells in a similar way. Although a variety of characterization procedures have already been described [31–34], only few surface markers are available for immunophenotyping due to the limited availability of suitable antibodies. Here, we characterized MSCs by demonstrating (i) plastic adherence, (ii) differentiation towards osteogenic (demonstrated by Alizarin Red staining) and adipogenic (demonstrated by Red Oil staining) lineage, and (iii) immunological characterization using CD105 and CD29, previously described as stable equine MSCs markers and widely used to characterize equine MSCs [35–38] and CD14 as a negative marker (Fig A and Table A in S1 Appendix). These have also been previously demonstrated to characterize equine monocytes (2013) [39]. We defined this characterization procedure as a minimum criterion for the use of equine MSCs within our study.

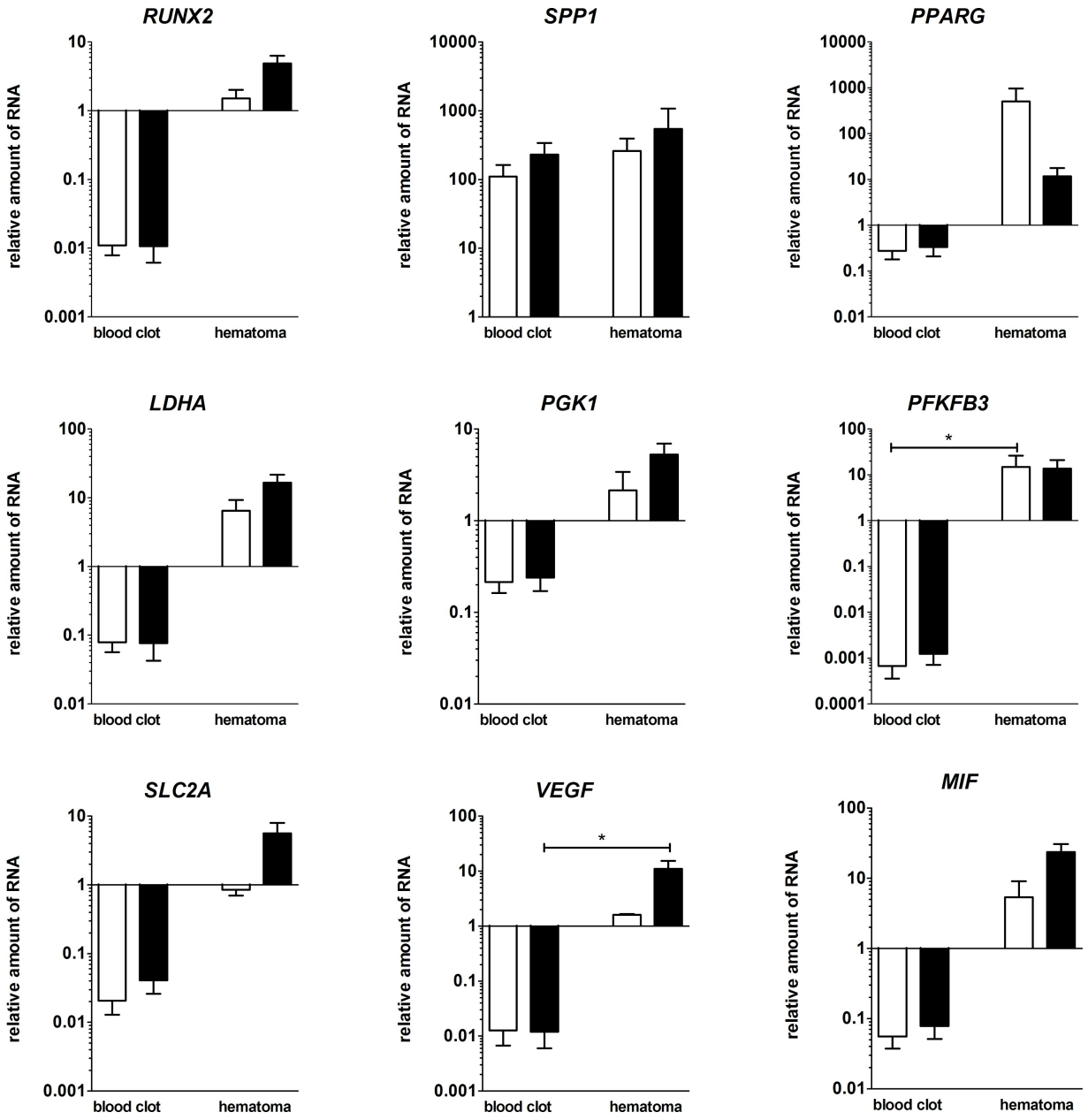

Fig 6. Osteogenic, hypoxia-induced and angiogenic markers were upregulated in the *in vitro* FH models compared to blood coagulates. Depicted is the relative RNA-expression of the osteogenic markers *RUNX2*, *SPP1*, the adipogenic marker *PPARG*, the hypoxia induced genes *LDHA*, *PGK1*, *PFKFB3*, *SLC2A1* and the angiogenic genes *VEGFA* and *MIF* within the FH models and the blood-only coagulates after cultivation in osteogenic differentiation medium for 48 h under either normoxic (white bars) or hypoxic conditions (black bars). All values are normalized to the "housekeeping gene" *B2M* and to 0 h (mean ± SEM, blood clots: n = 4, FH models: n = 3). Statistical analysis was conducted using Mann-Whitney U-test, $^{*}p<0.05$.

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For the characterization of equine immune cells, we used antibodies against CD4, CD8, and CD14, while granulocytes were determined by granularity and size using flow cytometry (Table A in Appendix 1). Separation of MSCs from the immune cell populations in the FH model was achieved by flow cytometry (Fig A in S1 Appendix).

Although *in vitro* FH models were generated in a standardized manner with regard to cell numbers and incubation times, we had to face limitations in terms of low cell recovery from coagulates after incubation, variations in hematoma size after coagulation and a massive impact on cell survival after incubation (Figs 2 and 3).

Analyzing the impact of a restricted microenvironment by focusing on hypoxia in our *in vitro* model, we did not observe any difference between normoxia and hypoxia concerning overall cell survival (Figs 2 and 3). However, we did observe a shift in the distribution of cell population towards an increase in the proportion of MSCs after incubation under hypoxic conditions (Fig 3). We assume that MSCs, well-known key players in the process of fracture healing [40–42], may promote the termination of the inflammatory phase especially in a hypoxic microenvironment, and this may resemble the *in vivo* situation at the fracture site [18]. Thus, MSCs can be assumed to be likely suitable candidates in cell-based therapeutic strategies to overcome fracture healing disorders in horses and humans [13, 14]. Additionally, we had observed previously that the osteogenic differentiation of human MSCs is enhanced in a hypoxic microenvironment but also that it does not influence cell survival or proliferation [43]. Conversely, Ranera et al. (2012) demonstrated that hypoxia limits the proliferation of equine MSCs in 2D cultures [34]. Whether or not these differences are species-specific needs to be clarified.

Focusing on myeloid cells such as monocytes and polymorph nuclear cells (PMNC), we observed a time-dependent decline of their frequencies within the *in vitro* FH model without any significant impact of hypoxia on cell survival (Figs 2–4). In general, the role of neutrophils with regard to fracture healing has to our knowledge been poorly investigated or is discussed controversially, although granulocytes are the most abundant cells in the early FH [44]. Groogard et al. (1990) reported that neutropenia in mice did not have any significant effect on fracture healing [45]. In addition, Chung et al. (2006) observed a slight increase of bony trabeculae when treating young rats with neutrophil-neutralizing antiserum in a growth plate injury model [46]. In the human *in vitro* FH model, which was performed recently from our group neutrophils seem to only marginally influence fracture healing, while being time-dependently depleted in the FH model, and even more strongly so under hypoxic conditions [47]. In contrast, a study of Kovtun et al. (2016) reveals a crucial role of neutrophils in bone healing [44]. They drastically reduced the number of neutrophils using a Ly-6G antibody in fractured mice and observed impaired bone healing after 21 d, with diminished bone content as well as impaired mechanical properties, implicating the important role of neutrophils in the very early phase of fracture healing [44]. With regard to PMNCs in our model, we could not determine any impact of hypoxia on the frequency of granulocytes (Figs 2–4). Neutrophils are primarily responsible for the removal of debris and spoilt cells in accordance with attracting monocytes to the fracture site [48] in the very early inflammatory phase, while their importance seems to be diminished in the ongoing process of fracture healing. In our study here, we could show the presence and survival of granulocytes within the first hours as well as their time-dependent decline of their frequencies within the *in vitro* FH models, which may resemble the *in vivo* situation (Figs 2–4). Although the frequency of granulocytes is time-dependently diminished, we cannot exclude, that granulocytes in apoptosis or netosis or degranulation processes have an influence on the ongoing process of fracture healing.

Among the adaptive immune cell populations, we observed a significantly enhanced hypoxia-mediated reduction in the frequencies of CD4+ and CD8+ cells after incubation of FH models for 72 h (Figs 2–4). In the line of our observation, reduction of an adaptive immune response has been reported to accelerate during bone healing [49, 50]. In more detail, Toben et al. demonstrated an accelerated fracture healing in recombination activating gene 1 knockout (RAG1(-/-)) mice lacking the adaptive immune system [49]. Although it has been

demonstrated that proliferation of CD8+ cells is diminished under hypoxic conditions [51], depletion of CD8+ T-cells in a mouse osteotomy model further has been reported to enhance fracture healing [50]. These findings and our findings presented here indicate that the continuous depletion of CD8+ T cells within the fracture site seems to be a feature beneficial for the fracture healing process. However, in an *ex vivo* human FH model, we demonstrated a decrease in lymphocyte survival after 24 h–independently of oxygen availability–although the frequencies of lymphocytes after hypoxic incubation increase. The latter can be explained by the relatively decreased granulocyte proportions after 24 h of incubation under hypoxia. Interestingly, we could detect a relevant expression of active caspases indicating apoptosis in granulocytes only after 24 h of incubation under hypoxia. However, human lymphocytes from ex vivo human FH models did express active caspases under all incubation conditions but with the highest expression after 24 h under hypoxia which is in the line with the findings in the equine FH model after 48 h [47].

Moreover, it has been well demonstrated that MSCs exhibit immunosuppressive functions [52], and that they are immunotolerant [53] and known to inhibit the proliferation of T-lymphocytes [42, 52, 54–56]. Here, we demonstrate that the increase in the frequency of MSCs over time is associated with a decrease of T-lymphocytes when investigating the incubation of these cells under hypoxia. This anti-correlative development may indicate that MSCs are limiting the initial inflammatory hypoxic phase of fracture healing in a human *ex vivo* FH model and also in an equine *in vitro* FH model.

In view of the impact of hypoxia on the expression of selected genes in our equine *in vitro* FH model, we observed that upregulation of osteogenic (*RUNX2*, *SPP1*), hypoxia-induced (*PGK1*, *LDHA*, *PFKFB3*, *SLC2A1*) and angiogenic (*VEGFA*, *MIF*) genes/factors essential for fracture healing after incubation for 48 h takes place at a higher extent under hypoxic than under normoxic conditions (Fig 5). In this line of observation, we could previously demonstrate a time-dependent increase in the expression of the osteogenic *RUNX2* and *SPP1* in a human *ex vivo* FHs [18]. Although we demonstrate an increase in the expression of *PPARG (*a key marker for the adipogenic differentiation of MSCs under normoxia), its upregulation is abolished under hypoxia. Thus, hypoxia seems to shift MSCs into the osteogenic lineage, as demonstrated previously [43].

To analyze the impact of MSCs, we compared the *in vitro* generated FH models (blood coagulates with MSCs) with blood coagulates without MSCs, focusing on the expression of selected genes (Fig 6). In general, MSCs contribute either directly or indirectly to the induction of gene expression from osteogenic (*RUNX2*, *SPP1*), hypoxia-induced (*PGK1*, *LDHA*, *PFKFB3*, *SLC2A1*) and angiogenic (*VEGFA*, *MIF*) factors essential for fracture healing. The contribution of MSCs in the equine *in vitro* FH models demonstrate a similar pattern of expression with regard to the hypoxia-induced genes *LDHA*, *PGK1* and *SLC2A1* after 48 h when compared to in human *ex vivo* as well as *in vitro* studies [18, 47]. The induction of these genes especially under hypoxia may reflect the adaptation towards a hypoxic environment and the shift towards anaerobic glycolysis [57–60]. All hypoxia-related genes were to a great extent more highly expressed in the *in vitro* hematomas when compared to blood coagulates, indicating the huge influence of MSCs on the expression of hypoxia-induced genes/factors in the FH models, which also involves the gene expression of *VEGFA* and *MIF* coding for angiogenic factors. *VEGFA* is known to be expressed locally by pre-osteoblasts [61] and MSCs, and also plays an important role in the fate of MSCs towards either adipocyte or osteoblast lineage [61, 62]. Several studies identified *VEGFA* as a key factor in osteogenesis as well as in angiogenesis [63–65]. Upregulation of *VEGFA* in our model may indicate the initiation of fracture healing-relevant processes involving angiogenesis and vascularization. *MIF* is also an essential molecule for fracture healing [25, 66] and several knock-out rat models have underlined the importance

of *MIF*, demonstrating a delayed fracture healing in the absence of *MIF* [67, 68]. *MIF* is secreted by MSCs [69] and is known to promote their survival [70].

Taken together, we observed in our present equine *in vitro* FH model profound similarities to our previously published results derived from a human *in vitro* FH model. These concern the expression of genes analyzed for the upregulation of angiogenic and hypoxia-induced markers, and the indication that they are more pronounced under hypoxic conditions [47].

Conclusion

In our study presented here, we characterized equine immune cells as well as MSCs and used these cells to establish an equine *in vitro* FH model. We demonstrate that hypoxia favors the survival of MSCs over that of immune cells and that the expression of fracture healing-relevant genes, most often enhanced by hypoxia, is widely induced. Compared to human *in vitro* and *ex vivo* data and *in vivo* data based on animal models, we could highlight significant similarities. However, further investigations of *ex vivo* equine fracture hematoma are needed to validate our approach and to clarify the cellular and molecular process of the initial phase of fracture healing in the "patient" horse more in detail.

Supporting information

S1 Appendix. File name: Supporting information.
(PDF)

Acknowledgments

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Funding acquisition: Frank Buttgereit, Timo Gaber, Annemarie Lang.

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Methodology: Moritz Pfeiffenberger, Janika Bartsch, Igor Ponomarev.

Project administration: Timo Gaber, Annemarie Lang.

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Writing – original draft: Moritz Pfeiffenberger, Janika Bartsch, Timo Gaber, Annemarie Lang.

Writing – review & editing: Paula Hoff, Igor Ponomarev, Dirk Barnewitz, Christa Thöne-Reineke, Frank Buttgereit.

References

1. Auer JA, Grainger DW. Fracture management in horses: Where have we been and where are we going? Veterinary journal (London, England: 1997). 2015; 206(1):5–14. Epub 2015/06/23. https://doi.org/10.1016/j.tvjl.2015.06.002 PMID: 26095036.
2. Rosanowski SM, Chang Y-M, Stirk AJ, Verheyen KLP. Risk factors for race-day fatality in flat racing Thoroughbreds in Great Britain (2000 to 2013). PLOS ONE. 2018; 13(3):e0194299. https://doi.org/10.1371/journal.pone.0194299 PMID: 29561898
3. Peloso JG, Mundy GD, Cohen ND. Prevalence of, and factors associated with, musculoskeletal racing injuries of thoroughbreds. Journal of the American Veterinary Medical Association. 1994; 204(4):620–6. Epub 1994/02/15. PMID: 8163419.
4. Maeda Y, Hanada M, Oikawa M-a. Epidemiology of racing injuries in Thoroughbred racehorses with special reference to bone fractures: Japanese experience from the 1980s to 2000s. Journal of Equine Science. 2016; 27(3):81–97. https://doi.org/10.1294/jes.27.81 PMC5048355. PMID: 27703403
5. Verheyen KL, Wood JL. Descriptive epidemiology of fractures occurring in British Thoroughbred racehorses in training. Equine veterinary journal. 2004; 36(2):167–73. Epub 2004/03/25. PMID: 15038441.
6. Janczarek I, Wilk I. Leisure riding horses: research topics versus the needs of stakeholders. Animal science journal = Nihon chikusan Gakkaiho. 2017; 88(7):953–8. Epub 2017/04/20. https://doi.org/10.1111/asj.12800 PMID: 28422370.
7. Mejdell CM, Jørgensen GHM, Rehn T, Fremstad K, Keeling L, Bøe KE. Reliability of an injury scoring system for horses. Acta Veterinaria Scandinavica. 2010; 52(1):68–. https://doi.org/10.1186/1751-0147-52-68 PMC3023730. PMID: 21194451
8. Auer JA. Chapter 81—Principles of Fracture Treatment. Equine Surgery (Third Edition). Saint Louis: W.B. Saunders; 2006. p. 1000–29.
9. Auer JA, Watkins JP. Treatment of radial fractures in adult horses: an analysis of 15 clinical cases. Equine veterinary journal. 1987; 19(2):103–10. Epub 1987/03/01. PMID: 3569192.
10. Jacobs Carrie C, Levine David G, Richardson Dean W. Use of locking compression plates in ulnar fractures of 18 horses*. Veterinary Surgery. 2017; 46(2):242–8. https://doi.org/10.1111/vsu.12607 PMID: 28146292
11. Turek B, Potynski A, Drewnowska O. Own-design external fixator for the treatment of diaphyseal fractures of the third metacarpal bone in horses. Med Weter. 2016; 72(3):197–202. WOS:000371281800010.
12. Cohen JM, Southwood LL, Engiles J, Leitch M, Nunamaker DM. Effects of a novel hydrogel on equine bone healing: a pilot study. Veterinary and comparative orthopaedics and traumatology: VCOT. 2012; 25(3):184–91. Epub 2012/03/01. https://doi.org/10.3415/VCOT-11-01-0006 PMID: 22366873.
13. Govoni KE. HORSE SPECIES SYMPOSIUM: Use of mesenchymal stem cells in fracture repair in horses. Journal of animal science. 2015; 93(3):871–8. Epub 2015/05/29. https://doi.org/10.2527/jas.2014-8516 PMID: 26020865.
14. Rosset P, Deschaseaux F, Layrolle P. Cell therapy for bone repair. Orthopaedics & traumatology, surgery & research: OTSR. 2014; 100(1 Suppl):S107–12. Epub 2014/01/15. https://doi.org/10.1016/j.otsr.2013.11.010 PMID: 24411717.
15. McDuffee LA, Pack L, Lores M, Wright GM, Esparza-Gonzalez B, Masaoud E. Osteoprogenitor cell therapy in an equine fracture model. Veterinary surgery: VS. 2012; 41(7):773–83. Epub 2012/07/19. https://doi.org/10.1111/j.1532-950X.2012.01024.x PMID: 22804243.
16. Murphey ED, Schneider RK, Adams SB, Santschi EM, Stick JA, Ruggles AJ. Long-term outcome of horses with a slab fracture of the central or third tarsal bone treated conservatively: 25 cases (1976–1993). Journal of the American Veterinary Medical Association. 2000; 216(12):1949–54. Epub 2000/06/23. PMID: 10863595.
17. Enneking WF, Burchardt H, Puhl JJ, Piotrowski G. Physical and biological aspects of repair in dog cortical-bone transplants. The Journal of bone and joint surgery American volume. 1975; 57(2):237–52. Epub 1975/03/01. PMID: 1089671.

18. Kolar P, Gaber T, Perka C, Duda GN, Buttgereit F. Human early fracture hematoma is characterized by inflammation and hypoxia. Clin Orthop Relat Res. 2011; 469(11):3118–26. Epub 2011/03/17. https://doi.org/10.1007/s11999-011-1865-3 PMID: 21409457; PubMed Central PMCID: PMCPMC3183184.

19. Schmidt-Bleek K, Schell H, Kolar P, Pfaff M, Perka C, Buttgereit F, et al. Cellular composition of the initial fracture hematoma compared to a muscle hematoma: a study in sheep. Journal of orthopaedic research: official publication of the Orthopaedic Research Society. 2009; 27(9):1147–51. Epub 2009/04/22. https://doi.org/10.1002/jor.20901 PMID: 19382195.

20. Martini L, Fini M, Giavaresi G, Giardino R. Sheep model in orthopedic research: a literature review. Comparative medicine. 2001; 51(4):292–9. Epub 2002/04/02. PMID: 11924786.

21. Holstein JH, Garcia P, Histing T, Kristen A, Scheuer C, Menger MD, et al. Advances in the establishment of defined mouse models for the study of fracture healing and bone regeneration. Journal of orthopaedic trauma. 2009; 23(5 Suppl):S31–8. Epub 2009/04/29. https://doi.org/10.1097/BOT.0b013e31819f27e5 PMID: 19390374.

22. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(9):3507–12. Epub 2013/02/13. https://doi.org/10.1073/pnas.1222878110 PMID: 23401516; PubMed Central PMCID: PMCPMC3587220.

23. Pearce AI, Richards RG, Milz S, Schneider E, Pearce SG. Animal models for implant biomaterial research in bone: a review. European cells & materials. 2007; 13:1–10. Epub 2007/03/06. PMID: 17334975.

24. Marsell R, Einhorn TA. The biology of fracture healing. Injury. 2011; 42(6):551–5. Epub 04/13. https://doi.org/10.1016/j.injury.2011.03.031 PMID: 21489527.

25. Hoff P, Gaber T, Strehl C, Jakstadt M, Hoff H, Schmidt-Bleek K, et al. A Pronounced Inflammatory Activity Characterizes the Early Fracture Healing Phase in Immunologically Restricted Patients. International journal of molecular sciences. 2017; 18(3). Epub 2017/03/12. https://doi.org/10.3390/ijms18030583 PMID: 28282868; PubMed Central PMCID: PMCPMC5372599.

26. Klenke S, Renckhoff K, Engler A, Peters J, Frey UH. Easy-to-use strategy for reference gene selection in quantitative real-time PCR experiments. Naunyn-Schmiedeberg's archives of pharmacology. 2016; 389(12):1353–66. Epub 2016/09/22. https://doi.org/10.1007/s00210-016-1305-8 PMID: 27650728.

27. Baddela VS, Baufeld A, Yenuganti VR, Vanselow J, Singh D. Suitable housekeeping genes for normalization of transcript abundance analysis by real-time RT-PCR in cultured bovine granulosa cells during hypoxia and differential cell plating density. Reproductive biology and endocrinology: RB&E. 2014; 12:118. Epub 2014/11/29. https://doi.org/10.1186/1477-7827-12-118 PMID: 25430436; PubMed Central PMCID: PMCPMC4280684.

28. Kawamoto T, Kawamoto K. Preparation of thin frozen sections from nonfixed and undecalcified hard tissues using Kawamot's film method (2012). Methods in molecular biology (Clifton, NJ). 2014; 1130:149–64. Epub 2014/02/01. https://doi.org/10.1007/978-1-62703-989-5_11 PMID: 24482171.

29. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006; 8(4):315–7. https://doi.org/10.1080/14653240600855905 WOS:000239953200002. PMID: 16923606

30. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284(5411):143–7. https://doi.org/10.1126/science.284.5411.143 WOS:000079509000053. PMID: 10102814

31. De Schauwer C, van de Walle GR, Piepers S, Hoogewijs MK, Govaere JL, Meyer E, et al. Successful isolation of equine mesenchymal stromal cells from cryopreserved umbilical cord blood-derived mononuclear cell fractions. Equine veterinary journal. 2013; 45(4):518–22. Epub 2012/12/05. https://doi.org/10.1111/evj.12003 PMID: 23206252.

32. Maia L, Landim-Alvarenga FC, Da Mota LS, De Assis Golim M, Laufer-Amorim R, De Vita B, et al. Immunophenotypic, immunocytochemistry, ultrastructural, and cytogenetic characterization of mesenchymal stem cells from equine bone marrow. Microscopy research and technique. 2013; 76(6):618–24. Epub 2013/03/28. https://doi.org/10.1002/jemt.22208 PMID: 23533133.

33. Radtke CL, Nino-Fong R, Gonzalez BPE, Stryhn H, McDuffee LA. Characterization and osteogenic potential of equine muscle tissue- and periosteal tissue-derived mesenchymal stem cells in comparison with bone marrow- and adipose tissue derived mesenchymal stem cells. American journal of veterinary research. 2013; 74(5):790–800. WOS:000318203500017. https://doi.org/10.2460/ajvr.74.5.790 PMID: 23627394

34. Ranera B, Remacha AR, Alvarez-Arguedas S, Romero A, Vazquez FJ, Zaragoza P, et al. Effect of hypoxia on equine mesenchymal stem cells derived from bone marrow and adipose tissue. BMC veterinary

research. 2012; 8:142. Epub 2012/08/24. https://doi.org/10.1186/1746-6148-8-142 PMID: 22913590; PubMed Central PMCID: PMCPMC3483288.

35. Barberini DJ, Freitas NPP, Magnoni MS, Maia L, Listoni AJ, Heckler MC, et al. Equine mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord: immunophenotypic characterization and differentiation potential. Stem Cell Research & Therapy. 2014; 5(1):25–. https://doi.org/10.1186/scrt414 PMC4055040. PMID: 24559797

36. Paebst F, Piehler D, Brehm W, Heller S, Schroeck C, Tarnok A, et al. Comparative immunophenotyping of equine multipotent mesenchymal stromal cells: an approach toward a standardized definition. Cytometry Part A: the journal of the International Society for Analytical Cytology. 2014; 85(8):678–87. Epub 2014/06/05. https://doi.org/10.1002/cyto.a.22491 PMID: 24894974.

37. Treonze KM, Alves K, Fischer P, Hagmann WK, Hora D, Kulick A, et al. Characterization of alpha(4) beta(1) (CD49d/CD29) on equine leukocytes: potential utility of a potent alpha(4)beta(1) (CD49d/CD29) receptor antagonist in the treatment of equine heaves (recurrent airway obstruction). Veterinary immunology and immunopathology. 2009; 130(1–2):79–87. Epub 2009/03/03. https://doi.org/10.1016/j.vetimm.2009.01.011 PMID: 19250687.

38. De Schauwer C, Piepers S, Van de Walle GR, Demeyere K, Hoogewijs MK, Govaere JL, et al. In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. Cytometry Part A: the journal of the International Society for Analytical Cytology. 2012; 81(4):312–23. Epub 2012/03/14. https://doi.org/10.1002/cyto.a.22026 PMID: 22411893.

39. Yeo WM, Osterrieder N, Stokol T. Equine herpesvirus type 1 infection induces procoagulant activity in equine monocytes. Veterinary research. 2013; 44:16. Epub 2013/03/19. https://doi.org/10.1186/1297-9716-44-16 PMID: 23497076; PubMed Central PMCID: PMCPMC3618259.

40. Keramaris NC, Kaptanis S, Moss HL, Loppini M, Pneumaticos S, Maffulli N. Endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) in bone healing. Current stem cell research & therapy. 2012; 7(4):293–301. Epub 2012/05/09. PMID: 22563666.

41. Knight MN, Hankenson KD. Mesenchymal Stem Cells in Bone Regeneration. Adv Wound Care (New Rochelle). 2013; 2(6):306–16. Epub 2014/02/15. https://doi.org/10.1089/wound.2012.0420 PMID: 24527352; PubMed Central PMCID: PMCPMC3842877.

42. Kovach TK, Dighe AS, Lobo PI, Cui Q. Interactions between MSCs and immune cells: implications for bone healing. Journal of immunology research. 2015; 2015:752510. Epub 2015/05/23. https://doi.org/10.1155/2015/752510 PMID: 26000315; PubMed Central PMCID: PMCPMC4427002.

43. Wagegg M, Gaber T, Lohanatha FL, Hahne M, Strehl C, Fangradt M, et al. Hypoxia promotes osteogenesis but suppresses adipogenesis of human mesenchymal stromal cells in a hypoxia-inducible factor-1 dependent manner. PloS one. 2012; 7(9):e46483. Epub 2012/10/03. https://doi.org/10.1371/journal.pone.0046483 PMID: 23029528; PubMed Central PMCID: PMCPMC3459928.

44. Kovtun A, Bergdolt S, Wiegner R, Radermacher P, Huber-Lang M, Ignatius A. The crucial role of neutrophil granulocytes in bone fracture healing. European cells & materials. 2016; 32:152–62. Epub 2016/07/28. PMID: 27452963.

45. Grogaard B, Gerdin B, Reikeras O. THE POLYMORPHONUCLEAR LEUKOCYTE—HAS IT A ROLE IN FRACTURE-HEALING. Arch Orthop Trauma Surg. 1990; 109(5):268–71. https://doi.org/10.1007/bf00419942 WOS:A1990DV58200007. PMID: 2271360

46. Chung R, Cool JC, Scherer MA, Foster BK, Xian CJ. Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats. Journal of leukocyte biology. 2006; 80(6):1272–80. Epub 2006/09/09. https://doi.org/10.1189/jlb.0606365 PMID: 16959896.

47. Hoff P, Maschmeyer P, Gaber T, Schutze T, Raue T, Schmidt-Bleek K, et al. Human immune cells' behavior and survival under bioenergetically restricted conditions in an in vitro fracture hematoma model. Cellular & molecular immunology. 2013; 10(2):151–8. Epub 2013/02/12. https://doi.org/10.1038/cmi.2012.56 PMID: 23396474; PubMed Central PMCID: PMCPMC4003042.

48. Soehnlein O, Lindbom L, Weber C. Mechanisms underlying neutrophil-mediated monocyte recruitment. Blood. 2009; 114(21):4613–23. Epub 2009/08/22. https://doi.org/10.1182/blood-2009-06-221630 PMID: 19696199.

49. Toben D, Schroeder I, El Khassawna T, Mehta M, Hoffmann JE, Frisch JT, et al. Fracture Healing Is Accelerated in the Absence of the Adaptive Immune System. J Bone Miner Res. 2011; 26(1):113–24. https://doi.org/10.1002/jbmr.185 WOS:000286035600014. PMID: 20641004

50. Reinke S, Geissler S, Taylor WR, Schmidt-Bleek K, Juelke K, Schwachmeyer V, et al. Terminally differentiated CD8(+) T cells negatively affect bone regeneration in humans. Science translational medicine. 2013; 5(177):177ra36. Epub 2013/03/22. https://doi.org/10.1126/scitranslmed.3004754 PMID: 23515078.

51. de Silly RV, Ducimetiere L, Maroun CY, Dietrich PY, Derouazi M, Walker PR. Phenotypic switch of CD8(+) T cells reactivated under hypoxia toward IL-10 secreting, poorly proliferative effector cells. Eur J

Immunol. 2015; 45(8):2263–75. https://doi.org/10.1002/eji.201445284 WOS:000359674200012. PMID: 25929785

52. De Miguel MP, Fuentes-Julian S, Blazquez-Martinez A, Pascual CY, Aller MA, Arias J, et al. Immunosuppressive properties of mesenchymal stem cells: advances and applications. Current molecular medicine. 2012; 12(5):574–91. Epub 2012/04/21. PMID: 22515979.

53. Faiella W, Atoui R. Immunotolerant Properties of Mesenchymal Stem Cells: Updated Review. Stem cells international. 2016; 2016:1859567. Epub 2016/02/04. https://doi.org/10.1155/2016/1859567 PMID: 26839557; PubMed Central PMCID: PMCPMC4709780.

54. Duffy MM, Ritter T, Ceredig R, Griffin MD. Mesenchymal stem cell effects on T-cell effector pathways. Stem Cell Res Ther. 2011; 2(4):34. Epub 2011/08/25. https://doi.org/10.1186/scrt75 PMID: 21861858; PubMed Central PMCID: PMCPMC3219065.

55. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 2007; 110 (10):3499–506. Epub 2007/08/01. https://doi.org/10.1182/blood-2007-02-069716 PMID: 17664353.

56. Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. Journal of immunology (Baltimore, Md: 1950). 2003; 171(7):3426–34. Epub 2003/09/23. PMID: 14500637.

57. Robey IF, Lien AD, Welsh SJ, Baggett BK, Gillies RJ. Hypoxia-Inducible Factor-1α and the Glycolytic Phenotype in Tumors. Neoplasia (New York, NY). 2005; 7(4):324–30. PMC1501147.

58. Ito K, Suda T. Metabolic requirements for the maintenance of self-renewing stem cells. Nature reviews Molecular cell biology. 2014; 15(4):243–56. Epub 2014/03/22. https://doi.org/10.1038/nrm3772 PMID: 24651542; PubMed Central PMCID: PMCPMC4095859.

59. Gaber T, Dziurla R, Tripmacher R, Burmester GR, Buttgereit F. Hypoxia inducible factor (HIF) in rheumatology: low O2! See what HIF can do! Annals of the rheumatic diseases. 2005; 64(7):971–80. Epub 2005/04/01. https://doi.org/10.1136/ard.2004.031641 PMID: 15800008; PubMed Central PMCID: PMCPMC1755583.

60. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annual review of cell and developmental biology. 1999; 15:551–78. Epub 1999/12/28. https://doi.org/10.1146/annurev.cellbio.15.1.551 PMID: 10611972.

61. Liu Y, Berendsen AD, Jia S, Lotinun S, Baron R, Ferrara N, et al. Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. The Journal of clinical investigation. 2012; 122 (9):3101–13. Epub 2012/08/14. https://doi.org/10.1172/JCI61209 PMID: 22886301; PubMed Central PMCID: PMCPMC3428080.

62. Berendsen AD, Olsen BR. How vascular endothelial growth factor-A (VEGF) regulates differentiation of mesenchymal stem cells. The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society. 2014; 62(2):103–8. Epub 2013/12/07. https://doi.org/10.1369/0022155413516347 PMID: 24309509; PubMed Central PMCID: PMCPMC3902099.

63. Kottstorfer J, Kaiser G, Thomas A, Gregori M, Kecht M, Domaszewski F, et al. The influence of non-osteogenic factors on the expression of M-CSF and VEGF during fracture healing. Injury. 2013; 44 (7):930–4. Epub 2013/04/11. https://doi.org/10.1016/j.injury.2013.02.028 PMID: 23570706.

64. Ochman S, Frey S, Raschke MJ, Deventer JN, Meffert RH. Local application of VEGF compensates callus deficiency after acute soft tissue trauma—results using a limb-shortening distraction procedure in rabbit tibia. Journal of orthopaedic research: official publication of the Orthopaedic Research Society. 2011; 29(7):1093–8. Epub 2011/02/02. https://doi.org/10.1002/jor.21340 PMID: 21284032.

65. Wang CJ, Huang KE, Sun YC, Yang YJ, Ko JY, Weng LH, et al. VEGF modulates angiogenesis and osteogenesis in shockwave-promoted fracture healing in rabbits. The Journal of surgical research. 2011; 171(1):114–9. Epub 2010/05/11. https://doi.org/10.1016/j.jss.2010.01.045 PMID: 20452608.

66. Schmidt-Bleek K, Schell H, Lienau J, Schulz N, Hoff P, Pfaff M, et al. Initial immune reaction and angiogenesis in bone healing. Journal of tissue engineering and regenerative medicine. 2014; 8(2):120–30. Epub 2012/04/13. https://doi.org/10.1002/term.1505 PMID: 22495762.

67. Kobayashi T, Onodera S, Kondo E, Tohyama H, Fujiki H, Yokoyama A, et al. Impaired fracture healing in macrophage migration inhibitory factor-deficient mice. Osteoporosis international: a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2011; 22(6):1955–65. Epub 2010/09/15. https://doi.org/10.1007/s00198-010-1385-0 PMID: 20838768.

68. Onodera S, Nishihira J, Yamazaki M, Ishibashi T, Minami A. Increased expression of macrophage migration inhibitory factor during fracture healing in rats. Histochemistry and cell biology. 2004; 121 (3):209–17. Epub 2004/02/10. https://doi.org/10.1007/s00418-004-0624-x PMID: 14767776.

69. Liu CH, Hwang SM. Cytokine interactions in mesenchymal stem cells from cord blood. Cytokine. 2005; 32(6):270–9. Epub 2005/12/27. https://doi.org/10.1016/j.cyto.2005.11.003 PMID: 16377203.

70. Xia W, Xie C, Jiang M, Hou M. Improved survival of mesenchymal stem cells by macrophage migration inhibitory factor. Molecular and cellular biochemistry. 2015; 404(1–2):11–24. Epub 2015/02/24. https://doi.org/10.1007/s11010-015-2361-y PMID: 25701358; PubMed Central PMCID: PMCPMC4544672.

Appendix A

Table A. List of antibodies with their specific reactivity and the dilution used to characterize the cell composition of equine blood and to further characterize equine mesenchymal stromal cells. Cells declared as non-specific were excluded from our study.

Antibody	Reactivity	Dilution	Comments	Refs.
Rat anti horse CD3:FITC, clone CD3-12, 1 mg/ml, Abcam plc.	horse-specific	1:20	specific binding, intracellular binding	[1]
Mouse anti horse CD8:PE, clone CVS21, 1 mg/ml, Bio-Rad AbD Serotec GmbH	horse-specific	1:20	specific binding	[2]
Mouse anti horse CD4:FITC, clone CVS4, 1 mg/ml, Bio-Rad AbD Serotec GmbH	horse-specific	1:500	specific binding	[2]
Mouse anti horse CD14:APC, clone 105, unknown conc., Cornell University	horse-specific	1:2.000	specific binding	[3]
Mouse anti equine Granulocytes:VioBlue, clone HACT39A, 1mg/ml, Kingfisher Biotech Inc.	horse-specific	1:1.200	specific binding, conjugation in house	-
Mouse anti horse CD16:PerCP, clone KD1, 1 mg/ml, Abcam plc.	horse-specific	-	non-specific binding, conjugation in house	[4]
Mouse anti human CD21, clone Bu33, 1 mg/ml Bio-Rad AbD Serotec GmbH	cross-reactive	-	non-specific binding	[5]
Mouse anti horse pan B-Cells:RPE, clone CVS36, 1 mg/ml Bio-Rad AbD Serotec GmbH	horse-specific	-	non-specific binding	-
Mouse anti human CD105:Alexa Fluor® 488, clone SN6, 1 mg/ml Bio-Rad AbD Serotec GmbH	cross-reactive	1:40	specific binding	[6]
Mouse anti human Integrin beta1/CD29:PE, clone 419127, 25 µg/ml, Bio-Techne GmbH	cross-reactive	1:20	specific binding	[7]
Mouse anti horse CD44:RPE, clone CVS18, 1 mg/ml, Bio-Rad AbD Serotec GmbH	horse-specific	-	non-specific binding	[8]
Mouse anti horse CD90:DyLight 405®, clone NS1, 1 mg/ml, Abcam plc.	horse-specific	(-)	specific binding, conjugation in house	[9]

Table B. Depicted is the data of the equine donors both for the blood clots and the *in vitro* fracture hematoma models. For the blood clots the blood of all 4 donors was coagulated (n=4). For the creation of the *in vitro* FH models either blood 1, 2 or 3 was mixed according to the protocol as described above with the MSC of the donor MSC1 (n=3).

Sample Name/Number	Age (years)	Sex	Race
Blood 1	6	mare	Trotter
Blood 2	14	gelding	Trotter
Blood 3	26	mare	Arabian horse
Blood 4	22	gelding	Icelander
MSC 1	13	gelding	Riding horse

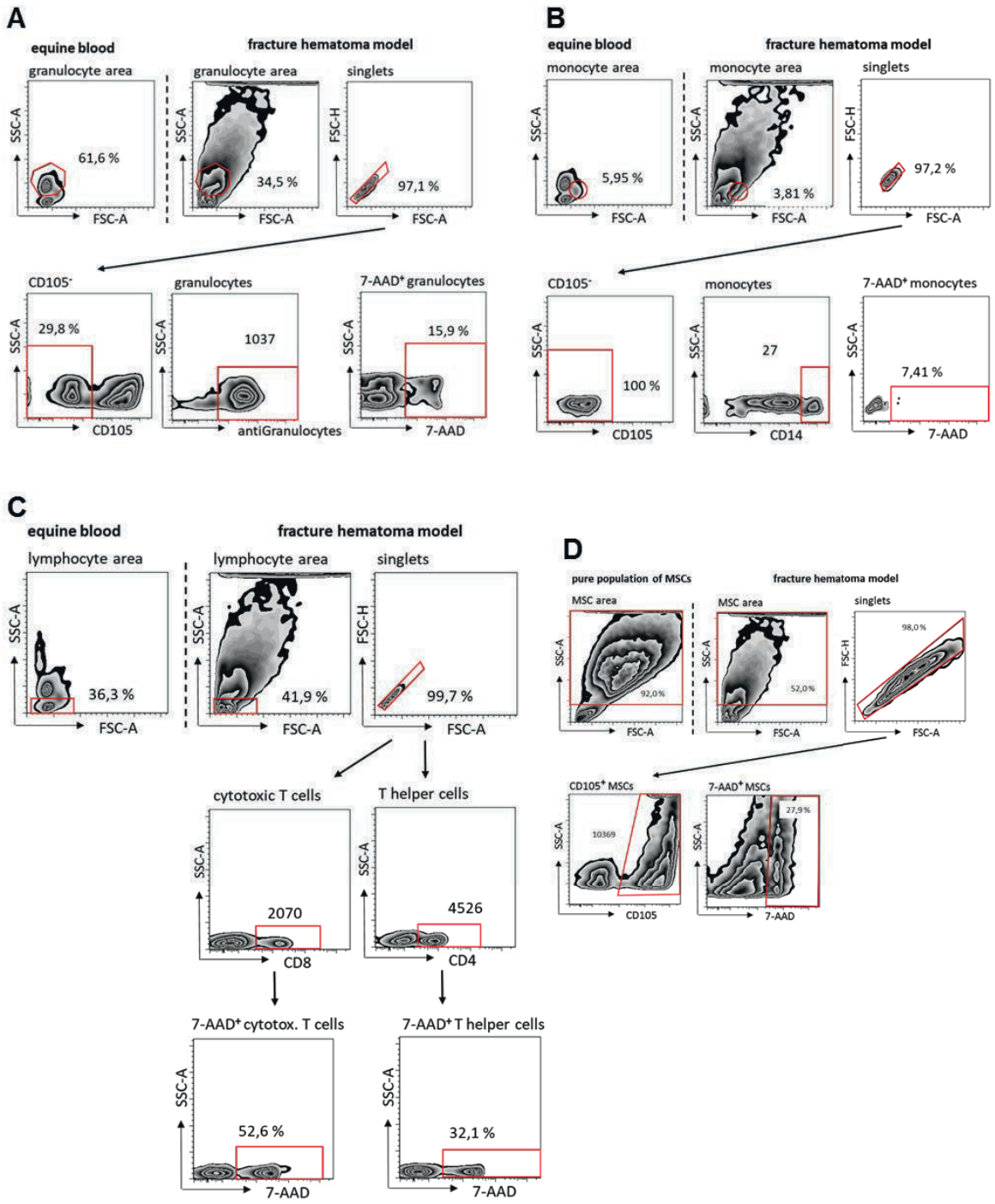

Fig A. Gating strategy for the flow cytometric characterization of cells (MSCs, granulocytes, monocytes, CD4+, and CD8+ cells), within the blood coagulates as well as in the *in vitro* FH models. (A) Gating strategy for the granulocyte population concerning their granularity and size, as well as the anti-granulocyte marker and the absence of the surface marker CD105 (to exclude MSCs). (B) Gating strategy of the monocyte population regarding the expression of CD14 and the lack of expression of CD105. (C) Gating of the lymphocyte fraction and concerning their typical surface markers T-helper-cells (CD4+) and cytotoxic T-cells (CD8+). (D) Gating of the MSC population regarding the typical surface marker CD105.

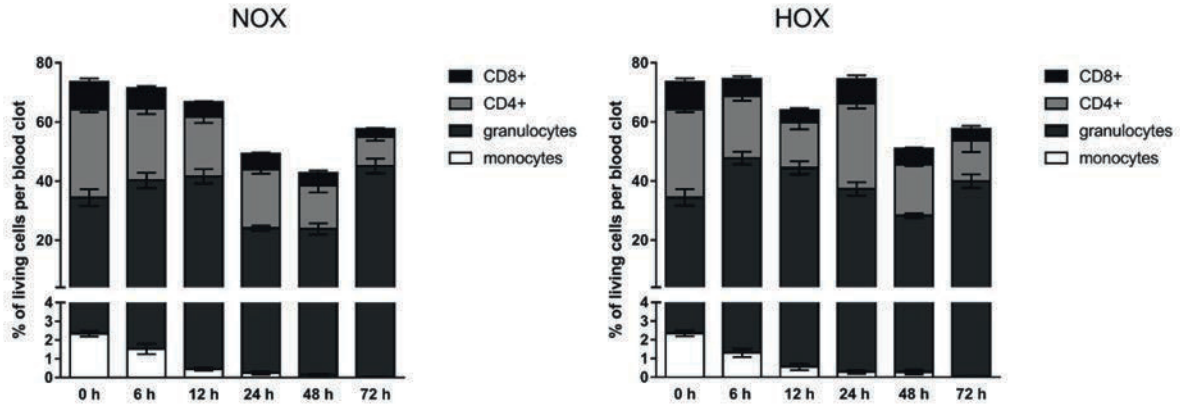

Fig B. Frequency of immune cell populations in blood clots without MSC. Frequency of granulocytes, CD14+ monocytes, CD4+ T cells, CD8+ T cells negative for 7-AAD present in the blood clots as incubated in osteogenic differentiation medium under either hypoxic or normoxic conditions (37 °C, 5% CO2, 18%/ 1% O_2) for 6, 12, 24, 48, and 72 h (mean ± SEM, n = 4).

References

1. Jones M, Cordell JL, Beyers AD, Tse AG, Mason DY. Detection of T and B cells in many animal species using cross-reactive anti-peptide antibodies. Journal of immunology (Baltimore, Md : 1950). 1993;150(12):5429-35. Epub 1993/06/15. PubMed PMID: 8515069.

2. Lunn DP, Holmes MA, Duffus WP. Three monoclonal antibodies identifying antigens on all equine T lymphocytes, and two mutually exclusive T-lymphocyte subsets. Immunology. 1991;74(2):251-7. Epub 1991/10/01. PubMed PMID: 1748472; PubMed Central PMCID: PMCPMC1384601.

3. Yeo WM, Osterrieder N, Stokol T. Equine herpesvirus type 1 infection induces procoagulant activity in equine monocytes. Veterinary research. 2013;44:16. Epub 2013/03/19. doi: 10.1186/1297-9716-44-16. PubMed PMID: 23497076; PubMed Central PMCID: PMCPMC3618259.

4. Elhmouzi-Younes J, Boysen P, Pende D, Storset AK, Le Vern Y, Laurent F, et al. Ovine CD16+/CD14- blood lymphocytes present all the major characteristics of natural killer cells. Veterinary research. 2010;41(1):4. Epub 2009/09/17. doi: 10.1051/vetres/2009052. PubMed PMID: 19755096; PubMed Central PMCID: PMCPMC2762129.

5. Mayall S, Siedek E, Hamblin AS. The anti-human CD21 antibody, BU33, identifies equine B cells. Journal of comparative pathology. 2001;124(1):83-7. Epub 2001/06/29. doi: 10.1053/jcpa.2000.0425. PubMed PMID: 11428193.

6. Braun J, Hack A, Weis-Klemm M, Conrad S, Treml S, Kohler K, et al. Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. American journal of veterinary research. 2010;71(10):1228-36. Epub 2010/10/06. doi: 10.2460/ajvr.71.10.1228. PubMed PMID: 20919912.

7. Treonze KM, Alves K, Fischer P, Hagmann WK, Hora D, Kulick A, et al. Characterization of alpha(4)beta(1) (CD49d/CD29) on equine leukocytes: potential utility of a potent alpha(4)beta(1) (CD49d/CD29) receptor antagonist in the treatment of equine heaves (recurrent airway obstruction). Veterinary immunology and immunopathology. 2009;130(1-2):79-87. Epub 2009/03/03. doi: 10.1016/j.vetimm.2009.01.011. PubMed PMID: 19250687.

8. De Schauwer C, Piepers S, Van de Walle GR, Demeyere K, Hoogewijs MK, Govaere JL, et al. In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. Cytometry Part A : the journal of the International Society for Analytical Cytology. 2012;81(4):312-23. Epub 2012/03/14. doi: 10.1002/cyto.a.22026. PubMed PMID: 22411893.

9. Marycz K, Kornicka K, Maredziak M, Golonka P, Nicpon J. Equine metabolic syndrome impairs adipose stem cells osteogenic differentiation by predominance of autophagy over selective mitophagy. Journal of cellular and molecular medicine. 2016;20(12):2384-404. Epub 2016/09/16. doi: 10.1111/jcmm.12932. PubMed PMID: 27629697; PubMed Central PMCID: PMCPMC5134411.

3.2 The *in vitro* human fracture hematoma model - a tool for preclinical drug testing

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Research Article

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

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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* (HIF-1; *HIF1A*) (Gross et al., 2005; Li et al., 2004). Furthermore, *matrix metalloproteinase2* (*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, distinctly 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

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granulocyte macrophage colony-stimulating factor (GM-CSF), *interleukin6* (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-Jawhari 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 Puleo, 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-Jawhari 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 adaptation 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 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-specific evolutionary pressure and as a result molecular and anatomical differences have to be considered carefully. This includes the lack of the Haversian system, a microtubule system, which provides the space for blood vessels and nerves to grow in (Bagi et al., 2011; Jowsey, 1966).

We have previously studied the cell composition, RNA and cytokine profile of human fracture hematoma obtained from patients (Hoff et al., 2016a; Hoff et al., 2013; Kolar et al., 2011b; Kolar et al., 2010). Based on these findings, we here developed a human *in vitro* model of the fracture hematoma, in order (i) to mimic the initial phase of fracture healing, (ii) to contextualize the obtained data to existing *ex vivo* and *in vivo* data and (iii) to use this system to analyze the impact of drugs/therapies 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 cm^2 cell tissue flask (Greiner Bio-one, Kremsmünster, Austria) and incubated in DMEM + GlutaMAX™ (Gibco, Carlsbad, USA) supplemented with 10% FCS (Biowest, 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% CO_2, 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). 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 $1x10^4$ 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

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given in Fig. S1A-D[1]). 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 H_2O_{dd} for 15 min at RT to visualize calcium deposition for microscopy (Fig. S1[1]).
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. S2[1]).

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 S2[1]).

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 allogenic combination with whole blood which was immediately processed. To this end, 2.5 x 10^5 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 $CaCl_2$ (solved in PBS). 100 µL of allogenic EDTA-blood was added and gently mixed by pipetting. After 30 min of incubation at 37 °C, 5% CO_2, the coagulated *in vitro* FH models were transferred into DMEM + GlutaMAX™ supplemented with 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin, 0.2% β-glycerophosphate (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% CO_2 and 1% O_2) or normoxia (37 °C, 5% CO_2 and 18% O_2) 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, Tuttlingen, Germany) and flushed with nitrogen. Normoxic and hypoxic conditions were constantly monitored using incubators equipped with CO_2–sensors and O_2 –sensors (Binder, Tuttlingen, 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

Donor	Cell type	Age	Sex	FH model	*Impact of hypoxia	*Impact of DFO/DEX
B1	blood	44	m	MSC1, MSC2	FC, GE, CCS	-
B2	blood	23	m	MSC1, MSC2	FC, GE, CCS	-
B3	blood	27	m	MSC1, MSC2	FC, GE, CCS	-
MSC1	bmMSCs	77	m	B1, B2, B3	FC, GE, CCS	-
MSC2	bmMSCs	75	m	B1, B2, B3	FC, GE, CCS	-
B4	blood	37	m	MSC3, MSC4	-	FC, GE, CCS
B5	blood	26	m	MSC3, MSC4	-	FC, GE, CCS
B6	blood	38	m	MSC3, MSC4	-	FC, GE, CCS
MSC3	bmMSCs	72	m	B4, B5, B6	-	FC, GE, CCS
MSC4	bmMSCs	62	m	B4, B5, B6	-	FC, GE, CCS

*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 two times 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 $KHCO_3$, 0.155 M NH_4Cl, 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 (ah) 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% NaN_3 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. S3[1].

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Tab. 2: Dilution for the antibodies used for the characterization of immune cells and MSCs

Antibody	Marker for	Manufacturer	Catalog Number	Species of origin	Dilution
Monoclonal anti-hCD3	T cells	Miltenyi Biotech	130-113-139	REAfinity™	1:100
Monoclonal anti-hCD4	T helper cells	Miltenyi Biotech	130-113-223	REAfinity™	1:100
Monoclonal anti-hCD8	Cytotoxic T cells	Miltenyi Biotech	130-110-684	REAfinity™	1:100
Monoclonal anti-hCD14	Monocytes	Miltenyi Biotech	130-110-521	REAfinity™	1:100
Monoclonal anti-hCD19	B cells	Miltenyi Biotech	130-113-649	REAfinity™	1:100
Monoclonal anti-hCD45	Pan-Leukocytes	Miltenyi Biotech	130-110-633	REAfinity™	1:50
Monoclonal anti-hCD73	MSCs	BioLegend®	3444004	Mouse	1:20
Monoclonal anti-hCD90	MSCs	BioLegend®	328114	Mouse	1:20

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 Transcription 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-α* (*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 (Curtis 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 used were purchased from TIB Molbiol (Berlin, Germany) and are listed in Tab. 3.

Tab. 3: Primers used

Gene symbol	Gene name	Forward primer	Reverse primer
SPP1	Secreted phosphoprotein 1	GCCGAGGTGATAGTGTGGTT	TGAGGTGATGTCCTCGTCGTCTG
VEGFA	Vascular endothelial growth factor A	AGCCTTGCCTTGCTGCTCTA	GTGCTGGCCTTGGTGAGG
RUNX2	Runt-related transcription factor 2	TTACTTACACCCCGCCAGTC	TATGGAGTGCTGCTGGTCTG
EF1A	Elongation factor 1-alpha	GTTGATATGGTTCCTGGCAAGC	TTGCCAGCTCCAGCAGCCT
MMP2	Matrix metalloproteinase-2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC
IL8	Interleukin 8	GGACCCCAAGGAAAACTGG	CAACCCTACAACAGACCCACAC
IL6	Interleukin 6	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTT
PGK1	Phosphoglycerate kinase 1	ATGGATGAGGTGGTGAAAGC	CAGTGCTCACATGGCTGACT
LDHA	Lactate dehydrogenase A	ACCCAGTTTCCACCATGATT	CCCAAAATGCAAGGAACACT
MMP9	Matrix metalloproteinase-9	CCTGGAGACCTGAGAACCAATC	CCACCCGAGTGTAACCATAGC
HIF1A	Hypoxia-inducible factor 1-alpha	CCATTAGAAAGCAGTTCCGC	TGGGTAGGAGATGGAGATGC

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-13 (7.21 pg/mL), IL-17 (24.44 pg/mL), interferon-gamma (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.

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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[1], 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% O_2, which is the average oxygen level measured in the incubator at 5% CO_2 and high humidity flushed with nitrogen) as compared to normoxia (18% O_2, which is the average oxygen level measured in the incubator at 5% CO_2 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 hypoxic 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.

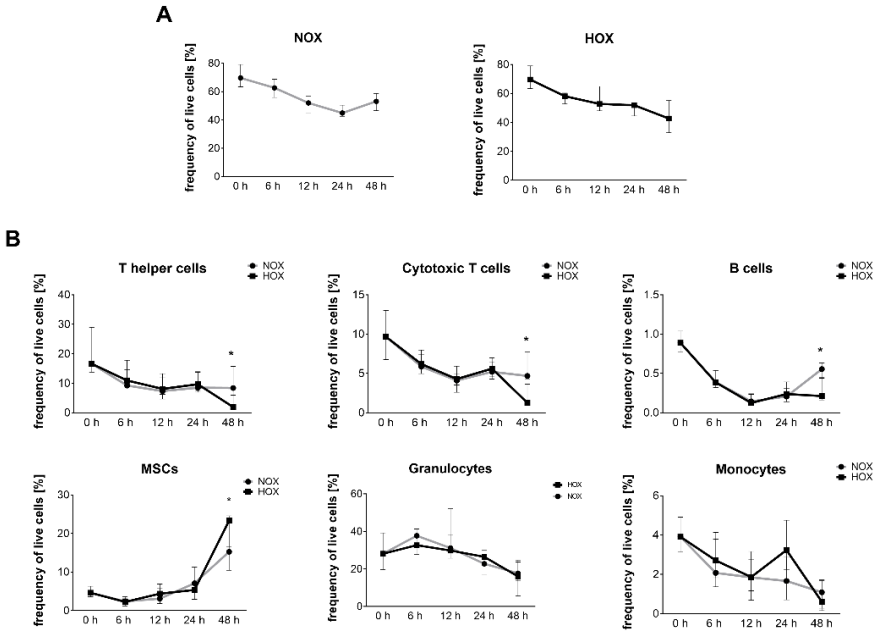

Fig. 1: Hypoxia favors survival of MSCs while T- and B-cell fractions significantly decreased

(A) Frequency of total cells negative for 7-AAD in the FH cultured in osteogenic differentiation medium under normoxic (NOX; 18% O_2) or hypoxic conditions (HOX; 1% O_2) 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).

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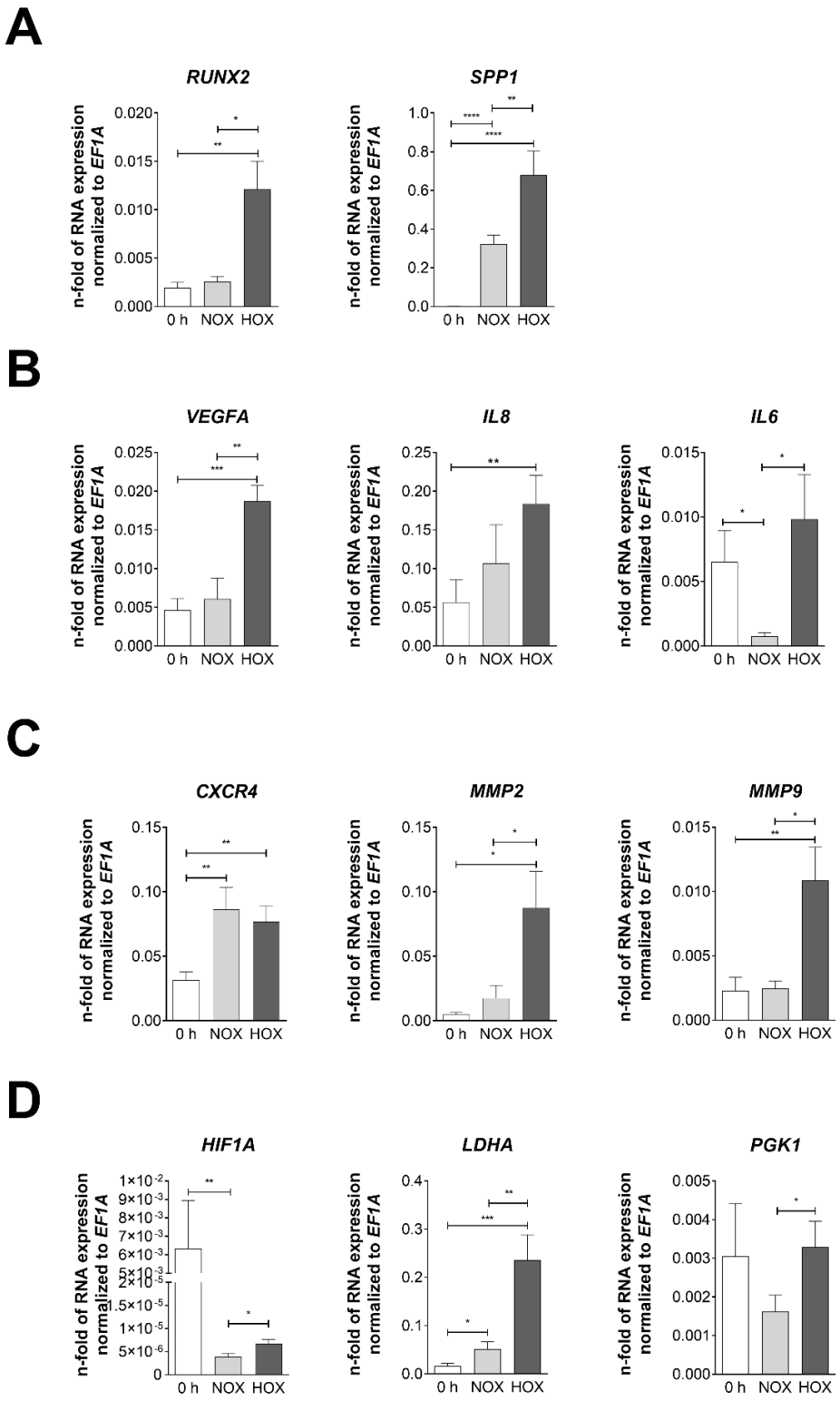

Fig. 2: Within the *in vitro* FHs, osteogenic, angiogenic, inflammatory, migration and metabolic markers were significantly upregulated after 48 h of incubation, more pronounced under hypoxia
Depicted is the relative RNA-expression of the osteogenic markers *RUNX2* and *SPP1* (A), the angiogenic/inflammatory 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).

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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, IL8, 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[1]), 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 ($p_{RUNX2} = 0.006$, $p_{SPP1} < 0.0001$) (Fig. 2A). Only *SPP1* was significantly induced after 48 h incubation under normoxic conditions ($p_{SPP1} < 0.0001$) but not *RUNX2*. Both osteogenic marker genes demonstrate a significant increase in gene expression under reduced oxygen availability ($p_{RUNX2} = 0.023$, $p_{SPP1} = 0.009$).

The angiogenic marker *VEGFA* was significantly up-regulated after 48 h incubation under hypoxia ($p_{VEGFA} = 0.0003$) 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 ($p_{VEGFA} = 0.0049$). The pro-inflammatory and pro-angiogenic *IL8* was significantly up-regulated under hypoxia when compared to normoxia ($p_{IL8} = 0.0017$), 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 ($p_{IL6} = 0.0138$), but remained unaltered after 48 h incubation under hypoxic conditions, resulting in a significant higher expression under hypoxic as compared to normoxic conditions after 48 h of incubation ($p_{IL6} = 0.03$; Fig. 2B).

The *CXCR4* transcript encodes the information of 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 ($p_{normoxia} = 0.005$; $p_{hypoxia} = 0.002$; 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 ($p_{MMP2}=0.0197$, $p_{MMP9} = 0.002$; Fig. 2C). Both genes were significantly higher expressed when comparing normoxia *vs.* hypoxia ($p_{MMP2} = 0.0244$, $p_{MMP9} = 0.0137$).

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 ($p_{LDHA} = 0.008$, $p_{PGK1} = 0.031$, $p_{HIF1A}= 0.027$; 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.

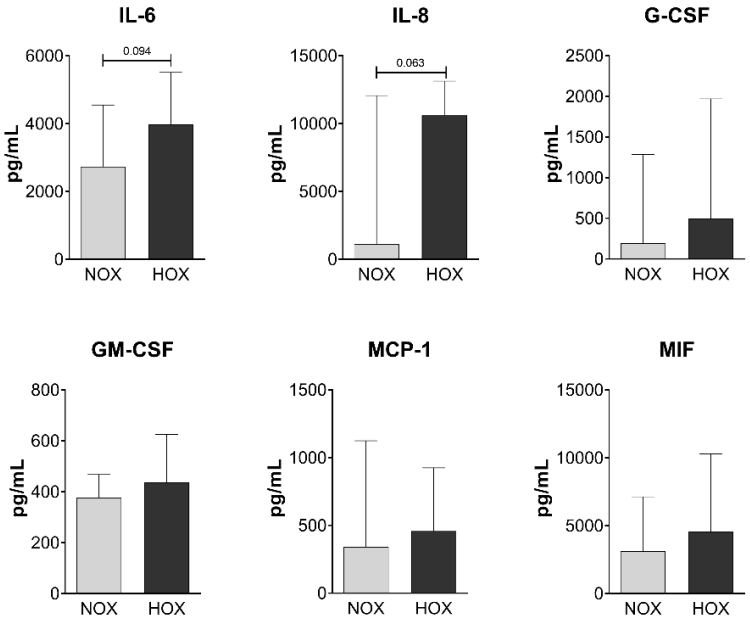

Fig. 3: The secretion of angiogenic as well as pro-inflammatory cytokines/chemokines was induced
In vitro FHs were incubated under normoxia (NOX) or hypoxia (HOX) for 48 h in osteogenic medium. Depicted is the concentration (median ± range, n=6) of secreted protein [pg/mL] in the supernatant. Statistical analysis via Wilcoxon signed rank test. p values indicate statistical trends (p<0.1).

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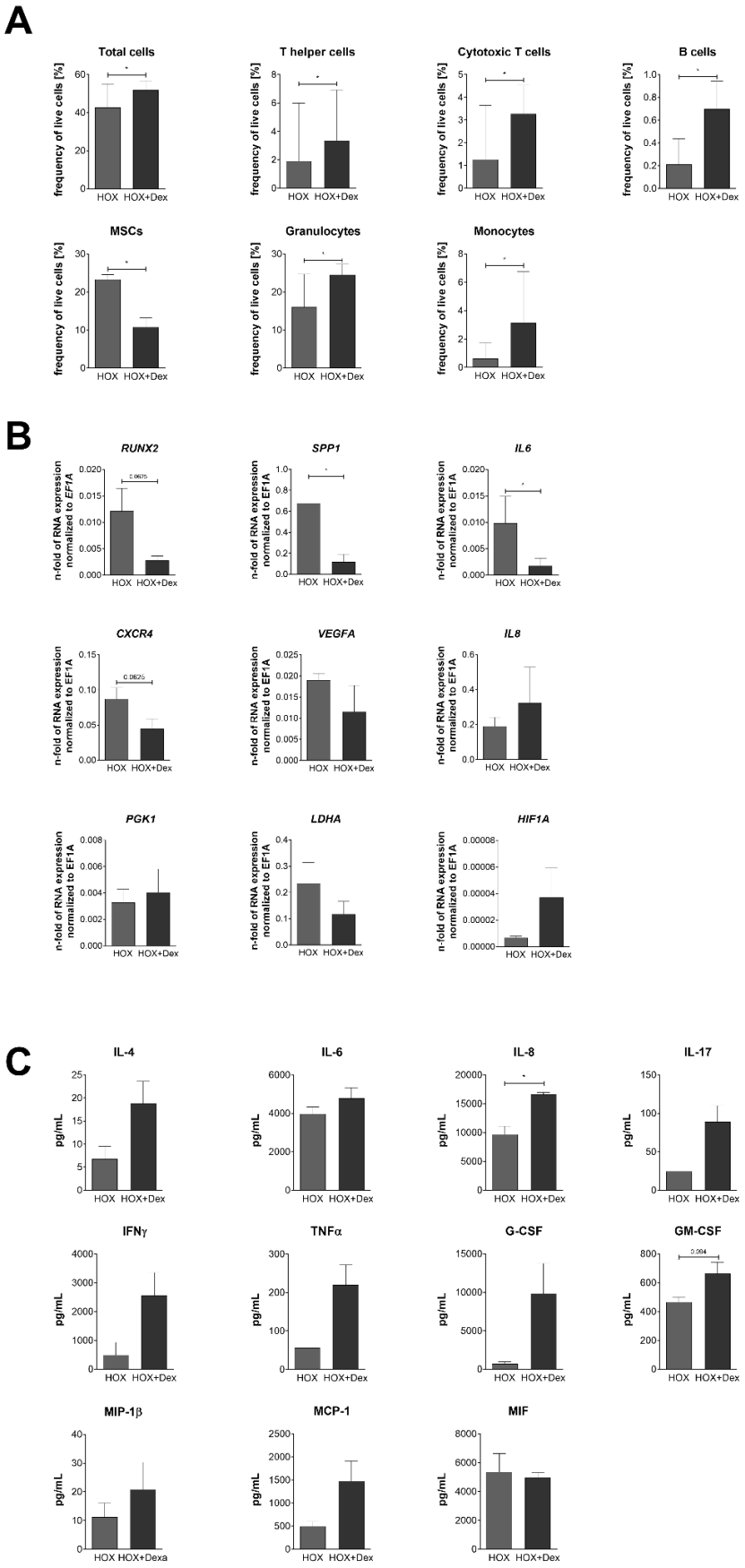

Fig. 4: Dexamethasone favored the survival of immune cells over MSCs, suppressed osteogenesis and enhanced the secretion of inflammatory cytokines

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).

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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, IL-17, IFNγ, TNFα, MCP-1, MIP-1β, G-CSF, and GM-CSF (listed in section 2.7). While IL1-β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, IL-17, TNFα 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 ($p_{IL\text{-}6} = 0.094$, $p_{IL\text{-}8} = 0.063$), 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^{-5} M DEX but not at clinically relevant dose of 10^{-7} M (Fig. S7[1]). 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, TNFα, 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 demonstrate a higher abundance 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 (Fig. 5) or left them untreated (treatment under hypoxic conditions are shown in Fig. S5[1]).

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 ($p_{PGK1} = 0.04$, $p_{HIF1A} = 0.03$). 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

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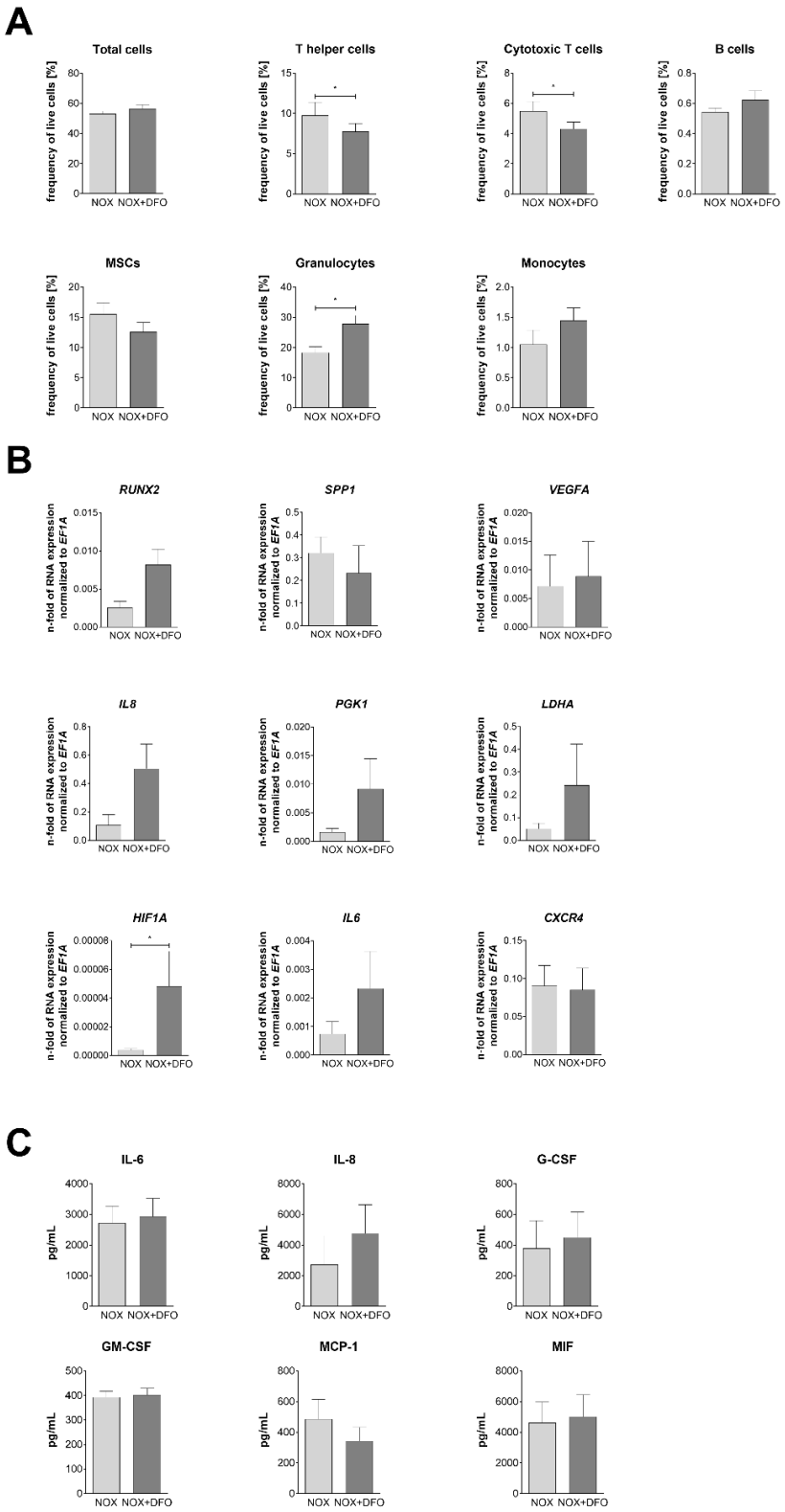

Fig. 5: DFO significantly increased the frequency of granulocytes, decreased the frequency of T cells and enhanced the expression of hypoxia-related and inflammatory markers
In vitro FH models were incubated under normoxia or normoxia with the supplementation of 250 µM DFO (dark grey bars) for 48 h in 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).

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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, Groogard et al. (1990) did not observe any impact of neutropenia 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 osteoblast/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 (Ankrum 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[1]).

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).

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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)[#]

Gene symbol	Importance in the fracture healing process	*in vitro* FH model (hypoxia for 48 h)	*ex vivo* primary human FHs (< 72 h)
RUNX2	key regulator that directs mesenchymal stromal cells towards the osteoblastic lineage (Vimalraj et al., 2015; Komori, 2010)	↑**	↑
SPP1	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).	↑****	↑*
VEGFA	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).	↑***	↑*
IL8	responsible for the activation and differentiation of leukocytes in an inflammatory environment (Herman et al., 2008)	↑**	↑***
IL6	key cytokines in the initial phase of fracture healing (Cassuto et al., 2018)	↑	↑***
CXCR4	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)	↑**	↑*
LDHA	(hypoxia-mediated) marker of inducted glycolysis, contributes to acidic pH, HIF-target gene (Semenza, 1998; Gaber et al., 2005)	↑***	↑**
PGK1	(hypoxia-mediated) marker of inducted glycolysis (Semenza, 1998; Gaber et al., 2005)	↑	↑
HIF1A	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)	↓	↑**
MMP2	remodeling of extracellular matrix, crucial for the survival of bone cells and vasculogenesis / angiogenesis and fracture healing (Stamenkovic, 2003; Paiva and Granjeiro, 2017; Varghese, 2006; Cui et al., 2017; Lieu et al., 2011)	↑*	n.a.
MMP9	remodeling of extracellular matrix, crucial for the survival of bone cells and vasculogenesis/angiogenesis and fracture healing (Stamenkovic, 2003; Paiva and Granjeiro, 2017; Varghese, 2006; Cui et al., 2017; Colnot et al., 2003)	↑**	n.a.

[#]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).

Cell type	CD surface marker	*in vitro* FH model (hypoxia for 48 h)	*ex vivo* primary human FHs (< 72 h)
T cells	CD45+CD3+	3.7% (2.4-10.6%)	8.2% (1.1-39.4%)
T helper cells	CD45+CD3+CD4+	1.9% (1.1-6%)	3.1% (1.4-15.4%)
Cytotoxic T cells	CD45+CD3+CD8+	1.3% (0.9-2.5%)	1.9% (0.2-12.6%)
Monocytes	Scatter and CD45+CD14+	0.6% (0.3-1.7%)	4.9% (0.1-38.5%)
B cells	CD45+CD3-CD19+	0.25% (0.2-0.4%)	0.9% (0.1-5.8%)
MSCs	CD45-CD73+CD90+	23.4% (16.6-24.5%)	n.a.
Granulocytes	Scatter and CD45+ vs. CD16+	16.1% (5.6-24.7%)	64.2% (0.8-94%)
Not assigned cells	n. a.	37.1% (27.6-45.8%)	12.2% (1.2-85.6%)

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).

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 (Fig. 4). Although DEX is well-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, 1996) as indicated also in the *in vitro* FH model by a reduced osteogenic gene expression (*RUNX2*, *SPP1*). We and other have also reported that DEX suppresses the hypoxia-induced HIF-target gene expression (Wu et al., 2014; Gaber

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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 prolyl 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[1]). 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 enhancement of inflammation 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

Ankrum, J. A., Ong, J. F. and Karp, J. M. (2014). Mesenchymal stem cells: Immune evasive, not immune privileged. *Nat Biotechnol 32*, 252-260. doi:10.1038/nbt.2816

ALTEX preprint
published June 9, 2020
doi:10.14573/altex.1910211

Annamalai, R. T., Turner, P. A., Carson, W. F. t. et al. (2018). Harnessing macrophage-mediated degradation of gelatin microspheres for spatiotemporal control of bmp2 release. *Biomaterials 161*, 216-227. doi:10.1016/j.biomaterials.2018.01.040

Bagi, C. M., Berryman, E. and Moalli, M. R. (2011). Comparative bone anatomy of commonly used laboratory animals: Implications for drug discovery. *Comparative medicine 61*, 76-85. doi:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3060425/pdf/cm2011000076.pdf

Baht, G. S., Vi, L. and Alman, B. A. (2018). The role of the immune cells in fracture healing. *Current osteoporosis reports 16*, 138-145. doi:10.1007/s11914-018-0423-2

Bastian, O. W., Koenderman, L., Alblas, J. et al. (2016). Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury. *Clin Immunol 164*, 78-84. doi:10.1016/j.clim.2016.02.001

Beamer, B., Hettrich, C. and Lane, J. (2010). Vascular endothelial growth factor: An essential component of angiogenesis and fracture healing. *Hss j 6*, 85-94. doi:10.1007/s11420-009-9129-4

Bocelli-Tyndall, C., Bracci, L., Spagnoli, G. et al. (2007). Bone marrow mesenchymal stromal cells (bm-mscs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes in vitro. *Rheumatology (Oxford) 46*, 403-408. doi:10.1093/rheumatology/kel267

Braham, M. V. J., Li Yim, A. S. P., Garcia Mateos, J. et al. (2019). A human hematopoietic niche model supporting hematopoietic stem and progenitor cells in vitro. *Adv Healthc Mater 8*, e1801444. doi:10.1002/adhm.201801444

Buron, F., Perrin, H., Malcus, C. et al. (2009). Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: An in vitro study using human cells. *Transplant Proc 41*, 3347-3352. doi:10.1016/j.transproceed.2009.08.030

Campbell, D. J., Kim, C. H. and Butcher, E. C. (2003). Chemokines in the systemic organization of immunity. *Immunol Rev 195*, 58-71. doi:10.1034/j.1600-065X.2003.00067.x

Canalis, E. (1996). Clinical review 83: Mechanisms of glucocorticoid action in bone: Implications to glucocorticoid-induced osteoporosis. *J Clin Endocrinol Metab 81*, 3441-3447. doi:10.1210/jcem.81.10.8855781

Canalis, E. (2003). Mechanisms of glucocorticoid-induced osteoporosis. *Curr Opin Rheumatol 15*, 454-457. doi:10.1186/ar1372

Cassuto, J., Folestad, A., Gothlin, J. et al. (2018). The key role of proinflammatory cytokines, matrix proteins, rankl/opg and wnt/beta-catenin in bone healing of hip arthroplasty patients. *Bone 107*, 66-77. doi:10.1016/j.bone.2017.11.004

Chen, X., Gan, Y., Li, W. et al. (2014). The interaction between mesenchymal stem cells and steroids during inflammation. *Cell death & disease 5*, e1009-e1009. doi:10.1038/cddis.2013.537

Claes, L., Recknagel, S. and Ignatius, A. (2012). Fracture healing under healthy and inflammatory conditions. *Nat Rev Rheumatol 8*, 133-143. doi:10.1038/nrrheum.2012.1

Colnot, C., Thompson, Z., Miclau, T. et al. (2003). Altered fracture repair in the absence of mmp9. *Development 130*, 4123-4133. doi:10.1242/dev.00559

Cramer, T., Yamanishi, Y., Clausen, B. E. et al. (2003). Hif-1alpha is essential for myeloid cell-mediated inflammation. *Cell 112*, 645-657. doi:10.1016/S0092-8674(03)00154-5

Cui, N., Hu, M. and Khalil, R. A. (2017). Biochemical and biological attributes of matrix metalloproteinases. *Prog Mol Biol Transl Sci 147*, 1-73. doi:10.1016/bs.pmbts.2017.02.005

Curtis, K. M., Gomez, L. A., Rios, C. et al. (2010). Ef1α and rpl13a represent normalization genes suitable for rt-qpcr analysis of bone marrow derived mesenchymal stem cells. *BMC Molecular Biology 11*, 61. doi:10.1186/1471-2199-11-61

Dengler, V. L., Galbraith, M. and Espinosa, J. M. (2014). Transcriptional regulation by hypoxia inducible factors. *Critical reviews in biochemistry and molecular biology 49*, 1-15. doi:10.3109/10409238.2013.838205

Denhardt, D. T. and Noda, M. (1998). Osteopontin expression and function: Role in bone remodeling. *J Cell Biochem Suppl 30-31*, 92-102. doi:10.1002/(SICI)1097-4644(1998)72:30/31+<92::AID-JCB13>3.0.CO;2-A

Dominici, M., Le Blanc, K., Mueller, I. et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy 8*, 315-317. doi:10.1080/14653240600855905

Donneys, A., Farberg, A. S., Tchanque-Fossuo, C. N. et al. (2012). Deferoxamine enhances the vascular response of bone regeneration in mandibular distraction osteogenesis. *Plastic and reconstructive surgery 129*, 850-856. doi:10.1097/PRS.0b013e31824422f2

Donneys, A., Ahsan, S., Perosky, J. E. et al. (2013a). Deferoxamine restores callus size, mineralization, and mechanical strength in fracture healing after radiotherapy. *Plastic and reconstructive surgery 131*, 711e-719e. doi:10.1097/PRS.0b013e3182865c57

Donneys, A., Ahsan, S., Perosky, J. E. et al. (2013b). Deferoxamine restores callus size, mineralization, and mechanical strength in fracture healing after radiotherapy. *Plast Reconstr Surg 131*, 711e-719e. doi:10.1097/PRS.0b013e3182865c57

Donneys, A., Weiss, D. M., Deshpande, S. S. et al. (2013c). Localized deferoxamine injection augments vascularity and improves bony union in pathologic fracture healing after radiotherapy. *Bone 52*, 318-325. doi:10.1016/j.bone.2012.10.014

Donneys, A., Nelson, N. S., Page, E. E. et al. (2015). Targeting angiogenesis as a therapeutic means to reinforce osteocyte survival and prevent nonunions in the aftermath of radiotherapy. *Head Neck 37*, 1261-1267. doi:10.1002/hed.23744

Donneys, A., Nelson, N. S., Perosky, J. E. et al. (2016). Prevention of radiation-induced bone pathology through combined pharmacologic cytoprotection and angiogenic stimulation. *Bone 84*, 245-252. doi:10.1016/j.bone.2015.12.051

Drager, J., Sheikh, Z., Zhang, Y. L. et al. (2016). Local delivery of iron chelators reduces in vivo remodeling of a calcium phosphate bone graft substitute. *Acta Biomater 42*, 411-419. doi:10.1016/j.actbio.2016.07.037

Drager, J., Ramirez-GarciaLuna, J. L., Kumar, A. et al. (2017). (*) hypoxia biomimicry to enhance monetite bone defect repair. *Tissue Eng Part A 23*, 1372-1381. doi:10.1089/ten.TEA.2016.0526

ALTEX preprint
published June 9, 2020
doi:10.14573/altex.1910211

El-Jawhari, J. J., Jones, E. and Giannoudis, P. V. (2016). The roles of immune cells in bone healing; what we know, do not know and future perspectives. *Injury 47*, 2399-2406. doi:10.1016/j.injury.2016.10.008

El Khassawna, T., Serra, A., Bucher, C. H. et al. (2017). T lymphocytes influence the mineralization process of bone. *Front Immunol 8*, 562. doi:10.3389/fimmu.2017.00562

Farberg, A. S., Jing, X. L., Monson, L. A. et al. (2012). Deferoxamine reverses radiation induced hypovascularity during bone regeneration and repair in the murine mandible. *Bone 50*, 1184-1187. doi:10.1016/j.bone.2012.01.019

Farberg, A. S., Sarhaddi, D., Donneys, A. et al. (2014). Deferoxamine enhances bone regeneration in mandibular distraction osteogenesis. *Plastic and reconstructive surgery 133*, 666-671. doi:10.1097/01.prs.0000438050.36881.a9

Foldager, C. B., Munir, S., Ulrik-Vinther, M. et al. (2009). Validation of suitable house keeping genes for hypoxia-cultured human chondrocytes. *BMC molecular biology 10*, 94-94. doi:10.1186/1471-2199-10-94

Frenkel, B., White, W. and Tuckermann, J. (2015). Glucocorticoid-induced osteoporosis. *Adv Exp Med Biol 872*, 179-215. doi:10.1007/978-1-4939-2895-8_8

Gaber, T., Dziurla, R., Tripmacher, R. et al. (2005). Hypoxia inducible factor (hif) in rheumatology: Low o2! See what hif can do! *Ann Rheum Dis 64*, 971-980. doi:10.1136/ard.2004.031641

Gaber, T., Schellmann, S., Erekul, K. B. et al. (2011). Macrophage migration inhibitory factor counterregulates dexamethasone-mediated suppression of hypoxia-inducible factor-1 alpha function and differentially influences human cd4+ t cell proliferation under hypoxia. *J Immunol 186*, 764-774. doi:10.4049/jimmunol.0903421

Gaston, M. S. and Simpson, A. H. (2007). Inhibition of fracture healing. *J Bone Joint Surg Br 89*, 1553-1560. doi:10.1302/0301-620x.89b12.19671

Gieseke, F., Schutt, B., Viebahn, S. et al. (2007). Human multipotent mesenchymal stromal cells inhibit proliferation of pbmcs independently of ifngammar1 signaling and ido expression. *Blood 110*, 2197-2200. doi:10.1182/blood-2007-04-083162

Gomez-Barrena, E., Rosset, P., Lozano, D. et al. (2015). Bone fracture healing: Cell therapy in delayed unions and nonunions. *Bone 70*, 93-101. doi:10.1016/j.bone.2014.07.033

Granero-Molto, F., Weis, J. A., Miga, M. I. et al. (2009). Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells 27*, 1887-1898. doi:10.1002/stem.103

Grogaard, B., Gerdin, B. and Reikeras, O. (1990). The polymorphonuclear leukocyte: Has it a role in fracture healing? *Arch Orthop Trauma Surg 109*, 268-271. doi:10.1007/BF00419942

Gross, T. S., King, K. A., Rabaia, N. A. et al. (2005). Upregulation of osteopontin by osteocytes deprived of mechanical loading or oxygen. *J Bone Miner Res 20*, 250-256. doi:10.1359/jbmr.041004

Grosso, A., Burger, M. G., Lunger, A. et al. (2017). It takes two to tango: Coupling of angiogenesis and osteogenesis for bone regeneration. *Front Bioeng Biotechnol 5*, 68. doi:10.3389/fbioe.2017.00068

Grundnes, O. and Reikeras, O. (1993). The importance of the hematoma for fracture healing in rats. *Acta Orthop Scand 64*, 340-342. doi:10.3109/17453679308993640

Guzey, S., Aykan, A., Ozturk, S. et al. (2016). The effects of desferroxamine on bone and bone graft healing in critical-size bone defects. *Ann Plast Surg 77*, 560-568. doi:10.1097/sap.0000000000000679

Haque, N., Rahman, M. T., Abu Kasim, N. H. et al. (2013). Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy. *ScientificWorldJournal 2013*, 632972. doi:10.1155/2013/632972

Hare, J. M., Fishman, J. E., Gerstenblith, G. et al. (2012). Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: The poseidon randomized trial. *JAMA 308*, 2369-2379. doi:10.1001/jama.2012.25321

Henle, P., Zimmermann, G. and Weiss, S. (2005). Matrix metalloproteinases and failed fracture healing. *Bone 37*, 791-798. doi:10.1016/j.bone.2005.06.015

Herman, S., Kronke, G. and Schett, G. (2008). Molecular mechanisms of inflammatory bone damage: Emerging targets for therapy. *Trends Mol Med 14*, 245-253. doi:10.1016/j.molmed.2008.04.001

Hileti, D., Panayiotidis, P. and Hoffbrand, A. V. (1995). Iron chelators induce apoptosis in proliferating cells. *Br J Haematol 89*, 181-187. doi:10.1111/j.1365-2141.1995.tb08927.x

Hoff, P., Gaber, T., Schmidt-Bleek, K. et al. (2011). Immunologically restricted patients exhibit a pronounced inflammation and inadequate response to hypoxia in fracture hematomas. *Immunol Res 51*, 116-122. doi:10.1007/s12026-011-8235-9

Hoff, P., Maschmeyer, P., Gaber, T. et al. (2013). Human immune cells' behavior and survival under bioenergetically restricted conditions in an in vitro fracture hematoma model. *Cell Mol Immunol 10*, 151-158. doi:10.1038/cmi.2012.56

Hoff, P., Gaber, T., Strehl, C. et al. (2016a). Immunological characterization of the early human fracture hematoma. *Immunol Res 64*, 1195-1206. doi:10.1007/s12026-016-8868-9

Hoff, P., Gaber, T., Strehl, C. et al. (2016b). Immunological characterization of the early human fracture hematoma. *Immunologic Research 64*, 1195-1206. doi:10.1007/s12026-016-8868-9

Jowsey, J. (1966). Studies of haversian systems in man and some animals. *J Anat 100*, 857-864. doi:https://wbrg.net/images/stories/references/jowsey_1966_haversian_systems.pdf

Kang, H., Yan, Y., Jia, P. et al. (2016). Desferrioxamine reduces ultrahigh-molecular-weight polyethylene-induced osteolysis by restraining inflammatory osteoclastogenesis via heme oxygenase-1. *Cell death & disease 7*, e2435-e2435. doi:10.1038/cddis.2016.339

Knight, M. N. and Hankenson, K. D. (2013). Mesenchymal stem cells in bone regeneration. *Adv Wound Care (New Rochelle) 2*, 306-316. doi:10.1089/wound.2012.0420

Kolar, P., Schmidt-Bleek, K., Schell, H. et al. (2010). The early fracture hematoma and its potential role in fracture healing. *Tissue Eng Part B Rev 16*, 427-434. doi:10.1089/ten.TEB.2009.0687

Kolar, P., Gaber, T., Perka, C. et al. (2011a). Human early fracture hematoma is characterized by inflammation and hypoxia. *Clin Orthop Relat Res 469*, 3118-3126. doi:10.1007/s11999-011-1865-3

ALTEX preprint
published June 9, 2020
doi:10.14573/altex.1910211

Kolar, P., Gaber, T., Perka, C. et al. (2011b). Human early fracture hematoma is characterized by inflammation and hypoxia. *Clinical orthopaedics and related research 469*, 3118-3126. doi:10.1007/s11999-011-1865-3

Komori, T. (2010). Regulation of osteoblast differentiation by runx2. *Adv Exp Med Biol 658*, 43-49. doi:10.1007/978-1-4419-1050-9_5

Konnecke, I., Serra, A., El Khassawna, T. et al. (2014). T and b cells participate in bone repair by infiltrating the fracture callus in a two-wave fashion. *Bone 64*, 155-165. doi:10.1016/j.bone.2014.03.052

Kovach, T. K., Dighe, A. S., Lobo, P. I. et al. (2015). Interactions between mscs and immune cells: Implications for bone healing. *J Immunol Res 2015*, 752510. doi:10.1155/2015/752510

Kovtun, A., Bergdolt, S., Wiegner, R. et al. (2016). The crucial role of neutrophil granulocytes in bone fracture healing. *Eur Cell Mater 32*, 152-162. doi:10.22203/ecm.v032a10

Krzywinska, E. and Stockmann, C. (2018). Hypoxia, metabolism and immune cell function. *Biomedicines 6*, doi:10.3390/biomedicines6020056

Kunkel, E. J. and Butcher, E. C. (2002). Chemokines and the tissue-specific migration of lymphocytes. *Immunity 16*, 1-4. doi:10.1016/S1074-7613(01)00261-8

Kusumbe, A. P., Ramasamy, S. K. and Adams, R. H. (2014). Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature 507*, 323-328. doi:10.1038/nature13145

Le Blanc, K., Tammik, L., Sundberg, B. et al. (2003). Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol 57*, 11-20. doi:10.1046/j.1365-3083.2003.01176.x

Le Blanc, K. and Davies, L. C. (2015). Mesenchymal stromal cells and the innate immune response. *Immunology Letters 168*, 140-146. doi:10.1016/j.imlet.2015.05.004

Lee, J., Byeon, J. S., Lee, K. S. et al. (2016). Chondrogenic potential and anti-senescence effect of hypoxia on canine adipose mesenchymal stem cells. *Vet Res Commun 40*, 1-10. doi:10.1007/s11259-015-9647-0

Lennon, D. P., Edmison, J. M. and Caplan, A. I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: Effects on in vitro and in vivo osteochondrogenesis. *J Cell Physiol 187*, 345-355. doi:10.1002/jcp.1081

Li, J., Fan, L., Yu, Z. et al. (2015). The effect of deferoxamine on angiogenesis and bone repair in steroid-induced osteonecrosis of rabbit femoral heads. *Exp Biol Med (Maywood) 240*, 273-280. doi:10.1177/1535370214553906

Li, P., Oparil, S., Feng, W. et al. (2004). Hypoxia-responsive growth factors upregulate periostin and osteopontin expression via distinct signaling pathways in rat pulmonary arterial smooth muscle cells. *J Appl Physiol (1985) 97*, 1550-1558; discussion 1549. doi:10.1152/japplphysiol.01311.2003

Lieu, S., Hansen, E., Dedini, R. et al. (2011). Impaired remodeling phase of fracture repair in the absence of matrix metalloproteinase-2. *Dis Model Mech 4*, 203-211. doi:10.1242/dmm.006304

Liu, W., Shen, S.-M., Zhao, X.-Y. et al. (2012). Targeted genes and interacting proteins of hypoxia inducible factor-1. *International journal of biochemistry and molecular biology 3*, 165-178. http://www.ijbmb.org/files/IJBMB1203001.pdf

Liu, X., Zhou, C., Li, Y. et al. (2013). Sdf-1 promotes endochondral bone repair during fracture healing at the traumatic brain injury condition. *PLoS One 8*, e54077. doi:10.1371/journal.pone.0054077

Liu, X., Tu, Y., Zhang, L. et al. (2014). Prolyl hydroxylase inhibitors protect from the bone loss in ovariectomy rats by increasing bone vascularity. *Cell Biochem Biophys 69*, 141-149. doi:10.1007/s12013-013-9780-8

Liu, Y. Z., Akhter, M. P., Gao, X. et al. (2018). Glucocorticoid-induced delayed fracture healing and impaired bone biomechanical properties in mice. *Clin Interv Aging 13*, 1465-1474. doi:10.2147/CIA.S167431

Luo, Y., He, D. L., Ning, L. et al. (2006). Over-expression of hypoxia-inducible factor-1alpha increases the invasive potency of lncap cells in vitro. *BJU Int 98*, 1315-1319. doi:10.1111/j.1464-410X.2006.06480.x

Madrigal, M., Rao, K. S. and Riordan, N. H. (2014). A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J Transl Med 12*, 260. doi:10.1186/s12967-014-0260-8

Martin, D., Galisteo, R. and Gutkind, J. S. (2009). Cxcl8/il8 stimulates vascular endothelial growth factor (vegf) expression and the autocrine activation of vegfr2 in endothelial cells by activating nfkappab through the cbm (carma3/bcl10/malt1) complex. *J Biol Chem 284*, 6038-6042. doi:10.1074/jbc.C800207200

Matsumoto, T. and Sato, S. (2015). Stimulating angiogenesis mitigates the unloading-induced reduction in osteogenesis in early-stage bone repair in rats. *Physiol Rep 3*, doi:10.14814/phy2.12335

Mestas, J. and Hughes, C. C. (2004). Of mice and not men: Differences between mouse and human immunology. *J Immunol 172*, 2731-2738. doi:10.4049/jimmunol.172.5.2731

Mizuno, K., Mineo, K., Tachibana, T. et al. (1990). The osteogenetic potential of fracture haematoma. Subperiosteal and intramuscular transplantation of the haematoma. *J Bone Joint Surg Br 72*, 822-829. doi:10.1302/0301-620X.72B5.2211764

Nauta, A. J. and Fibbe, W. E. (2007). Immunomodulatory properties of mesenchymal stromal cells. *Blood 110*, 3499-3506. doi:10.1182/blood-2007-02-069716

O'Toole, E. A., van Koningsveld, R., Chen, M. et al. (2008). Hypoxia induces epidermal keratinocyte matrix metalloproteinase-9 secretion via the protein kinase c pathway. *J Cell Physiol 214*, 47-55. doi:10.1002/jcp.21160

Paiva, K. B. S. and Granjeiro, J. M. (2017). Matrix metalloproteinases in bone resorption, remodeling, and repair. *Prog Mol Biol Transl Sci 148*, 203-303. doi:10.1016/bs.pmbts.2017.05.001

Perlman, R. L. (2016). Mouse models of human disease: An evolutionary perspective. *Evolution, medicine, and public health 2016*, 170-176. doi:10.1093/emph/eow014

Pfeiffenberger, M., Bartsch, J., Hoff, P. et al. (2019). Hypoxia and mesenchymal stromal cells as key drivers of initial fracture healing in an equine in vitro fracture hematoma model. *PLOS ONE 14*, e0214276. doi:10.1371/journal.pone.0214276

ALTEX preprint
published June 9, 2020
doi:10.14573/altex.1910211

Potian, J. A., Aviv, H., Ponzio, N. M. et al. (2003). Veto-like activity of mesenchymal stem cells: Functional discrimination between cellular responses to alloantigens and recall antigens. *J Immunol 171*, 3426-3434. doi:10.4049/jimmunol.171.7.3426

Qu, Z. H., Zhang, X. L., Tang, T. T. et al. (2008). Promotion of osteogenesis through beta-catenin signaling by desferrioxamine. *Biochem Biophys Res Commun 370*, 332-337. doi:10.1016/j.bbrc.2008.03.092

Reinke, S., Geissler, S., Taylor, W. R. et al. (2013). Terminally differentiated cd8(+) t cells negatively affect bone regeneration in humans. *Sci Transl Med 5*, 177ra136. doi:10.1126/scitranslmed.3004754

Rozier, P., Maria, A., Goulabchand, R. et al. (2018a). Mesenchymal stem cells in systemic sclerosis: Allogenic or autologous approaches for therapeutic use? *Front Immunol 9*, 2938. doi:10.3389/fimmu.2018.02938

Rozier, P., Maria, A., Goulabchand, R. et al. (2018b). Mesenchymal stem cells in systemic sclerosis: Allogenic or autologous approaches for therapeutic use? *Frontiers in immunology 9*, 2938-2938. doi:10.3389/fimmu.2018.02938

Sato, S., Kim, T., Arai, T. et al. (1986). Comparison between the effects of dexamethasone and indomethacin on bone wound healing. *Jpn J Pharmacol 42*, 71-78. doi:10.1254/jjp.42.71

Sawin, P. D., Dickman, C. A., Crawford, N. R. et al. (2001). The effects of dexamethasone on bone fusion in an experimental model of posterolateral lumbar spinal arthrodesis. *J Neurosurg 94*, 76-81. doi:10.3171/spi.2001.94.1.0076

Schallmoser, K. and Strunk, D. (2009). Preparation of pooled human platelet lysate (phpl) as an efficient supplement for animal serum-free human stem cell cultures. *Journal of visualized experiments : JoVE* 1523. doi:10.3791/1523

Schindeler, A., McDonald, M. M., Bokko, P. et al. (2008). Bone remodeling during fracture repair: The cellular picture. *Semin Cell Dev Biol 19*, 459-466. doi:10.1016/j.semcdb.2008.07.004

Schipani, E., Maes, C., Carmeliet, G. et al. (2009). Regulation of osteogenesis-angiogenesis coupling by hifs and vegf. *J Bone Miner Res 24*, 1347-1353. doi:10.1359/jbmr.090602

Semenza, G. L. (1998). Hypoxia-inducible factor 1: Master regulator of o2 homeostasis. *Curr Opin Genet Dev 8*, 588-594. doi:10.1016/S0959-437X(98)80016-6

Seok, J., Warren, H. S., Cuenca, A. G. et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A 110*, 3507-3512. doi:10.1073/pnas.1222878110

Shen, X., Wan, C., Ramaswamy, G. et al. (2009). Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice. *J Orthop Res 27*, 1298-1305. doi:10.1002/jor.20886

Sila-Asna, M., Bunyaratvej, A., Maeda, S. et al. (2007). Osteoblast differentiation and bone formation gene expression in strontium-inducing bone marrow mesenchymal stem cell. *Kobe J Med Sci 53*, 25-35. http://www.med.kobe-u.ac.jp/journal/contents/53/25.pdf

Soehnlein, O., Lindbom, L. and Weber, C. (2009). Mechanisms underlying neutrophil-mediated monocyte recruitment. *Blood 114*, 4613-4623. doi:10.1182/blood-2009-06-221630

Stamenkovic, I. (2003). Extracellular matrix remodelling: The role of matrix metalloproteinases. *J Pathol 200*, 448-464. doi:10.1002/path.1400

Stewart, R., Goldstein, J., Eberhardt, A. et al. (2011). Increasing vascularity to improve healing of a segmental defect of the rat femur. *J Orthop Trauma 25*, 472-476. doi:10.1097/BOT.0b013e31822588d8

Strehl, C., Ehlers, L., Gaber, T. et al. (2019). Glucocorticoids-all-rounders tackling the versatile players of the immune system. *Front Immunol 10*, 1744. doi:10.3389/fimmu.2019.01744

Tachibana, T., Matsubara, T., Mizuno, K. et al. (1991). Enhancement of new bone formation by hematoma at fracture site. *Nihon Seikeigeka Gakkai Zasshi 65*, 349-358.

Takao, K. and Miyakawa, T. (2015). Genomic responses in mouse models greatly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A 112*, 1167-1172. doi:10.1073/pnas.1401965111

Thomas, M. V. and Puleo, D. A. (2011). Infection, inflammation, and bone regeneration: A paradoxical relationship. *J Dent Res 90*, 1052-1061. doi:10.1177/0022034510393967

Toben, D., Schroeder, I., El Khassawna, T. et al. (2011). Fracture healing is accelerated in the absence of the adaptive immune system. *J Bone Miner Res 26*, 113-124. doi:10.1002/jbmr.185

Varghese, S. (2006). Matrix metalloproteinases and their inhibitors in bone: An overview of regulation and functions. *Front Biosci 11*, 2949-2966. doi:10.2741/2024

Vimalraj, S., Arumugam, B., Miranda, P. J. et al. (2015). Runx2: Structure, function, and phosphorylation in osteoblast differentiation. *Int J Biol Macromol 78*, 202-208. doi:10.1016/j.ijbiomac.2015.04.008

Wagegg, M., Gaber, T., Lohanatha, F. L. et al. (2012). Hypoxia promotes osteogenesis but suppresses adipogenesis of human mesenchymal stromal cells in a hypoxia-inducible factor-1 dependent manner. *PLoS One 7*, e46483. doi:10.1371/journal.pone.0046483

Wan, C., Gilbert, S. R., Wang, Y. et al. (2008). Role of hypoxia inducible factor-1 alpha pathway in bone regeneration. *J Musculoskelet Neuronal Interact 8*, 323-324. http://www.ismni.org/jmni/pdf/34/18WAN.pdf

Wang, G., Shen, G. and Yin, T. (2017a). In vitro assessment of deferoxamine on mesenchymal stromal cells from tumor and bone marrow. *Environ Toxicol Pharmacol 49*, 58-64. doi:10.1016/j.etap.2016.11.014

Wang, L., Jia, P., Shan, Y. et al. (2017b). Synergistic protection of bone vasculature and bone mass by desferrioxamine in osteoporotic mice. *Mol Med Rep 16*, 6642-6649. doi:10.3892/mmr.2017.7451

Waters, R. V., Gamradt, S. C., Asnis, P. et al. (2000). Systemic corticosteroids inhibit bone healing in a rabbit ulnar osteotomy model. *Acta Orthop Scand 71*, 316-321. doi:10.1080/000164700317411951

Wu, Y., Lucia, K., Lange, M. et al. (2014). Hypoxia inducible factor-1 is involved in growth factor, glucocorticoid and hypoxia mediated regulation of vascular endothelial growth factor-a in human meningiomas. *J Neurooncol 119*, 263-273. doi:10.1007/s11060-014-1503-5

Xing, K., Gu, B., Zhang, P. et al. (2015). Dexamethasone enhances programmed cell death 1 (pd-1) expression during t cell activation: An insight into the optimum application of glucocorticoids in anti-cancer therapy. *BMC immunology 16*, 39-39. doi:10.1186/s12865-015-0103-2

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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

Yellowley, C. (2013). Cxcl12/cxcr4 signaling and other recruitment and homing pathways in fracture repair. *Bonekey Rep 2*, 300. doi:10.1038/bonekey.2013.34

Zeng, H. L., Zhong, Q., Qin, Y. L. et al. (2011). Hypoxia-mimetic agents inhibit proliferation and alter the morphology of human umbilical cord-derived mesenchymal stem cells. *BMC Cell Biol 12*, 32. doi:10.1186/1471-2121-12-32

Zhang, J., Huang, X., Wang, H. et al. (2015). The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. *Stem Cell Res Ther 6*, 234. doi:10.1186/s13287-015-0240-9

Zhang, W., Li, G., Deng, R. et al. (2012). New bone formation in a true bone ceramic scaffold loaded with desferrioxamine in the treatment of segmental bone defect: A preliminary study. *J Orthop Sci 17*, 289-298. doi:10.1007/s00776-012-0206-z

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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Pfeiffenberger et al.:

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

Supplementary Data

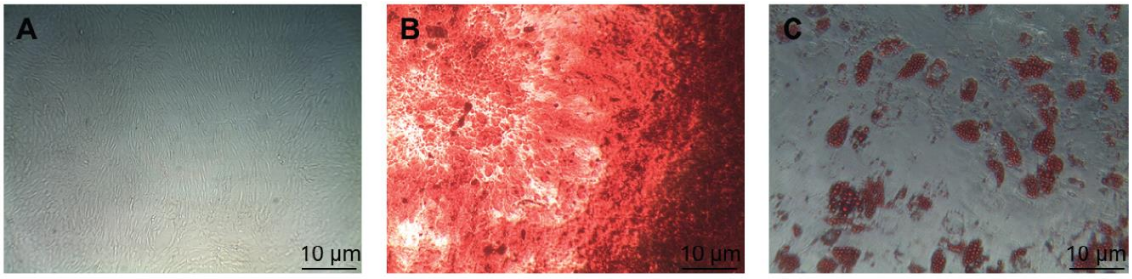

Fig. S1: **MSCs in monolayer, to prove the capacity to adhere to plastics (A), the differentiation of the MSCs towards the osteogenic lineage, demonstrated by Alizarin Red staining (B) and towards the adipogenic lineage, demonstrated by Red Oil staining (C)**

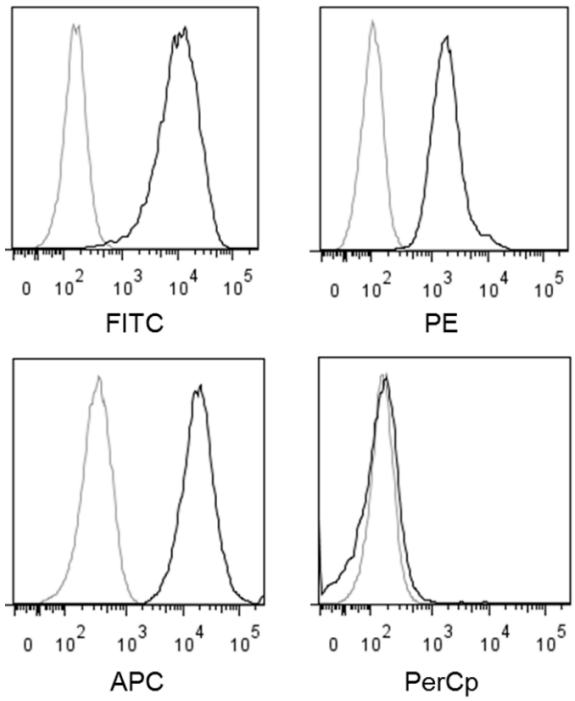

Fig. S2: **Surface marker-dependent characterization of hMSCs according to the "minimal criteria" for a common definition of hMSCs as defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006)**
Only hMSCs stained positive for CD73, CD90 and CD105 and negative for CD45, CD34, CD14, CD20 and HLA-DR surface antigens were used in the study as determined by flow cytometry after applying the MSC Phenotyping Kit (Miltenyi, Bergisch-Gladbach, Germany) according to the manufacturers´ protocols based on the phenotyping antibody cocktail including the following antibody conjugates: anti-CD73-FITC, anti-CD105-PE, anti-CD90-APC, anti-CD45-PerCp, anti-CD34-PerCp, anti-HLA-DR-PerCp, anti-CD20-PerCp, anti-CD14-PerCp and a corresponding isotype control cocktail.

doi:10.14573/altex.1910211s

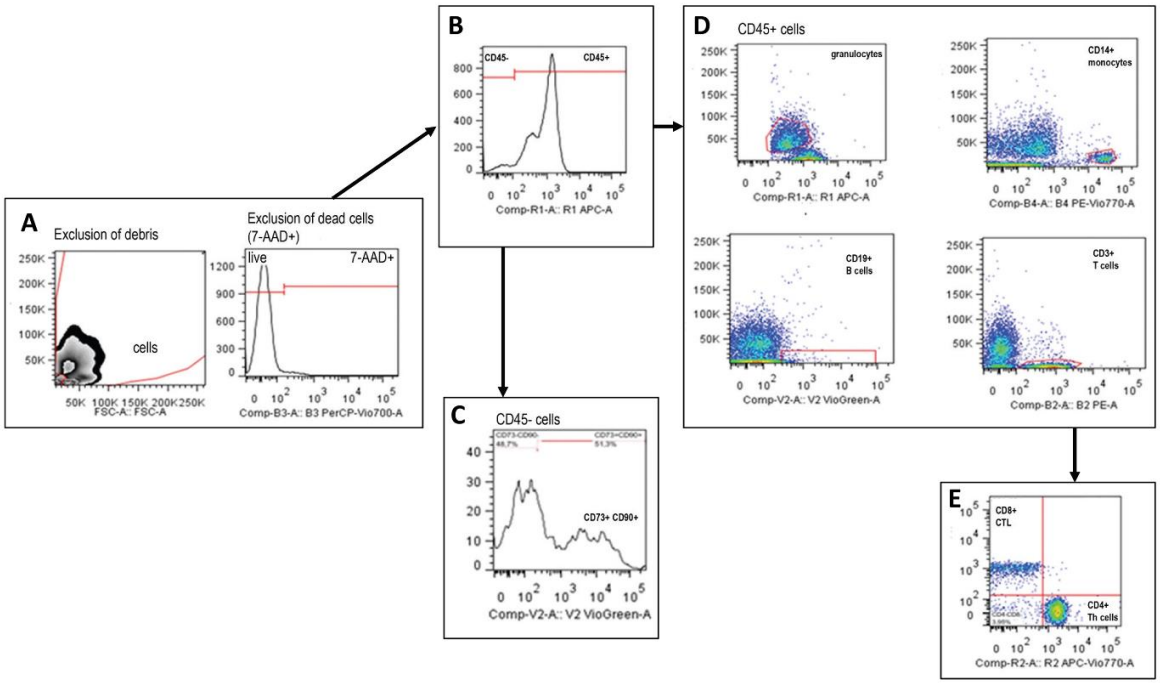

Fig. S3: **Gating strategy for evaluation of the cell composition within the FH models**
As a first step, cell debris and dead cells were excluded (dead cells positive for 7AAD, A). Then we differentiated between $CD45^+$ and $CD45^-$ cells (B). The $CD45^-CD73^+CD90^+$ cells were considered MSCs (C). Within the CD45+ gate, we characterized granulocytes based on size and granularity. $CD45^+CD19^+$ cells were considered B-cells, while $CD45^+CD14^+$ cells as monocytes (D). $CD45^+CD3^+$ cells were differentiated into $CD45^+CD3^+CD4^+$ and $CD45^+CD3^+CD8^+$ cells, considered as T helper or cytotoxic T cells respectively (E).

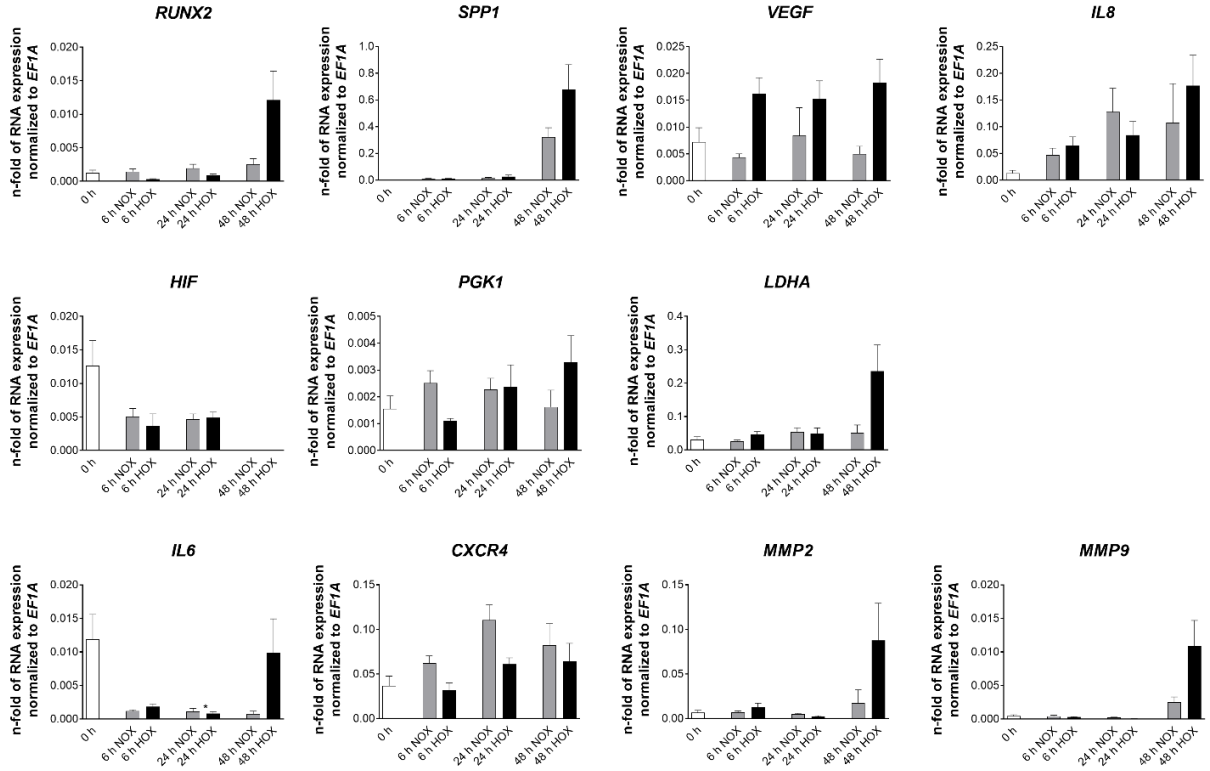

Fig. S4: **Time-dependent RNA expression of FH models incubated for 6, 12, 24 or 48 h under either normoxic (NOX) or hypoxic conditions (HOX; n=6)**
Depicted is the n-fold of the RNA (mean ± SEM). RNA expression was normalized to the gene expression of *EF1A* (ΔCt).

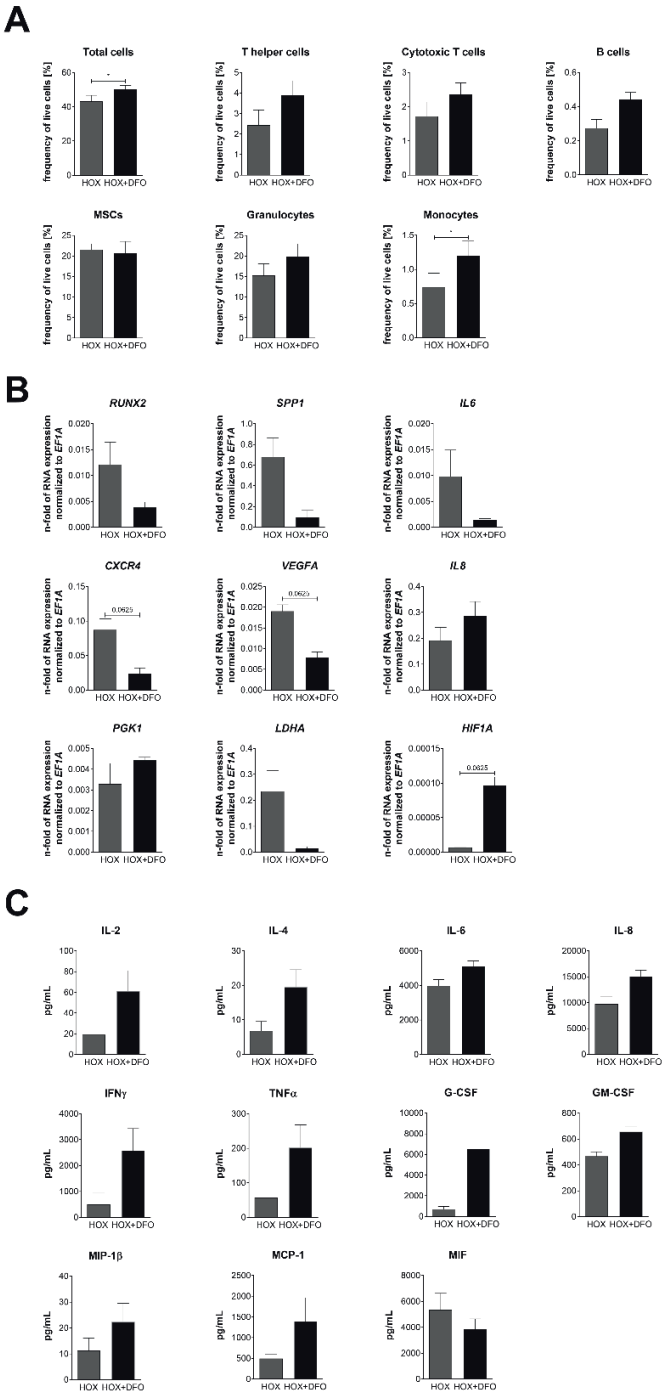

Fig. S5: ***In vitro FH*** **models were incubated under hypoxia (HOX; grey bars) or hypoxia with the supplementation of 250 µM DFO (NOX; dark grey bars) for 48 h in osteogenic medium**
Depicted is the frequency of total cells and single cell populations negative for 7-AAD in the FH (A), the relative 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.

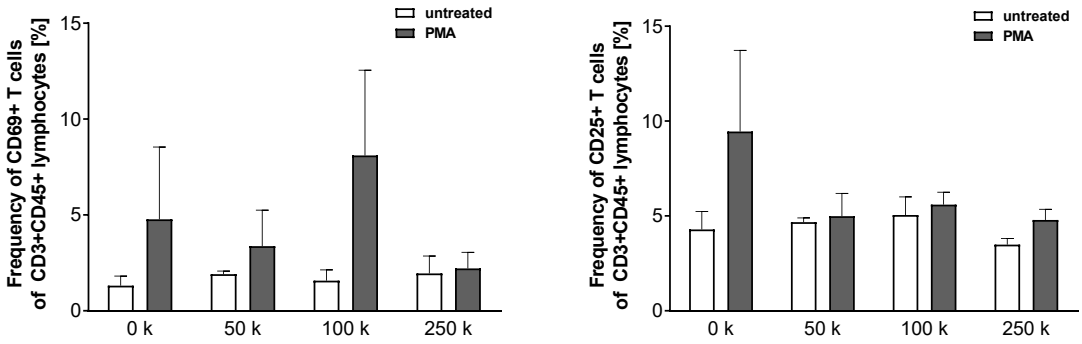

Fig. S6: Expression of early activation marker CD69 and late activation marker CD25 for CD45+CD3+ T cells after incubation of the FH models for 48 h in osteogenic induction medium as determined by flow cytometry (n=3, mean ± SD)
FH models were produced by combining either 0, 50,000, 100,000 or 250,000 human allogenic MSCs and 100 µL whole blood and subsequent coagulation of the combined cell mixtures. FH models were treated with PMA (50 ng/mL) or remained untreated.

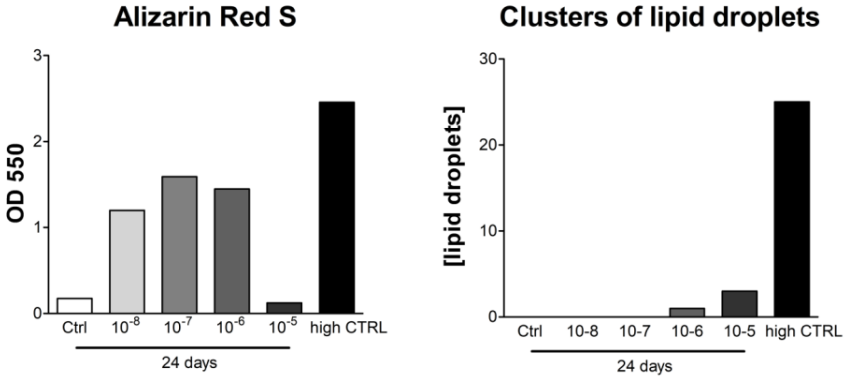

Fig. S7: OD550 values and the count of lipid droplet clusters after 24 days of differentiation of monolayer MSCs in either OD or AD (pilot experiment for n=1)
Here 10^4 MSCs per well were plated in a 96-well plate and incubated for up to 24 days in the respective induction medium. As a high control, MSCs were incubated with STEMMacs Osteo Diff or STEMMacs Adipo Diff medium respectively for 1 week. Osteogenic differentiation was measured at 550 nM using an ELISA plate reader. Adipogenic differentiation was analyzed microscopically.

3.3 Functional Scaffold-Free Bone Equivalents Induce Osteogenic and Angiogenic Processes in a Human In Vitro Fracture Hematoma Model

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Own contribution:
Study Design, Data Collection and Analysis, Data Discussion and Interpretation, Drafting Manuscript

You have to read this part online.

3.4 Comparison of both fracture hematoma models

In order to contextualize both *in vitro* models to an earlier study in our group, using *ex vivo* FHs up to 72 hours after surgery, the results were summarized in Tab. 3 (cellular composition) and Tab. 4 (gene expression data). Hypoxia favored the survival of MSCs over that of immune cells, resulting in that MSCs were the most prominent cell fraction after 48 h of incubation both in the equine and the human FH model (Pfeiffenberger, Bartsch et al. 2019, Pfeiffenberger, Hoff et al. 2020), not assessed in the *ex vivo* study. In the underlying studies, MSCs represent 23.4% (16.6-24.5%) in the human model and 34.7% (29.8- 38.3%) in the equine models of the whole cell population, while Hoff et al. could not assign 12.2% (1.2 - 85.6%) of the cells to a specific cell population. For the proportion of T cells, T helper cells and cytotoxic T cells, the studies show similar percentages. Granulocytes were at first sight more abundant in the *ex vivo* study as a percentage, but since the range is very broad; the percentages of the *in vitro* studies are comparable. Monocytes and B cells are additionally comparably present in the *ex vivo* and human *in vitro* study, yet not assigned within the equine model (Pfeiffenberger, Bartsch et al. 2019, Pfeiffenberger, Hoff et al. 2020) (Table 3).

Table 3: *Cell frequencies from an ex vivo study using primary human fracture hematomas obtained between 48 and 72 h after trauma (n=40), the in vitro human FH model (n=6, 48 h incubation under hypoxia) and the in vitro equine FH model (n=3, 48 h incubation under hypoxia), n.a. = not assigned, n.d. = not detectable.*

Cell type	CD surface marker	*ex vivo* FH (< 72 h)	*in vitro* human FH (48 h HOX)	*in vitro* equine FH (48 h HOX)
T cells	CD45+, CD3+	8.2% (1.1-39.4%)	3.7% (2.4-10.6%)	6.5% (5-7.1%)
T helper cells	CD45+, CD3+, CD4+	3.1% (1.4-15.4%)	1.9% (1.1-6%)	4.3% (4.1-4.6%)
Cytotoxic T cells	CD45+, CD3+, CD8+	1.9% (0.2-12.6%)	1.3% (0.9-2.5%)	1.2% (1.1-1.3%)
Monocytes	Scatter and CD45+, CD14+	4.9% (0.1-38.5%)	0.6% (0.3-1.7%)	n.d. after 48h
B cells	CD45+, CD3-, CD19+	0.9% (0.1-5.8%)	0.25% (0.2-0.4%)	n.a.
MSCs	CD45-, CD73+, CD90+	n.a.	23.4% (16.6-24.5%)	34.7% (29.8- 38.3%)
Granulocytes	Scatter and CD45+ vs. CD16+	64.2% (0.8-94%)	16.1% (5.6-24.7%)	5.3% (5.2-5.3%
Not assigned cells	n.a.	12.2% (1.2-85.6%)	37.1% (27.6-45.8%)	32.1% (24.6-38.1%)

Focusing on the gene expression, similar pattern between both FH models and the *ex vivo* study could be observed. Osteogenic genes such as *RUNX2* and *SPP1* were distinctly upregulated, indicating similar osteogenic induction within the three models. Early inflammatory markers (*IL6, IL8*) were upregulated in the human *in vitro* and *ex vivo* study, hence not as-

signed in the equine FH model. The similar adaptation towards a hypoxic microenvironment was also confirmed by the upregulation of significant marker genes (*LDHA*, *PGK1*, *PFKFB3* and *SLC2A*) as shown in Table 4 (Hoff, Gaber et al. 2016, Pfeiffenberger, Bartsch et al. 2019, Pfeiffenberger, Hoff et al. 2020).

Table 4: *Gene expression data from an ex vivo study using primary human fracture hematomas obtained between 48 and 72 h after trauma (n=40), the in vitro human FH model (n=6, 48 h incubation under hypoxia), and the in vitro equine FH model (n=3, 48 h incubation under hypoxia) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.a. = not assigned*

Gene symbol	*ex vivo* FHs (< 72 h)	*in vitro* human FHs (48 h HOX)	*in vitro* equine FHs (48 h HOX)
RUNX2	↑**	↑	↑
SPP1	↑*	↑****	↑
VEGFA	↑*	↑***	↑
IL8	↑***	↑**	n.a.
IL6	↑***	↑	n.a.
CXCR4	↑**	↑*	n.a.
LDHA	↑***	↑**	↑
PGK1	↑	↑	↑
HIF	↑**	↓	n.a.
MMP2	n.a.	↑**	n.a.
MMP9	n.a.	↑**	n.a.
PFKFB3	n.a.	n.a.	↑
SLC2A	n.a.	n.a.	↑
MIF	n.a.	n.a.	↑

4 Discussion

In order to provide a comprehensive preclinical tool capable of mimicking fundamental processes during the initial phase of fracture healing, a human *in vitro* fracture gap model was established. The developed model offers the possibility (i) to study cellular processes in detail, (ii) to gain further insights into adaptive processes to a restricted microenvironment, (iii) to test new therapeutic strategies with regard to fracture healing, (iv) to investigate the effectivity of therapeutics as well as pharmacological substances, thereby (v) effectively reducing the number of animal experiments, particularly in the preclinical phase. Since the bony ends and the fracture hematoma are the two main components involved in the initial phase of fracture healing, it was essential to establish and characterize two primary components. Representing the immune component, an *in vitro* fracture hematoma model (**FH**) was set up. For the bone component, scaffold-free bone like constructs (**SFBCs**), solely consisting of human MSCs, were developed, optimized and thoroughly characterized. Ultimately, these two components were combined, in order to provide a functional *in vitro* fracture gap model (**FGM**). Additionally, an equine *in vitro* fracture hematoma model was developed, which affords the opportunity to directly compare significant events during the initial phase between two species, both relevant to the treatment of fractures and emerging fracture healing disorders.

4.1 Development and characterization of the equine fracture hematoma model

The first step was to establish and characterize an equine *in vitro* FH model. The equine *in vitro* FH was considered an appropriate starting point for several reasons. An essential point can be derived from the “one health” approach, as contrary to rats and mice, the horse is an important patient, most notably in racing, but also with respect to popular and recreational sports (Ortved 2018, Moiroud, Coudry et al. 2019). Fractures in horses are often fatal and lead to subsequent euthanasia of affected horses, with far-reaching consequences, especially due to failures in horse racing (Riggs 2002). Focusing on the treatment of fractures in horses, great progress in the clinical development and testing of novel plates, nails and other fixation systems has been made in recent years (Nunamaker, Richardson et al. 1986). However, there is a lack of basic scientific knowledge on the mechanistic and temporal course of fracture healing in horses. The initial phase of fracture healing in equines is considered to be a phase of cleaning (elimination of debridement, dead cell and dead tissue) (Auer and Grainger 2015). Because to the often insufficient and delayed healing of equine fractures, Auer and Grainger proposed that immunological processes for initiating bone regeneration in the initial phase are altered in comparison to humans (Auer and Grainger 2015). Most findings and approaches to the course of fracture healing, especially in the initial phase, are based on investigations in animal models (mouse, rat, sheep) or humans (Schmidt-Bleek,

Schell et al. 2009, Hoff, Gaber et al. 2016, Yuasa, Saito et al. 2018). To date, there are barely any studies focused on the initial phase of fracture healing and the cellular composition or gene expression pattern in the equine FH. Collier and colleagues (1985) created a gap-healing model in horses. They set 4 mm defects, which were filled with implantable bone growth stimulators, in order to evaluate the effect of electric stimulation of osteogenesis. However, no effect was observed (Collier, Brighton et al. 1985).

Setting up FH models from two different species (horse and human) provides the opportunity to contextualize both *in vitro* FH models with existing *in vitro*, *ex vivo, in silico* and *in vivo* animal models in detail. Furthermore, our group and our cooperation partner already established and stratified scaffold-free cartilage transplants (Ponomarev 2013, Ponomarev, Kochneva et al. 2014, Weber, Fischer et al. 2020), which served as a basis for developing the bone component used in the 3D fracture gap model. To date, however, the complete 3D fracture gap model was only established in the human system.

As already outlined in the introduction, there is no existing gold-standard model for fracture healing research. Therefore, several approaches exist to shed light onto important aspects of bone healing. Most of the research is done using small animal models, especially rodents (mice and rats). Hence, considerable differences between these animals and humans exist, foremost in inflammation pathways and immunological functions, which complicates the translation of results obtained in animal models to the human system (Mestas and Hughes 2004, Seok, Warren et al. 2013, Warren, Tompkins et al. 2015). Moreover, obvious interspecies differences in bone composition, density and bone quality exist (Aerssens, Boonen et al. 1998), which must also be considered when interpreting results. Nevertheless, animal models clearly improved the general understanding of processes during fracture healing and paved the way for various therapeutic strategies in bone related diseases. Rats in particular have proven to be a reliable and useful model for conducting basic research involving the skeleton (Bagi, Berryman et al. 2011).

Most research regarding bone healing focuses on the later phases of endochondral ossification, soft and hard callus formation and remodeling, especially in veterinary practice, but also in human healthcare. However, studies within the horse are rare. In the underlying study, the focus was drawn towards the initial phase after fracture. The central event is the formation of a FH were cells released from ruptured blood vessels (leukocytes, erythrocytes) and cells originating from the bone marrow (leukocytes, progenitor cells such as MSCs or HSCs) coagulate in the fracture gap (Hoff, Gaber et al. 2016, Walters, Pountos et al. 2018). This hematoma formation is known to be the most important stage of fracture healing, since initiated processes in the FH orchestrate the cellular activation and migration as well as the inflammatory response, in order to form a new callus, regenerate bone tissue and restore functionality

(Walters, Pountos et al. 2018). The importance of the FH was underlined by the studies of Grundnes et al. and Schell et al. They removed the FH after fracture in rats, resulting in diminished bone formation and prolongation of the inflammatory phase (Grundnes and Reikerås 1993, Schell, Duda et al. 2017). Additionally, Street et al. revealed that the initial FH is inherently angiogenic, therefore comprising crucial cues for revascularization (Street, Winter et al. 2000). One of the challenges of the FH-containing cells is the hostile microenvironment after fracture, foremost hypoxia. Therefore, the *in vitro* equine FH models were either cultivated under normoxic conditions (control) or hypoxic conditions (approximation towards the situation *in vivo*). For establishing the *in vitro* equine FHs, 100 µL EDTA-blood was mixed with 250.000 MSCs and subsequently coagulated with 10 mM $CaCl_2$ (Pfeiffenberger, Bartsch et al. 2019). The proportion of MSCs in the FH in comparison to other cells has never been evaluated, thus the amount of MSCs was titrated on account of technical reasons. Since the composition of the cells in the FH was analyzed by flow cytometry, a reasonable amount of MSCs had to be implemented to obtain measurable results. After incubation for 12, 24 or 48 h under normoxic or hypoxic conditions, cell composition and mRNA expression within the equine FHs was evaluated (Pfeiffenberger, Bartsch et al. 2019). Generally, the immunophenotype of equine MSCs is maintained and the gene expression pattern is very similar comparing hypoxic or normoxic cell culture conditions (Ranera, Remacha et al. 2012). MSCs - which are considered to be the most important cell type, particularly in the initial phase of fracture healing both in horses and humans, are very comparable with regard to their differentiation properties and functionality (Hillmann, Ahrberg et al. 2016). Moreover, MSCs are frequently used with great success in the treatment of orthopedic injuries (De Schauwer, Van de Walle et al. 2013), tendinopathies (Smith 2008) and wound healing (Textor, Clark et al. 2018).

Regarding the cell composition, hypoxia favored the survival of MSCs over immune cells and MSCs were the most abundant cell population after 48 h of incubation (hypoxia) (Pfeiffenberger, Bartsch et al. 2019). MSCs are osteogenic progenitor cells and undeniably the driving force behind bone regeneration and fracture healing (Majidinia, Sadeghpour et al. 2018). Moreover, adequate numbers of MSCs within the micro-environment of the fracture are capable of recruiting essential cells towards the fracture site (Wang, Wang et al. 2013). This may explain the increasing abundance of MSCs within the FH, which may be crucial for the ongoing process of bone regeneration and bone healing reflected within this study and underlines the therapeutic potential of MSCs. The huge influence of MSCs was confirmed, as blood clots without the addition of MSCs resulted in a diminished expression of the analyzed genes, except for *SPP1 (*Pfeiffenberger, Bartsch et al. 2019). However, *SPP1* is an omnipresent protein, involved in diverse biological events, such as immunological responses, wound healing and developmental processes. Hence, it is not unique to bone regeneration,

but rather linked to various activation pathways (Sodek, Ganss et al. 2000). Bussche and van de Walle revealed the strong angiogenic potential of MSCs. In detail, they analyzed the presence of crucial angiogenic factors in the conditioned medium of equine MSCs and their effect on endothelial cells. They observed both the induction of angiogenic processes in the endothelial cells and the high expression of angiogenic marker genes such as *VEGFA* (Bussche and Van de Walle 2014). Interestingly, *PPARγ* is also upregulated after 48 h of incubation and thus, to a lower extent under hypoxic conditions. At first glance, this was unexpected as PPARγ is well-known to inhibit osteogenesis and promote adipogenesis (Lin, Yang et al. 2007, Marciano, Kuruvilla et al. 2015). However, different isoforms of PPARγ exist. In fact, several studies revealed that PPARγ-2 but not PPARγ-1 is responsible for adipogenic induction (Ren, Collingwood et al. 2002, Li, Zhang et al. 2016). Since the used primer covers both isoforms, conclusions whether adipogenesis was induced cannot be drawn. Additionally, the expression of *macrophage migration inhibitory factor* (MIF*)* was analyzed, which was expressed higher after 48 h compared to 0 h {Pfeiffenberger, 2019 #150}. Kobayashi et al. revealed the importance of MIF for fracture healing, since *MIF*-null mice showed impaired fracture healing (Kobayashi, Onodera et al. 2011). Generally, the role of MIF in horses is still elusive. However, Klein and Troedsson observed the distinct mRNA expression of MIF in the endometrium of horses, suggesting an essential role in reproduction and developmental processes (Klein and Troedsson 2013). Altogether, the presented equine FH model is capable of mimicking several key features of the initial phase of fracture healing and may reflect the situation *in vivo* (Pfeiffenberger, Bartsch et al. 2019).

- **Limitations:**

The predominant cells in the FH right after fracture are MSCs and immune cells. To distinguish between cell populations present in the FH via flow cytometry, the expression of cell-typical surface markers (cluster of differentiation (CD) markers) is used. That way, cells like T (CD3) and B (CD19) cells or monocytes (CD14) can be identified and unequivocally assigned. However, in contrast to humans, mice and rats, only a few equine-specific antibodies are commercially available. Several antibodies were tested in the course of establishing the equine FH model, in order to assign pan-T, T helper, cytotoxic T cells, B cells, granulocytes and monocytes, representing the immune cells in the peripheral blood. However, no marker could be established for B cells, as no commercially available marker showed specific binding. Hence, B cells were excluded from the analysis. Nevertheless, specifically binding antibodies for pan-T cells (CD3), T helper cells (CD4), cytotoxic T cells (CD8), granulocytes (pan-granulocyte marker) and monocytes (CD14) have been established and were used to analyze the cell composition in the models (Pfeiffenberger, Bartsch et al. 2019). In terms of MSCs, there is no acknowledged surface marker pattern as defined for human MSCs

(CD73+, CD90+, CD105+, CD45-, CD34-, CD20-, CD14-, MHCII-) (Dominici, Le Blanc et al. 2006). Other stemness markers include CD44 and CD29, which have been defined for human MSCs (Maleki, Ghanbarvand et al. 2014). Neither CD73 nor CD90 surface marker antibodies are commercially available for horses. Until 2011, only antibodies recognizing the equine epitopes CD13, CD44 and MHC-II were commercially available for the characterization of equine MSCs (De Schauwer, Meyer et al. 2011). In the underlying studies, only cells which were positive for both CD29 and CD105 but negative for CD14 were considered MSCs in the flow cytometry analysis (Pfeiffenberger, Bartsch et al. 2019). Before using the MSCs in the equine FH model, the cells were characterized by the marker panel described above. Additionally, the differentiation potential towards osteogenic and adipogenic lineage was identified. In contrast to human MSCs, equine MSCs could not be differentiated in their adipogenic lineage by using the commercially available adipogenic differentiation medium AdipoDiff™ by Miltenyi. Therefore, the differentiation medium was established according to the protocol of Vidal et al. (Vidal, Kilroy et al. 2007). Using this protocol, the adipogenic differentiation of MSCs was confirmed.

4.2 Development and characterization of the human fracture hematoma model

Generally, the reference values for blood count and differential blood count in humans and horses are very comparable (Laboklin). Therefore, the same cell amounts and coagulation parameters defined for the equine FH model were used for the human FH model. The FH models were cultivated under normoxic or hypoxic conditions for up to 48 h. Focusing on the cell populations, the proportion of MSCs was most abundant at 48 h under hypoxic conditions, whereas the frequency of leukocytes continuously regressed (comparable to the results of the equine FHs) (Pfeiffenberger, Hoff et al. 2020). Another study of the cellular composition in a sheep FH, which was sampled 4 h after fracture by Schmidt-Bleek et al., showed that lymphocytes and granulocytes are the most abundant cells in the FH right after fracture. Yet, the cells were pre-gated with CD45 (Pan leukocyte marker), which excludes some cell types, including MSCs, from the analysis (Schmidt-Bleek, Schell et al. 2009). Furthermore, Hoff et al. also observed granulocytes as the most abundant cell population in *ex vivo* FHs up to 72 h after fracture. However, MSCs were not assigned in their analysis either (Hoff, Gaber et al. 2016). Generally, the analytical separation of MSCs and e.g. leukocytes via flow cytometry is challenging, since MSCs do not have one particular surface marker that clearly defines them as MSCs. In the underlying study, MSCs were tracked using CD45 as a negative as well as CD73 and CD90 as positive markers, which allows for distinguishing MSCs from leukocytes (Pfeiffenberger, Hoff et al. 2020). Finally, it is very challenging to obtain clear-cut FHs through surgery. Thus, cells surrounding FHs could explain the diverse results in the underlying study and the mentioned previous studies.

The adaptive processes towards a hypoxic microenvironment differ notably between various cell types. Cells with many mitochondria usually have an intrinsic need to be sufficiently supplied by oxygen and undergo apoptosis when challenged with hypoxic conditions (e.g. neuronal cells). However, the surviving capacity and activation status of immune cells (e.g. T cells, B cells, monocytes, neutrophils) is increased under hypoxia, because of the activation/stabilization of the HIF-signaling pathway in an oxygen-low microenvironment (Krzywinska and Stockmann 2018). Regarding the survival of MSCs, hypoxia seems to favor MSCs and enhance the differentiation towards the osteogenic and chondrogenic cells (Wagegg, Gaber et al. 2012, Lee, Byeon et al. 2016). However, whether inflammatory cells and MSCs differ in their tolerance of oxygen is yet to be evaluated.

Since chondrocytes and osteoblasts are the main cells involved in bone regeneration (Kolar, Gaber et al. 2011, Knight and Hankenson 2013, Hoff, Gaber et al. 2016) and capable of ceasing the inflammatory phase (Kolar, Gaber et al. 2011), it is not surprising that MSCs, their progenitor cells, are the most abundant cells in the established FH model after 48 h (Pfeiffenberger, Hoff et al. 2020). MSCs are considered to be immunomodulatory by inhibiting the proliferation of cytotoxic T cells, T helper cells and natural killer cells, suppressing the immunoglobulin production of plasma cells and stimulating regulatory T cell proliferation, thereby actively contributing to the termination of the inflammatory phase (Sargent and Miller 2016). In contrast, the exact role of immune cells such as lymphocytes and granulocytes in fracture healing is still controversially discussed (Grogaard, Gerdin et al. 1990, Toben, Schroeder et al. 2011, Reinke, Geissler et al. 2013, Konnecke, Serra et al. 2014, Kovtun, Bergdolt et al. 2016, El Khassawna, Serra et al. 2017). In the present study, the proportion of T and B cells time-dependently decreased (Pfeiffenberger, Hoff et al. 2020). El Khassawna and colleagues revealed the crucial role of mature T and B cells on fracture healing. They used an osteotomy model of RAG1-deficient mice, which lack mature T and B cells, therefore lacking an appropriate adaptive immune system and could show highly dysregulated collagen deposition and disturbed distribution of osteoblasts (El Khassawna, Serra et al. 2017), also confirmed by Nam et al. (Nam, Mau et al. 2012). Accordingly, Reinke et al. depleted cytotoxic T cells in a mouse osteotomy model, which resulted in enhanced fracture regeneration, while the transfer of cytotoxic T cells significantly impaired the healing (Reinke, Geissler et al. 2013). Interestingly, Schlundt et al. studied the interplay between CD8+ effector cells, which impair the regeneration process and CD4+ regulatory T cells. They hypothesized that CD4+ regulatory T cells could counteract the inflammatory potential of CD8+ effector cells. While the transfer of CD4+ regulatory T cells to mice with low amounts of CD8+ effector cells resulted in improved bone healing, the transfer to mice with a high amount of CD8+ effector cells revealed heterogeneous bone regeneration. Hence, it can be postulated that the ratio of

these cells is crucial for appropriate bone healing (Schlundt, Reinke et al. 2019). However, the contribution of T and B cells to a higher quality of bone, by orchestrating the collagen deposition process in later phases of fracture healing, are undisputed. These cell populations invade the fracture site through newly formed blood vessels (Konnecke, Serra et al. 2014).

The exact role of granulocytes during fracture healing remains elusive and has been discussed controversially. However, granulocytes are important in the initial phase, since they remove spoilt cells and debris in the fracture gap (Baht, Vi et al. 2018). Neutrophils are also capable of synthesizing an "emergency extracellular matrix“, which allows further MSCs to migrate in the FH (Bastian, Koenderman et al. 2016). While Grogard et al. did not detect any impact of neutropenia on the callus formation in rats (Grogaard, Gerdin et al. 1990), Kovtun et al. observed impaired bone healing in a mouse model using an anti-Ly-6G antibody, in order to significantly diminish the number of neutrophils, therefore suggesting an important role of granulocytes (Kovtun, Bergdolt et al. 2016). However, Bastian and colleagues could show that high neutrophil numbers inhibit the synthesis of mineralized extracellular matrix of MSCs *in vitro (Bastian, Croes et al. 2018)*. Furthermore, granulocytes secrete cytokines (e.g. IL-1, IL-6, IL-10, TNFα, MCP-1, CXCL1α, MIP-1), that attract monocytes and macrophages towards the fracture site, which exhibit an essential role during the following phases of fracture healing (Baht, Vi et al. 2018). Hoff et al. reported a decrease of granulocytes over time, when cultivating *ex vivo* FHs under hypoxic conditions (Hoff, Maschmeyer et al. 2013), which is in accordance to the results obtained in this present study. It can be postulated, that granulocytes may be crucial in the early phase of inflammation. Hence, their time-dependent reduction might be important for an appropriate process of fracture healing.

Regarding the mRNA expression pattern, osteogenic markers, inflammatory markers and hypoxia-adaptation markers were expressed significantly higher after 48 h of incubation and to a higher extent when cultivated under hypoxia (Pfeiffenberger, Hoff et al. 2020). As for the cellular composition, normoxic conditions are considered a control setup, while the focus of the discussion is drawn towards the results after 48 h under hypoxia, since they most adequately mimic the restrictive microenvironment after fracture. Genes responsible for the induction of osteogenesis (*RUNX2*, *SPP1* and *VEGF*) are distinctly upregulated after 48 h (Pfeiffenberger, Hoff et al. 2020). Since *RUNX2* and *SPP1* (Jensen, Gopalakrishnan et al. 2010) as well as *VEGF* (Yang, Tan et al. 2012) are marker genes for the onset of osteogenesis, indicating the induction of osteogenesis. Additionally, Liu et al. could show that VEGF is responsible for the fate of MSCs differentiation to either adipocytes or osteoblasts. They conditionally deleted *VEGF* using a cre-lox system in mice or knocked-down *VEGF* in osteoblasts of mice *in vitro*. Thereby, they observed increased bone marrow fat and reduced bone density *in vivo* as well as a significant increase of adipogenesis and reduction of osteogene-

sis *in vitro* (Liu, Berendsen et al. 2012). Furthermore, VEGF has proven to be the key inducer and mediator of angiogenesis (Hoeben, Landuyt et al. 2004, Shibuya 2011), coupling osteogenesis and angiogenesis (Gerber, Vu et al. 1999), which indicates the induction of osteogenesis and angiogenesis in the *in vitro* FHs. This could also be confirmed by the increased expression of *IL8*, a potent proangiogenic factor, which promotes the activation of VEGF and triggers the upregulation of VEGF on mRNA and protein levels in an autocrine manner (Martin, Galisteo et al. 2009).

The initial phase of fracture healing is characterized by an acute inflammation and the inflammatory response is one of the initiating events for bone healing, as inflammatory conditions modulate the primary interplay and response of osteoprogenitor cells, MSCs, osteoblasts, chondrocytes and immune cells. This is mainly mediated through pro-inflammatory cytokines (e.g. IL1β, IL-6 and TNFα) and growth factors such as BMPs or MMPs (Pape, Marcucio et al. 2010, Claes, Recknagel et al. 2012). The secretion of cytokines such as IL-6 leads to the recruitment of monocytes and macrophages towards the fracture site. Monocytes differentiate into osteoclasts, which resorb necrotic bone aggregates and the necrotic regions of the bony ends, while macrophages remove the provisional "emergency extracellular matrix“, thereby cleaning the fracture gap (Loi, Córdova et al. 2016). Furthermore, MMPs are highly expressed by inflammatory cells and have a crucial role during bone regeneration. Lieu et al. revealed the importance of MMPs on appropriate fracture healing. The treatment of mice with a general MMP inhibitor (GM6001) resulted in delayed bone formation, while in specific *MMP2*- and *MMP9*-null mice both mutations affected the bony callus and resulted in impaired bone remodeling (Lieu, Hansen et al. 2011), also reported by Colnot and colleagues (Colnot, Thompson et al. 2003). In the *in vitro* FH model, *IL6* was upregulated similarly to *ex vivo* models (Hoff, Gaber et al. 2016), while *MMP2* and *MMP9* were significantly upregulated, indicating an adequate inflammatory response in the FHs (Pfeiffenberger, Hoff et al. 2020). Another crucial factor for proper fracture healing is CXCR4 (SDF-1 receptor), which is expressed on granulocytes, monocytes, lymphocytes as well as stem cells and reflects the migratory capacity (Kolar, Gaber et al. 2011, Edderkaoui 2017). Hosogane et al. could demonstrate that CXCR4 expressing cells recruit MSCs to the injured bone, subsequently resulting in enhanced bone formation (Hosogane, Huang et al. 2010). This was also verified by the study of Kitaori and colleagues, who registered an inhibition of new bone formation in mice after the administration of an anti-SDF-1 antibody, while the administration of SDF-1 increased MSC chemotaxis in an *in vitro* model (Kitaori, Ito et al. 2009). The upregulation of genes relevant to the adaptation towards hypoxia such as *PGK1* and *LDHA*, which are highly expressed, shows that the cells within the FHs adapt to the hypoxic microenvironment and change their metabolism towards glycolysis (Nagao, Kobayashi et al. 2019).

Additionally, using Multiplex ELISA, the protein secretion of the cells in the FHs was analyzed in the supernatant. The secretion of pro-angiogenic IL-8 and pro-inflammatory IL-6 confirmed the findings on mRNA expression level. Moreover, granulocyte-colony stimulating factor (G-CSF), GM-CSF and MIP were abundantly released, which is in accordance with the analysis of Hoff et al. (Hoff, Gaber et al. 2016, Pfeiffenberger, Hoff et al. 2020). The positive effect of G-CSF was confirmed by the studies of Bozlar et al. and Herrmann and colleagues (Bozlar, Aslan et al. 2005, Herrmann, Zeiter et al. 2018). Bozlar et al. administered recombinant G-CSF to a group of Sprague Dawley rats and observed higher mechanical parameters (max-load) compared to the control group (Bozlar, Aslan et al. 2005). Herrmann et al. observed an increased bone formation and reduced gap size in the G-CSF treatment group in a rat segmental bone defect model (Herrmann, Zeiter et al. 2018). GM-SCF and MIP, mainly secreted by MSCs, are known to enhance the recruitment of immune cells and maintenance of macrophages as well as increase the migration capacity, enhance the activation and phagocytosis potential and thereby promote the survival of neutrophils (Le Blanc and Davies 2015).

Overall, the profile of the cell composition, mRNA expression and protein pattern show profound similarities to experimental studies in the literature and therefore resemble the *in vivo* situation (Pfeiffenberger, Hoff et al. 2020).

- **Limitations:**

To produce the human *in vitro* FHs, peripheral blood from healthy donors is mixed with MSCs of other donors, making it an allogenic approach (Pfeiffenberger, Hoff et al. 2020). For technical and ethical reasons, it was not possible to obtain blood and MSCs from the same donor. However, since MSCs are considered to be immunoprivileged and commonly used in clinics when transplanting MSCs and show no adverse effects, this should not be a problem. Additionally, our group and other groups could show that allogenic MSCs do not activate T cells or monocytes *in vitro* (Hare, Fishman et al. 2012, Zhang, Huang et al. 2015, Rozier, Maria et al. 2018). For technical reasons, other cells such as HSCs, which are represented in the FH, were not included, since only a considerably small number of these cells is present in the FH and they are very challenging to isolate. In addition, fully differentiated cells such as macrophages or osteoblasts, which are part of the initial fracture hematoma to a certain degree, are not included in the FHs presented here. Yet, their progenitor cells were implemented.

Another limitation is that the approach is not completely xeno-free, since fetal calf serum (FCS) was used for the cultivation of the MSCs and the established models. FCS is used extensively worldwide to enhance the proliferation of various cell types. Beyond ethical con-

siderations, mainly due to the harvesting procedure, scientific issues should also be considered (Jochems, van der Valk et al. 2002). In general, the composition of FCS is unknown and varies greatly between individual batches (Honn, Singley et al. 1975). Moreover, Knepper and colleagues found various transcription factors present in different batches of FCS, which could influence cell culture (Knepper, Mayanil et al. 1998). In addition, Yokoyama et al. could show that FCS components increased the chondrogenic differentiation potential of human MSCs *in vitro*, while decreasing the expression of the stemness marker CD90, and found a morphological change of the MSCs to larger polygonal cells (Yokoyama, Miwa et al. 2008). To minimize these effects and avoid batch-to-batch variations, FCS from one large batch was used in this study. To completely replace FCS, several approaches exist. For instance, Popov et al. used human serum, which did not alter the proliferation capacity of human MSCs, yet enhanced their osteogenic differentiation potential (Popov, Scotchford et al. 2019). Another approach is the use of human platelet lysate, which showed promising results and could fully replace FCS in MSC cell culture (Fernandez-Rebollo, Mentrup et al. 2017, Tylek, Schilling et al. 2019). However, human platelet lysate is ill-defined (broad range of growth factors) and batch-to-batch variations are obvious (Hemeda, Giebel et al. 2014). A chemically defined medium for the cultivation of MSCs is still elusive.

4.3 Stratification of the human fracture hematoma model – Proof of concept

In order to stratify the FH model and validate the applicability for therapeutic approaches, the **human** *in vitro* FH models were either treated with deferoxamine (DFO, fosters fracture healing) or dexamethasone (DEX, impairs fracture healing). Hereby, DFO was administered to FHs cultivated under normoxic conditions, in order to mimic impaired bone healing conditions, while DEX was administered to FHs under hypoxic conditions, in order to mimic normal bone healing conditions (Pfeiffenberger, Hoff et al. 2020). To simulate the clinical application of DFO to counter-regulate an inadequate hypoxic response in patients that are susceptible to impaired bone healing, as demonstrated by Kolar et al. (Kolar, Gaber et al. 2011), the *in vitro* FHs were treated with 250 µM DFO under normoxic conditions. DFO has been reported to support angiogenesis (Farberg, Sarhaddi et al. 2014), to enhance the osteogenic differentiation of MSCs (Jia, Chen et al. 2016), to augment revascularization after fracture (Donneys, Farberg et al. 2012, Matsumoto and Sato 2015) and to boost the mineralization and later restoration of the callus (Shen, Wan et al. 2009, Grewal, Keller et al. 2014), making it an attractive off-label therapeutic target to foster fracture healing. Our group could also observe the enhanced osteogenic differentiation and calcification of MSCs *in vitro*, while application of DFO *in vivo* in a mouse osteotomy model lead to accelerated callus mineralization and subsequent vessel formation (Lang, Helfmeier et al. 2020). DFO stabilizes HIF1 by suppressing PHDs, which are responsible for tagging HIF1 for degradation through proteasomes, there-

fore mimicking hypoxic conditions and significantly improving osteogenesis (Qu, Zhang et al. 2008) and bone regeneration (Wan, Gilbert et al. 2008). When treated with DFO, the composition of cells within the FH model shifts towards a higher frequency of granulocytes, while the frequency of cytotoxic T cells and T helper was significantly diminished and the frequency of MSCs was slightly diminished (Pfeiffenberger, Hoff et al. 2020). DFO has been shown to inhibit the proliferation of T cells, by preventing the cells to complete the S phase of the cell proliferation cycle (Lederman, Cohen et al. 1984) or inducing the p53-mediated apoptosis pathway (Kim, Choi et al. 2007), with barely any effect on granulocytes (Hileti, Panayiotidis et al. 1995). This may explain the diminished frequencies of T cells but not granulocytes observed in the underlying study. Furthermore, studies could show that DFO augmented the functional capacity of neutrophils by means of the induced formation of neutrophil extracellular traps (Vollger, Akong-Moore et al. 2016) and enhanced phagocytosis (Cantinieaux, Hariga et al. 1990). With regard to MSCs, Wang et al. revealed an antiproliferative effect of DFO on MSCs *in vitro* (Wang, Shen et al. 2017), which may explain the slightly diminished frequency of MSCs. However, DFO seems to trigger the upregulation of *RUNX2* (Pfeiffenberger, Hoff et al. 2020) and DFO has been shown to induce osteogenesis and enhance the expression of *RUNX2* in MSCs (Qu, Zhang et al. 2008) and human periodontal ligament cells (Mu, Guo et al. 2017) *in vitro*. Interestingly, the expression of *SPP1*, which is inter alia activated through *RUNX2* and a late marker of osteogenic processes, is expressed on a basal level, indicating a delay of osteogenesis (Pfeiffenberger, Hoff et al. 2020). Additionally, marker genes for the adaptation towards hypoxia (*HIF*, *LDHA*, *PGK1*) are higher expressed when the FHs were treated with DFO compared to normoxia (Pfeiffenberger, Hoff et al. 2020), suggesting adaptive processes of the implemented cells, which can be explained by the stabilization of HIF, thereby preventing its degradation (Qu, Zhang et al. 2008). With respect to the secretion of cytokines/chemokines, IL-6 and IL-8 were both similarly secreted compared to normoxia. Furthermore, the treatment with DFO did not lead to the increased secretion of G-CSF, GM-CSF, MCP, MIF and MIP. Thus, DFO did not cause an enhanced inflammatory response (Pfeiffenberger, Hoff et al. 2020).

Glucocorticoids such as DEX are immunosuppressive, anti-allergic, anti-inflammatory and analgetic drugs (Jokela, Ahonen et al. 2009, Strehl, Ehlers et al. 2019), which inhibit the expression of inflammatory mediators. Hence, they are used to tackle immune-mediated (auto) inflammatory diseases in a variety of treatment strategies (Abraham, Lawrence et al. 2006). However, besides other side effects (Polderman, Farhang-Razi et al. 2018), DEX is considered to negatively influence fracture healing by delaying bone union (Snäll, Apajalahti et al. 2015), inhibiting osteogenesis (Li, Wang et al. 2012), for instance by triggering apoptosis in osteoblasts and osteocytes (Brabnikova Maresova, Pavelka et al. 2013) or enhancing bone

resorption by an induced osteoclastogenesis (Takuma, Kaneda et al. 2003). In the underlying study, the FH models were treated with a therapeutic dose of DEX (10^{-7} M) for 48 h under hypoxic conditions, while the non-treated FH under hypoxia served as a control. After incubation, the cellular composition shifted towards a significantly diminished proportion of MSCs, while immune cells were significantly higher compared to the untreated control. Additionally, osteogenic marker genes (*RUNX2* and *SPP1*) were expressed to a lower extent, while the secretion of inflammatory cytokines was induced (Pfeiffenberger, Hoff et al. 2020). Most notably, the increase in immune cells was unexpected at first glance, since DEX is well-known to act anti-inflammatory by inducing apoptosis, particularly in T cells, and actively suppresses T cell activation (Xing, Gu et al. 2015). However, two independent studies reported that DEX induces the proliferation and reverses the immunosuppressive effect of MSCs, if peripheral blood mononuclear cells are co-cultivated with MSCs (Buron, Perrin et al. 2009, Chen, Gan et al. 2014). In a mouse model, Chen et al. observed that DEX reversed the anti-inflammatory effect of transplanted MSCs (Chen, Gan et al. 2014). The finding here, that the expression of osteogenic marker genes is diminished, is in accordance with several studies (Canalis 1996, Liu, Akhter et al. 2018) and indicates delayed osteogenesis within the *in vitro* FH model. Additionally, the expression of HIF-induced genes such as *VEGF*, *CXCR4* and *LDHA* is reduced in the DFO-treated *in vitro* models (Pfeiffenberger, Hoff et al. 2020), which is also in accordance with studies by our group and other groups (Gaber, Schellmann et al. 2011, Wu, Lucia et al. 2014).

Taken together, the treatment of the *in vitro* FH model with DEX showed several features of a delayed fracture healing process and therefore, might be used as an *in vitro* model to mimic the situation of delayed fracture healing (Pfeiffenberger, Hoff et al. 2020).

Challenging the *in vitro* FH models with both DFO and DEX revealed the possibility of using the introduced model as a platform to test new therapeutic strategies regarding fracture healing-related disorders.

- **Limitations:**

In order to stratify the FH model with regard to its response to the treatment with therapeutics that affect fracture-healing, the FH models were challenged either with DFO or DEX (Pfeiffenberger, Hoff et al. 2020). Within the DFO treatment studies, the FHs were challenged with 250 µM DFO, which is in the range of other studies evaluating the influence of DFO on fracture healing in rats (Farberg, Sarhaddi et al. 2012, Donneys, Yang et al. 2019), rabbits (Li, Fan et al. 2015) and *in vitro* approaches (Wang, Shen et al. 2017). However, Wang et al. revealed a dose-dependent increase of tubule formation of HUVECs *in vitro* (Wang, Shen et al. 2017, Wang, Shen et al. 2017). In the underlying study, only one specific

concentration of DFO was used. Thus, no dose-dependent effect on the FH models could be evaluated (Pfeiffenberger, Hoff et al. 2020), which could be an important task for further approaches. Dose-dependent influences have also been reported for DEX (Chen, Gan et al. 2014). Consequently, the influence of different doses of DEX could also be of interest to future studies and was not analyzed here (Pfeiffenberger, Hoff et al. 2020).

4.4 Comparison of both fracture hematoma models

As discussed above, hypoxia favored the survival of MSCs over that of immune cells, resulting in that MSCs were the most prominent cell fraction after 48 h of incubation both in the equine and the human FH model (Pfeiffenberger, Bartsch et al. 2019, Pfeiffenberger, Hoff et al. 2020). A direct comparison to an earlier study in our group by Hoff et al. using human *ex vivo* FHs < 72 h after fracture, with regard to the MSC population within the FH is challenging, since in this study MSCs were not included in the flow cytometric analysis. Hence, in the underlying studies MSCs represent 23.4% (16.6-24.5%) in the human model and 34.7% (29.8- 38.3%) of the whole cell population, while Hoff et al. could not assign 12.2% (1.2 - 85.6%) of the cells to a specific cell population, which fits perfectly in the range of MSCs revealed in the presented *in vitro* study (Hoff, Gaber et al. 2016). For the proportion of T cells, T helper cells and cytotoxic T cells, the studies show similar percentages. Granulocytes were at first sight more abundant in the *ex vivo* study as a percentage, but since the range is very broad; the percentages of the *in vitro* studies are comparable. Monocytes and B cells are additionally comparably present in the *ex vivo* and human *in vitro* study, yet not assigned within the equine model (Pfeiffenberger, Bartsch et al. 2019, Pfeiffenberger, Hoff et al. 2020) (Table 3). Focusing on the gene expression, similar pattern between both FH models and the *ex vivo* study could be observed. Osteogenic genes such as *RUNX2* and *SPP1* were distinctly upregulated, indicating similar osteogenic induction within the three models. Early inflammatory markers (*IL6, IL8*) were upregulated in the human *in vitro* and *ex vivo* study, hence not assigned in the equine FH model. The similar adaptation towards a hypoxic microenvironment was also confirmed by the upregulation of significant marker genes (*LDHA*, *PGK1*, *PFKFB3* and *SLC2A*) as shown in Table 4 (Hoff, Gaber et al. 2016, Pfeiffenberger, Bartsch et al. 2019, Pfeiffenberger, Hoff et al. 2020).

Taken together, both *in vitro* models could be stratified using the *ex vivo* data and showed very similar progression with regard to the cell composition and gene expression pattern. Interestingly, profound inter-species similarities have been revealed, indicating similar processes in the adaptation to traumatic events such as fractures in horses and humans.

4.5 Development and characterization of scaffold-free bone-like constructs (SFBCs)

In general, the multitude of studies focused on bone development and bone regeneration are performed using small animal models. These studies offer a profound insight into important processes of fracture healing. However, particularly *in vitro* approaches in the field of BTE provide promising tools to reduce the use of animals, while allowing sophisticated research, in order to understand elementary processes during bone regeneration (Amini, Laurencin et al. 2012). Generally, BTE is based on the three pillars: biomaterials, biodegradable scaffolds and cellular approaches. In brief, biomaterials such as natural and synthetic ceramics or hydrogels are osteo-inductive materials, which are capable to act as osteogenic inducers and enhance bone formation, while biodegradable scaffolds like TCP provide temporary mechanical integrity, in order to allow attached cells to regenerate or repair bone tissue. Cellular approaches mostly rely on the transplantation of stem cells into the defect, which differentiate *in situ* and induce or rather enhance bone healing properties (Amini, Laurencin et al. 2012). In order to generate tissue-like structures, the cultivation of three-dimensional cell pellets, consisting of primary cells or cell lines with self-produced matrices and without exogenous scaffolds, are of emerging interest in the field of bone regeneration (DuRaine, Brown et al. 2015), most often using MSCs (Sart, Tsai et al. 2014). Another promising cellular bottom-up approach is the generation of spheroids, with the aim to precondition cells to form modular tissue units as building blocks for tissue engineering approaches (Baptista, Kronemberger et al. 2018). Generation of these 3D cell models is based on hanging droplet techniques, rotation cultures or the usage of pelleting and subsequent cultivation in non-adhesive cell culture plates (Hildebrandt, Büth et al. 2011, Lee, Pathak et al. 2019), most often using MSCs or adipose stem cells (Baptista, Kronemberger et al. 2018).

In the underlying study, the first step was to produce, establish and characterize scaffold-free bone-like constructs, in order to simulate the bony end present after fracture. These constructs should implement essential osteo-inductive cues, represented by the bony ends after fracture *in vivo* (Pfeiffenberger, Damerau et al. 2020). The SFBCs were produced by refining a patented technique introduced by Ponomarev et al., which is based on self-organization of equine MSCs, in order to produce cartilage-like transplants (Ponomarev 2013, Ponomarev, Kochneva et al. 2014). In our group, this technique was already translated to human MSCs to generate a model of osteoarthritis (Weber, Fischer et al. 2020). In the underlying study, the established and characterized models were produced in a similar way, with the distinction that the constructs were cultivated in osteogenic induction medium, which stimulates the osteogenic differentiation of MSCs. MSCs, in particular, are capable of self-assembly and self-organization under the appropriate conditions (DuRaine, Brown et al. 2015). Bhumiratana et

al. and Ghosh et al. provided functional *in vitro* cartilage models using mesenchymal condensation (Ghosh, Laha et al. 2009, Bhumiratana, Eton et al. 2014), showing the bridging towards ectopic endochondral bone formation after transplantation. Furthermore, Scotti et al. could replicate endochondral bone formation in a nude mouse model after subcutaneously implanting human MSCs. This effect was significantly enhanced by pre-maturation of the cells *in vitro*, allowing the formation of hypertrophic tissue structures using a trans-well *in vitro* system (Scotti, Tonnarelli et al. 2010). Interestingly, Akiyama et al. produced cell pellets of periosteal cells through centrifugation and propagation in medium containing ascorbic acid. These pre-differentiated cells also resulted in new bone formation, areas of calcification and even vascular invasion after subcutaneous transplantation on the back of nude mice (Akiyama, Nonomura et al. 2006). However, these models focus on the recapitulation of bone formation or bone regeneration processes after transplantation, therefore tackling the capability of implemented cells to eventually provide functionality using *in vivo* setups. Furthermore, these pellet models and other bone models using spheroids share the disadvantage of low cell numbers, unphysiological size, unphysiological geometrical arrangement of the cells as well as a lack in density and matrix formation. Within this thesis, the focus was directed on developing and establishing an *in vitro* macroscale model (SFBCs) to capture fundamental events of fracture healing, particularly the initial phase, when combined with the FH model to represent a complete fracture gap model (Pfeiffenberger, Damerau et al. 2020).

As verified by *in vitro* µCT, the SFBCs were calcified and mineralized throughout the complete tissue constructs, however, to a lesser degree in the central region (Pfeiffenberger, Damerau et al. 2020). Nevertheless, H&E staining revealed no necrotic regions, which is often a problem in spheroid culturing (Freyer 1988). Hence, oxygen-permeable spheroid culture systems (Anada, Fukuda et al. 2012) and sophisticated bioreactor platforms with preferable arrays (Barisam, Saidi et al. 2018) have been developed, in order to tackle the insufficient oxygen and nutrient supply in the center region. For that reason, the hypothesis is raised that the cultivation of the SFBCs for a longer time period could eventually lead to full calcification. However, the approach here was to establish a model functioning as an osteogenic inducer as part of a more complex 3D model of the fracture gap, representing the bony ends right after fracture. The calcification was confirmed histologically by Alizarin Red S staining, which stains for calcium-complexes. Mineralization was confirmed via µCT and von Kossa staining, which specifically reacts to phosphate-complexes. Additionally, histology/immunohistochemistry revealed the distinct expression of ALP and COL1 with no expression of COL2 (Pfeiffenberger, Damerau et al. 2020), which is in accordance with the results of other studies that characterized MSC aggregates at comparable time points (Muraglia, Corsi et al. 2003, Frith, Thomson et al. 2010, Kabiri, Kul et al. 2012). Interestingly, we were

able to detect a contrary dissemination of COL1 and COL2, with COL2 as the primarily expressed protein and a lower expression of COL1 in a previous study, using a similar MSC condensation technique, but cultivating the constructs in chondrogenic differentiation medium. This illustrates the switch towards osteogenic development in the SFBCs (Weber, Fischer et al. 2020). Furthermore, scanning with electron microscopy exposed the structure and morphology of the SFBCs with a dense, lining structure at the surface and layer-like structures in the inner regions (Pfeiffenberger, Damerau et al. 2020), resembling native, woven bone (Parrilli, Pagani et al. 2014), (Erli, Marx et al. 2003). These layer-like structures were also obvious in the H&E, Alizarin Red S and von Kossa stainings. In order to identify the most abundant typical bone cells, such as osteoblasts and osteoblast-derived osteocytes (Paic, Igwe et al. 2009, Florencio-Silva, Sasso et al. 2015), immunofluorescence stainings for OPN and OC were applied (Pfeiffenberger, Damerau et al. 2020). During the differentiation and maturation of osteoblasts a specific gene and protein expression pattern is obvious (Weinreb, Shinar et al. 1990, Granchi, Ochoa et al. 2010, Wang, Li et al. 2015). OPN, a highly phosphorylated glycophosphoprotein (Icer and Gezmen-Karadag 2018), is particularly expressed in pre-osteoblasts or immature osteoblasts, while COL1 expression is mainly found in stage 2 osteoblasts and OC is found in mature osteoblasts during their transformation into osteocytes (Rutkovskiy, Stensløkken et al. 2016). The distinct expression of the different specific proteins indicates the presence of a heterogeneous cell composition in various differentiation and maturation stages within the SFBCs. Therefore, it can be stated that the differentiation/maturation of the implemented cells is functional and very similar to bone development/regeneration *in vivo*.

On mRNA-level, *SPP1*, *DLX5* and *VEGF* were expressed to a higher extent when compared to monolayer MSC cultures, once again indicating osteogenic differentiation processes within the SFBCs, while *RUNX2*, a specific osteogenesis transcription factor (Xu, Li et al. 2015) was only slightly higher expressed (Pfeiffenberger, Damerau et al. 2020). *RUNX2* is mainly involved in the early mesenchymal condensation and osteoblast differentiation, and orchestrates the expression of downstream genes such as COL1, OPN and OC. Hence, *RUNX2* actually has to be downregulated for further maturation of osteoblasts (Bruderer, Richards et al. 2014). Consequently, the significant upregulation of *RUNX2* in the SFBCs was unexpected. Single-cell RNA-Seq could be implemented in the near future to allocate cells with different expression statuses of *RUNX2* in the SFBCs, in order to assign the cells directly to their maturation status. With regard to the enhanced expression of *SPP1,* the gene coding for OPN and *DLX5,* a master regulator of osteogenesis (Heo, Lee et al. 2017), other studies using condensation/aggregation techniques observed comparable results (Wang, Itaka et al. 2009, Jing and Jian-Xiong 2011, Ma, Zhong et al. 2011, Cheng, Wang et al. 2012). The im-

portance of *DLX5* for an appropriate osteogenesis was confirmed by studies in mice lacking the *DLX5* gene, since *DLX5*-null mice showed a drastic reduction of trabecular and total bone volume (Samee, Geoffroy et al. 2008). Interestingly, *VEGF* is significantly more expressed in the SFBCs compared to monolayer MSCs. Primarily, *VEGF* is known to play a pivotal role during angiogenesis and revascularization (Hoeben, Landuyt et al. 2004, Chung, Kim et al. 2010). Thus, *VEGF* is also required for coupling osteogenesis and angiogenesis in bone regeneration (Grosso, Burger et al. 2017) and endochondral ossification (Gerber, Vu et al. 1999, Dai and Rabie 2007). Additionally, *VEGF* is known to enhance the osteogenic differentiation of stem cells *in vitro* (I, Nargi et al. 2011, Clark, Wang et al. 2015). Furthermore, *VEGF* is a downstream target of the HIF1α pathway (Irwin, McCord et al. 2009, Maes, Carmeliet et al. 2012). Since the SFBCs are macroscale models, the inner area could be hypoxic; therefore, *VEGF* could be upregulated due to the activation of the HIF1α pathway.

- **Limitations:**

Focusing on the SFBCs, the main limitation is the time-consuming process of production. At least three months pass between the isolation of the MSCs from bone marrow and the fully-derived SFBCs, which makes it difficult to achieve a high number of n_s in the experiments, thereby limiting the throughput of the system. Since the MSCs are mostly derived from elderly people undergoing hip replacement surgery, the proliferation capacity of some MSC lines is limited, which also makes the propagation time-consuming in some cases. Generally, MSCs (and other cells) expanded in high passages *in vitro* undergo senescence, known as the Hayflick effect (Liu, Ding et al. 2020). In terms of MSCs, Banfi et al. showed that MSCs in high passages undergo replicative aging; osteogenic differentiation was induced, and telomere length decreased over time. They could also show that this effect is enhanced in older donors (Banfi, Bianchi et al. 2002). In addition, Oja and colleagues found morphological differences of MSCs between early and later passages. MSCs in early passages revealed their typical fibroid morphology, while cells in later passages were enlarged and flattened out. However, they could show that MSCs between passages 1-3 did not alter their morphology (Oja, Komulainen et al. 2018). To minimize senescent effects, only cells from early passages (3-4) were used to produce the SFBCs in the underlying study.

Mature and healthy bone consists of osteoblasts, osteocytes and osteoclasts in a highly balanced equilibrium. Thereby, osteoblasts form and osteoclasts resorb bone in a highly orchestrated manner. Osteoblasts derive from MSCs, whereas osteoclasts originate from mononuclear cells of the hematopoietic stem cell lineage (Florencio-Silva, Sasso et al. 2015). In the underlying study, the SFBCs were produced solely of MSCs, therefore no osteoclasts were included, and remodeling of the bone was not possible. However, right after fracture, the bone is broken and not fully functional in terms of its structural properties, as the bone is

shattered by the trauma. Nevertheless, fracture healing-inducing signals could be communicated through autocrine or paracrine mechanisms, which could influence processes within the FH in the fracture gap without significant impact by means of the presence of osteoclasts. Moreover, healthy bone is highly vascularized to supply the bone with oxygen, nutrients, hormones and growth factors; vascularization plays a profound role in bone growth, bone development, bone regeneration and remodeling (Filipowska, Tomaszewski et al. 2017). Several approaches exist to implement the vasculature component in bone models. For instance, Zhang et al. developed a double-cell sheet complex *in vitro*, whereby the first cell sheet was composed of an osteogenic cell layer (osteogenic potential) and the second of vascular endothelial cells (blood vessel potential), which was transplanted to nude mice. Twelve weeks after transplantation, they revealed both osteogenic and blood vessel formation potential *in vivo* (Zhang, Zhou et al. 2018). In another approach, Chiesa et al. developed a vascularized bone model *in vitro* using 3D bioprinting techniques. They combined pre-differentiated osteogenic MSCs and HUVECs on a gelatin-nanohydroxyapatite bioprinted scaffold. After 4 weeks of cultivation, the HUVECs formed tubular-like structure and a capillary-like network within the bone constructs, along with ongoing osteogenesis (Chiesa, De Maria et al. 2020).

In the introduced model, the functional part of the SFBCs was to act as an osteogenic cue, in order to induce osteogenic processes within the FHs. Therefore, a completely functional bone construct involving all bone-specific cell types was obsolete. Since vasculature is at least compromised by means of ruptured blood vessels after trauma, vascularized bone was also not necessary for the SFBCs presented here. Additionally, vascularization of the bone does not occur in the initial phase of fracture healing but in subsequent phases.

4.6 Development and characterization of the *in vitro* fracture gap model

After thoroughly characterizing the two main components, the SFBC and the *in vitro* FH, the models were combined, in order to establish a model capable of mimicking the initial phase of fracture healing *in vitro*. As the results of the *in vitro* FH models studies clearly showed the enormous and essential influence of hypoxia on setting up an appropriate model to reflect the situation found *in vivo* best, the co-cultivation of SFBCs and the FH was performed under hypoxic conditions. In order to assess if the SFBC implements all the osteogenic cues to induce processes such as osteogenesis, angiogenesis, metabolic adaptation and acute inflammation, the fracture gap models were incubated in medium without osteogenic inductive supplements (and with supplements as control). The discussion therefore focuses on the results after 48 h in medium without supplementation. The SFBC and the FH model were cultivated in close contact, which allows direct cell-cell and cell-matrix contact of the two

components, allowing direct cell-cell communication and activation, as well as through metabolites in the surrounding medium. After incubation, H&E staining revealed that the two components remained closely stuck together and the cells were similarly distributed as seen in the FHs and SFBCs when cultivated alone. Via von Kossa and Alizarin Red S staining, the calcification of the SFBC was confirmed, while calcification was not obvious in the FH (Pfeiffenberger, Damerau et al. 2020). However, a functional bridging was not to be expected after such a short cultivation period, since the formation of the soft callus in humans takes approximately 3 weeks, while hard callus formation can be observed after 3-4 months (Tian, Tang et al. 2019). Nevertheless, the mRNA expression in the FHs displayed the distinct upregulation of osteogenic (*RUNX2*, *SPP1*), angiogenic (*VEGF*, *IL8*), inflammatory (*IL6*, *MMP2*) and metabolic (*LDHA*, *PGK1*) genes compared to 0 h (Pfeiffenberger, Damerau et al. 2020). The comparison with the *in vitro* FH models and the *ex vivo* study of Hoff et al. demonstrates impressive similarities. Since the *in vitro* FH models were incubated in osteogenic induction medium, this should be the driving force to induce osteogenesis within the FH. Thus, the co-cultivation of the FH with the SFBC in normal medium resulted in similar results, the SFBC functions as osteo-inductive cue. Osteogenic induction of MSCs *in vitro* by various materials was verified by many groups. For instance, Weisgerber et al. used a mineralized collagen-GAG scaffold, which was sufficient to induce and enhance osteogenic differentiation and matrix remodeling of MSCs without any supplements (Weisgerber, Caliari et al. 2015), while Martins et al. and Xi et al. showed similar results using electrospun scaffolds on polycaprolactone (PCL) nanofiber or Poly-d,l-lactide-*co*-glycolide (PLGA) nanofiber basis (Xin, Hussain et al. 2007, Martins, Duarte et al. 2010). The osteo-inductive capacity of various MSC-seeded scaffolds was also verified *in vivo* using animal models (Mauney, Jaquiery et al. 2005, Yuan, Rezzadeh et al. 2015). However, the osteogenic induction potential of scaffolds or scaffold-free bone constructs on a 3D model *in vitro* is mostly elusive. In a study using bone chips immediately after fracture, Burska et al. could reveal the distinct expression of inflammatory markers such as *IL6* and *IL8*, migration markers such as *CXCR4* and *MMP9* and osteogenic markers like *SPP1* or osteonectin (Burska, Giannoudis et al. 2020). In the SFBCs, mRNA expression analysis revealed the induced expression of *VEGF* and *SPP1*; indicating that osteogenic processes remain unchanged within the SFBC and a self-sustaining equilibrium of important processes within the SFBCs. With regard to the protein secretion, IL-6, IL-8, G-SCF, GM-CSF and MIP were abundantly present in the supernatants (Pfeiffenberger, Damerau et al. 2020), which are in accordance with the human FH model and several other studies (Hoff, Gaber et al. 2016, Hoff, Gaber et al. 2017). In order to validate the model in its applicability to respond to fracture healing-relevant drugs, the models were challenged with 250 mM DFO (Pfeiffenberger, Damerau et al. 2020). DFO has been verified to augment vascularity, callus formation and mineralization in long-bone fracture

models (Donneys, Ahsan et al. 2013) and significantly enhance bone regeneration (Wan, Gilbert et al. 2008). Since the positive effect of DFO is attributed to the activation of the HIF1α-pathway, the upregulation of HIF1α-controlled genes was to be expected. Upon treatment, the mRNA expression of *SPP1*, *VEGF*, *MMP2*, *IL6* and *LDHA* was upregulated compared to the non-treatment group, while *RUNX2*, *IL8* and *PGK1* were expressed on a basal level. Hence, DFO triggered osteogenesis, angiogenesis and inflammatory conditions within the FHs, presumably by activating the HIF1α-pathway (Pfeiffenberger, Damerau et al. 2020).

Taken together, within the 3D *in vitro* fracture gap model presented here, key features of the initial phase of fracture healing could be mimicked soundly. The established model can be used i) to investigate probable underlying mechanisms of fracture healing disorders, particularly focusing on immunologically restricted patients (Hoff, Gaber et al. 2011, Hoff, Gaber et al. 2017), compelling the crosstalk between immune cells and bone, and ii) as a prediction tool for potential therapeutic strategies, while actively implementing the 3R principle.

- **Limitations:**

With regard to the fracture gap model, we observed a drastic decline of living cells after 72 h of incubation. Consequently, the analysis after 72 h could not be included in the underlying study. This is mainly due to the steady-state system used here, which could be overcome by cultivation in a perfused system, maybe prolonging the timeframe for analysis. Additionally, in contrast to e.g. mouse models, the presented study is not a systemic approach. For one, mechanical influences, which are particularly essential in the initial phase of fracture healing, have to be considered. Kenwright and Gardner, using a tibial diaphyseal fracture model treated by external skeletal fixation, observed that external fixation should allow amplitudes of movement to improve an appropriate healing process in the first 4-6 weeks after fracture (Kenwright and Gardner 1998). In order to overcome this limitation, our group plans to implement a pressurized cell chamber system within a bioreactor platform, providing the opportunity to implement defined scenarios of external pressure and analyze the influence of mechanics on fracture healing *in vitro*. As already discussed above, the allogenic approach and the use of FCS must also be considered as a limitation of the FGM.

4.7 Positioning of the thesis in the fields of 3R research and comparative medicine

The book "*The Principles of Humane Experimental Technique"* published by Russell and Burch (1959) is well known in the scientific world. Generally, the principles of the 3R´s include the (i) refinement of the experimental methods with regard to animal welfare aspects, (ii) reduction of animal numbers for experiments and (iii) complete replacement of animal use

by *in vitro* methods, *in silico* methods or animals of a lower phylum (Reifenrath, Angrisani et al. 2014). Despite the age of this book, most of the facts written down there still have an enormous influence on the scientific community and beyond. The groundbreaking ideas derived from it fundamentally changed the scientific working ethos and procedures. For instance, every study implementing animal experiment applications has to refer to the principle of the 3R´s (Refine, Reduce, Replace). Briefly, every animal experiment conducted in a new study has to be justified, by means that no validated alternative method exists to adequately answer the scientific question. As a consequence, animal welfare moved into the spotlight. For the protection of animals used for scientific purposes, the European Parliament and the council of the European Union (EU) ratified the directive 2010/63/EU to ensure high ethical standards of welfare for laboratory animals in science (Olsson, Silva et al. 2017). In Germany, the judicial transposition of this directive was achieved through a revision of the German Animal Welfare Act and the adoption of the Ordinance on the Protection of Experimental Animals in 2013 (Verbraucherschutz 2013, Verbraucherschutz 2020). Furthermore, the EU Cosmetics Directive prohibited the use of animal-tested cosmetics in Europe in 2013 (Adler, Basketter et al. 2011). Following the agenda, the new European Chemicals Legislation (REACH) promotes and calls for the implementation of alternative methods, if validated and appropriate. Interestingly, alternative methods in the status of pre-validation or currently in the validation process may be used under certain conditions, which has led to considerable progress in the establishment of alternative methods, particularly with regard to methods predicting local toxicity effects or genotoxicity (Lilienblum, Dekant et al. 2008). The validation of alternative methods is defined as a process by which the relevance and reliability of a specific method or methodology is established for a defined purpose. In the process of validation, the within- and between-laboratory reproducibility as well as the transferability of the method or methodology in different laboratories (reliability) and the biological relevance and predictive capacity (relevance) has to be irrefutably demonstrated. These validation steps accelerate and facilitate the regulatory acceptance of alternative test methods by legislative authorities like the Organization for Economic Co-operation and Development (OECD) (Griesinger, Desprez et al. 2016). To date, few *in vitro* methods capable of completely replacing animal experiments have been approved by the OECD, all of which can be used in the field of toxicology. However, with regard to the validation and approval, the replacement of animal trials in basic science is even more challenging and there are concepts on the process and implementation as well as the standardization of these methods (Griesinger, Desprez et al. 2016). Yet, animal experiments are worth discussing, not only in the context of ethical justification or moral aspects, but also in light of good scientific practice. In line with this, Paul Cotton introduced “Animals and Science Benefit from 'Replace, Reduce, Refine'

Effort" (Cotton 1993), also supported by Poole (Poole 1997), which implements an important detail often neglected in discussions on animal experiments.

With respect to fracture healing models, no general consensus is stated on the gold standard. Different animal models to study fracture healing have been used, which impairs the possibility of inter-study comparisons (Mills and Simpson 2012, Reifenrath, Angrisani et al. 2014). Therefore, various organizations actively involved and experienced in musculoskeletal research in Europe, namely AOVET (the veterinary specialty group of the AO Foundation), the AO Research Institute (ARI) and the European Academy for the Study of Scientific and Technological Advance, created a list with 10 golden rules for animal experimentation in this field (Auer, Goodship et al. 2007). Briefly, these suggestions include a thoughtful study design (including adequate sample size calculation and precise formulation of hypothesis beforehand), defined endpoints, minimal severity for the animals used, a detailed description of material and methods as well as confounding variables in publications, post-operative pain management, detailed study protocols and surgical expertise of operating persons (Auer, Goodship et al. 2007). Interestingly, these expert opinions and the elaborated guidelines were not exclusively related to persons in the area of 3R research, yet bring up several very important aspects regarding the 3R`s. This example impressively demonstrates the close connection between good scientific practice and the idea of 3R research. Especially the accurate and reliable statistical planning is capable of saving many animals. Studies without appropriate animal numbers lack statistical significance and, together with the strict practice of holding back negative results, cause unnecessary repetitions of studies on the same topic, which in turn are responsible for the use of many animals that could have been spared.

With regard to fracture healing, a lot of *in silico* research is implied to reduce animal testing, mainly before starting experiments *in vivo.* Therefore, computational models have been developed, ranging from mechanoregulatory computational healing models to healing models that incorporate certain cell activities (Wang, Yang et al. 2017). These *in silico* devices provide unique insights, particularly into the various tiers on organ, tissue, cellular and intracellular levels, thereby using numerous modeling approaches such as finite element techniques, agent-based models and partial differential equations (PDE) (Borgiani, Duda et al. 2017). As refinement strategies, pain management studies, in particular, have been conducted (Auer, Goodship et al. 2007, Moreno-Jiménez, Hulsart-Billstrom et al. 2016, Jirkof, Durst et al. 2019).

Using biological system models, particularly animal models, commences complex variables that complicate the potential for successful reproducibility of experiments. In conjunction with the selection of the most appropriate model, the risk of failed reproducibility can be mini-

mized by ensuring consistent experimental methods and methodologies, the involvement of appropriately trained and qualified investigators and controlling and reducing uncontrolled variables. As a consequence, the CONSORT, PREPARE and ARRIVE guidelines were launched, in order to include details on animal use and care, implementing reporting consistency in scientific research (Schulz, Altman et al. 2010, Macy and Horvath 2017, Smith, Clutton et al. 2018, Percie du Sert, Hurst et al. 2019). The reproducibility of experimental findings is not only an issue within the same species but even more across the species. Moreover, it is closely linked to the difficulties with regard to the translation of experimental findings towards the human. Thus, a great majority of drugs or therapeutic strategies that work perfectly in mice or other small animal models fail in clinical trials. This can be a result of already described differences between animals and humans (Seok, Warren et al. 2013). Hence, the assignment of the appropriate model to the scientific question is of enormous importance, if the translation to humans is requested and no basic scientific question has to be tackled. However, while there are apparent differences between some of the diseases of humans and those of other mammalian species, the similarities and resemblances exist in much greater numbers. As a consequence, human and veterinary medicine are often faced with the same questions and problems, and employ similar means for their solution (Bradley 1927). Comparative medicine is defined as a scientific discipline, which relates differences and similarities between animals and humans, closely linking veterinarians and physicians in order to understand the mechanism of diseases (Macy and Horvath 2017). Thus, a better understanding of animal diseases could directly lead to the better understanding of human-related diseases. An important aspect of comparative medicine is the choice of the model. For instance, choosing animal models with very similar pathology and pathophysiology could lead to better translatability, and as a result, spare animals. Additionally, choosing animal models with naturally occurring diseases, for instance fracture healing disorders in horses, could also help the involved animals as patients.

4.8 Outlook

Within this thesis, an *in vitro* fracture gap model was established to mimic the initial phase of fracture healing. The initial phase takes place within the first few days and several processes of this phase could be simulated here. However, several approaches to further optimize this project will be implemented in future studies of our group. Therefore, we are planning to introduce our model in a newly developed bioreactor platform. This platform will give us the ability (i) to perfuse 4 models at the same time (while separately controllable) with fresh medium, simultaneously discard metabolites in the surrounding medium and (ii) to apply targeted pressure with the aid of a pressure unit, integrated in the bioreactor. The system will also allow us to produce SFBCs on our own in a highly standardized manner. The possibility to

cultivate 4 models and apply different supplements (growth factors, therapeutics), allows us to directly compare different approaches using the same setup. Additionally, integrated sensors provide the opportunity to analyze changes in the pH value and lactate/glucose production over time online. Furthermore, the perfusion of the models will allow us to substantially prolong the incubation time and to possibly demonstrate the transition of fracture healing into the second phase and the transition from pro-inflammatory to anti-inflammatory phase *in vitro*.

In another approach, the focus will be on the replacement of FCS as medium supplement with platelet derived human platelet lysate (pHPL). We have already established a protocol to produce pHPL from healthy donor blood and were able to show that MSCs proliferate when FCS is fully replaced with pHPL. However, so far, we failed to completely replace FCS, due to the fact that freshly isolated MSCs initially did not adhere to plastics when no FCS was present, presumably due to the fact that pHPL does not contain enough fibronectin. In the near future, we plan to optimize the cultivation of MSCs in FCS-free medium.

Within this study, the influence of DFO and DEX on the human FH model and DFO on the FGM was analyzed (Pfeiffenberger, Damerau et al. 2020, Pfeiffenberger, Hoff et al. 2020). However, there are many medications (e. g. NSAIDs, statins), not-described drugs (e.g. alcohol) and therapeutics (e.g. antibiotics, anticoagulants) that are known to influence the outcome after fracture (Gaston and Simpson 2007). The effects (positive or negative) of these and other substances, particularly on the initial phase of fracture healing, could be tested in the presented models, in order to further stratify the model on the way to OECD validation.

5 Summary

The initial phase after a fracture is particularly susceptible to fracture healing disorders. This is noticeable both in horses and humans. In order to better understand processes, especially during this phase, there are a number of different research approaches. While animal models, mostly conducted in small animals like mice and rats, were the only available option, possibilities to generate research results using 2D and 3D *in vitro* models have arisen more recently. These offer the possibility to transfer the obtained results into the human preclinical stage. The aim of this thesis was to develop an *in vitro* model that will help to develop new therapeutic strategies focused on fracture healing. First, an equine and a human fracture hematoma (FH model) were developed. The models show a clear overlap with *ex vivo* and *in vivo* experiments in animal and human models. Thus, essential processes (osteogenesis, inflammation, adaptation to hypoxia and angiogenesis) could be shown on the cell, RNA and protein level for the initial phase. In addition, three-dimensional scaffold-free bone constructs (bone model, SFBC) were developed, which are very similar to native initial bone in structure, cell composition and mineralization. In the final step, the immune component (FH model) was co-cultured with the bone component (SFBC) to simulate the fracture gap. This also shows significant overlap with *ex vivo* and *in vivo* experiments in animals and humans. The complete model makes it possible to mimic the initial phase of fracture healing. The later use of the model aims to provide a platform for the development of new therapeutic strategies for fracture healing and to enable drug testing, especially prior to animal testing, in order to evaluate a suitable candidate group prior to animal testing.

6 Zusammenfassung

Simulation der Anfangsphase der Frakturheilung *in vitro*

Besonders die initiale Phase nach einer Fraktur ist anfällig für Frakturheilungsstörungen. Dies ist sowohl im Pferd als auch im Menschen auffällig. Um Prozesse speziell während dieser Phase besser zu verstehen, gibt es eine Vielzahl verschiedener Forschungsansätze. Während früher lediglich Tiermodelle, zumeist im Kleintier (Maus und Ratte), aber auch im Großtier zur Verfügung standen, ergeben sich in der jüngeren Vergangenheit Möglichkeiten anhand von 2D und 3D *in vitro* Modellen Forschungsergebnisse zu generieren. Diese bieten die Möglichkeit erhaltene Ergebnisse besser in die humane Prä-klinik zu transferieren. Ziel der vorliegenden Arbeit war es *in vitro* ein Modell zu entwickeln, mit dessen Hilfe es möglich sein wird, neue therapeutische Strategien mit dem Fokus auf die Frakturheilung entwickeln zu können. Zunächst wurde ein equines sowie ein humanes Frakturhämatom (FH-Modell) etabliert. Die Modelle zeigen dabei deutliche Überschneidungen mit *ex vivo* sowie *in vivo* Versuchen im Tiermodell sowie Menschen. So konnten auf Zell-, RNA-, und Protein-Ebene für die initiale Phase essentielle Prozesse (Osteogenese, Entzündung, Anpassung an Hypoxie und Angiogenese) gezeigt werden. Zusätzlich wurden dreidimensionale trägerfreie Knochenkonstrukte (Knochenmodell, SFBC) entwickelt, die in ihrer Struktur, Zellzusammensetzung und Mineralisierung nativem, initialem Knochen stark ähneln. Im finalen Schritt, wurde die Immunkomponente (FH-Modell) mit der Knochenkomponente (SFBC) co-kultiviert, um den Frakturspalt zu simulieren. Hier zeigen sich ebenfalls deutliche Überschneidungen mit *ex vivo* sowie *in vivo* Versuchen im Tiermodell sowie im Menschen. Das komplette Modell macht es möglich die initiale Phase der Frakturheilung darzustellen. Die spätere Verwendung des Modells zielt darauf ab, eine Plattform zu etablieren, die zur Entwicklung neuer therapeutischer Strategien in Bezug auf die Frakturheilung und zur präklinischen Medikamentenentwicklung und -testung beiträgt.

7 References

Abraham, S. M., T. Lawrence, A. Kleiman, P. Warden, M. Medghalchi, J. Tuckermann, J. Saklatvala and A. R. Clark (2006). "Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1." The Journal of experimental medicine **203**(8): 1883-1889.

Adili, A., M. Bhandari and E. H. Schemitsch (2002). "The biomechanical effect of high-pressure irrigation on diaphyseal fracture healing in vivo." J Orthop Trauma **16**(6): 413-417.

Adler, S., D. Basketter, S. Creton, O. Pelkonen, J. van Benthem, V. Zuang, K. E. Andersen, A. Angers-Loustau, A. Aptula, A. Bal-Price, E. Benfenati, U. Bernauer, J. Bessems, F. Y. Bois, A. Boobis, E. Brandon, S. Bremer, T. Broschard, S. Casati, S. Coecke, R. Corvi, M. Cronin, G. Daston, W. Dekant, S. Felter, E. Grignard, U. Gundert-Remy, T. Heinonen, I. Kimber, J. Kleinjans, H. Komulainen, R. Kreiling, J. Kreysa, S. B. Leite, G. Loizou, G. Maxwell, P. Mazzatorta, S. Munn, S. Pfuhler, P. Phrakonkham, A. Piersma, A. Poth, P. Prieto, G. Repetto, V. Rogiers, G. Schoeters, M. Schwarz, R. Serafimova, H. Tähti, E. Testai, J. van Delft, H. van Loveren, M. Vinken, A. Worth and J.-M. Zaldivar (2011). "Alternative (non-animal) methods for cosmetics testing: current status and future prospects—2010." Archives of Toxicology **85**(5): 367-485.

Aerssens, J., S. Boonen, G. Lowet and J. Dequeker (1998). "Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research." Endocrinology **139**(2): 663-670.

Akiyama, M., H. Nonomura, S. H. Kamil and R. A. Ignotz (2006). "Periosteal cell pellet culture system: a new technique for bone engineering." Cell Transplant **15**(6): 521-532.

Al-Waeli, H., B. Nicolau, L. Stone, L. Abu Nada, Q. Gao, M. N. Abdallah, E. Abdulkader, M. Suzuki, A. Mansour, A. Al Subaie and F. Tamimi (2020). "Chronotherapy of Non-Steroidal Anti-Inflammatory Drugs May Enhance Postoperative Recovery." Scientific Reports **10**(1): 468.

Alblowi, J., R. A. Kayal, M. Siqueira, E. McKenzie, N. Krothapalli, J. McLean, J. Conn, B. Nikolajczyk, T. A. Einhorn, L. Gerstenfeld and D. T. Graves (2009). "High levels of tumor necrosis factor-alpha contribute to accelerated loss of cartilage in diabetic fracture healing." The American journal of pathology **175**(4): 1574-1585.

Alblowi, J., C. Tian, M. F. Siqueira, R. A. Kayal, E. McKenzie, Y. Behl, L. Gerstenfeld, T. A. Einhorn and D. T. Graves (2013). "Chemokine expression is upregulated in chondrocytes in diabetic fracture healing." Bone **53**(1): 294-300.

Almond, G. W. (1996). "Research applications using pigs." Vet Clin North Am Food Anim Pract **12**(3): 707-716.

Amin, S., S. J. Achenbach, E. J. Atkinson, S. Khosla and L. J. Melton, 3rd (2014). "Trends in fracture incidence: a population-based study over 20 years." Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research **29**(3): 581-589.

Amini, A. R., C. T. Laurencin and S. P. Nukavarapu (2012). "Bone tissue engineering: recent advances and challenges." Critical reviews in biomedical engineering **40**(5): 363-408.

Anada, T., J. Fukuda, Y. Sai and O. Suzuki (2012). "An oxygen-permeable spheroid culture system for the prevention of central hypoxia and necrosis of spheroids." Biomaterials **33**(33): 8430-8441.

Annamalai, R. T., P. A. Turner, W. F. t. Carson, B. Levi, S. Kunkel and J. P. Stegemann (2018). "Harnessing macrophage-mediated degradation of gelatin microspheres for spatiotemporal control of BMP2 release." Biomaterials **161**: 216-227.

Aprile, M., M. R. Ambrosio, V. D'Esposito, F. Beguinot, P. Formisano, V. Costa and A. Ciccodicola (2014). "PPARG in Human Adipogenesis: Differential Contribution of Canonical Transcripts and Dominant Negative Isoforms." PPAR research **2014**: 537865-537865.

Auer, J. A., A. Goodship, S. Arnoczky, S. Pearce, J. Price, L. Claes, B. von Rechenberg, M. Hofmann-Amtenbrinck, E. Schneider, R. Muller-Terpitz, F. Thiele, K. P. Rippe and D. W. Grainger (2007). "Refining animal models in fracture research: seeking consensus in optimising both animal welfare and scientific validity for appropriate biomedical use." BMC Musculoskelet Disord **8**: 72.

Auer, J. A. and D. W. Grainger (2015). "Fracture management in horses: Where have we been and where are we going?" Vet J **206**(1): 5-14.

Bagi, C. M., E. Berryman and M. R. Moalli (2011). "Comparative bone anatomy of commonly used laboratory animals: implications for drug discovery." Comparative medicine **61**(1): 76-85.

Baht, G. S., L. Vi and B. A. Alman (2018). "The Role of the Immune Cells in Fracture Healing." Current osteoporosis reports **16**(2): 138-145.

Baldwin, P., D. J. Li, D. A. Auston, H. S. Mir, R. S. Yoon and K. J. Koval (2019). "Autograft, Allograft, and Bone Graft Substitutes: Clinical Evidence and Indications for Use in the Setting of Orthopaedic Trauma Surgery." J Orthop Trauma **33**(4): 203-213.

Banfi, A., G. Bianchi, R. Notaro, L. Luzzatto, R. Cancedda and R. Quarto (2002). "Replicative Aging and Gene Expression in Long-Term Cultures of Human Bone Marrow Stromal Cells." Tissue Engineering **8**(6): 901-910.

Baptista, L. S., G. S. Kronemberger, I. Côrtes, L. E. Charelli, R. A. M. Matsui, T. N. Palhares, J. Sohier, A. M. Rossi and J. M. Granjeiro (2018). "Adult Stem Cells Spheroids to Optimize Cell Colonization in Scaffolds for Cartilage and Bone Tissue Engineering." International journal of molecular sciences **19**(5): 1285.

Barisam, M., M. S. Saidi, N. Kashaninejad and N.-T. Nguyen (2018). "Prediction of Necrotic Core and Hypoxic Zone of Multicellular Spheroids in a Microbioreactor with a U-Shaped Barrier." Micromachines **9**(3): 94.

Barnes, H. G., R. L. Tucker, B. D. Grant, G. D. Roberts and M. Prades (1995). "Lag screw stabilization of a cervical vertebral fracture by use of computed tomography in a horse." J Am Vet Med Assoc **206**(2): 221-223.

Bastian, O. W., M. Croes, J. Alblas, L. Koenderman, L. P. H. Leenen and T. J. Blokhuis (2018). "Neutrophils Inhibit Synthesis of Mineralized Extracellular Matrix by Human Bone Marrow-Derived Stromal Cells In Vitro." Front Immunol **9**: 945.

Bastian, O. W., L. Koenderman, J. Alblas, L. P. Leenen and T. J. Blokhuis (2016). "Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury." Clin Immunol **164**: 78-84.

Bernhardt, W. M., C. Warnecke, C. Willam, T. Tanaka, M. S. Wiesener and K. U. Eckardt (2007). Organ Protection by Hypoxia and Hypoxia-Inducible Factors. Methods in Enzymology, Academic Press. **435:** 219-245.

Bez, M., D. Sheyn, W. Tawackoli, P. Avalos, G. Shapiro, J. C. Giaconi, X. Da, S. B. David, J. Gavrity, H. A. Awad, H. W. Bae, E. J. Ley, T. J. Kremen, Z. Gazit, K. W. Ferrara, G. Pelled and D. Gazit (2017). "In situ bone tissue engineering via ultrasound-mediated gene delivery to endogenous progenitor cells in mini-pigs." Science Translational Medicine **9**(390): eaal3128.

Bhandari, M., K. Fong, S. Sprague, D. Williams and B. Petrisor (2012). "Variability in the definition and perceived causes of delayed unions and nonunions: a cross-sectional, multinational survey of orthopaedic surgeons." J Bone Joint Surg Am **94**(15): e1091-1096.

Bhumiratana, S., R. E. Eton, S. R. Oungoulian, L. Q. Wan, G. A. Ateshian and G. Vunjak-Novakovic (2014). "Large, stratified, and mechanically functional human cartilage grown in vitro by mesenchymal condensation." Proceedings of the National Academy of Sciences **111**(19): 6940.

Bonewald, L. F. and M. L. Johnson (2008). "Osteocytes, mechanosensing and Wnt signaling." Bone **42**(4): 606-615.

Bonnarens, F. and T. A. Einhorn (1984). "Production of a standard closed fracture in laboratory animal bone." J Orthop Res **2**(1): 97-101.

Borgiani, E., G. N. Duda and S. Checa (2017). "Multiscale Modeling of Bone Healing: Toward a Systems Biology Approach." Frontiers in Physiology **8**(287).

Bossard, M. J., T. A. Tomaszek, S. K. Thompson, B. Y. Amegadzie, C. R. Hanning, C. Jones, J. T. Kurdyla, D. E. McNulty, F. H. Drake, M. Gowen and M. A. Levy (1996). "Proteolytic activity of human osteoclast cathepsin K - Expression, purification, activation, and substrate identification." Journal of Biological Chemistry **271**(21): 12517-12524.

Boyce, B. F. and L. Xing (2007). "Biology of RANK, RANKL, and osteoprotegerin." Arthritis Res Ther **9 Suppl 1**: S1.

Bozlar, M., B. Aslan, A. Kalaci, L. Baktiroglu, A. N. Yanat and A. Tasci (2005). "Effects of human granulocyte-colony stimulating factor on fracture healing in rats." Saudi Med J **26**(8): 1250-1254.

Brabnikova Maresova, K., K. Pavelka and J. J. Stepan (2013). "Acute effects of glucocorticoids on serum markers of osteoclasts, osteoblasts, and osteocytes." Calcif Tissue Int **92**(4): 354-361.

Bradley, O. C. (1927). "What is Comparative Medicine?" Proceedings of the Royal Society of Medicine **21**(1): 129-134.

Brommage, R. and C. Ohlsson (2020). "High Fidelity of Mouse Models Mimicking Human Genetic Skeletal Disorders." Frontiers in Endocrinology **10**(934).

Bruderer, M., R. G. Richards, M. Alini and M. J. Stoddart (2014). "Role and regulation of RUNX2 in osteogenesis." Eur Cell Mater **28**: 269-286.

Buron, F., H. Perrin, C. Malcus, O. Hequet, O. Thaunat, M. N. Kholopp-Sarda, F. T. Moulin and E. Morelon (2009). "Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: an in vitro study using human cells." Transplant Proc **41**(8): 3347-3352.

Burska, A. N., P. V. Giannoudis, B. H. Tan, D. Ilas, E. Jones and F. Ponchel (2020). "Dynamics of Early Signalling Events during Fracture Healing and Potential Serum Biomarkers of Fracture Non-Union in Humans." Journal of clinical medicine **9**(2): 492.

Bussche, L. and G. R. Van de Walle (2014). "Peripheral Blood-Derived Mesenchymal Stromal Cells Promote Angiogenesis via Paracrine Stimulation of Vascular Endothelial Growth Factor Secretion in the Equine Model." Stem Cells Transl Med **3**(12): 1514-1525.

Canalis, E. (1996). "Clinical review 83: Mechanisms of glucocorticoid action in bone: implications to glucocorticoid-induced osteoporosis." J Clin Endocrinol Metab **81**(10): 3441-3447.

Cantinieaux, B., C. Hariga, P. Fondu, A. Ferster and M. Toppet (1990). "Desferrioxamine improves neutrophil phagocytosis in thalassemia major." American Journal of Hematology **35**(1): 13-17.

Chen, X., Y. Gan, W. Li, J. Su, Y. Zhang, Y. Huang, A. I. Roberts, Y. Han, J. Li, Y. Wang and Y. Shi (2014). "The interaction between mesenchymal stem cells and steroids during inflammation." Cell death & disease **5**(1): e1009-e1009.

Cheng, N. C., S. Wang and T. H. Young (2012). "The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities." Biomaterials **33**(6): 1748-1758.

Cheung, K. M., K. Kaluarachi, G. Andrew, W. Lu, D. Chan and K. S. Cheah (2003). "An externally fixed femoral fracture model for mice." J Orthop Res **21**(4): 685-690.

Chiesa, I., C. De Maria, A. Lapomarda, G. M. Fortunato, F. Montemurro, R. Di Gesù, R. S. Tuan, G. Vozzi and R. Gottardi (2020). "Endothelial cells support osteogenesis in an in vitro vascularized bone model developed by 3D bioprinting." Biofabrication **12**(2): 025013.

Chinwalla, A. T., L. L. Cook, K. D. Delehaunty, G. A. Fewell, L. A. Fulton, R. S. Fulton, T. A. Graves, L. W. Hillier, E. R. Mardis, J. D. McPherson, T. L. Miner, W. E. Nash, J. O. Nelson, M. N. Nhan, K. H. Pepin, C. S. Pohl, T. C. Ponce, B. Schultz, J. Thompson, E. Trevaskis, R. H. Waterston, M. C. Wendl, R. K. Wilson, S.-P. Yang, P. An, E. Berry, B. Birren, T. Bloom, D. G. Brown, J. Butler, M. Daly, R. David, J. Deri, S. Dodge, K. Foley, D. Gage, S. Gnerre, T. Holzer, D. B. Jaffe, M. Kamal, E. K. Karlsson, C. Kells, A. Kirby, E. J. Kulbokas, E. S. Lander, T. Landers, J. P. Leger, R. Levine, K. Lindblad-Toh, E. Mauceli, J. H. Mayer, M. McCarthy, J. Meldrim, J. Meldrim, J. P. Mesirov, R. Nicol, C. Nusbaum, S. Seaman, T. Sharpe, A. Sheridan, J. B. Singer, R. Santos, B. Spencer, N. Stange-Thomann, J. P. Vinson, C. M. Wade, J. Wierzbowski, D. Wyman, M. C. Zody, E. Birney, N. Goldman, A. Kasprzyk, E. Mongin, A. G. Rust, G. Slater, A. Stabenau, A. Ureta-Vidal, S. Whelan, R. Ainscough, J. Attwood, J. Bailey, K. Barlow, S. Beck, J. Burton, M. Clamp, C. Clee, A. Coulson, J. Cuff, V. Curwen, T. Cutts, J. Davies, E. Eyras, D. Grafham, S. Gregory, T. Hubbard, A. Hunt, M. Jones, A. Joy, S. Leonard, C. Lloyd, L. Matthews, S. McLaren, K. McLay, B. Meredith, J. C. Mullikin, Z. Ning, K. Oliver, E. Overton-Larty, R. Plumb, S. Potter, M. Quail, J. Rogers, C. Scott, S. Searle, R. Shownkeen, S. Sims, M. Wall, A. P. West, D. Willey, S. Williams, J. F. Abril, R. Guigó, G. Parra, P. Agarwal, R. Agarwala, D. M. Church, W. Hlavina, D. R. Maglott, V. Sapojnikov, M. Alexandersson, L. Pachter, S. E. Antonarakis, E. T. Dermitzakis, A. Reymond, C. Ucla, R. Baertsch, M. Diekhans, T. S. Furey, A. Hinrichs, F. Hsu, D. Karolchik, W. J. Kent, K. M. Roskin, M. S. Schwartz, C. Sugnet, R. J. Weber, P. Bork, I. Letunic, M. Suyama, D. Torrents, E. M. Zdobnov, M. Botcherby, S. D. Brown, R. D. Campbell, I. Jackson, N. Bray, O. Couronne, I. Dubchak, A. Poliakov, E. M. Rubin, M. R. Brent, P. Flicek, E. Keibler, I. Korf, S. Batalov, C. Bult, W. N. Frankel, P. Carninci, Y. Hayashizaki, J. Kawai, Y. Okazaki, S. Cawley, D. Kulp, R. Wheeler, F. Chiaromonte, F. S. Collins, A. Felsenfeld, M. Guyer, J. Peterson, K. Wetterstrand, R. R. Copley, R. Mott, C. Dewey, N. J. Dickens, R. D. Emes, L. Goodstadt, C. P. Ponting, E. Winter, D. M. Dunn, A. C. von Niederhausern, R. B. Weiss, S. R. Eddy, L. S. Johnson, T. A. Jones, L. Elnitski, D. L. Kolbe, P. Eswara, W. Miller, M. J. O'Connor, S. Schwartz, R. A. Gibbs, D. M. Muzny, G. Glusman, A. Smit, E. D. Green, R. C. Hardison, S. Yang, D. Haussler, A. Hua, B. A. Roe, R. S. Kucherlapati, K. T. Montgomery, J. Li, M. Li, S. Lucas, B. Ma, W. R. McCombie, M.

Morgan, P. Pevzner, G. Tesler, J. Schultz, D. R. Smith, J. Tromp, K. C. Worley, E. S. Lander, J. F. Abril, P. Agarwal, M. Alexandersson, S. E. Antonarakis, R. Baertsch, E. Berry, E. Birney, P. Bork, N. Bray, M. R. Brent, D. G. Brown, J. Butler, C. Bult, F. Chiaromonte, A. T. Chinwalla, D. M. Church, M. Clamp, F. S. Collins, R. R. Copley, O. Couronne, S. Cawley, J. Cuff, V. Curwen, T. Cutts, M. Daly, E. T. Dermitzakis, C. Dewey, C. Mouse Genome Sequencing, C. Genome Sequencing, M. I. T. C. f. G. R. Whitehead Institute, I. European Bioinformatics, I. Wellcome Trust Sanger, I. Research Group in Biomedical, Bioinformatics, I. National Center for Biotechnology, M. Department of, G. Division of Medical, S. Center for Biomolecular, Engineering, Embl, U. M. M. S. Consortium, L. Lawrence Berkeley National, S. Department of Computer, S. School of Computer, L. The Jackson, E. Laboratory for Genome, I. Affymetrix, S. Departments of, S. Health Evaluation, I. National Human Genome Research, G. Wellcome Trust Centre for Human, E. Department of Electrical, A. Department of Human, Genetics, G. Department of Human, I. Howard Hughes Medical, G. Department of, B. Departments of, B. Molecular, S. Computer, Engineering, S. Department of Computer, Engineering, M. Baylor College of, B. The Institute for Systems, B. Department of, B. Molecular, I. Howard Hughes Medical, C. Department of, Biochemistry, G. Departments of, Medicine, G. Harvard-Partners Center for, Genomics, S. Department of, U. D. J. G. Institute, L. Cold Spring Harbor, T. Wellcome, G. Max Planck Institute for Molecular, C. Genome Therapeutics, I. Bioinformatics Solutions, M. Department of, G. Human, B. Department of and G. Members of the Mouse Genome Analysis (2002). "Initial sequencing and comparative analysis of the mouse genome." Nature **420**(6915): 520-562.

Chung, Y. I., S. K. Kim, Y. K. Lee, S. J. Park, K. O. Cho, S. H. Yuk, G. Tae and Y. H. Kim (2010). "Efficient revascularization by VEGF administration via heparin-functionalized nanoparticle-fibrin complex." J Control Release **143**(3): 282-289.

Claes, L., N. Maurer-Klein, T. Henke, H. Gerngross, M. Melnyk and P. Augat (2006). "Moderate soft tissue trauma delays new bone formation only in the early phase of fracture healing." J Orthop Res **24**(6): 1178-1185.

Claes, L., S. Recknagel and A. Ignatius (2012). "Fracture healing under healthy and inflammatory conditions." Nature Reviews Rheumatology **8**(3): 133-143.

Clark, D., X. Wang, S. Chang, A. Czajka-Jakubowska, B. H. Clarkson and J. Liu (2015). "VEGF promotes osteogenic differentiation of ASCs on ordered fluorapatite surfaces." Journal of biomedical materials research. Part A **103**(2): 639-645.

Clarke, B. (2008). "Normal bone anatomy and physiology." Clinical journal of the American Society of Nephrology : CJASN **3 Suppl 3**(Suppl 3): S131-S139.

Cohn Yakubovich, D., D. Sheyn, M. Bez, Y. Schary, E. Yalon, A. Sirhan, M. Amira, A. Yaya, S. De Mel, X. Da, S. Ben-David, W. Tawackoli, E. J. Ley, D. Gazit, Z. Gazit and G. Pelled (2017). "Systemic administration of mesenchymal stem cells combined with parathyroid hormone therapy synergistically regenerates multiple rib fractures." Stem Cell Res Ther **8**(1): 51.

Collier, M. A., C. T. Brighton, R. Norrdin, A. R. Twardock and V. T. Rendano (1985). "Direct current stimulation of bone production in the horse: preliminary study with a "gap healing" model." Am J Vet Res **46**(3): 610-621.

Colnot, C., Z. Thompson, T. Miclau, Z. Werb and J. A. Helms (2003). "Altered fracture repair in the absence of MMP9." Development **130**(17): 4123.

Connolly, C. K., G. Li, J. R. Bunn, M. Mushipe, G. R. Dickson and D. R. Marsh (2003). "A reliable externally fixated murine femoral fracture model that accounts for variation in movement between animals." J Orthop Res **21**(5): 843-849.

Cotton, P. (1993). "Animals and Science Benefit From 'Replace, Reduce, Refine' Effort." JAMA **270**(24): 2905-2907.

Cottrell, J. and J. P. O'Connor (2010). "Effect of Non-Steroidal Anti-Inflammatory Drugs on Bone Healing." Pharmaceuticals (Basel, Switzerland) **3**(5): 1668-1693.

Crawford, W. H. and P. B. Fretz (1985). "Long Bone Fractures in Large Animals A Retrospective Study." Veterinary Surgery **14**(4): 295-302.

Crotti, T. N., S. M. Sharma, J. D. Fleming, M. R. Flannery, M. C. Ostrowski, S. R. Goldring and K. P. McHugh (2008). "PU.1 and NFATc1 mediate osteoclastic induction of the mouse β3 integrin promoter." Journal of Cellular Physiology **215**(3): 636-644.

Cullinane, D. M. (2002). "The role of osteocytes in bone regulation: mineral homeostasis versus mechanoreception." J Musculoskelet Neuronal Interact **2**(3): 242-244.

Curry, A. S., N. W. Pensa, A. M. Barlow and S. L. Bellis (2016). "Taking cues from the extracellular matrix to design bone-mimetic regenerative scaffolds." Matrix biology : journal of the International Society for Matrix Biology **52-54**: 397-412.

Dai, J. and A. B. Rabie (2007). "VEGF: an essential mediator of both angiogenesis and endochondral ossification." J Dent Res **86**(10): 937-950.

De Schauwer, C., E. Meyer, G. R. Van de Walle and A. Van Soom (2011). "Markers of stemness in equine mesenchymal stem cells: a plea for uniformity." Theriogenology **75**(8): 1431-1443.

De Schauwer, C., G. R. Van de Walle, A. Van Soom and E. Meyer (2013). "Mesenchymal stem cell therapy in horses: useful beyond orthopedic injuries?" Veterinary Quarterly **33**(4): 234-241.

De Witte, T.-M., L. E. Fratila-Apachitei, A. A. Zadpoor and N. A. Peppas (2018). "Bone tissue engineering via growth factor delivery: from scaffolds to complex matrices." Regenerative biomaterials **5**(4): 197-211.

Decker, S., J. Reifenrath, M. Omar, C. Krettek and C. W. Müller (2014). "Non-osteotomy and osteotomy large animal fracture models in orthopedic trauma research." Orthopedic reviews **6**(4): 5575-5575.

Dengler, V. L., M. Galbraith and J. M. Espinosa (2014). "Transcriptional regulation by hypoxia inducible factors." Critical reviews in biochemistry and molecular biology **49**(1): 1-15.

Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop and E. Horwitz (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.

Donneys, A., S. Ahsan, J. E. Perosky, S. S. Deshpande, C. N. Tchanque-Fossuo, B. Levi, K. M. Kozloff and S. R. Buchman (2013). "Deferoxamine restores callus size, mineralization, and mechanical strength in fracture healing after radiotherapy." Plastic and reconstructive surgery **131**(5): 711e-719e.

Donneys, A., A. S. Farberg, C. N. Tchanque-Fossuo, S. S. Deshpande and S. R. Buchman (2012). "Deferoxamine enhances the vascular response of bone regeneration in mandibular distraction osteogenesis." Plastic and reconstructive surgery **129**(4): 850-856.

Donneys, A., Q. Yang, M. L. Forrest, N. S. Nelson, T. Zhang, R. Ettinger, K. Ranganathan, A. Snider, S. S. Deshpande, M. S. Cohen and S. R. Buchman (2019). "Implantable hyaluronic acid-deferoxamine conjugate prevents nonunions through stimulation of neovascularization." npj Regenerative Medicine **4**(1): 11.

DuRaine, G. D., W. E. Brown, J. C. Hu and K. A. Athanasiou (2015). "Emergence of scaffold-free approaches for tissue engineering musculoskeletal cartilages." Annals of biomedical engineering **43**(3): 543-554.

Edderkaoui, B. (2017). "Potential Role of Chemokines in Fracture Repair." Frontiers in endocrinology **8**: 39-39.

Einhorn, T. A. (1998). "The cell and molecular biology of fracture healing." Clin Orthop Relat Res(355 Suppl): S7-21.

Einhorn, T. A. and L. C. Gerstenfeld (2015). "Fracture healing: mechanisms and interventions." Nat Rev Rheumatol **11**(1): 45-54.

El-Jawhari, J. J., E. Jones and P. V. Giannoudis (2016). "The roles of immune cells in bone healing; what we know, do not know and future perspectives." Injury **47**(11): 2399-2406.

El Khassawna, T., A. Serra, C. H. Bucher, A. Petersen, C. Schlundt, I. Konnecke, D. Malhan, S. Wendler, H. Schell, H. D. Volk, K. Schmidt-Bleek and G. N. Duda (2017). "T Lymphocytes Influence the Mineralization Process of Bone." Front Immunol **8**: 562.

Elefteriou, F. and X. Yang (2011). "Genetic mouse models for bone studies--strengths and limitations." Bone **49**(6): 1242-1254.

Elliott, N. T. and F. Yuan (2011). "A review of three-dimensional in vitro tissue models for drug discovery and transport studies." J Pharm Sci **100**(1): 59-74.

Erli, H. J., R. Marx, O. Paar, F. U. Niethard, M. Weber and D. C. Wirtz (2003). "Surface pretreatments for medical application of adhesion." BioMedical Engineering OnLine **2**: 15-15.

Farberg, A. S., D. Sarhaddi, A. Donneys, S. S. Deshpande and S. R. Buchman (2014). "Deferoxamine enhances bone regeneration in mandibular distraction osteogenesis." Plastic and reconstructive surgery **133**(3): 666-671.

Farberg, A. S., D. Sarhaddi, E. R. Razdolsky, A. Donneys, S. S. Deshpande and S. R. Buchman (2012). "Deferoxamine Enhances Bone Regeneration in Irradiated Mandibular Distraction Osteogenesis." Plastic and Reconstructive Surgery **130**(5S-1).

Faugere, M. C., R. M. Friedler, P. Fanti and H. H. Malluche (1990). "Bone changes occurring early after cessation of ovarian function in beagle dogs: a histomorphometric study employing sequential biopsies." J Bone Miner Res **5**(3): 263-272.

Fazzalari, N. L. (2011). "Bone fracture and bone fracture repair." Osteoporosis International **22**(6): 2003-2006.

Fernandez-Rebollo, E., B. Mentrup, R. Ebert, J. Franzen, G. Abagnale, T. Sieben, A. Ostrowska, P. Hoffmann, P.-F. Roux, B. Rath, M. Goodhardt, J.-M. Lemaitre, O. Bischof, F. Jakob and W. Wagner

(2017). "Human Platelet Lysate versus Fetal Calf Serum: These Supplements Do Not Select for Different Mesenchymal Stromal Cells." Scientific Reports **7**(1): 5132.

Filipowska, J., K. A. Tomaszewski, Ł. Niedźwiedzki, J. A. Walocha and T. Niedźwiedzki (2017). "The role of vasculature in bone development, regeneration and proper systemic functioning." Angiogenesis **20**(3): 291-302.

Florencio-Silva, R., G. R. d. S. Sasso, E. Sasso-Cerri, M. J. Simões and P. S. Cerri (2015). "Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells." BioMed research international **2015**: 421746-421746.

Foundation, I. O. (2019, 2019). "Fracture Epidemiology." Retrieved 19.08.2020, 2020, from https://www.capturethefracture.org/fracture-epidemiology.

Fragogeorgi, E. A., M. Rouchota, M. Georgiou, M. Velez, P. Bouziotis and G. Loudos (2019). "In vivo imaging techniques for bone tissue engineering." Journal of tissue engineering **10**: 2041731419854586-2041731419854586.

Freigang, V., F. Gschrei, H. Bhayana, P. Schmitz, J. Weber, M. Kerschbaum, M. Nerlich and F. Baumann (2019). "Risk factor analysis for delayed union after subtrochanteric femur fracture: quality of reduction and valgization are the key to success." BMC Musculoskeletal Disorders **20**(1): 391.

Freyer, J. P. (1988). "Role of necrosis in regulating the growth saturation of multicellular spheroids." Cancer Res **48**(9): 2432-2439.

Frith, J. E., B. Thomson and P. G. Genever (2010). "Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential." Tissue Eng Part C Methods **16**(4): 735-749.

Gaber, T., R. Dziurla, R. Tripmacher, G. R. Burmester and F. Buttgereit (2005). "Hypoxia inducible factor (HIF) in rheumatology: low O2! See what HIF can do!" Annals of the rheumatic diseases **64**(7): 971-980.

Gaber, T., S. Schellmann, K. B. Erekul, M. Fangradt, K. Tykwinska, M. Hahne, P. Maschmeyer, M. Wagegg, C. Stahn, P. Kolar, R. Dziurla, M. Lohning, G. R. Burmester and F. Buttgereit (2011). "Macrophage migration inhibitory factor counterregulates dexamethasone-mediated suppression of hypoxia-inducible factor-1 alpha function and differentially influences human CD4+ T cell proliferation under hypoxia." J Immunol **186**(2): 764-774.

Garcia, P., S. Herwerth, R. Matthys, J. H. Holstein, T. Histing, M. D. Menger and T. Pohlemann (2011). "The LockingMouseNail--a new implant for standardized stable osteosynthesis in mice." J Surg Res **169**(2): 220-226.

Garcia, P., V. Speidel, C. Scheuer, M. W. Laschke, J. H. Holstein, T. Histing, T. Pohlemann and M. D. Menger (2011). "Low dose erythropoietin stimulates bone healing in mice." J Orthop Res **29**(2): 165-172.

Gasser, J. and M. Kneissel (2017). Bone Physiology and Biology**:** 27-94.

Gaston, M. S. and A. H. R. W. Simpson (2007). "Inhibition of fracture healing." The Journal of Bone and Joint Surgery. British volume **89-B**(12): 1553-1560.

Gerber, H. P., T. H. Vu, A. M. Ryan, J. Kowalski, Z. Werb and N. Ferrara (1999). "VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation." Nat Med **5**(6): 623-628.

Ghiasi, M. S., J. Chen, A. Vaziri, E. K. Rodriguez and A. Nazarian (2017). "Bone fracture healing in mechanobiological modeling: A review of principles and methods." Bone reports **6**: 87-100.

Ghosh, S., M. Laha, S. Mondal, S. Sengupta and D. L. Kaplan (2009). "In vitro model of mesenchymal condensation during chondrogenic development." Biomaterials **30**(33): 6530-6540.

Giannotti, S., V. Bottai, G. Dell'osso, E. Pini, G. De Paola, G. Bugelli and G. Guido (2013). "Current medical treatment strategies concerning fracture healing." Clinical cases in mineral and bone metabolism : the official journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases **10**(2): 116-120.

Grabowski, P. (2009). "Physiology of bone." Endocr Dev **16**: 32-48.

Granchi, D., G. Ochoa, E. Leonardi, V. Devescovi, S. R. Baglio, L. Osaba, N. Baldini and G. Ciapetti (2010). "Gene expression patterns related to osteogenic differentiation of bone marrow-derived mesenchymal stem cells during ex vivo expansion." Tissue Eng Part C Methods **16**(3): 511-524.

Granero-Molto, F., J. A. Weis, M. I. Miga, B. Landis, T. J. Myers, L. O'Rear, L. Longobardi, E. D. Jansen, D. P. Mortlock and A. Spagnoli (2009). "Regenerative effects of transplanted mesenchymal stem cells in fracture healing." Stem Cells **27**(8): 1887-1898.

Grewal, B. S., B. Keller, P. Weinhold and L. E. Dahners (2014). "Evaluating effects of deferoxamine in a rat tibia critical bone defect model." J Orthop **11**(1): 5-9.

Griesinger, C., B. Desprez, S. Coecke, W. Casey and V. Zuang (2016). "Validation of Alternative In Vitro Methods to Animal Testing: Concepts, Challenges, Processes and Tools." Adv Exp Med Biol **856**: 65-132.

Grogaard, B., B. Gerdin and O. Reikeras (1990). "The polymorphonuclear leukocyte: has it a role in fracture healing?" Arch Orthop Trauma Surg **109**(5): 268-271.

Gross, T. S., K. A. King, N. A. Rabaia, P. Pathare and S. Srinivasan (2005). "Upregulation of osteopontin by osteocytes deprived of mechanical loading or oxygen." J Bone Miner Res **20**(2): 250-256.

Grosso, A., M. G. Burger, A. Lunger, D. J. Schaefer, A. Banfi and N. Di Maggio (2017). "It Takes Two to Tango: Coupling of Angiogenesis and Osteogenesis for Bone Regeneration." Frontiers in Bioengineering and Biotechnology **5**(68).

Grundnes, O. and O. Reikeras (1993). "The importance of the hematoma for fracture healing in rats." Acta Orthop Scand **64**(3): 340-342.

Grundnes, O. and O. Reikerås (1993). "The role of hematoma and periosteal sealing for fracture healing in rats." Acta Orthopaedica Scandinavica **64**(1): 47-49.

Habermann, B., C. Eberhardt, M. Feld, L. Zichner and A. A. Kurth (2007). "Tartrate-resistant acid phosphatase 5b (TRAP 5b) as a marker of osteoclast activity in the early phase after cementless total hip replacement." Acta Orthopaedica **78**(2): 221-225.

Haffner-Luntzer, M., A. Kovtun, A. E. Rapp and A. Ignatius (2016). "Mouse Models in Bone Fracture Healing Research." Current Molecular Biology Reports **2**(2): 101-111.

Haffner-Luntzer, M., F. Müller-Graf, R. Matthys, Y. Hägele, V. Fischer, R. Jonas, A. Abaei, F. Gebhard, V. Rasche and A. Ignatius (2017). "Evaluation of high-resolution In Vivo MRI for longitudinal analysis of endochondral fracture healing in mice." PloS one **12**(3): e0174283-e0174283.

Hardingham, T. E., R. A. Oldershaw and S. R. Tew (2006). "Cartilage, SOX9 and Notch signals in chondrogenesis." Journal of anatomy **209**(4): 469-480.

Hare, J. M., J. E. Fishman, G. Gerstenblith, D. L. DiFede Velazquez, J. P. Zambrano, V. Y. Suncion, M. Tracy, E. Ghersin, P. V. Johnston, J. A. Brinker, E. Breton, J. Davis-Sproul, I. H. Schulman, J. Byrnes, A. M. Mendizabal, M. H. Lowery, D. Rouy, P. Altman, C. Wong Po Foo, P. Ruiz, A. Amador, J. Da Silva, I. K. McNiece, A. W. Heldman, R. George and A. Lardo (2012). "Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial." JAMA **308**(22): 2369-2379.

Hayman, A. R. (2008). "Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy." Autoimmunity **41**(3): 218-223.

Hemeda, H., B. Giebel and W. Wagner (2014). "Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells." Cytotherapy **16**(2): 170-180.

Hemmann, P., P. Ziegler, C. Konrads, A. Ellmerer, T. Klopfer, A. J. Schreiner and C. Bahrs (2020). "Trends in fracture development of the upper extremity in Germany-a population-based description of the past 15 years." J Orthop Surg Res **15**(1): 65.

Henle, P., G. Zimmermann and S. Weiss (2005). "Matrix metalloproteinases and failed fracture healing." Bone **37**(6): 791-798.

Heo, J. S., S. G. Lee and H. O. Kim (2017). "Distal-less homeobox 5 is a master regulator of the osteogenesis of human mesenchymal stem cells." International journal of molecular medicine **40**(5): 1486-1494.

Herrmann, M., S. Zeiter, U. Eberli, M. Hildebrand, K. Camenisch, U. Menzel, M. Alini, S. Verrier and V. A. Stadelmann (2018). "Five Days Granulocyte Colony-Stimulating Factor Treatment Increases Bone Formation and Reduces Gap Size of a Rat Segmental Bone Defect: A Pilot Study." Frontiers in bioengineering and biotechnology **6**: 5-5.

Hildebrandt, C., H. Büth and H. Thielecke (2011). "A scaffold-free in vitro model for osteogenesis of human mesenchymal stem cells." Tissue Cell **43**(2): 91-100.

Hileti, D., P. Panayiotidis and A. V. Hoffbrand (1995). "Iron chelators induce apoptosis in proliferating cells." Br J Haematol **89**(1): 181-187.

Hillmann, A., A. B. Ahrberg, W. Brehm, S. Heller, C. Josten, F. Paebst and J. Burk (2016). "Comparative Characterization of Human and Equine Mesenchymal Stromal Cells: A Basis for Translational Studies in the Equine Model." Cell Transplant **25**(1): 109-124.

Hiltunen, A., E. Vuorio and H. T. Aro (1993). "A standardized experimental fracture in the mouse tibia." J Orthop Res **11**(2): 305-312.

Hino, K., A. Saito, M. Kido, S. Kanemoto, R. Asada, T. Takai, M. Cui, X. Cui and K. Imaizumi (2014). "Master regulator for chondrogenesis, Sox9, Regulates Transcriptional Activation of the ER Stress Transducer BBF2H7/CREB3L2 in Chondrocytes." Journal of Biological Chemistry.

Histing, T., P. Garcia, J. H. Holstein, M. Klein, R. Matthys, R. Nuetzi, R. Steck, M. W. Laschke, T. Wehner, R. Bindl, S. Recknagel, E. K. Stuermer, B. Vollmar, B. Wildemann, J. Lienau, B. Willie, A. Peters, A. Ignatius, T. Pohlemann, L. Claes and M. D. Menger (2011). "Small animal bone healing models: standards, tips, and pitfalls results of a consensus meeting." Bone **49**(4): 591-599.

Histing, T., P. Garcia, R. Matthys, M. Leidinger, J. H. Holstein, A. Kristen, T. Pohlemann and M. D. Menger (2010). "An internal locking plate to study intramembranous bone healing in a mouse femur fracture model." Journal of Orthopaedic Research **28**(3): 397-402.

Hoeben, A., B. Landuyt, M. S. Highley, H. Wildiers, A. T. Van Oosterom and E. A. De Bruijn (2004). "Vascular endothelial growth factor and angiogenesis." Pharmacol Rev **56**(4): 549-580.

Hoff, P., T. Gaber, K. Schmidt-Bleek, U. Senturk, C. L. Tran, K. Blankenstein, S. Lutkecosmann, J. Bredahl, H. J. Schuler, P. Simon, G. Wassilew, F. Unterhauser, G. R. Burmester, G. Schmidmaier, C. Perka, G. N. Duda and F. Buttgereit (2011). "Immunologically restricted patients exhibit a pronounced inflammation and inadequate response to hypoxia in fracture hematomas." Immunol Res **51**(1): 116-122.

Hoff, P., T. Gaber, C. Strehl, M. Jakstadt, H. Hoff, K. Schmidt-Bleek, A. Lang, E. Rohner, D. Huscher, G. Matziolis, G. R. Burmester, G. Schmidmaier, C. Perka, G. N. Duda and F. Buttgereit (2017). "A Pronounced Inflammatory Activity Characterizes the Early Fracture Healing Phase in Immunologically Restricted Patients." Int J Mol Sci **18**(3).

Hoff, P., T. Gaber, C. Strehl, K. Schmidt-Bleek, A. Lang, D. Huscher, G. R. Burmester, G. Schmidmaier, C. Perka, G. N. Duda and F. Buttgereit (2016). "Immunological characterization of the early human fracture hematoma." Immunologic Research **64**(5): 1195-1206.

Hoff, P., P. Maschmeyer, T. Gaber, T. Schütze, T. Raue, K. Schmidt-Bleek, R. Dziurla, S. Schellmann, F. L. Lohanatha, E. Röhner, A. Ode, G.-R. Burmester, G. N. Duda, C. Perka and F. Buttgereit (2013). "Human immune cells' behavior and survival under bioenergetically restricted conditions in an in vitro fracture hematoma model." Cellular & molecular immunology **10**(2): 151-158.

Holstein, J. H., M. D. Menger, U. Culemann, C. Meier and T. Pohlemann (2007). "Development of a locking femur nail for mice." J Biomech **40**(1): 215-219.

Honn, K. V., J. A. Singley and W. Chavin (1975). "Fetal bovine serum: a multivariate standard." Proc Soc Exp Biol Med **149**(2): 344-347.

Hosogane, N., Z. Huang, B. A. Rawlins, X. Liu, O. Boachie-Adjei, A. L. Boskey and W. Zhu (2010). "Stromal derived factor-1 regulates bone morphogenetic protein 2-induced osteogenic differentiation of primary mesenchymal stem cells." Int J Biochem Cell Biol **42**(7): 1132-1141.

I, D. A., E. Nargi, F. Mastrangelo, G. Falco, P. Lanuti, M. Marchisio, S. Miscia, I. Robuffo, M. Capogreco, S. Buccella, S. Caputi, F. Caciagli, S. Tete and R. Ciccarelli (2011). "Vascular endothelial growth factor enhances in vitro proliferation and osteogenic differentiation of human dental pulp stem cells." J Biol Regul Homeost Agents **25**(1): 57-69.

Icer, M. A. and M. Gezmen-Karadag (2018). "The multiple functions and mechanisms of osteopontin." Clin Biochem **59**: 17-24.

Irwin, D. C., J. M. McCord, E. Nozik-Grayck, G. Beckly, B. Foreman, T. Sullivan, M. White, J. T. Crossno, D. Bailey, S. C. Flores, S. Majka, D. Klemm and M. C. Tissot van Patot (2009). "A potential role for reactive oxygen species and the HIF-1α–VEGF pathway in hypoxia-induced pulmonary vascular leak." Free Radical Biology and Medicine **47**(1): 55-61.

Jäger, M., P. Hernigou, C. Zilkens, M. Herten, X. Li, J. Fischer and R. Krauspe (2010). "Cell therapy in bone healing disorders." Orthopedic reviews **2**(2): e20-e20.

Jahagirdar, R. and B. E. Scammell (2009). "Principles of fracture healing and disorders of bone union." Surgery (Oxford) **27**(2): 63-69.

James, A. W., G. LaChaud, J. Shen, G. Asatrian, V. Nguyen, X. Zhang, K. Ting and C. Soo (2016). "A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2." Tissue engineering. Part B, Reviews **22**(4): 284-297.

Jensen, E. D., R. Gopalakrishnan and J. J. Westendorf (2010). "Regulation of gene expression in osteoblasts." BioFactors (Oxford, England) **36**(1): 25-32.

Jia, P., H. Chen, H. Kang, J. Qi, P. Zhao, M. Jiang, L. Guo, Q. Zhou, N. D. Qian, H. B. Zhou, Y. J. Xu, Y. Fan and L. F. Deng (2016). "Deferoxamine released from poly(lactic-co-glycolic acid) promotes healing of osteoporotic bone defect via enhanced angiogenesis and osteogenesis." J Biomed Mater Res A **104**(10): 2515-2527.

Jing, Y. and Y. Jian-Xiong (2011). "3-D spheroid culture of bone marrow mesenchymal stem cell of rhesus monkey with improved multi-differentiation potential to epithelial progenitors and neuron in vitro." Clin Exp Ophthalmol **39**(8): 808-819.

Jirkof, P., M. Durst, R. Klopfleisch, R. Palme, C. Thone-Reineke, F. Buttgereit, K. Schmidt-Bleek and A. Lang (2019). "Administration of Tramadol or Buprenorphine via the drinking water for post-operative analgesia in a mouse-osteotomy model." Sci Rep **9**(1): 10749.

Jochems, C. E. A., J. B. F. van der Valk, F. R. Stafleu and V. Baumans (2002). "The Use of Fetal Bovine Serum: Ethical or Scientific Problem?" Alternatives to Laboratory Animals **30**(2): 219-227.

Johnson, R. B., J. A. Gilbert, R. C. Cooper, D. E. Parsell, B. A. Stewart, X. Dai, T. G. Nick, C. F. Streckfus, R. A. Butler and J. G. Boring (2002). "Effect of estrogen deficiency on skeletal and alveolar bone density in sheep." J Periodontol **73**(4): 383-391.

Jokela, R. M., J. V. Ahonen, M. K. Tallgren, P. C. Marjakangas and K. T. Korttila (2009). "The effective analgesic dose of dexamethasone after laparoscopic hysterectomy." Anesth Analg **109**(2): 607-615.

Jurgensen, J. S., C. Rosenberger, M. S. Wiesener, C. Warnecke, J. H. Horstrup, M. Grafe, S. Philipp, W. Griethe, P. H. Maxwell, U. Frei, S. Bachmann, R. Willenbrock and K. U. Eckardt (2004). "Persistent induction of HIF-1alpha and -2alpha in cardiomyocytes and stromal cells of ischemic myocardium." FASEB J **18**(12): 1415-1417.

Kabiri, M., B. Kul, W. B. Lott, K. Futrega, P. Ghanavi, Z. Upton and M. R. Doran (2012). "3D mesenchymal stem/stromal cell osteogenesis and autocrine signalling." Biochem Biophys Res Commun **419**(2): 142-147.

Kent Leach, J., D. Kaigler, Z. Wang, P. H. Krebsbach and D. J. Mooney (2006). "Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration." Biomaterials **27**(17): 3249-3255.

Kenwright, J. and T. Gardner (1998). "Mechanical Influences on Tibial Fracture Healing." Clinical Orthopaedics and Related Research (1976-2007) **355**.

Kidd, L. J., A. S. Stephens, J. S. Kuliwaba, N. L. Fazzalari, A. C. Wu and M. R. Forwood (2010). "Temporal pattern of gene expression and histology of stress fracture healing." Bone **46**(2): 369-378.

Kim, B. M., J. Y. Choi, Y. J. Kim, H. D. Woo and H. W. Chung (2007). "Desferrioxamine (DFX) has genotoxic effects on cultured human lymphocytes and induces the p53-mediated damage response." Toxicology **229**(3): 226-235.

Kitaori, T., H. Ito, E. M. Schwarz, R. Tsutsumi, H. Yoshitomi, S. Oishi, M. Nakano, N. Fujii, T. Nagasawa and T. Nakamura (2009). "Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model." Arthritis Rheum **60**(3): 813-823.

Klein, C. and M. H. T. Troedsson (2013). "Macrophage migration inhibitory factor is expressed by equine conceptuses and endometrium." Reproduction in domestic animals = Zuchthygiene **48**(2): 297-304.

Klotz, B. J., K. S. Lim, Y. X. Chang, B. G. Soliman, I. Pennings, F. P. W. Melchels, T. B. F. Woodfield, A. J. Rosenberg, J. Malda and D. Gawlitta (2018). "Engineering of a complex bone tissue model with endothelialised channels and capillary-like networks." Eur Cell Mater **35**: 335-348.

Knepper, P. A., C. S. Mayanil, W. Goossens, D. G. McLone and E. Hayes (1998). "The presence of transcription factors in fetal bovine sera." In Vitro Cellular & Developmental Biology - Animal **34**(2): 170-173.

Knight, M. N. and K. D. Hankenson (2013). "Mesenchymal Stem Cells in Bone Regeneration." Adv Wound Care (New Rochelle) **2**(6): 306-316.

Kobayashi, T., S. Onodera, E. Kondo, H. Tohyama, H. Fujiki, A. Yokoyama and K. Yasuda (2011). "Impaired fracture healing in macrophage migration inhibitory factor-deficient mice." Osteoporos Int **22**(6): 1955-1965.

Kolar, P., T. Gaber, C. Perka, G. N. Duda and F. Buttgereit (2011). "Human early fracture hematoma is characterized by inflammation and hypoxia." Clinical orthopaedics and related research **469**(11): 3118-3126.

Kolar, P., K. Schmidt-Bleek, H. Schell, T. Gaber, D. Toben, G. Schmidmaier, C. Perka, F. Buttgereit and G. N. Duda (2010). "The early fracture hematoma and its potential role in fracture healing." Tissue Eng Part B Rev **16**(4): 427-434.

Kolios, L., A. K. Hoerster, S. Sehmisch, M. C. Malcherek, T. Rack, M. Tezval, D. Seidlova-Wuttke, W. Wuttke, K. M. Stuermer and E. K. Stuermer (2010). "Do estrogen and alendronate improve metaphyseal fracture healing when applied as osteoporosis prophylaxis?" Calcif Tissue Int **86**(1): 23-32.

Konnecke, I., A. Serra, T. El Khassawna, C. Schlundt, H. Schell, A. Hauser, A. Ellinghaus, H. D. Volk, A. Radbruch, G. N. Duda and K. Schmidt-Bleek (2014). "T and B cells participate in bone repair by infiltrating the fracture callus in a two-wave fashion." Bone **64**: 155-165.

Kovtun, A., S. Bergdolt, R. Wiegner, P. Radermacher, M. Huber-Lang and A. Ignatius (2016). "The crucial role of neutrophil granulocytes in bone fracture healing." Eur Cell Mater **32**: 152-162.

Krischak, G. D., P. Augat, T. Sorg, R. Blakytny, L. Kinzl, L. Claes and A. Beck (2007). "Effects of diclofenac on periosteal callus maturation in osteotomy healing in an animal model." Arch Orthop Trauma Surg **127**(1): 3-9.

Krzywinska, E. and C. Stockmann (2018). "Hypoxia, Metabolism and Immune Cell Function." Biomedicines **6**(2).

Laboklin, L. f. k. D. G. C. K. "Hund, Katze, Pferd (1) Klinisch-chemische Werte." Retrieved 20.08.2020, 2020, from https://laboklin.com/de/infothek/referenzwerte/hund-katze-pferd/.

Lang, A., S. Helfmeier, J. Stefanowski, A. Kuppe, V. Sunkara, M. Pfeiffenberger, A. Wolter, A. Damerau, S. Hemmati-Sadeghi, J. Ringe, R. Haag, A. E. Hauser, M. Löhning, C. Perka, G. N. Duda, P. Hoff, K. Schmidt-Bleek, T. Gaber and F. Buttgereit (2020). "HIF-stabilization prevents delayed fracture healing." bioRxiv: 2020.2007.2002.182832.

Le Blanc, K. and L. C. Davies (2015). "Mesenchymal stromal cells and the innate immune response." Immunology Letters **168**(2): 140-146.

Lederman, H. M., A. Cohen, J. W. Lee, M. H. Freedman and E. W. Gelfand (1984). "Deferoxamine: a reversible S-phase inhibitor of human lymphocyte proliferation." Blood **64**(3): 748-753.

Lee, D., S. Pathak and J.-H. Jeong (2019). "Design and manufacture of 3D cell culture plate for mass production of cell-spheroids." Scientific Reports **9**(1): 13976.

Lee, J., J. S. Byeon, K. S. Lee, N. Y. Gu, G. B. Lee, H. R. Kim, I. S. Cho and S. H. Cha (2016). "Chondrogenic potential and anti-senescence effect of hypoxia on canine adipose mesenchymal stem cells." Vet Res Commun **40**(1): 1-10.

Leszczynska, J., B. Zyzynska-Granica, K. Koziak, S. Ruminski and M. Lewandowska-Szumiel (2013). "Contribution of endothelial cells to human bone-derived cells expansion in coculture." Tissue Eng Part A **19**(3-4): 393-402.

Li, D., F. Zhang, X. Zhang, C. Xue, M. Namwanje, L. Fan, M. P. Reilly, F. Hu and L. Qiang (2016). "Distinct functions of PPARγ isoforms in regulating adipocyte plasticity." Biochemical and Biophysical Research Communications **481**(1): 132-138.

Li, J., L. Fan, Z. Yu, X. Dang and K. Wang (2015). "The effect of deferoxamine on angiogenesis and bone repair in steroid-induced osteonecrosis of rabbit femoral heads." Experimental biology and medicine (Maywood, N.J.) **240**(2): 273-280.

Li, J., X. Wang, C. Zhou, L. Liu, Y. Wu, D. Wang and H. Jiang (2012). "Perioperative glucocorticosteroid treatment delays early healing of a mandible wound by inhibiting osteogenic differentiation." Injury **43**(8): 1284-1289.

Li, P., S. Oparil, W. Feng and Y. F. Chen (2004). "Hypoxia-responsive growth factors upregulate periostin and osteopontin expression via distinct signaling pathways in rat pulmonary arterial smooth muscle cells." J Appl Physiol (1985) **97**(4): 1550-1558; discussion 1549.

Lieu, S., E. Hansen, R. Dedini, D. Behonick, Z. Werb, T. Miclau, R. Marcucio and C. Colnot (2011). "Impaired remodeling phase of fracture repair in the absence of matrix metalloproteinase-2." Disease Models & Mechanisms **4**(2): 203.

Lilienblum, W., W. Dekant, H. Foth, T. Gebel, J. G. Hengstler, R. Kahl, P. J. Kramer, H. Schweinfurth and K. M. Wollin (2008). "Alternative methods to safety studies in experimental animals: role in the risk assessment of chemicals under the new European Chemicals Legislation (REACH)." Archives of Toxicology **82**(4): 211-236.

Lin, T.-H., R.-S. Yang, C.-H. Tang, C.-P. Lin and W.-M. Fu (2007). "PPARγ inhibits osteogenesis via the down-regulation of the expression of COX-2 and iNOS in rats." Bone **41**(4): 562-574.

Lissenberg-Thunnissen, S. N., D. J. J. de Gorter, C. F. M. Sier and I. B. Schipper (2011). "Use and efficacy of bone morphogenetic proteins in fracture healing." International orthopaedics **35**(9): 1271-1280.

Liu, J., Y. Ding, Z. Liu and X. Liang (2020). "Senescence in Mesenchymal Stem Cells: Functional Alterations, Molecular Mechanisms, and Rejuvenation Strategies." Frontiers in Cell and Developmental Biology **8**(258).

Liu, W., S.-M. Shen, X.-Y. Zhao and G.-Q. Chen (2012). "Targeted genes and interacting proteins of hypoxia inducible factor-1." International journal of biochemistry and molecular biology **3**(2): 165-178.

Liu, Y., A. D. Berendsen, S. Jia, S. Lotinun, R. Baron, N. Ferrara and B. R. Olsen (2012). "Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation." The Journal of Clinical Investigation **122**(9): 3101-3113.

Liu, Y. Z., M. P. Akhter, X. Gao, X. Y. Wang, X. B. Wang, G. Zhao, X. Wei, H. J. Wu, H. Chen, D. Wang and L. Cui (2018). "Glucocorticoid-induced delayed fracture healing and impaired bone biomechanical properties in mice." Clin Interv Aging **13**: 1465-1474.

Loi, F., L. A. Córdova, J. Pajarinen, T.-h. Lin, Z. Yao and S. B. Goodman (2016). "Inflammation, fracture and bone repair." Bone **86**: 119-130.

Luo, Y., D. L. He, L. Ning, S. L. Shen, L. Li, X. Li, H. E. Zhau and L. W. Chung (2006). "Over-expression of hypoxia-inducible factor-1alpha increases the invasive potency of LNCaP cells in vitro." BJU Int **98**(6): 1315-1319.

Ma, D., C. Zhong, H. Yao, Y. Liu, F. Chen, J. Li, J. Zhao, T. Mao and L. Ren (2011). "Engineering injectable bone using bone marrow stromal cell aggregates." Stem Cells Dev **20**(6): 989-999.

Mackey, V. S., D. R. Trout, D. M. Meagher and W. J. Hornof (1987). "STRESS FRACTURES OF THE HUMERUS, RADIUS, AND TIBIA IN HORSES." Veterinary Radiology **28**(1): 26-31.

Mackie, E. J., Y. A. Ahmed, L. Tatarczuch, K. S. Chen and M. Mirams (2008). "Endochondral ossification: How cartilage is converted into bone in the developing skeleton." International Journal of Biochemistry & Cell Biology **40**(1): 46-62.

Macy, J. and T. L. Horvath (2017). "Comparative Medicine: An Inclusive Crossover Discipline." The Yale journal of biology and medicine **90**(3): 493-498.

Maes, C., G. Carmeliet and E. Schipani (2012). "Hypoxia-driven pathways in bone development, regeneration and disease." Nat Rev Rheumatol **8**(6): 358-366.

Majidinia, M., A. Sadeghpour and B. Yousefi (2018). "The roles of signaling pathways in bone repair and regeneration." J Cell Physiol **233**(4): 2937-2948.

Maleki, M., F. Ghanbarvand, M. Reza Behvarz, M. Ejtemaei and E. Ghadirkhomi (2014). "Comparison of mesenchymal stem cell markers in multiple human adult stem cells." International journal of stem cells **7**(2): 118-126.

Manigrasso, M. B. and J. P. O'Connor (2004). "Characterization of a closed femur fracture model in mice." J Orthop Trauma **18**(10): 687-695.

Marciano, D. P., D. S. Kuruvilla, S. V. Boregowda, A. Asteian, T. S. Hughes, R. Garcia-Ordonez, C. A. Corzo, T. M. Khan, S. J. Novick, H. Park, D. J. Kojetin, D. G. Phinney, J. B. Bruning, T. M. Kamenecka and P. R. Griffin (2015). "Pharmacological repression of PPARγ promotes osteogenesis." Nature Communications **6**(1): 7443.

Marsell, R. and T. A. Einhorn (2011). "The biology of fracture healing." Injury **42**(6): 551-555.

Marsh, D. (1998). "Concepts of Fracture Union, Delayed Union, and Nonunion." Clinical Orthopaedics and Related Research® **355**.

Martin, D., R. Galisteo and J. S. Gutkind (2009). "CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex." J Biol Chem **284**(10): 6038-6042.

Martin, R. K., J. P. Albright, W. S. Jee, G. N. Taylor and W. R. Clarke (1981). "Bone loss in the beagle tibia: influence of age, weight, and sex." Calcif Tissue Int **33**(3): 233-238.

Martins, A., A. R. C. Duarte, S. Faria, A. P. Marques, R. L. Reis and N. M. Neves (2010). "Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release functionality." Biomaterials **31**(22): 5875-5885.

Masoud, G. N. and W. Li (2015). "HIF-1α pathway: role, regulation and intervention for cancer therapy." Acta pharmaceutica Sinica. B **5**(5): 378-389.

Mathew, G. and B. P. Hanson (2009). "Global burden of trauma: Need for effective fracture therapies." Indian journal of orthopaedics **43**(2): 111-116.

Matsumoto, T. and S. Sato (2015). "Stimulating angiogenesis mitigates the unloading-induced reduction in osteogenesis in early-stage bone repair in rats." Physiol Rep **3**(3).

Matthys, R. and S. M. Perren (2009). "Internal fixator for use in the mouse." Injury **40 Suppl 4**: S103-109.

Mauney, J. R., C. Jaquiery, V. Volloch, M. Heberer, I. Martin and D. L. Kaplan (2005). "In vitro and in vivo evaluation of differentially demineralized cancellous bone scaffolds combined with human bone marrow stromal cells for tissue engineering." Biomaterials **26**(16): 3173-3185.

McKenzie, J. A. and M. J. Silva (2011). "Comparing histological, vascular and molecular responses associated with woven and lamellar bone formation induced by mechanical loading in the rat ulna." Bone **48**(2): 250-258.

Mestas, J. and C. C. W. Hughes (2004). "Of Mice and Not Men: Differences between Mouse and Human Immunology." The Journal of Immunology **172**(5): 2731.

Mills, L. A. and A. H. Simpson (2012). "In vivo models of bone repair." J Bone Joint Surg Br **94**(7): 865-874.

Mizuno, K., K. Mineo, T. Tachibana, M. Sumi, T. Matsubara and K. Hirohata (1990). "The osteogenetic potential of fracture haematoma. Subperiosteal and intramuscular transplantation of the haematoma." J Bone Joint Surg Br **72**(5): 822-829.

Mohamed, A. M. (2008). "An overview of bone cells and their regulating factors of differentiation." The Malaysian journal of medical sciences : MJMS **15**(1): 4-12.

Moiroud, C. H., V. Coudry and J. M. Denoix (2019). "Distribution of Pelvic Fractures in Racing and Non-racing Sport Horses: A Retrospective Study of 86 Cases Examined in a Referral Centre." Vet Comp Orthop Traumatol **32**(3): 215-221.

Moreno-Jiménez, I., G. Hulsart-Billstrom, S. A. Lanham, A. A. Janeczek, N. Kontouli, J. M. Kanczler, N. D. Evans and R. O. C. Oreffo (2016). "The chorioallantoic membrane (CAM) assay for the study of human bone regeneration: a refinement animal model for tissue engineering." Scientific Reports **6**(1): 32168.

Morgan, E. F., A. De Giacomo and L. C. Gerstenfeld (2014). "Overview of skeletal repair (fracture healing and its assessment)." Methods in molecular biology (Clifton, N.J.) **1130**: 13-31.

Morrison, S. J. and D. T. Scadden (2014). "The bone marrow niche for haematopoietic stem cells." Nature **505**(7483): 327-334.

Mu, S., S. Guo, X. Wang, Y. Zhan, Y. Li, Y. Jiang, R. Zhang and B. Zhang (2017). "Effects of deferoxamine on the osteogenic differentiation of human periodontal ligament cells." Mol Med Rep **16**(6): 9579-9586.

Muraglia, A., A. Corsi, M. Riminucci, M. Mastrogiacomo, R. Cancedda, P. Bianco and R. Quarto (2003). "Formation of a chondro-osseous rudiment in micromass cultures of human bone-marrow stromal cells." J Cell Sci **116**(Pt 14): 2949-2955.

Nagao, A., M. Kobayashi, S. Koyasu, C. C. T. Chow and H. Harada (2019). "HIF-1-Dependent Reprogramming of Glucose Metabolic Pathway of Cancer Cells and Its Therapeutic Significance." International journal of molecular sciences **20**(2): 238.

Nam, D., E. Mau, Y. Wang, D. Wright, D. Silkstone, H. Whetstone, C. Whyne and B. Alman (2012). "T-lymphocytes enable osteoblast maturation via IL-17F during the early phase of fracture repair." PloS one **7**(6): e40044-e40044.

Noel, D., F. Djouad and C. Jorgense (2002). "Regenerative medicine through mesenchymal stem cells for bone and cartilage repair." Curr Opin Investig Drugs **3**(7): 1000-1004.

Nunamaker, D. M., D. W. Richardson, D. M. Butterweck, M. T. Provost and R. D. Sigafoos (1986). "A New External Skeletal Fixation Device* That Allows Immediate Full Weightbearing Application in the Horse." Veterinary Surgery **15**(5): 345-355.

O'Toole, E. A., R. van Koningsveld, M. Chen and D. T. Woodley (2008). "Hypoxia induces epidermal keratinocyte matrix metalloproteinase-9 secretion via the protein kinase C pathway." J Cell Physiol **214**(1): 47-55.

Oe, K., M. Miwa, Y. Sakai, S. Y. Lee, R. Kuroda and M. Kurosaka (2007). "An in vitro study demonstrating that haematomas found at the site of human fractures contain progenitor cells with multilineage capacity." J Bone Joint Surg Br **89**(1): 133-138.

Oja, S., P. Komulainen, A. Penttilä, J. Nystedt and M. Korhonen (2018). "Automated image analysis detects aging in clinical-grade mesenchymal stromal cell cultures." Stem Cell Research & Therapy **9**(1): 6.

Olsen, B. R., A. M. Reginato and W. F. Wang (2000). "Bone development." Annual Review of Cell and Developmental Biology **16**: 191-220.

Olsson, I. A. S., S. P. d. Silva, D. Townend and P. Sandøe (2017). "Protecting Animals and Enabling Research in the European Union: An Overview of Development and Implementation of Directive 2010/63/EU." ILAR Journal **57**(3): 347-357.

Ortved, K. F. (2018). "Regenerative Medicine and Rehabilitation for Tendinous and Ligamentous Injuries in Sport Horses." Vet Clin North Am Equine Pract **34**(2): 359-373.

Paic, F., J. C. Igwe, R. Nori, M. S. Kronenberg, T. Franceschetti, P. Harrington, L. Kuo, D. G. Shin, D. W. Rowe, S. E. Harris and I. Kalajzic (2009). "Identification of differentially expressed genes between osteoblasts and osteocytes." Bone **45**(4): 682-692.

Pape, H. C., R. Marcucio, C. Humphrey, C. Colnot, M. Knobe and E. J. Harvey (2010). "Trauma-induced inflammation and fracture healing." J Orthop Trauma **24**(9): 522-525.

Parrilli, A., S. Pagani, M. C. Maltarello, S. Santi, A. Salerno, P. A. Netti, R. Giardino, L. Rimondini and M. Fini (2014). "Three-dimensional cellular distribution in polymeric scaffolds for bone regeneration: a microCT analysis compared to SEM, CLSM and DNA content." J Microsc **255**(1): 20-29.

Pearce, A. I., R. G. Richards, S. Milz, E. Schneider and S. G. Pearce (2007). "Animal models for implant biomaterial research in bone: a review." Eur Cell Mater **13**: 1-10.

Percie du Sert, N., V. Hurst, A. Ahluwalia, S. Alam, M. T. Avey, M. Baker, W. J. Browne, A. Clark, I. C. Cuthill, U. Dirnagl, M. Emerson, P. Garner, S. T. Holgate, D. W. Howells, N. A. Karp, K. Lidster, C. J. MacCallum, M. Macleod, O. Petersen, F. Rawle, P. Reynolds, K. Rooney, E. S. Sena, S. D. Silberberg, T. Steckler and H. Würbel (2019). "The ARRIVE guidelines 2019: updated guidelines for reporting animal research." bioRxiv: 703181.

Pfeiffenberger, M., J. Bartsch, P. Hoff, I. Ponomarev, D. Barnewitz, C. Thöne-Reineke, F. Buttgereit, T. Gaber and A. Lang (2019). "Hypoxia and mesenchymal stromal cells as key drivers of initial fracture healing in an equine in vitro fracture hematoma model." PLoS One **14**(4): e0214276.

Pfeiffenberger, M., A. Damerau, I. Ponomarev, C. H. Bucher, Y. Chen, D. Barnewitz, C. Thöne-Reineke, P. Hoff, F. Buttgereit, T. Gaber and A. Lang (2020). "An in vitro human-based fracture gap model – Mimicking the crosstalk between bone and immune cells." bioRxiv: 2020.2006.2026.165456.

Pfeiffenberger, M., P. Hoff, C. Thöne-Reineke, F. Buttgereit, A. Lang and T. Gaber (2020). "The in vitro human fracture hematoma model - a tool for preclinical drug testing." ALTEX.

Polderman, J. A., V. Farhang-Razi, S. Van Dieren, P. Kranke, J. H. DeVries, M. W. Hollmann, B. Preckel and J. Hermanides (2018). "Adverse side effects of dexamethasone in surgical patients." The Cochrane database of systematic reviews **8**(8): CD011940-CD011940.

Ponomarev, I. (2013). "A New Technology in Cartilage Tissue Engineering: Scaffold - Free Cartilage Transplantats (Sfct)." Biomed Tech (Berl) **58 Suppl 1**.

Ponomarev, I. V., L. M. Kochneva and D. Barnewitz (2014). "Effect of 3D chondrocyte culturing conditions on the formation of extracellular matrix in cartilage tissue-engineering constructs." Bull Exp Biol Med **156**(4): 548-555.

Pool, M., H. Leuvenink and C. Moers (2019). "Reparative and Regenerative Effects of Mesenchymal Stromal Cells-Promising Potential for Kidney Transplantation?" Int J Mol Sci **20**(18).

Poole, T. (1997). "Happy animals make good science." Lab Anim **31**(2): 116-124.

Popov, A., C. Scotchford, D. Grant and V. Sottile (2019). "Impact of Serum Source on Human Mesenchymal Stem Cell Osteogenic Differentiation in Culture." Int J Mol Sci **20**(20).

Qu, Z. H., X. L. Zhang, T. T. Tang and K. R. Dai (2008). "Promotion of osteogenesis through beta-catenin signaling by desferrioxamine." Biochem Biophys Res Commun **370**(2): 332-337.

Ranera, B., A. R. Remacha, S. Álvarez-Arguedas, A. Romero, F. J. Vázquez, P. Zaragoza, I. Martín-Burriel and C. Rodellar (2012). "Effect of hypoxia on equine mesenchymal stem cells derived from bone marrow and adipose tissue." BMC Vet Res **8**: 142.

Reifenrath, J., N. Angrisani, M. Lalk and S. Besdo (2014). "Replacement, refinement, and reduction: Necessity of standardization and computational models for long bone fracture repair in animals." Journal of Biomedical Materials Research Part A **102**(8): 2884-2900.

Reinke, S., S. Geissler, W. R. Taylor, K. Schmidt-Bleek, K. Juelke, V. Schwachmeyer, M. Dahne, T. Hartwig, L. Akyuz, C. Meisel, N. Unterwalder, N. B. Singh, P. Reinke, N. P. Haas, H. D. Volk and G. N. Duda (2013). "Terminally differentiated CD8(+) T cells negatively affect bone regeneration in humans." Sci Transl Med **5**(177): 177ra136.

Reinwald, S. and D. Burr (2008). "Review of Nonprimate, Large Animal Models for Osteoporosis Research." Journal of Bone and Mineral Research **23**(9): 1353-1368.

Ren, D., T. N. Collingwood, E. J. Rebar, A. P. Wolffe and H. S. Camp (2002). "PPARgamma knockdown by engineered transcription factors: exogenous PPARgamma2 but not PPARgamma1 reactivates adipogenesis." Genes Dev **16**(1): 27-32.

Riggs, C. M. (2002). "Fractures – A Preventable Hazard of Racing Thoroughbreds?" The Veterinary Journal **163**(1): 19-29.

Rocca, M., M. Fini, G. Giavaresi, N. N. Aldini and R. Giardino (2002). "Osteointegration of hydroxyapatite-coated and uncoated titanium screws in long-term ovariectomized sheep." Biomaterials **23**(4): 1017-1023.

Rosenberger, C., W. Griethe, G. Gruber, M. Wiesener, U. Frei, S. Bachmann and K. U. Eckardt (2003). "Cellular responses to hypoxia after renal segmental infarction." Kidney Int **64**(3): 874-886.

Rossignol, F., O. Brandenberger and C. Mespoulhes-Riviere (2016). "Internal Fixation of Cervical Fractures in Three Horses." Vet Surg **45**(1): 104-109.

Rozier, P., A. Maria, R. Goulabchand, C. Jorgensen, P. Guilpain and D. Noel (2018). "Mesenchymal Stem Cells in Systemic Sclerosis: Allogenic or Autologous Approaches for Therapeutic Use?" Front Immunol **9**: 2938.

Rucci, N. (2008). "Molecular biology of bone remodelling." Clinical cases in mineral and bone metabolism : the official journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases **5**(1): 49-56.

Rutkovskiy, A., K.-O. Stensløkken and I. J. Vaage (2016). "Osteoblast Differentiation at a Glance." Medical science monitor basic research **22**: 95-106.

Samee, N., V. Geoffroy, C. Marty, C. Schiltz, M. Vieux-Rochas, G. Levi and M.-C. de Vernejoul (2008). "Dlx5, a positive regulator of osteoblastogenesis, is essential for osteoblast-osteoclast coupling." The American journal of pathology **173**(3): 773-780.

Sargent, A. and R. H. Miller (2016). "MSC Therapeutics in Chronic Inflammation." Current stem cell reports **2**(2): 168-173.

Sart, S., A. C. Tsai, Y. Li and T. Ma (2014). "Three-dimensional aggregates of mesenchymal stem cells: cellular mechanisms, biological properties, and applications." Tissue Eng Part B Rev **20**(5): 365-380.

Schaller, B., J. P. Matthias Burkhard, M. Chagnon, S. Beck, T. Imwinkelried and M. Assad (2018). "Fracture Healing and Bone Remodeling With Human Standard-Sized Magnesium Versus Polylactide–Co-Glycolide Plate and Screw Systems Using a Mini-Swine Craniomaxillofacial Osteotomy Fixation Model." Journal of Oral and Maxillofacial Surgery **76**(10): 2138-2150.

Scheinpflug, J., M. Pfeiffenberger, A. Damerau, F. Schwarz, M. Textor, A. Lang and F. Schulze (2018). "Journey into Bone Models: A Review." Genes **9**(5): 247.

Schell, H., G. N. Duda, A. Peters, S. Tsitsilonis, K. A. Johnson and K. Schmidt-Bleek (2017). "The haematoma and its role in bone healing." Journal of experimental orthopaedics **4**(1): 5-5.

Schindeler, A., M. M. McDonald, P. Bokko and D. G. Little (2008). "Bone remodeling during fracture repair: The cellular picture." Semin Cell Dev Biol **19**(5): 459-466.

Schlegel, K. A., F. R. Kloss, P. Kessler, S. Schultze-Mosgau, E. Nkenke and J. Wiltfang (2003). "Bone conditioning to enhance implant osseointegration: an experimental study in pigs." Int J Oral Maxillofac Implants **18**(4): 505-511.

Schlundt, C., S. Reinke, S. Geissler, C. H. Bucher, C. Giannini, S. Märdian, M. Dahne, C. Kleber, B. Samans, U. Baron, G. N. Duda, H.-D. Volk and K. Schmidt-Bleek (2019). "Individual Effector/Regulator T Cell Ratios Impact Bone Regeneration." Frontiers in immunology **10**: 1954-1954.

Schmidt-Bleek, K., H. Schell, P. Kolar, M. Pfaff, C. Perka, F. Buttgereit, G. Duda and J. Lienau (2009). "Cellular composition of the initial fracture hematoma compared to a muscle hematoma: a study in sheep." J Orthop Res **27**(9): 1147-1151.

Schulz, K. F., D. G. Altman and D. Moher (2010). "CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials." PLoS Med **7**(3): e1000251.

Scotti, C., B. Tonnarelli, A. Papadimitropoulos, A. Scherberich, S. Schaeren, A. Schauerte, J. Lopez-Rios, R. Zeller, A. Barbero and I. Martin (2010). "Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering." Proceedings of the National Academy of Sciences **107**(16): 7251.

Seok, J., H. S. Warren, A. G. Cuenca, M. N. Mindrinos, H. V. Baker, W. Xu, D. R. Richards, G. P. McDonald-Smith, H. Gao, L. Hennessy, C. C. Finnerty, C. M. Lopez, S. Honari, E. E. Moore, J. P. Minei, J. Cuschieri, P. E. Bankey, J. L. Johnson, J. Sperry, A. B. Nathens, T. R. Billiar, M. A. West, M. G. Jeschke, M. B. Klein, R. L. Gamelli, N. S. Gibran, B. H. Brownstein, C. Miller-Graziano, S. E. Calvano, P. H. Mason, J. P. Cobb, L. G. Rahme, S. F. Lowry, R. V. Maier, L. L. Moldawer, D. N. Herndon, R. W. Davis, W. Xiao and R. G. Tompkins (2013). "Genomic responses in mouse models poorly mimic human inflammatory diseases." Proc Natl Acad Sci U S A **110**(9): 3507-3512.

Shapiro, F. (2008). "Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts." European Cells & Materials **15**: 53-76.

Sharp, F. R., M. Bergeron and M. Bernaudin (2001). "Hypoxia-inducible factor in brain." Adv Exp Med Biol **502**: 273-291.

Sheen, J. R. and V. V. Garla (2020). Fracture Healing Overview. StatPearls. Treasure Island FL, StatPearls Publishing LLC.

Shen, V., D. W. Dempster, R. Birchman, R. W. Mellish, E. Church, D. Kohn and R. Lindsay (1992). "Lack of changes in histomorphometric, bone mass, and biochemical parameters in ovariohysterectomized dogs." Bone **13**(4): 311-316.

Shen, X., C. Wan, G. Ramaswamy, M. Mavalli, Y. Wang, C. L. Duvall, L. F. Deng, R. E. Guldberg, A. Eberhart, T. L. Clemens and S. R. Gilbert (2009). "Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice." Journal of orthopaedic research : official publication of the Orthopaedic Research Society **27**(10): 1298-1305.

Shibuya, M. (2011). "Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies." Genes & cancer **2**(12): 1097-1105.

Shiozawa, Y., A. M. Havens, K. J. Pienta and R. S. Taichman (2008). "The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites." Leukemia **22**(5): 941-950.

Sirén, A. L., M. Fratelli, M. Brines, C. Goemans, S. Casagrande, P. Lewczuk, S. Keenan, C. Gleiter, C. Pasquali, A. Capobianco, T. Mennini, R. Heumann, A. Cerami, H. Ehrenreich and P. Ghezzi (2001). "Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress." Proceedings of the National Academy of Sciences of the United States of America **98**(7): 4044-4049.

Smith, A. J., R. E. Clutton, E. Lilley, K. E. A. Hansen and T. Brattelid (2018). "PREPARE: guidelines for planning animal research and testing." Lab Anim **52**(2): 135-141.

Smith, R. K. W. (2008). "Mesenchymal stem cell therapy for equine tendinopathy." Disability and Rehabilitation **30**(20-22): 1752-1758.

Snäll, J., S. Apajalahti, A.-L. Suominen, J. Törnwall and H. Thorén (2015). "Influence of perioperative dexamethasone on delayed union in mandibular fractures: A clinical and radiological study." Medicina oral, patologia oral y cirugia bucal **20**(5): e621-e626.

Sodek, J., B. Ganss and M. D. McKee (2000). "Osteopontin." Crit Rev Oral Biol Med **11**(3): 279-303.

Sohn, H.-S. and J.-K. Oh (2019). "Review of bone graft and bone substitutes with an emphasis on fracture surgeries." Biomaterials Research **23**(1): 9.

Steinbrech, D. S., B. J. Mehrara, P. B. Saadeh, G. Chin, M. E. Dudziak, R. P. Gerrets, G. K. Gittes and M. T. Longaker (1999). "Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts in vitro." Plastic and reconstructive surgery **104**(3): 738-747.

Street, J., D. Winter, J. H. Wang, A. Wakai, A. McGuinness and H. P. Redmond (2000). "Is human fracture hematoma inherently angiogenic?" Clin Orthop Relat Res(378): 224-237.

Strehl, C., L. Ehlers, T. Gaber and F. Buttgereit (2019). "Glucocorticoids-All-Rounders Tackling the Versatile Players of the Immune System." Front Immunol **10**: 1744.

Swiontkowski, M. F., S. Tepic, B. A. Rahn, J. Cordey and S. M. Perren (1993). "The effect of fracture on femoral head blood flow: Osteonecrosis and revascularization studied in miniature swine." Acta Orthopaedica Scandinavica **64**(2): 196-202.

Tachibana, T., T. Matsubara, K. Mizuno and K. Hirohata (1991). "Enhancement of new bone formation by hematoma at fracture site." Nihon Seikeigeka Gakkai Zasshi **65**(5): 349-358.

Takuma, A., T. Kaneda, T. Sato, S. Ninomiya, M. Kumegawa and Y. Hakeda (2003). "Dexamethasone enhances osteoclast formation synergistically with transforming growth factor-beta by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts." J Biol Chem **278**(45): 44667-44674.

Teti, A. (2011). "Bone development: overview of bone cells and signaling." Curr Osteoporos Rep **9**(4): 264-273.

Textor, J. A., K. C. Clark, N. J. Walker, F. A. Aristizobal, A. Kol, S. S. LeJeune, A. Bledsoe, A. Davidyan, S. N. Gray, L. K. Bohannon-Worsley, K. D. Woolard and D. L. Borjesson (2018). "Allogeneic Stem Cells Alter Gene Expression and Improve Healing of Distal Limb Wounds in Horses." STEM CELLS Translational Medicine **7**(1): 98-108.

Thomas, M. V. and D. A. Puleo (2011). "Infection, inflammation, and bone regeneration: a paradoxical relationship." J Dent Res **90**(9): 1052-1061.

Thompson, M. S., D. R. Epari, F. Bieler and G. N. Duda (2010). "In vitro models for bone mechanobiology: applications in bone regeneration and tissue engineering." Proc Inst Mech Eng H **224**(12): 1533-1541.

Tian, L., N. Tang, T. Ngai, C. Wu, Y. Ruan, L. Huang and L. Qin (2019) "Hybrid fracture fixation systems developed for orthopaedic applications: A general review." Journal of orthopaedic translation **16**, 1-13 DOI: 10.1016/j.jot.2018.06.006.

Toben, D., I. Schroeder, T. El Khassawna, M. Mehta, J. E. Hoffmann, J. T. Frisch, H. Schell, J. Lienau, A. Serra, A. Radbruch and G. N. Duda (2011). "Fracture healing is accelerated in the absence of the adaptive immune system." J Bone Miner Res **26**(1): 113-124.

Tylek, T., T. Schilling, K. Schlegelmilch, M. Ries, M. Rudert, F. Jakob and J. Groll (2019). "Platelet lysate outperforms FCS and human serum for co-culture of primary human macrophages and hMSCs." Scientific Reports **9**(1): 3533.

Tzioupis, C. and P. V. Giannoudis (2007). "Prevalence of long-bone non-unions." Injury **38 Suppl 2**: S3-9.

Verbraucherschutz, B. d. J. u. f. (2013). "Verordnung zum Schutz von zu Versuchszwecken oder zu anderen wissenschaftlichen Zwecken verwendeten Tieren (Tierschutz-Versuchstierverordnung - TierSchVersV)." Retrieved 20.08.2020, 2020, from https://www.gesetze-im-internet.de/tierschversv/BJNR312600013.html.

Verbraucherschutz, B. d. J. u. f. (2020, 2020). "Tierschutzgesetz." Retrieved 18.08.2020, 2020, from https://www.gesetze-im-internet.de/tierschg/BJNR012770972.html.

Vidal, M. A., G. E. Kilroy, M. J. Lopez, J. R. Johnson, R. M. Moore and J. M. Gimble (2007). "Characterization of Equine Adipose Tissue-Derived Stromal Cells: Adipogenic and Osteogenic Capacity and Comparison with Bone Marrow-Derived Mesenchymal Stromal Cells." Veterinary Surgery **36**(7): 613-622.

Vollger, L., K. Akong-Moore, L. Cox, O. Goldmann, Y. Wang, S. T. Schafer, H. Y. Naim, V. Nizet and M. von Kockritz-Blickwede (2016). "Iron-chelating agent desferrioxamine stimulates formation of neutrophil extracellular traps (NETs) in human blood-derived neutrophils." Biosci Rep **36**(3).

Wagegg, M., T. Gaber, F. L. Lohanatha, M. Hahne, C. Strehl, M. Fangradt, C. L. Tran, K. Schonbeck, P. Hoff, A. Ode, C. Perka, G. N. Duda and F. Buttgereit (2012). "Hypoxia promotes osteogenesis but suppresses adipogenesis of human mesenchymal stromal cells in a hypoxia-inducible factor-1 dependent manner." PLoS One **7**(9): e46483.

Walters, G., I. Pountos and P. V. Giannoudis (2018). "The cytokines and micro-environment of fracture haematoma: Current evidence." J Tissue Eng Regen Med **12**(3): e1662-e1677.

Wan, C., S. R. Gilbert, Y. Wang, X. Cao, X. Shen, G. Ramaswamy, K. A. Jacobsen, Z. S. Alaql, A. W. Eberhardt, L. C. Gerstenfeld, T. A. Einhorn, L. Deng and T. L. Clemens (2008). "Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration." Proc Natl Acad Sci U S A **105**(2): 686-691.

Wang, G., G. Shen and T. Yin (2017). "In vitro assessment of deferoxamine on mesenchymal stromal cells from tumor and bone marrow." Environmental Toxicology and Pharmacology **49**: 58-64.

Wang, G., G. Shen and T. Yin (2017). "In vitro assessment of deferoxamine on mesenchymal stromal cells from tumor and bone marrow." Environ Toxicol Pharmacol **49**: 58-64.

Wang, L., Z.-y. Li, Y.-p. Wang, Z.-h. Wu and B. Yu (2015). "Dynamic Expression Profiles of Marker Genes in Osteogenic Differentiation of Human Bone Marrow-derived Mesenchymal Stem Cells." Chinese Medical Sciences Journal **30**(2): 108-113.

Wang, M., N. Yang and X. Wang (2017). "A review of computational models of bone fracture healing." Med Biol Eng Comput **55**(11): 1895-1914.

Wang, W., K. Itaka, S. Ohba, N. Nishiyama, U. I. Chung, Y. Yamasaki and K. Kataoka (2009). "3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells." Biomaterials **30**(14): 2705-2715.

Wang, X., Y. Wang, W. Gou, Q. Lu, J. Peng and S. Lu (2013). "Role of mesenchymal stem cells in bone regeneration and fracture repair: a review." Int Orthop **37**(12): 2491-2498.

Warren, H. S., R. G. Tompkins, L. L. Moldawer, J. Seok, W. Xu, M. N. Mindrinos, R. V. Maier, W. Xiao and R. W. Davis (2015). "Mice are not men." Proc Natl Acad Sci U S A **112**(4): E345.

Weber, M. C., L. Fischer, A. Damerau, I. Ponomarev, M. Pfeiffenberger, T. Gaber, S. Götschel, J. Lang, S. Röblitz, F. Buttgereit, R. Ehrig and A. Lang (2020). "Macroscale mesenchymal condensation to study cytokine-driven cellular and matrix-related changes during cartilage degradation." Biofabrication.

Weinreb, M., D. Shinar and G. A. Rodan (1990). "Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization." J Bone Miner Res **5**(8): 831-842.

Weisgerber, D. W., S. R. Caliari and B. A. Harley (2015). "Mineralized collagen scaffolds induce hMSC osteogenesis and matrix remodeling." Biomater Sci **3**(3): 533-542.

Wilson, S. R., C. Peters, P. Saftig and D. Brömme (2009). "Cathepsin K activity-dependent regulation of osteoclast actin ring formation and bone resorption." The Journal of biological chemistry **284**(4): 2584-2592.

Wu, Y., K. Lucia, M. Lange, D. Kuhlen, G. K. Stalla and U. Renner (2014). "Hypoxia inducible factor-1 is involved in growth factor, glucocorticoid and hypoxia mediated regulation of vascular endothelial growth factor-A in human meningiomas." J Neurooncol **119**(2): 263-273.

Xin, X., M. Hussain and J. J. Mao (2007). "Continuing differentiation of human mesenchymal stem cells and induced chondrogenic and osteogenic lineages in electrospun PLGA nanofiber scaffold." Biomaterials **28**(2): 316-325.

Xing, K., B. Gu, P. Zhang and X. Wu (2015). "Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: an insight into the optimum application of glucocorticoids in anti-cancer therapy." BMC immunology **16**: 39-39.

Xiong, J. and C. A. O'Brien (2012). "Osteocyte RANKL: new insights into the control of bone remodeling." Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research **27**(3): 499-505.

Xu, J., Z. Li, Y. Hou and W. Fang (2015). "Potential mechanisms underlying the Runx2 induced osteogenesis of bone marrow mesenchymal stem cells." American journal of translational research **7**(12): 2527-2535.

Yang, Y.-Q., Y.-Y. Tan, R. Wong, A. Wenden, L.-K. Zhang and A. B. M. Rabie (2012). "The role of vascular endothelial growth factor in ossification." International journal of oral science **4**(2): 64-68.

Yin, J., S. Qiu, B. Shi, X. Xu, Y. Zhao, J. Gao, S. Zhao and S. Min (2018). "Controlled release of FGF-2 and BMP-2 in tissue engineered periosteum promotes bone repair in rats." Biomedical Materials **13**(2): 025001.

Yokoyama, M., H. Miwa, S. Maeda, S. Wakitani and M. Takagi (2008). "Influence of fetal calf serum on differentiation of mesenchymal stem cells to chondrocytes during expansion." J Biosci Bioeng **106**(1): 46-50.

Yuan, N., K. S. Rezzadeh and J. C. Lee (2015). "Biomimetic Scaffolds for Osteogenesis." Receptors & clinical investigation **2**(3): 898.

Yuasa, M., M. Saito, C. Molina, S. N. Moore-Lotridge, M. A. Benvenuti, N. A. Mignemi, A. Okawa, T. Yoshii, H. S. Schwartz, J. S. Nyman and J. G. Schoenecker (2018). "Unexpected timely fracture union in matrix metalloproteinase 9 deficient mice." PLoS One **13**(5): e0198088.

Zhang, H., Y. Zhou, W. Zhang, K. Wang, L. Xu, H. Ma and Y. Deng (2018). "Construction of vascularized tissue-engineered bone with a double-cell sheet complex." Acta Biomater **77**: 212-227.

Zhang, J., X. Huang, H. Wang, X. Liu, T. Zhang, Y. Wang and D. Hu (2015). "The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy." Stem Cell Res Ther **6**: 234.

Ziello, J. E., I. S. Jovin and Y. Huang (2007). "Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia." The Yale journal of biology and medicine **80**(2): 51-60.

8 Publications, Congress Presentations, Awards

8.1 Publications from the presented work (Peer reviewed)

Moritz Pfeiffenberger*, Janika Bartsch*, Igor Ponomarev, Dirk Barnewitz, Paula Hoff, Christa Thöne Reineke, Frank Buttgereit, Timo Gaber* & Annemarie Lang* (2019), Hypoxia and mesenchymal stromal cells as key drivers of initial fracture healing in an equine *in vitro* fracture hematoma model. *PLOS ONE* *contributed equally, DOI: 10.1371/journal.pone.0214276

Moritz Pfeiffenberger, Paula Hoff, Christa Thöne-Reineke, Frank Buttgereit, Annemarie Lang & Timo Gaber (2020), The *in vitro* human fracture hematoma model - a tool for preclinical drug testing. *ALTEX*, DOI: 10.14573/altex.1910211

Moritz Pfeiffenberger, Alexandra Damerau, Igor Ponomarev, Christian H. Bucher, Yuling Chen, Dirk Barnewitz, Christa Thöne-Reineke, Paula Hoff, Frank Buttgereit, Timo Gaber & Annemarie Lang (2020), Functional Scaffold-Free Bone Equivalents Induce Osteogenic and Angiogenic Processes in a Human In Vitro Fracture Hematoma Model, *JBMR*, DOI: 10.1002/jbmr.4267

8.2 Publications from additional work (Peer reviewed)

Timo Gaber, Antonia Brinkman, Justyna Pienczikowski, Karoline Diesing, Alexandra Damerau, **Moritz Pfeiffenberger**, Annemarie Lang, Sarah Ohrndorf, Gerd Burmester, Frank Buttgereit & Paula Hoff (2020) Impact of Janus Kinase Inhibition with Tofacitinib on Fundamental Processes of Bone Healing. *Int J Mol Sci.*

Annemarie Lang, Marieluise Kirchner, Jonathan Stefanowski, Mattea Durst, Marie Weber, **Moritz Pfeiffenberger**, Alexandra Damerau, Andrea Hauser, Paula Hoff, Georg Duda, Frank Buttgereit, Katharina Schmidt-Bleek & Timo Gaber (2019), Collagen I-based scaffolds negatively impact fracture healing in a mouse-osteotomy-model although used routinely in research and clinical application. *Acta Biomater.*

Timo Gaber, Kerstin Schönbeck, Holger Hoff, Cam Loan Tran, Cindy Strehl, Annemarie Lang, Sarah Ohrndorf, **Moritz Pfeiffenberger**, Eric Röhner, Georg Matziolis, Gerd-R. Burmester, Frank Buttgereit & Paula Hoff (2018) CTLA-4 Mediates Inhibitory Function of Mesenchymal Stem/Stromal Cells (MSCs). *IJMS*

Julia Scheinpflug*, **Moritz Pfeiffenberger***, Alexandra Damerau, Martin Textor, Franziska Schwarz, Annemarie Lang* & Frank Schulze* (2018) Journey into Bone Models: A Review. *Genes*. *contributed equally

Florian Weigold, Jeannine Günther, **Moritz Pfeiffenberger,** Otavio Marques, Elise Siegert, Duska Dragun, Aurelie Philippe, Ann-Kathrin Regensburger, Andreas Recke, Xinhua Yu, Frank Petersen, Rusan Catar, Robert Biesen, Falk Hiepe, Gerd Burmester, Harald Heidecke & Gabriela Riemekasten (2018) Antibodies against chemokine receptors CXCR3 and CXCR4 predict progressive deterioration of lung function in patients with systemic sclerosis. *Arthritis & Res. Ther*.

Annemarie Lang, Johannes Neuhaus, **Moritz Pfeiffenberger**, Erik Schröder, Yvonne Weber, Igor Ponomarev, Timo Gaber & M.F.G. Schmidt (2014) Use of an equine cell culture model for controlled gene therapy approaches to treat osteoarthritis. *Journal of Gene Medicine*.

8.3 Participation in Conferences, symposia and congresses

EULAR 2020 – Annual European Congress of Rheumatology, 2020

Moritz Pfeiffenberger, Alexandra Damerau, Paula Hoff, Annemarie Lang, Frank Buttgereit & Timo Gaber: A preclinical testing tool: The *in vitro* 3D fracture gap model. Oral Presentation

EUSAAT 21nd European Congress on Alternatives to Animal Testing, 2019

Moritz Pfeiffenberger, Alexandra Damerau, Karoline Diesing, Paula Hoff, Frank Buttgereit, Timo Gaber & Annemarie Lang: An *in vitro* fracture hematoma model as a tool for preclinical drug testing. Oral Presentation

Moritz Pfeiffenberger, Alexandra Damerau, Wiktor Burdzinski, Karoline Diesing, Frank Buttgereit, Timo Gaber & Annemarie Lang: Using platelet-rich human platelet lysate to substitute fetal calf serum for cultivation of mesenchymal stromal cells. Oral Presentation

Moritz Pfeiffenberger, Alexandra Damerau, Karoline Diesing, Paula Hoff, Frank Buttgereit, Annemarie Lang & Timo Gaber: An *in vitro* 3D fracture gap model as a tool for preclinical testing procedures. Poster Presentation

Osteologie-Kongress OSTAK, 2019

Moritz Pfeiffenberger, Annemarie Lang, A, Paula Hoff, Frank Buttgereit & Timo Gaber: Das Frakturhämatom: Simulation der initialen Phase der Frakturheilung *in vitro*. Oral Presentation and Poster

Moritz Pfeiffenberger, Annemarie Lang, Alexandra Damerau, Igor Ponomarev, Frank Buttgereit & Timo Gaber: *In vitro* Modell für endochondrale Ossifikation. Oral Presentation and Poster

Lange Nacht der Wissenschaften, 2018

Moritz Pfeiffenberger & Annemarie Lang: Modelling of musculoskeletal disorders *in vitro* -with complex processes to convincing systems. Invited Oral Presentation

3R Webinar "Alternatives to animal use in research and education - Refine, Reduce, Replace, 2018

Moritz Pfeiffenberger, Alexandra Damerau & Annemarie Lang: Modelling of musculoskeletal disorders *in vitro* -with complex processes to convincing systems. Invited Oral Presentation

EUSAAT 21nd European Congress on Alternatives to Animal Testing, 2018

Moritz Pfeiffenberger, Alexandra Damerau, Frank Buttgereit, Timo Gaber & Annemarie Lang: Modelling the initial phase of fracture healing *in vitro*. Oral Presentation

3R Symposium Copenhagen, 2017

Moritz Pfeiffenberger, Annemarie Lang, Alexandra Damerau, Igor Ponomarev, Frank Buttgereit & Timo Gaber: The *in vitro* fracture hematoma model, Poster Presentation

10th World Congress on Alternatives and Animal Use in the Life Sciences (WC10), 2017

Moritz Pfeiffenberger, Igor Ponomarev, Janika Bartsch, Alexandra Damerau, Frank Buttgereit, Timo Gaber & Annemarie Lang: Modelling the initial phase of fracture healing *in vitro*. Oral Presentation

Annemarie Lang, Janika Bartsch, **Moritz Pfeiffenberger**, Timo Gaber & Frank Buttgereit: Characterization of an equine 3D *in vitro* fracture hematoma. Poster Presentation

ORS 2017 Annual Meeting - Orthopaedic Research Society, 2017

Moritz Pfeiffenberger, Igor Ponomarev, Dirk Barnewitz, Frank Buttgereit, Timo Gaber & Annemarie Lang: Modelling the Initial Phase of Fracture Healing *in vitro* – 3D Bone-like Models of Endochondral Ossification. Poster Presentation

EULAR 2017 - Annual European Congress of Rheumatology, 2017

Moritz Pfeiffenberger, Igor Ponomarev, Dirk Barnewitz, Frank Buttgereit, Timo Gaber & Annemarie Lang: Modelling the Initial Phase of Fracture Healing *in vitro* – 3D Bone-like Models of Endochondral Ossification. Poster Presentation

DRS Doktorandensymposium, 2016

Moritz Pfeiffenberger, Igor Ponomarev, Dirk Barnewitz, Frank Buttgereit, Timo Gaber & Annemarie Lang: Modelling the Initial Phase of Fracture Healing *in vitro*. Oral Presentation

Osteologie Kongress 2016 OSTAK, 2016

Moritz Pfeiffenberger, Annemarie Lang, Paula Hoff, Igor Ponomarev, Frank Buttgereit & Timo Gaber: *In vitro* Modellierung der initialen Phase der Frakturheilung - Generierung der ersten Bausteine. Poster Tour

Wissenschaftliches Symposium – 30 Jahre Biotechnologie – der BHT Berlin, 2016

Moritz Pfeiffenberger, Igor_Ponomarev, Dirk Barnewitz, Frank Buttgereit, Timo Gaber & Annemarie Lang: Modellierung der initialen Phase der Frakturheilung *in vitro*. Oral Presentation

BSRT Symposium, 2016

Moritz Pfeiffenberger, Annemarie_Lang, Paula Hoff, Johannes Neuhaus, Igor Ponomarev, Dirk Barnewitz, Frank Buttgereit & Timo Gaber: Modelling the first phase of fracture healing *in vitro*. Poster Presentation

DRS Doktorandensymposium, 2015

Moritz Pfeiffenberger, Annemarie Lang, Paula Hoff, Igor Ponomarev, Frank Buttgereit & Timo Gaber: *In vitro* Modellierung der initialen Phase der Frakturheilung, Poster Presentation

8.4 Awards and Scholarships

Travel Award by Deutsche Tierseuchenforschungsstiftung 500 Euro

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11 Conflict of Interest

There is no conflict of interest through financial support of the work

12 Selbständigkeitserklärung/Anteilserklärung

Anteilserklärung von Moritz Pfeiffenberger an der vorliegenden Monografie gemäß § 8 Abschnitt 3 Promotionsordnung des Fachbereichs Veterinärmedizin der Freien Universität Berlin (Stand 14.09.2011)

Moritz Pfeiffenberger hat die Experimente, Ergebnisse und Abbildungen/Tabellen, die in dieser Thesis dargestellt sind, selbständig durchgeführt, analysiert und erstellt. Abbildungen/Tabellen und Ergebnisse, die in Zusammenarbeit mit anderen Wissenschaftlern entstanden und in anderen Arbeiten/Veröffentlichungen publiziert sind, werden im Folgenden detailliert aufgelistet:

Moritz Pfeiffenberger*, Janika Bartsch*, Igor Ponomarev, Dirk Barnewitz, Paula Hoff, Christa Thöne Reineke, Frank Buttgereit, Timo Gaber* & Annemarie Lang* (2019), Hypoxia and mesenchymal stromal cells as key drivers of initial fracture healing in an equine *in vitro* fracture hematoma model. *PLOS ONE* *contributed equally

Die Publikation -Hypoxia and mesenchymal stromal cells as key drivers of initial fracture healing in an equine *in vitro* fracture hematoma model- wurde von Moritz Pfeiffenberger und Janika Bartsch als geteilte Erstautorenschaft in PLOS ONE veröffentlicht. Aus den erhobenen Daten entstand ebenfalls eine Masterarbeit von Frau Janika Bartsch an der Beuth-Hochschule für Technik in Berlin. Moritz Pfeiffenberger war vor allem bei der Versuchsidee und der Planung der Versuche und der Anleitung der praktischen Versuche federführend. Bei der Etablierung von Antikörpern für die Zellpopulationsanalyse und Primern für die Genexpressionsanalyse war Moritz Pfeiffenberger sowohl bei der Planung als auch der Etablierung mittels Durchflusszytometrie und qPCR in der Praxis beteiligt. Das Manuskript zur Publikation wurde vorrangig auf kurierten und aufbereiteten Daten der Masterarbeit von Frau Janika Bartsch aufgebaut und wurde von Moritz Pfeiffenberger geschrieben.

Moritz Pfeiffenberger, Alexandra Damerau, Igor Ponomarev, Christian H. Bucher, Yuling Chen, Dirk Barnewitz, Christa Thöne-Reineke, Paula Hoff, Frank Buttgereit, Timo Gaber & Annemarie Lang (2020), An *in vitro* human-based fracture gap model – Mimicking the crosstalk between bone and immune cells, ***preprint in biorxiv*** **doi:** https://doi.org/10.1101/2020.06.26.165456

Die Analyse via µCT der SFBCs in der Publikation -An *in vitro* human-based fracture gap model - Mimicking the crosstalk between bone and immune cells- wurde mit Hilfe von Frau Dr. Annemarie Lang durchgeführt.

Hiermit bestätige ich, dass ich die hier vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Moritz Pfeiffenberger Berlin, den 20.05.2021

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