Aus der Chirurgischen Klinik und Poliklinik – Innenstadt,

der Ludwig-Maximilians-Universität München,

Experimentelle Chirurgie und Regenerative Medizin, Experimed

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Investigation of the effect of low oxygen tension on the osteogenic differentiation of human mesenchymal stem cells

Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

(Dr. rer. biol. hum.)

an der Medizinischen Fakultät

der Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus Wiesbaden

2010

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Tag der mündlichen Prüfung:	19.07.2010

For my parents

"Education is a companion which no misfortune can depress,

no crime can destroy, no enemy can alienate, no despotism can enslave.

At home, a friend, abroad, an introduction, in solitude a solace and in society an ornament.

It chastens vice, it guides virtue, it gives at once grace and government to genius.

Without it, what is man? A splendid slave, a reasoning savage."

Joseph Addison (1672-1719)

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1 Introduction

1.1 Clinical relevance: bone defects

Bone defects can be either inherited, occur following trauma, surgical correction of hereditary defects, through infection, congenital anomalies but can also arise due to operative intervention such as cyst and tumor resections (1). In general bone defects can be classified based on their morphological characteristics such as length, breadth and depth of injury. In addition to this the localisation of the defect plays an important role in the treatment of the defect. One differentiates between cortical, cancellous and cortico-cancellous defects with and without implication of the joint. For the treatment of osseous defects one has also to take into account the structure and biomechanical properties of the adjacent bones, and also the surrounding connective tissue such as muscle, tendon and skin in order to obtain a differential therapeutic diagnosis. Apart from this the local vascularisation plays a crucial role in the process of bone healing (2).

1.2 Bone fracture healing

Bone has the distinct feature to regenerate itself if the fracture is within a certain critical size. Bone fracture healing represents a unique physiological process to repair and restore bone function. In the human body bone healing resembles the stages of embryonic bone development. In the well orchestrated regenerative process of the bone four components at the injury site contribute to the healing at various extents; the cortex, the periosteum, the bone marrow, and the external soft tissues. The extent to which each of the compartments contributes to the process of regeneration depends on a number of factors at and around the injured site. The

factors can vary from cytokines, hormones, nutrient and oxygen supply, pH to mechanical stability of the fracture (3). Histologically, fracture repair can be differentiated into two different processes namely: primary (direct or cortical) and secondary (indirect or combined endochondral and intramembranous) healing.

1.2.1 Primary fracture healing

Primary fracture healing occurs only if the apposed fractured areas can be directly approximated. In real life this can be achieved if the anatomical space between the two ends is reduced and rigid fixation gives the required stability. The primary fracture healing takes place mainly through cortical involvement via the reestablishment of new Haversian systems by forming discrete remodelling units known as “cutting cones”. This is aimed to restore mechanical continuity (4). Mesenchymal stem cells and vascular endothelial cells produce osteoprogenitor cells that differentiate into osteoblast that eventually produce an osteon and subsequently a woven bone is formed. The latter is substituted by lamellar bone. In order to guarantee a successful primary fracture healing the distance between the fracture ends should be less than 2 mm and requires minimal interfragmentary strain (5-8). There is no callus formation in primary fracture healing.

1.2.2 Secondary fracture healing

Secondary fracture healing is the prevailing mode of repair in the majority of fractures, and it is characterized by a callus formation which emanates from relative stability and motion at the fracture site (3). As the name already denotes in the indirect fracture healing a cartilage is initially formed which later on in the process of

healing gets replaced by bone. Mesenchymal stem cells get recruited to the site of fracture where they proliferate and differentiate into cartilage which becomes calcified and eventually replaced by bone. Intramembranous periosteal bone formation leads to the formation of hard callus, whereas the endochondral ossification results in soft callus formation. Following a bone fracture, a haematoma is formed and an inflammatory response arises. This triggers an array of processes to take place, starting with angiogenesis followed by cartilage formation, cartilage calcification, cartilage removal and ending with bone formation and bone remodelling (9, 10).

If the size of a bony defect is beyond a critical size the bone does not regenerate on itself. In such scenario the resultant defects present a major predicament for orthopaedic surgeons. In recent years the rise in bone defects has not only given a dilemma to the surgeons but represents now a substantial biomedical burden to the healthcare system (1). The demography of the population of the industrialized countries is shifting towards an older profile, making injury and disease of the skeletal system a more relevant clinical issue. It has been predicted that the percentage of persons over 50 years of age affected by bone diseases will double by 2020 (11). In case of large skeletal defects, the “gold standard” to treat such bony defects, is the use of autogenous bone grafts (12).

1.3 Bone grafting

1.3.1 Principle

The principle aim of bone repair is to promote and accelerate the bone healing, in order to obtain a bone that can withhold the mechanical load and provide stability to the skeleton. This is primarily achieved by means of a bone graft whose functions are to promote bone formation or osteogenesis and provide structural support.

An ideal bone graft should possess the following properties:

1.) Osteoinduction refers to a process whereby mesenchymal stem cells are recruited and prompted to differentiate into the osteogenic lineage, giving rise to osteoblasts (bone forming cells). A material is said to be osteoinductive if it has the property to induce bone formation at an ectopic site. One example of such a material is demineralised bone matrix (DBM).

2.) Osteoconduction denotes a process where a bone graft acts as a platform on which cells required for bone formation can attach and proliferate. These materials offer an appropriate topology on which MSC can attach, spread, migrate and proliferate.

3.) Osteogenity refers to generation of bone-forming cells. A graft or material is said to be osteogenic if it is capable of supplying and supporting bone forming cells (13, 14).

1.3.2 Types of bone grafts

The process of transplanting bone from one atomic site to another atomic site within the same individual is referred to as autogenous bone grafting. The sites where bone is usually harvested are proximal femur or tibia, femoral head, iliac crest and a resected rib. Autogenous bone graft remains until today the “gold standard” to treat bony defects as it possesses osteogenic properties (marrow-derived osteoblastic cells and preosteoblastic precursor cells), osteoinductive properties (non collagenous bone matrix and growth factors), and osteoconductive properties (bone mineral and collagen). Autogenous grafts are completely histocompatible, nullifying the fear of immune rejection and rendering immuno-suppression unnecessary. All these attributes make autogenous grafts the primary choice for graft materials for bone reconstructions (15). However, autogenous bone grafting is limited by restricted supply and donor site morbidity, including bleeding, hematoma, infection, muscle weakness, nerve injury and chronic pain (16, 17).

The process of transplanting bone from one individual to another individual of the same species is referred to as allogenous bone grafting. Allografting is primarily done to support mechanical loads and resist failure at site where structural integrity is required (16). Allografts have grown over the years as evident through the rise in bone tissue banks across the world. The greatest disadvantage of using allografts is the possibility of transmitting viral diseases such as hepatitis B, hepatitis C and human immunodeficiency virus (HIV; 18). By thorough screening of the donors at the tissue banks one tries to reduce the risk of viral transmission. Besides this there are various methods now employed to sterilize bone grafts such as low dose irradiation,

physical debridement and ultrasonic washes to mention a few of the widely used techniques (19). Another main objective of sterilizing allografts is to obtain a more histocompatible bone by removing the antigen components of the graft that might elicit an immune response in the host and that still retains its biological and biomechanical properties.

Although autogenous and allogenous bone grafts are a helpful tool in treating bony defects, due to their distinct disadvantages there is a need to look out for other conventional methods to treat bone defects in the future (16). In recent years a multitude of bionatural and synthetic bone graft substitutes have made their way to the market. Among the popular ones are calcium-phosphates, ceramics, demineralised bone matrix (DBM), and composite grafts (20, 21). The attractiveness of these bone graft substitutes is that they are devoid of surface antigens and hence do not mount an immunologic graft rejection, eliminate the risk of disease transmission as seen with allografts, and above all there is an infinite supply of the graft material. Bone graft substitutes though promising have their drawbacks like contour irregularities and structural failure, and mostly lack the osteoinductive property.

The vast amount of techniques and materials available to the surgeon to treat bone defect reflects both the inadequate supply of individual bone and the necessity to find new tactics for reconstructive surgery of bone defects. Cell based tissue engineering might be an alternative solution for this dilemma and might overcome the numerous drawbacks as seen with the traditional graft material as mentioned above.

1.4 Tissue Engineering

Tissue Engineering, an emerging field of the 21st century and one of the frontiers in biotechnology is an interdisciplinary field that involves the fusion of biomaterial science and life sciences (22). Tissue engineering has come a long way, and in order to make reasonable speculations on where this ever developing field is heading towards, it is important to understand where it had been. In the early years of tissue engineering around 1970 Green and colleagues at the children's hospital Boston, tried to generate new cartilage *in vivo* by seeding chondrocytes onto spicules of bone and thereafter implanting the cell-seeded 3D construct in nude mice. Though this experiment failed, he concluded that with the advancement of technology and the development of innovative biocompatible material it would be possible to generate new tissue *in vivo* (23). Most of the subsequent and important work in the field of tissue engineering was carried out around Boston, and it is considered that the modern roots of tissue engineering are deeply seated in Boston. Other eminent personalities who contributed to the field of tissue engineering at the early stages were Burke and Yannos who were able to produce a skin graft through the use of dermal fibroblast grown on collagen gels. One of the key moments in the history of modern tissue engineering was when Vacanti and Langer collaborated to design an appropriate scaffold for cell based tissue engineering (23). This new approach gave tissue engineering a whole new side as it attempted to seed cells on a synthetically created scaffold with known physical and chemical parameters, rather than in the older times where cells were seeded on naturally occurring materials. Scaffold development has from there onwards been an ever growing field, which gives tissue

engineering new perspectives and possibilities that would have been not achievable with biologically occurring materials.

Cell based tissue engineering involves the construction of a three-dimensional scaffold that acts as a mechanical support on which the desired cells are grown followed by the implantation of the seeded scaffold to induce and direct the growth of new, healthy tissue (Fig.1; 24, 25). The Scaffold acts as a temporary matrix mimicking the structural and biomechanical properties of the extracellular matrix of the damaged tissue. *In vitro* cultured cells adhere and grow on the scaffold which will be then implanted on to the damaged site where it will favour the regeneration of new cells. As the cells grow *in vivo* they start generating their own extracellular matrix (ECM) and the scaffold starts to degenerate. In theory tissue engineering enables us to generate a scaffold of controlled size, shape, strength and composition that can be suited to replace an extracellular matrix of a tissue with specific biomechanical properties (26, 27).

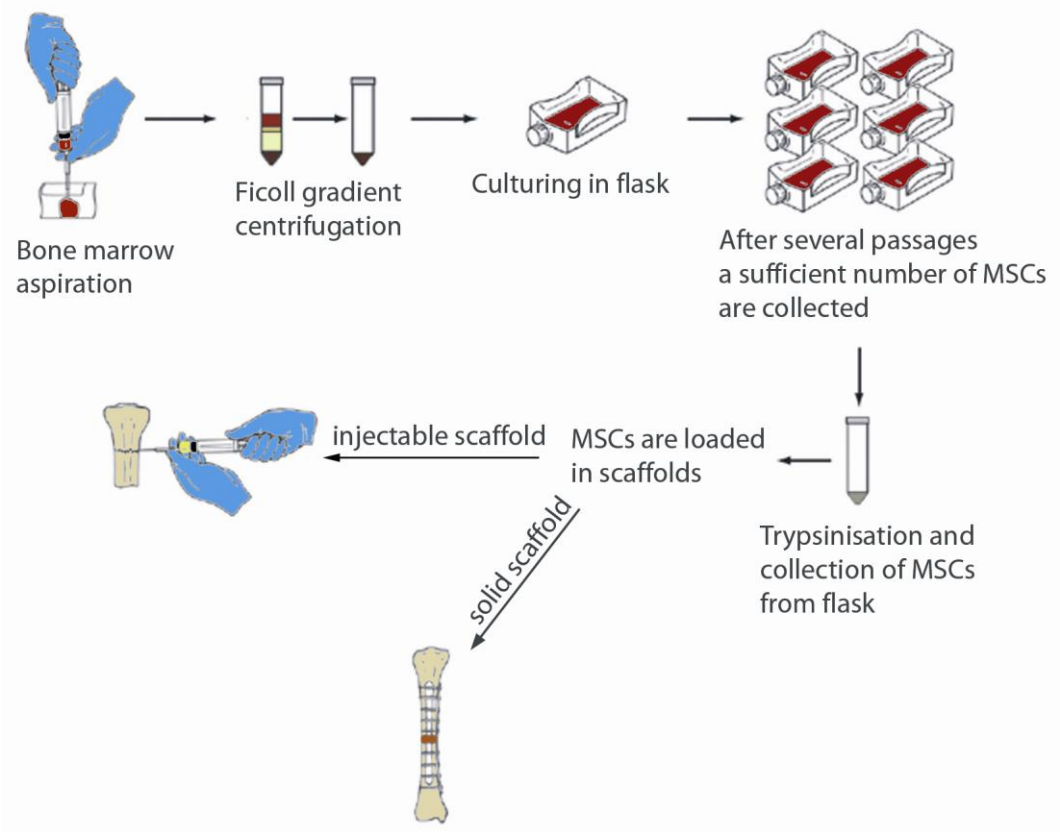


Figure 1 Concept of cell based tissue engineering. Autologous stem cells are obtained via bone marrow aspiration. Mesenchymal stem cells are isolated via Ficoll gradient centrifugation and stem cell characteristics are determined. They are expanded *in vitro* to the desired cell number and thereafter put into the site of fracture either via injection or seeded on biocompatible scaffolds and implanted at the site of fracture where they will aid in regenerating new healthy tissue. (Adapted and modified from Pountos et al., 2006 (28))

1.4.1 Bone Tissue Engineering

1.4.1.1 Bone - Structure and Function

Bone is a vascularised tissue that provides mechanical stability to the skeleton that is required for locomotion, load bearing and protection of the brain, spinal cord, heart and lungs. Furthermore it serves as a mineral reservoir for calcium and serves as an attachment ground for muscles, ligaments and tendons (29). It is composed of three different cell types, namely osteoblasts, osteocytes and osteoclasts that are confined in a highly organised extracellular matrix (ECM).

Osteoblasts are derived from mesenchymal stem cells and their primary function is to synthesize the major protein content of the ECM. In addition to this they induce and downregulate osteoclasts and express genes required for calcification. Osteoblasts are large polyhedron shaped cells of 20-30 µm in size. Typical markers for osteoblast differentiation are type I collagen (COL I), alkaline phosphatase (ALP), runt related protein 2 (RUNX 2) and osterix (OSX) for early stage and osteocalcin (OCN), osteopontin (OPN), osteonectin (ON) and bone sialoprotein (BSP) for the late stage of osteoblastic differentiation (30). Osteoblasts once trapped in the ECM mature into osteocytes that are the most numerous and longest-lived cells in bone. Osteocytes are stellate and found in the interior of bone inside lacunae, whereas osteoblast and osteoclast are situated on the bone surface. They are assumed to sense bone deformation and send out signals for requirement of adequate bone modelling (31, 32). Osteoclasts are derived from monocytes and their primary

functions are bone resorption and bone turnover. The balanced interplay between bone formation through osteoblasts and bone resorption through osteoclasts is important to maintain bone strength in adulthood. The osteoblast and osteoclast communicate with each other through transmembrane proteins or integrins, that either link cells or the cells to the extracellular matrix and through secreted signalling proteins such as RANK-ligand which regulate osteoclastic activity.

The ECM consists of an organic and an inorganic phase. The organic part also known as the osteoid material is fundamentally formed from collagen type I. Apart from this other constituents of the organic part are proteoglycans, glycoproteins, proteins from plasma, growth factors and proteins with g-carboxyglutamic acid. The inorganic part is formed through hydroxyapatite crystals which consist of calciumphosphate and in smaller quantities by magnesium, sodium, potassium, manganese and fluoride (33).

In adults one can distinguish between two types of bone depending on the architecture of the tissue within the bone. In Cancellous bone, the tissue is arranged in a trabecular pattern and in cortical bone the bone tissue is arranged in a compact pattern. Cancellous bone is more porous and flexible compared to cortical bone and are present in the metaphysis of long bones, iliac crest and the vertebral bodies. Cortical bone is ubiquitously present in long, short and flat bones (17).

1.4.2 Scaffolds for bone tissue engineering

Biomaterials as scaffolds for bone tissue engineering serve certain biological functions by substituting or repairing different bone in the skeleton or by even guiding

bone repair. Over the past 60 years the field of material science revolving around scaffold development has grown tremendously. One can distinguish between three different generations of biomaterials: First generation biomaterials were developed during 1960s and 1970s and their main function was to resemble the physical properties of the bone to be replaced and to cause minimal toxic effect to the host (34). Examples include commercially pure Ti (CP Ti®) and Ti6Al4V (ASTM F136®) the most commonly used titanium alloys in orthopaedics (11). With the design of the second generation biomaterials one tried to produce a more sophisticated scaffold by moving from an inert biomaterial to a more bioactive and biodegradable material. One of the first second generation biomaterial was bioactive glass followed by synthetic hydroxyapatite (HA) ceramics which were routinely used in the mid 1980s as powder, porous implant and coatings on metallic prostheses to provide bioactive fixation (35). Finally the third generation of biomaterials appeared which tried to stimulate specific cellular responses at the molecular level (35). For these biomaterials the attributes of bioactivity and biodegradability are combined. A popular example of this group is demineralised bone matrix (DBM), which is widely used in clinical applications as an alternative to autografts for filling bone defects (36).

Scaffolds play a very important role in cell based tissue engineering, they act as temporary matrices on which desired cells can attach, spread, proliferate and eventually differentiate into a specific lineage. The scaffold should ideally be designed in a way that it matches the geometry and size of the defect. Besides its external appearance it should facilitate the ingrowth of capillaries and vessels (angiogenesis) so that cells residing in the interior of the scaffolds are sufficiently supplied with oxygen and nutrient. This is warranted by micro - and macroporosity of the scaffold. A pore size of approximately 10 µm is required for capillary ingrowth and

cell-matrix interaction whereas a pore size of 150-900 µm allows for nutrient supply and waste removal (37-40).

One can group scaffolds into organic and inorganic matrices. Organic scaffolds include natural graft materials and biodegradable polymers, either synthetic or naturally occurring ones. Within the natural biodegradable polymers one can find among others collagen, fibrinogen, chitosan, hyaluronic acid and starch. These naturally occurring polymers have distinct advantages like low immunogenic potential. Synthetic biodegradable polymers are more widely used in the biomedical engineering field. Well known examples are for instance poly (ε-caprolactone), poly (propylene fumarate), and poly (α-hydroxyacids). Polylactic acid (PLA) and polyglycolic acid (PGA) belong to the family of poly (α-hydroxyacids) and are widely used in the production of 3D scaffolds. They have been also approved by the Food and Drug administration to be used as bone fixation devices such as pins and screws (ReFIX Xtremi-T®, LLC®) and PDS/PGA staple (Mitek®), to name a few. Their application is restricted to fractures with low mechanical modulus (20). The key advantages of using biodegradable materials over inorganic substance in bone repair are: (i) a second surgery is not required to remove the implant, as it will degrade on its own over the period of regeneration and the degradation products are excreted from the body through natural pathways, (ii) the progressive loss of implant material results in the induction of bone formation by osteoblasts (41). Inorganic scaffolds include calciumphosphate-based ceramic or cement and calciumsulphate-based scaffolds and bioactive glasses (42). Calciumphosphate-based ceramics are currently employed by orthopaedic surgeons in reconstructive surgery. Several studies have shown that good results could be obtained with ceramic implants used for bone defects (43, 44). Most notably calciumphosphate-based ceramics have been

used as bone substitutes either in bulk form or as granules and in combination with cells from the bone marrow. The benefit of using ceramic based implant lies in their distinct properties of being biocompatible, bioactive and osteoinductive. However due to their low tensile strength and brittle nature they can not be employed at sites of significant torsion, bending, or shear stress (11, 38, 45).

Ideally scaffolds should have the following properties to be used in cell based tissue engineering applications: First and foremost the scaffold should be biocompatible. It should not mount an immune response within the host nor should it be cytotoxic. In addition the scaffold should possess adequate mechanical strength to sustain handling and during the patients normal activities. The scaffolds must be sterilizable to prevent any sort of infection or transmission of viral diseases. Furthermore the porosity of the scaffold is important. The scaffold requires an interconnected porous architecture that supports vascularisation and exchange of nutrient and oxygen. In addition scaffolds must be biodegradable and the byproducts generated during degradation should be non toxic to the host and easily removable by the body’s own metabolic processes. The scaffold should optimally resorb at the same rate as the tissue is repaired (46, 47). Another desirable property would be the molding of the scaffold to match the size and geometry of the defect and as well a radioopaque nature in order to discriminate between bone and implant via radiography (45, 48). Scaffolds derived from native osseous tissue, are preferentially used in bone tissue engineering because of their close resemblance in structure and function to autologous bone (49). Nowadays bone tissue engineering is focusing more on scaffolds that are biological in nature and are able to carry cells and provide an environment for them to proliferate and differentiate towards the osteogenic lineage. One such type of scaffold is the demineralised bone matrix (DBM).

Demineralised bone matrix (DBM) is a third generation biomaterial and has been already successfully used in clinical applications, mostly to fill in bone defects (11). Unlike synthetic materials, DBM is known to be osteoinductive and stimulate bone formation both in heterotopic and orthotopic implant sites. In 1965, Marshall Urist discovered the osteoinductive properties of DBM scaffolds. She was able to see bone formation when DBM scaffolds were placed ectopically in subcutaneous tissue (50). Their osteoinductive nature is attributed to residual matrix incorporated osteogenic factors such as bone morphogenetic proteins (BMPs) and other non collagenous proteins retained within these scaffolds (15). It has been shown that BMSC grown on DBM have an increased potential to differentiate into the osteogenic lineage.

1.4.3 Stem cells

Stem cells are found in almost all multicellular organisms and are characterized by their self-renewal potential and their ability to differentiate into a wide variety of cells. They can be differentiated into embryonic stem cells and adult stem cells.

1.4.3.1 Embryonic stem cells

Embryonic stem cells (ES) are touted for their pluripotency and are harvested from the inner cell mass of blastocyst during gastrulation, an early stage during embryonic development. ES have the unique ability to give rise to all tissues derived from the 3 embryonic germ layers namely: ectoderm, endoderm, and mesoderm. They have been shown to differentiate into the osteogenic lineage both *in vitro* and *in vivo* (51-

53). The pluripotency of ES is governed by a set of transcription factors, namely OCT 4 (Pou5f1), SOX2 and NANOG (54-57). Even though their pluripotent nature makes them an ideal candidate for tissue engineering applications, however their predisposition for teratoma formation and their isolation from embryos makes them a distant cell source (1). Therefore, research has made a significant effort to identify a postnatal cell source that is multipotent.

1.4.3.2 Human mesenchymal stem cells

Human mesenchymal stem cells (hMSC) are widely used in tissue engineering applications because of their multipotentiality, relative ease of isolation and their high proliferation profile. They were first described by Friedenstein and colleagues in 1970 as colony-forming unit-fibroblast (CFU-F; 58). Their ability to differentiate into the 3 classic lineages, that is to say chondrogenic, adipogenic and osteogenic were first shown by Pittenger et al. in 1999 (Fig. 2; 59, 60). Primarily MSC are obtained from the bone marrow but can also be derived from adipose tissue, umbilical cord, amniotic fluid, skeletal muscle and, in low numbers from peripheral blood (61).

Over the years our understanding over these unique cells has taken great strides forward, but nevertheless we still have not identified a specific marker that could distinguish this subset of cells from the remainder. Currently we are following a series of criteria set by the International Society for Cellular Therapy (ISCT) that defines MSC. According to these criteria MSC must be plastic-adherent, >95% of the MSC population must express the surface antigens CD105, CD73 and CD90 and must lack the expression of CD45, CD34 and CD14. MSC must also be able to differentiate *in vitro* into the osteogenic, adipogenic and chondrogenic lineage and

this must be demonstrated via staining (62, 63). The search for a specific marker remains a hot topic in the present time.

BMSC isolated both from animals and humans have been already administered in combination with 3D construct to sites of muscoskeletal defects in experimental animals to regenerate bone. Bruder et al. for instance delivered hMSC loaded on ceramic into critical sized defects in the femur of adult athymic mice. Their study showed that the ceramic carrier with cells resulted in a better bone formation than ceramic carrier without cells after 12 weeks of implantation (64). Another group reported successful regeneration of large bone defects in humans through the implantation of autologous hMSC seeded on macroporous hydroxyapatite scaffolds (44). This group also followed up this study (post surgery) and checked for durability of the regenerated bone after 6 to 7 years. They have observed a good integrity of the implants was maintained even after 7 years (65). In contrast several other groups failed in regenerating large bony defects indicating that the optimal combination of scaffold material, design and cells has yet to be found (66).

1.4.3.2.1 The stem cell niche

The concept of stem cell niche was first introduced by Schofield in 1978 (67). He suggested that haematopoietic stem cells (HSC) could proliferate infinitely without losing their stem cell characteristic when they reside at a particular area he coined stem cell niche. Cipolleschi described that the stem cell niche is an area of extreme low oxygen (68, 69). Therefore mesenchymal stem cells that are derived from the bone marrow are assumed to reside in an hypoxic microenvironment (70). They coexist with HSC (71). It is believed that immature progenitors are located in areas of

low oxygen whereas more committed progenitors exhibit places with more oxygen in either closer to blood vessels.

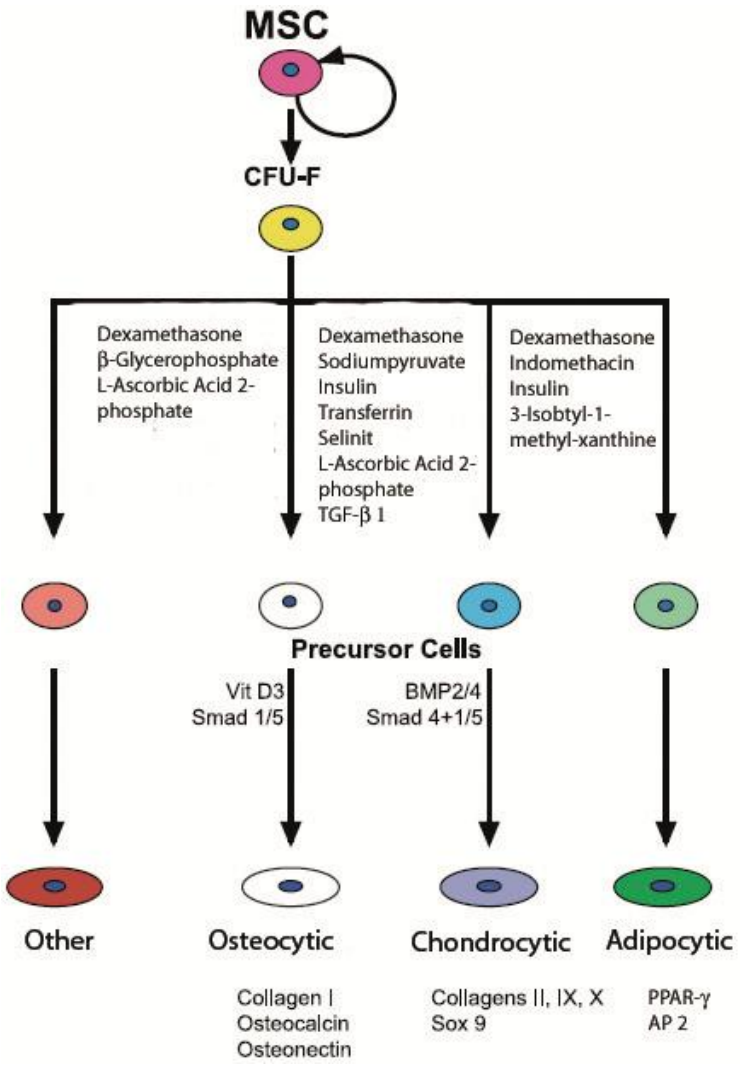


Figure 2 Characteristics of MSC. MSC are able to self renew themselves and give rise to colony forming unit-fibroblasts (CFU-F). Each of this unit is able to differentiate into a variety of lineages depending on the stimulus given. Here we show their classical differentiation ability towards bone, cartilage and fat respectively. (Adapted and modified from Otto et al., 2004 (72))

1.4.3.3 Immortalised hMSC (SCP-1)

Though hMSC have their advantages of being a suitable candidate for bone tissue engineering applications, only 0.001-0.1% of nucleated cells derived from the bone marrow are MSC. These low numbers necessitates the need of culture expansion *in vitro* before clinical use. Cell based tissue engineering requires a high number of cells, MSC tend to reach replicative senescence before they can grow to the required cell number (73). It has been reported that hMSC will loose their proliferation potential, homing and differentiation potential once they reach a maximum of 20-40 population doublings *in vitro* (9, 74, 75). Numerous studies have revealed that telomere shortening plays a crucial role in the life span of somatic cells in culture including MSC (76, 77). During cell division the telomere length shortens and results in senescence and growth arrest. Telomere length can be maintained despite cell division through a ribonuclear protein complex telomerase which serves as a telomeric template and a subunit that has a reverse transcriptase activity. Human MSC are known to lack telomerase activity *in vitro* (78, 79). Therefore numerous groups immortalised hMSC through the incorporation of the human telomerase reverse transcriptase (hTERT) transgene under the control of a constitutive promoter that would prevent hMSC to enter senescence-associated growth arrest. They were also able to show that even after immortalisation the hMSC retained their ability to differentiate into several lineages (80-83). Nakahara and colleagues could show that immortalised hMSC were able to form bone in mice (84).

Our Lab has created an immortalised hMSC line through the ectopic expression of hTERT using lentiviral gene transfer, that we call SCP-1 (Single cell picked-clone 1).

We were able to show that even after long term culture (2 years) the cells did not show any malignant transformations (85).

1.5 Hypoxia in tissue engineering and regenerative medicine

Though tissue engineering has been in existence for decades it's still a mere vision of putting the concept of tissue engineering into reality. One of the major drawbacks in tissue engineering is the occurrence of a reduced oxygen tension within the interiors of a scaffold. The lack of vessel formation within a scaffold during the initial stages of implantation leads to an inadequate supply of oxygen, nutrients and a hindered waste removal that additively leads to the death of cells. Apart from the death of cells it also brings about the uneven distribution of cells within the scaffold that might result in a poor quality of the resulting tissue (86). In living organisms the distance between cells and capillaries, that on one hand supplies nutrient and oxygen to the cells and on the other hand helps in the removal of metabolic waste products, lies between 20 and 200 µm (87). Therefore cells residing within the core of large 3D constructs will face suboptimal oxygen supply (17).

Likewise, at the site of fractured bone, a milieu of reduced oxygen triggers bone healing further linking hypoxia to osteogenic differentiation of bone precursor cells (88). It is thus evident that cells that are utilized to regenerate bone tissue will face hypoxic conditions, either when used in the setting of bone tissue engineering or whenever a fracture has occurred (86, 89, 90).

When exposed to hypoxia, cells respond via a complex signalling cascade, in which the hypoxia-inducible factor 1α (HIF-1α) plays a predominant role (91-94). HIF-1α

leads to an upregulation of genes, which orchestrate cell metabolism and survival under low oxygen conditions, including anaerobic glycolysis, formation of new blood vessels, or ultimately induce apoptosis (91-95).

1.5.1 HIF-1α

HIF-1α is a transcription factor that belongs to the basic helix-loop-helix-Per-ARNT-SIM (bHLH-PAS) protein family. It is required for attaining homeostasis under reduced oxygen tension and was discovered in 1992 (96). HIF-1α is stably expressed under reduced oxygen tension. It heterodimerises with HIF-1β which is constitutively expressed and is independent of oxygen concentration (97). Once the heterodimer is formed there is a configurational change taking place within the dimer allowing it to bind to the hypoxia responsive element (HRE), a DNA sequence residing on the target gene. This in turn will trigger the activation of transcription of genes involved in various biological processes that are vital for sustaining homeostasis in hypoxic milieu, such as erythropoiesis and glycolysis, which instantly counteracts oxygen deprivation. Angiogenesis, in either sprouting of vessels from pre-existing vessels is prompted through the expression of vascular endothelial growth factor (VEGF), which is a long term adaptation to low oxygen tension (Fig. 3).

Hif-1α gets degraded through its oxygen dependent degradation domain (ODDD) via the E3 ubiquitination pathway. In order for Hif-1α to be degraded, proline residues situated at the ODDD need to be hydroxylated by prolyl hydroxylase domain-containing proteins (PHD). Following the hydroxylation the von Hippel-Lindau tumor suppressor protein (pVhL) is able to bind to the site orchestrating a cascade of reaction to take place winding up in the proteosomal degradation of the Hif-1α via the

E3 ligase complex (91, 98). The proteosomal degradation of Hif-1α can be inhibited through substances such as desferrioxamine (DFO) that renders PHD inactive. PHD requires besides molecular oxygen, iron as a co-factor to hydroxylate the proline residues. DFO being an iron chelator takes away the iron from PHD and hence making it inactive, and therefore Hif-1α degradation is inhibited even under normoxia (Fig. 3; 99). The transcription product of both HIF-1α and HIF-1β are ubiquitously expressed and is independent of oxygen concentration.

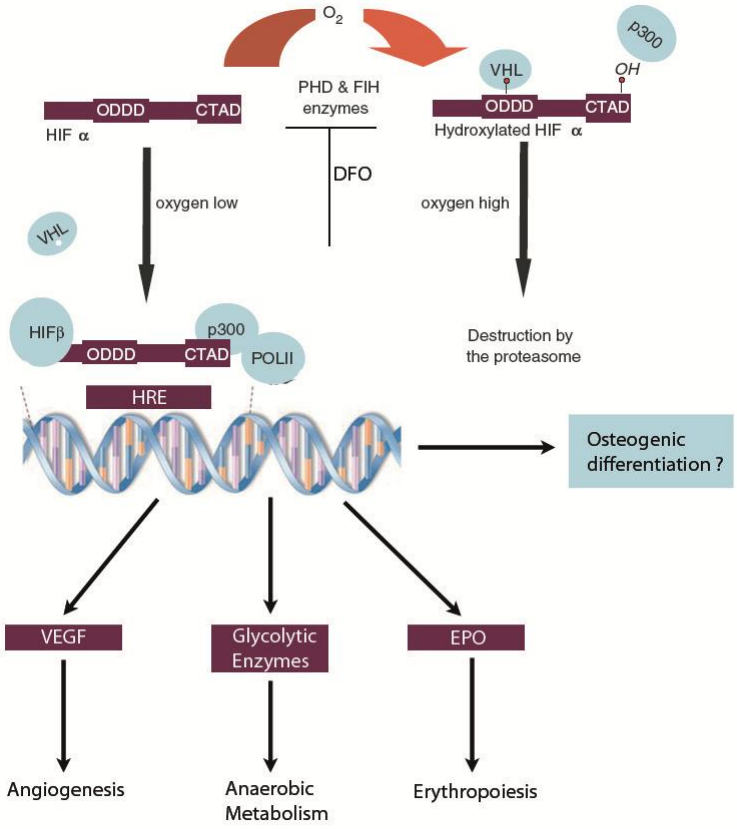


Figure 3 Regulation of HIF-1α by oxygen concentration. During low oxygen tension HIF-1α is stable and gets translocated into the nucleus where it will bind to HIF-1β. The hetero dimerization brings about a configurational change within the protein complex allowing it to bind to a specific DNA sequence on the target gene and thereby initiating the transcription of various genes that mediate hypoxia response mechanisms. Under high oxygen tension Hif-1α gets hydroxylated at its

oxygen dependent degradation domain (ODDD) and C terminal transactivation domain (CTAD) via PHD and FIH enzymes respectively. Hydroxylation enables the von-hippel-lindau tumor suppressor protein (VhL) to bind to Hif-1α triggering its ubiquitylation and destruction. (Adapted and modified from Maxwell, P.H., 2008 (100))

1.5.2 Hypoxia and its role in osteogenic differentiation

Lately, it was shown that differentiation processes are also affected by hypoxia (101-107). Malladi and colleagues for instance reported that osteogenic differentiation was reduced when cells were differentiated under 2% oxygen (104). Several articles have since provided proof that osteogenic differentiation may be negatively affected by hypoxia (104, 106-111). It was shown that hypoxia brings about a decrease in the expression level of key transcription factors and osteogenic marker genes, such as RUNX 2, OCN, and COL 1 (106, 107, 109, 111).

Although it remains to be determined what true hypoxic conditions are, it is now widely accepted that 21% of oxygen as commonly used in cell culture is rather a state of artificial hyperoxia (103, 112, 113). In consequence, low oxygen tensions are considered to be a more physiological milieu for stem cells (103, 112, 114-116). In accordance with these considerations, a recent study provided evidence that preconditioning hMSC in low oxygen improves their therapeutic potential (117). It was shown that hypoxic preconditioning improved the homing and tissue-repairing capacity of hMSC, and besides this, cells preconditioned in hypoxia displayed an increased motility. Another study documented that MSC would survive longer after an assault of deadly hypoxia if preconditioned in low oxygen (118). In addition, it was

proposed that activation of the HIF-1α pathway accelerates bone regeneration *in vivo*, suggesting that hypoxia is an important co-factor in bone healing (119).

Wan and colleagues conducted experiments in osteoblasts of mice that lacked pVHL and therefore had a constitutive HIF-1α activation and observed that these mice had an increased vascularity and produced more bone in response to distraction osteogenisis compared to mice that lacked HIF-1α which had an impaired angiogenesis and bone healing (119). Similarly Wang et al. showed that mice overexpressing HIF-1α in osteoblasts through the deletion of the von Hippel-Lindau gene (VHL) expressed high levels of vascular endothelial growth factor (VEGF) and developed extremely dense, heavily vascularised long bones. In contrast they observed in mice lacking HIF-1α in osteoblasts an opposite skeletal phenotype; in either the long bones were substantially thinner and less vascularised. In mice that lacked both VHL and HIF-1α the phenotype of the long bone was intermediate between the mice lacking HIF-1α through the deletion of VHL and the mice overexpressing HIF-1α (94). Interestingly Wang et al. were able to show that upon HIF-1α knockdown the mice produced an elevated level of HIF-2α and as both HIF-1α and HIF-2α have overlapping function, HIF-2α might substitute partially for the loss of function of HIF-1α. This compensatory mechanism might also explain why HIF-1α knockdown mice were still able to develop functional bone (94). Taken together the results of Wang and Wan depicts HIF-1α pathway as a critical mediator of neoangiogenesis that is required for bone development and regeneration. Their studies collectively imply a possible therapeutic application of HIF-1 activators to improve bone repair.

1.6 Aim of this study

As hMSC used in tissue engineering are subjected to low oxygen concentrations, it is of critical importance to investigate how cells respond to hypoxia and how their regenerative and differentiation potential can be maintained despite exposure to hypoxia (120).

In this study we therefore first asked whether constant exposure of cells to low oxygen (2%) affects proliferation of hMSC. We then assessed whether the constant exposure to 2% O_2 would inhibit osteogenic differentiation of hMSC. We then analysed whether preconditioning hMSC in hypoxia would restore the differentiation potential. To address this question, we have investigated two different setups of differentiation; one, where cells were grown in 2% oxygen prior to osteogenic differentiation under 2% O_2. Despite the claim that low oxygen may be more representative of physiological conditions, this setup will be referred to as hypoxic preconditioning for consistency with conventional terminology. In the second case, cells were expanded under a so-called normoxic environment (21% O_2). Thereafter, they were osteogenically induced under 2% O_2.

We then addressed the question why osteogenic differentiation is delayed under hypoxia. In order to answer this question we have performed a clonogenic assay and semi quantitative RT-PCR for embryonic stem cell markers. We further asked what the role of HIF-1α is during hypoxic preconditioning with respect to the osteogenic differentiation potential of hMSC. To solve this question we transiently knocked down

HIF-1α using silencing RNA in hypoxia or stabilised Hif-1α using DFO treatment in normoxia.

As a next step, we wanted to see whether SCP-1 can be used as a model system for hMSC. For that we first characterised SCP-1 with respect to their growth kinetics, DNA synthesis, clonogenic potential and their stem cell characteristics in both normoxia and hypoxia. SCP-1 were then differentiated into the osteogenic and adipogenic lineages using the hypoxic preconditioned and normoxia setup. Finally we screened for the potential of hypoxia preconditioned SCP-1 to survive and proliferate on DBM scaffolds and their oxygen consumption in 2D versus 3D.

2 Materials and Methods

2.1 Cell culture

Table 1 Characteristics of hMSC donors

Donor	hMSC lot number	Age	Sex	Race
XI	1F2155	24	male	Other*
XIII	4F0591	32	male	caucasian
XIV	4F0760	25	female	caucasian
XV	6F3837	34	female	caucasian

*Other refers to mixed ethical background

Human mesenchymal stem cells were purchased from Lonza (Verviers, Belgium) at passage 2 (P2). The cells were expanded in minimum essential medium alpha with L-glutamine (αMEM; Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS; Sigma, Munich, Germany) and 40 IU/ml penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria) at 37˚C in a 5% carbon dioxide environment in a standard cell culture incubator (21% O_2). Cell stocks were frozen at P5 in a -80˚C freezer. Cells were not grown beyond 60% confluency during expansion culture. Table 1 reflects the various donors used in this project. Donor XV was the central donor for the studies.

SCP-1 was derived from donor XIV through a lentiviral transduction of the transgene hTERT. This cell line was derived from a single cell-picked clone of hTERT-immortalized hMSC and was therefore named ‘‘single cell picked-clone 1’’ (SCP-1). These cells were shown to differentiate toward the adipogenic, chondrogenic, and

osteoblastic lineage like their non immortalized counterparts (85). One important feature of SCP-1 is that they proliferate faster than primary hMSC. We have selected passage 72 for SCP-1 as early passages tended to be in their lag phase. Passages around 70 were in their exponential phase of growth and were also shown to differentiate into the 3 lineages (osteogenic, adipogenic and chondrogenic). In addition to this it was shown that SCP-1 do not produce any neoplastic transformations, when implanted subcutaneously into immuno-deficient athymic nude rats (85).

2.2 3D culture

Static three-dimensional (3D) culture was performed as described previously (86). In brief, cylindrical bovine demineralised bone matrix (DBM; Tutogen, Neunkirchen, Germany) scaffolds of 9mm in diameter and 5mm of height (Fig. 4) were seeded with 1 million hMSC/SCP-1 cells according to a standardized protocol (121). The scaffold was initially centrifuged in complete medium at 500 x g for 5 minutes to remove air that was trapped within the scaffold and to get rid of any chemical residues that were left on the scaffold from the demineralisation step. Initial centrifugation of the scaffold with medium resulted in discolouration of medium from light red to yellow. The centrifugation step of scaffold with alpha MEM was repeated as often as required until the medium retained its colour indicating that the pH remained constant. The DBMs were then placed into 48 well plates. Thereafter 500 µl of cell suspension containing 1 million cells were pipetted onto the scaffold. Every 20 minutes, during the first 2 hours, the 3D construct was turned over and cell suspension was reseeded on top of the scaffold. In this way it was made sure that the majority of the cells adhered to the scaffold rather than to the plastic surface of the 48 well. With this

seeding technique a seeding efficiency of 90% was achieved. The cell-scaffold constructs were then transferred to a 24-well-dish (Nunc, Wiesbaden, Germany) and cultured under standard conditions.

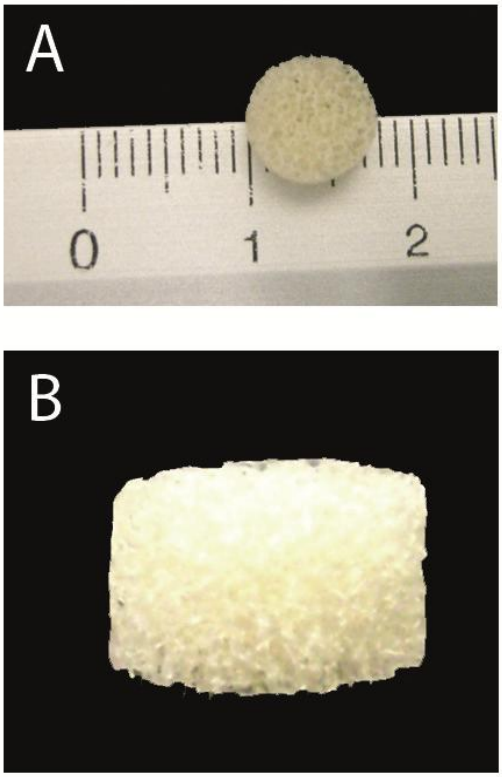


Figure 4 DBM Scaffold (A) Bird view of scaffold. (B) Side view of scaffold.

2.3 Hypoxia

To study the effect of hypoxia on human mesenchymal stem cells, hMSC were kept in a multi-gas incubator (MCO-5M, Sanyo, Pfaffenhofen, Germany) that maintained a gas mixture composed of 93% N_2, 5% CO_2 and 2% O_2. The oxygen concentration was maintained at 2% by the delivery of nitrogen. If O_2 percentage rose above the desired level, N_2 gas was automatically injected into the system to displace the excess oxygen. We have used 2% oxygen for our experiments as hMSC in 3D culture are exposed to as little as 2% of oxygen *in vitro*. However, we are well aware

that 2% oxygen may be more physiologic for certain cell types than the so-called normoxia (21%). Despite the claim that low oxygen may be more representative of physiological conditions, 2% of oxygen will be referred to as hypoxia for consistency with conventional terminology.

2.4 Passaging of cells

Cells were passaged at around 60% confluency into fresh culture flasks. Initially the spent medium was aspirated off and cells were washed with PBS in order to remove the last bit of medium remains. Thereafter cells were trypsinised with 1x trypsin for approximately 5 minutes at 37˚C. Trypsin is a serine proteinase that detaches the cells from the surface of flask by cleaving the proteins that bind the cells to the plastic surface. Trypsin was neutralised by adding double the amount of fresh medium into the flasks. Cells were resuspended thoroughly in order to get single cells and then were counted using a Neubauer chamber. The cell suspension was transferred into a suitable falcon tube and centrifuged at 500 x g for 5 minutes. The supernatant was aspirated off and the pellet was resuspended in fresh medium, to get rid off the trypsin. Finally the cells were plated at a density of 2500 to 6500 cells/cm^2 into a sterile culture flask, so that on the next day the confluency of the cells was around 20% to 50% respectively.

2.5 Cell counting using Neubauer cell chamber

Cells were counted using the Neubauer cell chamber. Following trypsinisation and resuspension 10 µl was pipetted on either one of the chambers of the neubauer cell chamber. Afterwards the 4 external quadrants (A, B, C, D) were counted and thereby

care was taken not to count cells that either lied on the right and top margins of each quadrant. The final cell count was determined using the following formula:

$[(A+B+C+D)/4] \times 10^4$ = total cell number/ml

2.6 Cryopreservation and thawing of cells

Cells were trypsinised and counted as above and around 0.5×10^6 cells were frozen using freezing medium in a cryovial. The freezing medium consisted of 70% culture medium, 20% FBS and 10% dimethyl sulfoxide (DMSO). DMSO is an important constituent of cryoprotectant vitrification mixture, which prevents ice crystal formation, and thereby protects cells from shear strain. Cryovials were stored for around 15 minutes in dry ice until cell suspension within cryovials solidified and then transferred either to -80˚C or into liquid nitrogen tank for prolonged preservation.

In order to thaw cells, vials from nitrogen tank or -80˚C were thawed in a 37˚C water bath until the cell suspension became liquid. Following this the suspension was transferred into a 15ml falcon tube, containing 2 ml of fresh medium and centrifuged at 500 x g for 5 minutes. The supernatant was aspirated off and the pellet was resuspended with fresh warm culture medium and finally transferred into culture flask. After 24 hours of incubation medium was changed, in order to remove the non adherent cells, which have succumbed during the process of cryopreserving and thawing.

2.7 Induction of osteogenic differentiation

For osteogenic differentiation, P5 vials were thawed and cultured as described above. After trypsinization, P6 hMSC were plated in a 6 well plate (NUNC, Wiesbaden, Germany) at a density of 3000 cells/cm^2 (122). They were cultured under normoxia until subconfluency (70-80 %) was reached. Subconfluency was usually attained 3 days after plating. Following subconfluency, 3 out of the 6 wells in each plate were induced to differentiate by adding osteogenic differentiation medium as described previously (121). The differentiation medium consisted of DMEM high glucose medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FBS, 40 IU/ml penicillin/streptomycin, 100 nM dexamethasone (Sigma, Munich, Germany), 10 mM β-glycerophosphate (Sigma, Munich, Germany) and 50µM L-ascorbic acid 2-phosphate (Sigma, Munich, Germany). As a negative control, the remaining 3 wells were cultured further with complete medium. After induction, the plates were either incubated in a low oxygen atmosphere of 2% O_2 for the remaining period of differentiation (hypoxic samples; Fig. 5B) or they were kept under normoxic conditions (normoxic control samples; Fig 5A). Every 3 days, a medium change was carried out.

2.8 Hypoxic preconditioning

Hypoxic preconditioning was established by expanding P6 hMSC under hypoxic conditions (2% O_2) until subconfluency was reached. Osteogenic differentiation was then carried out under hypoxia (2% O_2) in the same way as described for the hypoxic samples (Fig. 5C).

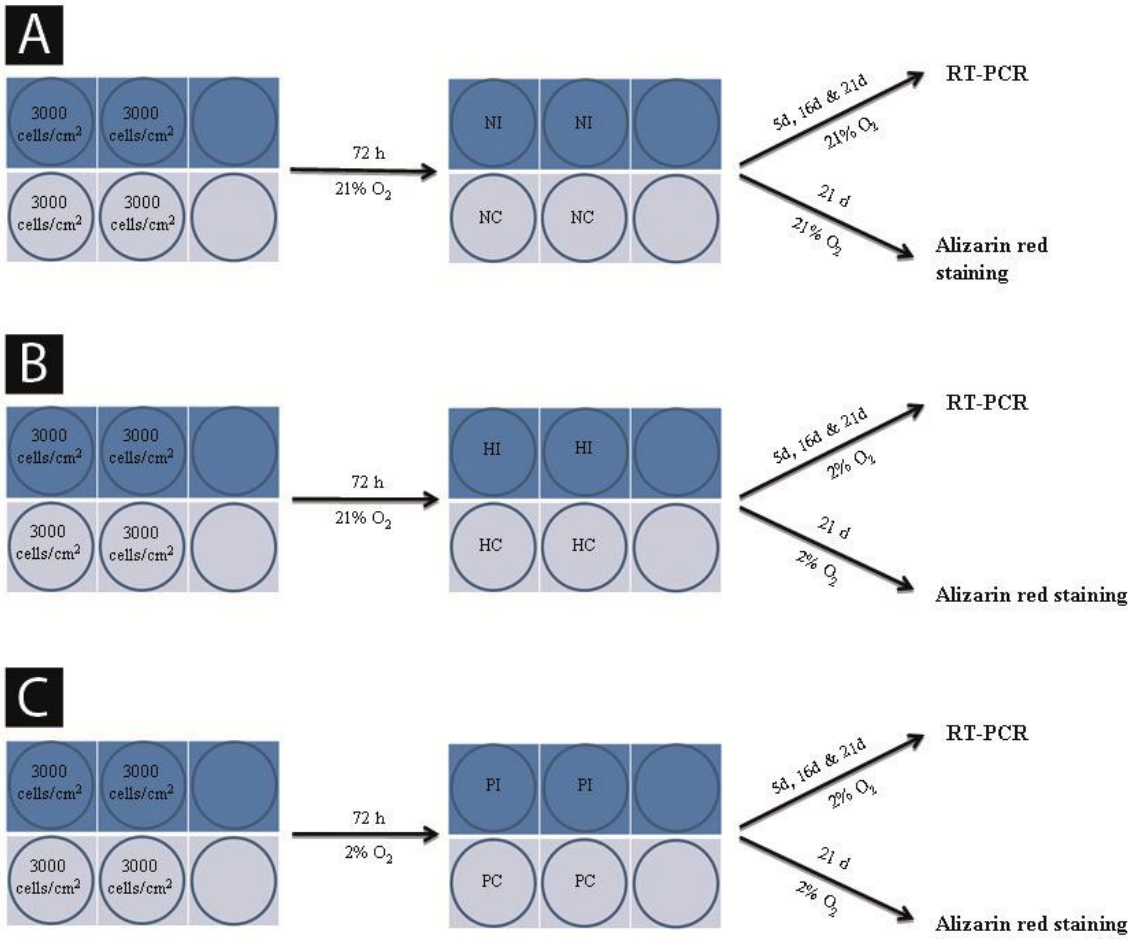


Figure 5 Schematic representation of osteogenic differentiation of hMSC. (A) Shows normoxic osteogenic differentiation. (B) Shows hypoxic osteogenic differentiation. (C) Shows hypoxic preconditioned osteogenic differentiation. Initially 3000 cells/cm^2 were seeded out in 6 wells and cultured for 3 days under normoxia and hypoxia. The cells were then induced into the osteogenic lineage for 21 days either by maintaining them at 21% O_2 (A) or 2% O_2 (C) or were transferred from normoxia to hypoxia (B). Abbreviations: NI: normoxia induced; NC: normoxia control; HI: hypoxia induced; HC: hypoxia control; PI: preconditioned induced; PC: preconditioned control.

2.9 Induction of adipogenic differentiation

For adipogenic differentiation, SCP-1 were seeded in 6 well plates (NUNC, Wiesbaden, Germany) at a density of 5000 cells/cm^2. They were cultured under normoxia or hypoxia until they were 90% confluent, which was reached after 3-4 days in culture. Following 90% confluency, 3 out of the 6 wells in each plate were induced to differentiate by adding the adipogenic differentiation medium. The differentiation medium consisted of DMEM high glucose medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FBS, 40 IU/ml penicillin/streptomycin, 4mM L-Glutamine, 1 µM Dexamethasone, 0,2 mM Indomethacin, 0,01 mg/ml insulin and 1mM 3-isobutyl-1-methyl-xanthine. Following 5 days of induction cells were maintained for 2 days using the maintenance medium which consisted of DMEM high glucose supplemented with 10% FBS, 40 IU/ml penicillin/streptomycin, 4mM L-glutamine and 0,01 mg/ml of insulin. Induction followed by maintenance was alternatively changed for a period of 16 days. As a negative control, the remaining 3 wells were cultured further with complete medium. Every 3 days a medium change was carried out.

2.10 Oxygen Measurements

2.10.1 Principle of oxygen measurement

The principle behind the oxygen measurement relies on two different dyes. One is the oxygen indicator whose phosphorescence intensity I_{ind}, is dependent on the oxygen concentration whereas the other dye acts as the reference whose

fluorescence intensity, I_{ref}, is independent of the oxygen concentration. The ratio of the luminescence intensities of the 2 dyes gives rise to the reference signal I_R which corresponds to the concentration of O_2 (123).

$$I_R = \frac{I_{ind}}{I_{ref}}$$

2.10.2 2D oxygen measurement

The oxygen was measured in 2D using a SensorDish Reader (SDR, Presens, Regensburg, Germany). In brief 100.000 SCP-1 were seeded in duplicates into a sterile polystyrene 24-well multidish with integrated optical-chemical sensor that measures dissolved oxygen (OxoDish; Fig. 6). The oxygen sensors were already calibrated using a two point calibration by the manufacturer. Thereafter the oxodish was placed on the SDR and placed in the incubator and the oxygen was measured every hour over a period of 7 days. For illustrative purposes the data obtained from 24 hours was pooled together and a standard deviation was calculated. The detailed mechanism is described in Volkmer et al., 2008 (86).

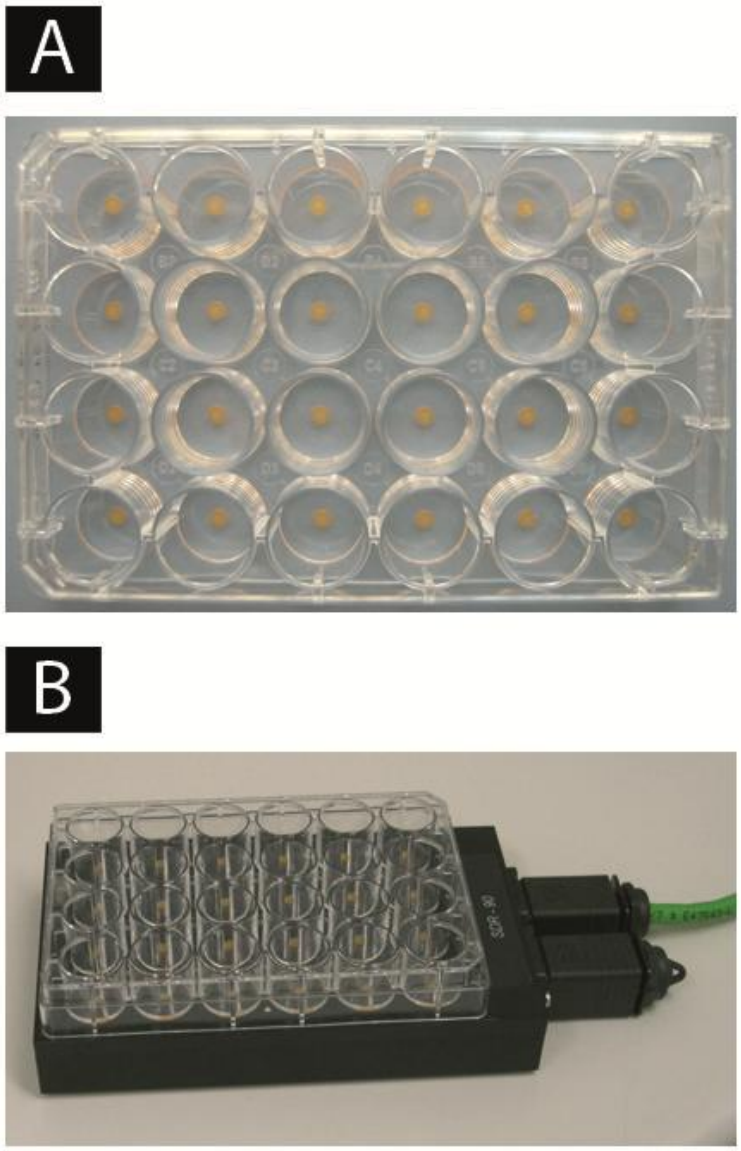


Figure 6 Oxygen measurement device for 2D culture. (A) Shows a 24-well multidish with integrated optical-chemical sensor that measures dissolved oxygen. (B) Shows an oxodish placed on a sensor dish reader (SDR).

2.10.3 3D oxygen measurement

Oxygen measurements in the centre of three-dimensional (3D) cell culture constructs were performed as described previously (Fig. 7C; 86). In brief, a needle type oxygen micro sensor (PreSens, Regensburg, Germany) was introduced into the geometric centre of a static 3D culture seeded with either 1 million P8-hMSC or with 1 million

SCP-1 cells (Fig. 7B). These oxygen micro sensors have a very thin and fragile tip with a diameter of 50 µm. The microsensor is housed in a commercially available hollow needle of diameter 0.4 mm (Fig 7A), in order to prevent any mechanical damages to the sensor while introducing it into the scaffold. Oxygen sensors were calibrated using a 2 point calibration with 100% CO_2 equalling 0% O_2 and surrounding air equalling 21% O_2. For comparability with our previous studies, oxygen measurements were carried out over a period of 7 days (86). To avoid disturbance of O_2 measurements, in these experiments medium was changed only every 4 days.

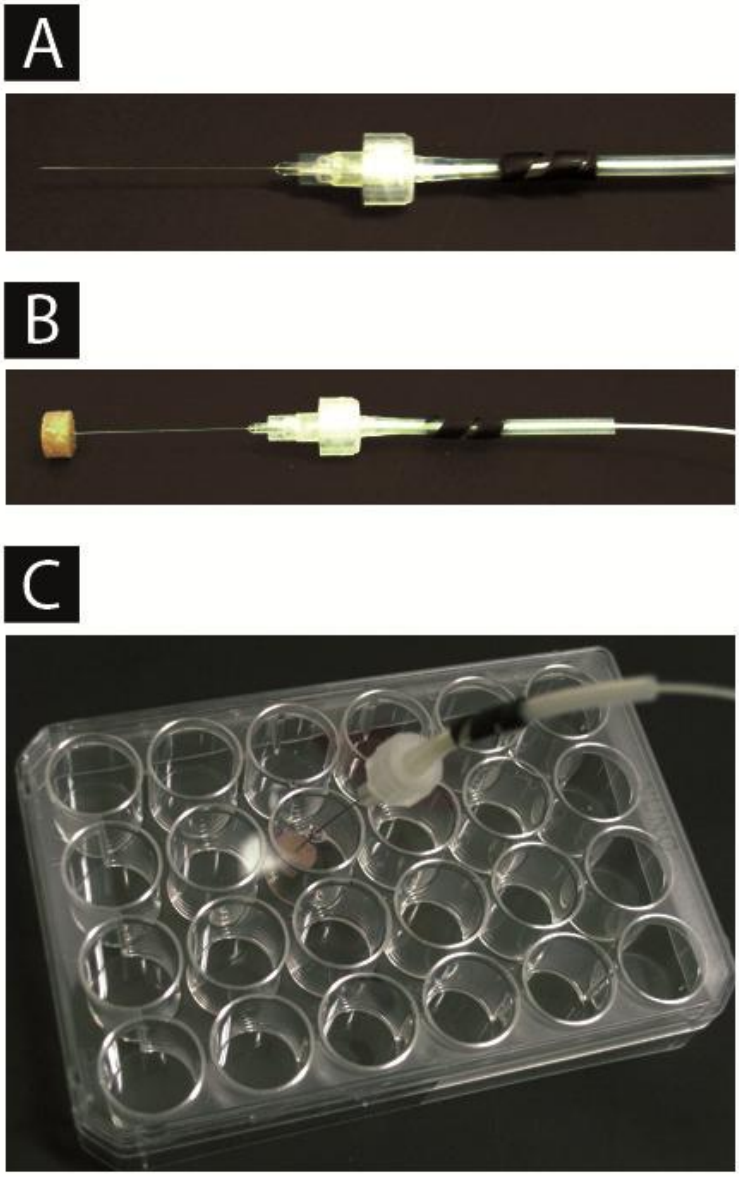


Figure 7 Static oxygen measurement in DBM scaffolds. (A) Shows a needle type oxygen microsensor (B) Shows a microsensor tip placed in the geometric centre of a scaffold. (C) Shows an experimental setup of oxygen measurement within a scaffold.

2.11 Live-dead-assay

To assess survival of cells on scaffolds, fluorescence microscopy based on incubation of cells with fluoresceindiacetate (FDA) and propidium iodide (PI; Fluka/Sigma, Munich, Germany) was performed as described previously (86). In brief, a stock solution of FDA was prepared by freshly dissolving 10 mg of FDA in 2

ml of pure acetone and by diluting this mix 1:500 with phosphate buffered saline (PBS). To obtain the final FDA / PI staining solution, the two components were mixed at a ratio of 1:1. Prior to evaluation by fluorescence microscopy, scaffolds were cut in halves. For staining, medium was removed and samples were washed with 0.5 ml PBS. Each sample was incubated for 1 minute with 500 µl FDA/PI staining solution. After discarding the dye, the wells were again washed with 0.5 ml PBS. Subsequently, samples were analysed by fluorescence microscopy using an Axiovert 100 microscope equipped with a 75 W mercury lamp (Zeiss, Munich, Germany). To detect red and green fluorescence of dead and alive cells, respectively, the Zeiss filter sets #10 and #15 were used. Pictures were taken with a Zeiss black and white digital camera (AxioCam MRm) and processed with the Zeiss Axiovision software (AxioVs40 V 4.5.0.0).

2.12 Hypoxia detection assay

hMSC (P8) were seeded at a density of 1000 cells/cm^2 on glass slides and incubated for 48 hours under either normoxia or hypoxia. Afterwards cells were treated with 200 µM of pimonidazole hydrochloride dissolved in medium and incubated for another 2 hours at the respective oxygen condition. Thereafter, the cells were washed with PBS, fixed in 4% paraformaldehyde and the immuno-staining was performed as indicated by the manufacturer's protocol (Hypoxyprobe^{TM}-1 Plus Kit, Millipore, Schwalbach, Germany). For immunostaining a primary fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody which was directed against pimonidazole protein adducts and a secondary mouse anti-FITC monoclonal antibody conjugated to horseradish peroxidase (HRP) were used.

2.13 Hif-1α western blot

For direct detection of the Hif-1α Protein, a Hif-1α Western Blot was performed. Therefore, following exposure to hypoxia or normoxia (controls) for 72 hours, whole cell lysates were obtained. In brief, cells were washed in ice cold PBS and treated with 1x Laemmli Buffer and 0.2M DTT at a ratio of 1:4 and incubated for 2 minutes at room temperature. Thereafter, cells were harvested using a cell scraper and homogenised by sonification. Following this procedure, proteins were denatured at 99˚C for 5 minutes. Prior to loading the cell lysates onto the gel, they were centrifuged at 10.000 x g for 10 minutes at 4˚C. 50 µl of the protein extract were then separated onto an 8% acrylamide SDS mini gel and transferred electrophoretically onto polyvinylidene fluoride membranes (PVDF, Roche Diagnostics, GmbH, Mannheim, Germany) using a Bio-Rad transferring unit (BioRad, München, Germany). The membranes were then blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 2 hours at room temperature. Subsequently, membranes were incubated overnight at 4°C with the primary antibody anti-Hif-1α (catalogue no. AB1536, R&D Systems, Wiesbaden, Germany) mixed in TBST in a 1:1000 dilution. After that, the membranes were washed thoroughly with TBST and incubated with a horseradish peroxidase-conjugated (HRP-conjugated) secondary antibody for 1.5 hours at room temperature. The membrane was finally washed with TBST. The protein bands were visualized by treating the secondary antibody with the HRP substrate (Immobilon Western, Millipore, Schwalbach, Germany). This treatment resulted in a chemiluminescent signal, which was detected using X-ray film. To confirm loading of the protein, the membranes were stripped and probed for β-actin (catalogue no. sc-47778, Santa Cruz biotechnology, Heidelberg, Germany) mixed at a 1:4000 dilution in TBST.

2.14 WST-1 assay

A WST-assay was performed in order to monitor the cell's metabolic activity during a prolonged exposure to hypoxia. For each time point (1, 3, 5, 7, 10, 13, 16, 18 and 21 days) hMSC (P8) were seeded in duplicates in 6 well plates at a density of 500 cells/cm^2 and incubated under either normoxia or hypoxia. Thereafter, the cells were treated with the WST-I reagent (Roche Diagnostics, Mannheim, Germany) mixed with medium at a ratio of 1:10 and incubated for 4 hrs under normoxia. Subsequently, 100 µl of the medium from each well were transferred into a 96 well plate and the optical density was measured at 450 nm using an ELISA reader.

2.15 Growth kinetics

For each time point (1, 3, 5, 7, 10, 13, 16, 18 and 21 days), hMSC (P8) were seeded in duplicates in 6 well plates at a density of 500 cells/cm^2. Upon evaluation, cells were trypsinised, resuspended and counted using a standard haemocytometer.

2.16 5-bromo-2'deoxyuridine (BrdU) assay

The BrdU assay represents a calorimetric immunoassay for the quantification of DNA synthesis, based on the measurement of BrdU incorporation during DNA synthesis. The assay was performed as according to the manufacturer's protocol. In brief cells were cultured in 24 well plates either in normoxia or hypoxia for a certain period of time. Twenty four hours prior to fixation, BrdU is supplemented into the culture medium for both normoxia and hypoxia samples. BrdU is a pyrimidine analogue and gets incorporated in place of thymidine into the DNA of proliferating cells. Following

24 hours incubation with BrdU cells are fixed and denatured and thereafter anti-BrdU peroxidase (POD) is given to the denatured cells that bind to the BrdU which have been incorporated into the newly synthesised, cellular DNA. The immune complexes are detected by the subsequent substrate reaction and quantitatively measured via a scanning multi well spectrophotometer at a wavelength of 450 nm and a reference wavelength of 690 nm.

2.17 Alizarin red staining

After a 20-day period of osteogenic differentiation in either normoxia or hypoxia cells were assessed for calcium depositions using the Alizarin red staining. The staining and the subsequent quantification were performed using an osteogenesis quantitation kit (Millipore, Schwalbach, Germany). In brief, cells were washed with PBS and then fixed at room temperature with 10% paraformaldehyde for 15 min. After fixation cells were washed with excess of distilled water and stained with 40 mM of Alizarin red solution for 20 minutes at room temperature. Thereafter cells were washed with distilled water to remove excess stain. Cells were lastly pictured under the above mentioned Carl Zeiss microscope and quantification was performed following the manufacturer's protocol. The extent of osteogenesis was quantified by extracting the stain and subsequent measurement of Alizarin red uptake against a standard curve of Alizarin red serial dilution.

2.18 Oil red O staining

After 16 days of adipogenic stimulation the cells were assessed for intracellular lipid droplets using Oil red O staining. Oil red O is a lysochrome diazo dye that stains

lipids and triglcerides on frozen sections. Cells were initially washed with PBS and then fixed with 4% formaldehyde for 2 minutes at -20˚C. Thereafter the excess of formalin was washed off with 50% ice cold ethanol. Cells were then stained with 0.2 % Oil red O staining for 20 minutes at room temperature. Afterwards cells were washed with 50% ice cold ethanol followed by a washing step with distilled water and then pictured under the previously mentioned Carl Zeiss microscope.

2.19 RT-PCR assays

In order to investigate the temporal expression pattern of important osteogenic markers during the osteogenic differentiation reverse-transcription polymerase chain reaction (RT-PCR) was performed. To this end, cells derived from normoxic, hypoxic and preconditioned samples were plated in 6 well plates and the total RNA was isolated after different time periods (5, 16 and 21 days after induction of osteogenic differentiation). Total mRNA from cells was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany). Following RNA isolation, hexamer-primed reverse transcription was performed using the cloned AMV first strand cDNA synthesis kit (Invitrogen, Karlsruhe, Germany). To detect the mRNA expression of OPN, ALP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), RT-PCR was employed using the following primers:

ALP forward- TACAACACCAATGCCCAGGT,
ALP reverse- TTCCACCAGCAAGAAGAAGC (approximately 696 bps);

OPN forward- CTGATGAACTGGTCACTGATTTTC,
OPN reverse- CCGCTTATATAATCTGGACTGCTT (approximately 359 bps);

GAPDH forward- CAACTACATGGTTTACATGTTC,

GAPDH reverse- GCCAGTGGACTCCACGAC (approximately 181 bps);

To address the stemness profile of hMSC/SCP-1 under normoxia or hypoxia, cells were seeded at a density of 3000 cells/cm^2 into 6 well plates. Total RNA was extracted after 21 days in culture. mRNA isolation and cDNA synthesis was performed as above mentioned. Semi quantitative RT-PCR against embryonic stem cell markers octamer 4 (OCT4) and NANOG were performed using the following primers:

OCT 4 forward- GGGTGGAGGAAGCTGACAAC

OCT 4 reverse- GCATAGTCGCTGCTTGATCG (259 bps)

NANOG forward- ACCTCAGCCTCCAGCAGATG

NANOG reverse- TGCACCAGGTCTGAGTGTTC (327 bps)

To see whether hTERT expression was upregulated under hypoxia we've seeded SCP-1 in 6 well plates at a density of 3000 cells/cm^2 and cultured them for 21 days under normoxia or hypoxia respectively. Total RNA was extracted after 21 days. mRNA isolation and cDNA synthesis was performed as above mentioned. Semi quantitative RT-PCR was performed using the following primer pair:

hTERT forward- ATGTGACGGGCGCGTACGAC

hTERT reverse- GGCTGGAGGTCTGTCAAG (413 bps)

The PCR products were analysed on a 1.5% agarose gel and subsequently the bands for OPN and ALP were quantified using the freeware image J

(http://rsbweb.nih.gov/ij/download.html). These readings were normalized to the GAPDH expression level, and the results were reported as relative expression.

2.20 hTERT staining

In order to validate whether hTERT was expressed in hypoxia, we performed an immuno staining against hTERT protein. SCP-1 were seeded at a density of 1000 cells/cm^2 on glass slides and incubated for 48 hours in normoxia or hypoxia. Afterwards cells were washed with PBS and fixed in 100% methanol for 10 minutes at -20˚C. Thereafter immuno staining was performed as described by Böcker et al., 2008 (85). In brief cells were washed in PBS and thereafter blocked with 3% BSA at room temperature for 2 hours. Subsequently primary antibody anti-hTERT rabbit polyclonal antibody (Merck; CalBiochem, Darmstadt, Germany) at a dilution of 1:10 was applied and incubated over night at 4˚C. The glass slides were washed with PBS to remove the excess primary antibody and incubated with the secondary antibody (Alexa Fluor 488 donkey anti-rabbit IgG, Invitrogen, Karlsruhe, Germany) at a dilution of 1:500 at room temperature for 1 hour. Thereafter glass slides were washed with PBS and the nuclei were counter stained with DAPI (Invitrogen, Karlsruhe, Germany) for 5 minutes at room temperature.

2.21 Clonogenic assay

The CFU-F (Colony forming unit-fibroblast) protocol was adopted from Grayson et al. (102). In brief hMSC/SCP-1 were cultured for time periods of 5, 10, 16 and 21 days either in normoxic (21%) or hypoxic (2%) conditions. Afterwards they were trypsinised and plated into 10 cm petri dishes at a density of 14 cells/cm^2, and incubated at normoxic conditions for 16 (hMSC) or 12 (SCP-1) days. Following their

respective incubation period the cells were washed with PBS and stained with 0.05% crystal violet solution for 30 minutes at room temperature. Afterwards the excess of crystal violet was washed off carefully with tap water and colonies were counted with the naked eye.

2.22 Transient knockdown of HIF-1α with siRNA during osteogenic differentiation of hMSC

In order to determine whether the differentiation as seen in preconditioned hypoxia samples were due to HIF-1α, we transiently downregulated HIF-1α using silencing RNA (siRNA). HIF-1α was selectively downregulated during the expansion culture of preconditioned samples (Fig. 8). Subsequently cells were osteogenically stimulated for 20 days or cultured with complete medium. On day 21 the success of differentiation was determined through Alizarin Red staining.

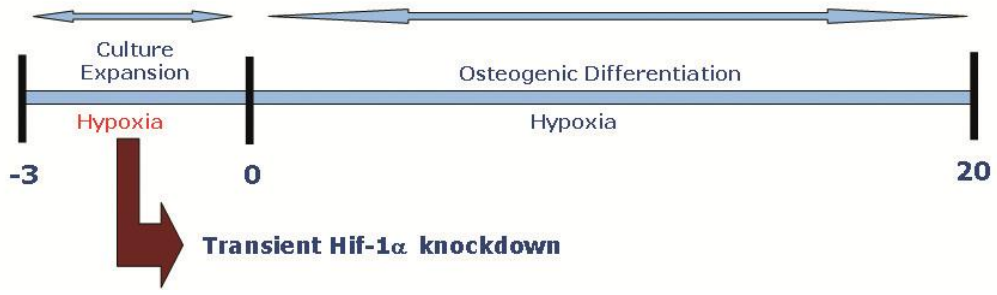


Figure 8 Overview representation of osteogenic differentiation using siRNA against HIF-1α. The role of HIF-1α on the osteogenic differentiation of hMSC was determined by transiently knocking down HIF-1 α using silencing RNA during the expansion culture of preconditioned samples.

HIF-1α siRNA (h):sc-35561 was purchased from Santa Cruz biotechnology (Santa Cruz, California, USA), and the hMSC were transfected using lipofectamine 2000

from invitrogen using the latter's manufacturer's protocol. In brief 3000 cells/cm^2 were plated out in 6 wells and incubated overnight at the respective oxygen conditions. The cells were transiently transfected as follows: 100 pmol of siRNA was dissolved in 250 µl of Opti-MEM (Invitrogen, Karlsruhe, Germany) giving rise to solution A. 5µl of lipofectamine was dissolved in 250 µl of Opti-MEM giving rise to solution B. Solution B was incubated for 5 minutes at room temperature. Solution A and B were mixed together and left to stand for 20 minutes at room temperature. The oligomer-lipofectamine 2000 complex was then added to the 6 well and incucbated at the respective oxygen concentration. After 5 hours of incubation medium was replaced with normal culture expansion medium. The cells were kept for 2 more days in culture before start of osteogenic induction. To determine the transfection efficiency, RNA was isolated from control samples and transfected cells, prior to differentiation. cDNA was synthesized and semi quantitative RT-PCR against HIF-1α was performed in order to detect the mRNA expression level of HIF-1α gene. Osteogenic differentiation (normoxia and hypoxia preconditioned) was carried out as previously described and the success of differentiation was evaluated through Alizarin red staining (Fig. 9).

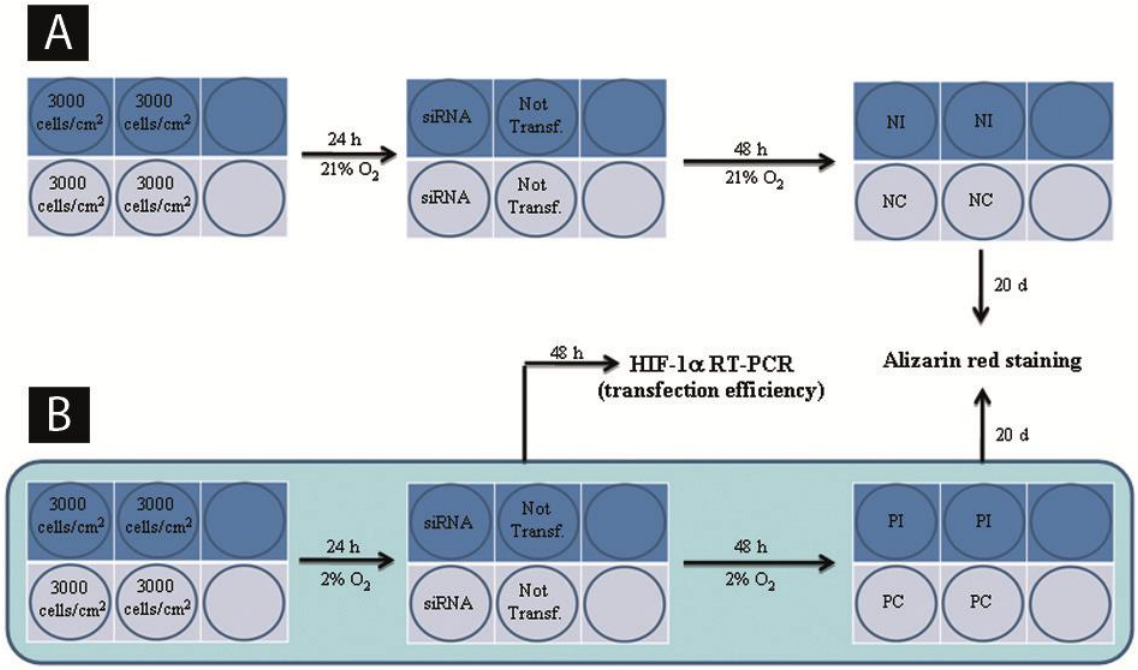


Figure 9 Schematic representation of siRNA transfection experiment. Initially 3000 cells/cm^2 were seeded out in 6 wells and cultured for 24 hours under normoxia or hypoxia. Thereafter cells were transfected with siRNA against HIF-1α. Fourty eight hours later the transfection efficiency was determined in the preconditioned samples. Cells were osteogenically induced for 20 days and on day 21 the success of differentiation was determined through Alizarin red staining.

2.23 Stabilisation of Hif-1α with DFO during osteogenic differentiation of hMSC

The hypoxia mimetic agent desferrioxamine (DFO) was used in order to test whether hypoxic samples were able to differentiate into the osteogenic lineage, if Hif-1α protein would be stabilised during the expansion culture period in normoxia (Fig. 10).

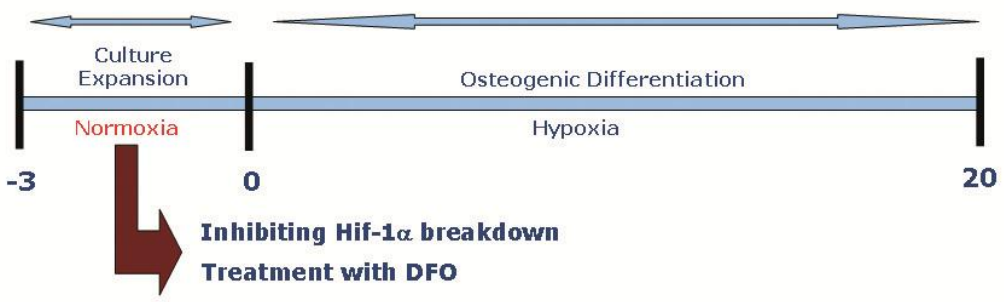


Figure 10 Overview representation of osteogenic differentiation using DFO. The role of Hif-1α on the osteogenic differentiation of hMSC was determined by applying DFO to cells during the expansion culture of hypoxic samples.

50 and 100 µM solutions of DFO dissolved in alpha MEM supplemented with 10% FBS and 1% PenStrep were prepared. Cells were seeded out in the normal fashion for osteogenic differentiation. Twenty four hours after plating cells were expanded in either 50 µM or 100 µM of DFO medium for an additional 2 days before they were stimulated to differentiate into the osteogenic lineage. Following 3 days of expansion culture some cells were used to obtain proteins. Proteins were isolated from hMSC in normoxia that were treated with 50 µM, 100 µM and 0 µM of DFO. Proteins were also isolated from cells that were cultured for 3 days under hypoxia. Western blot analysis against Hif-1α was performed using the protein samples. After the expansion culture cells were induced into the osteogenic lineage. For induction normal osteogenic differentiation medium without DFO was used. Cells which were used as control were cultured using complete medium without DFO supplements. Cells were induced for 20 days and then hMSC were stained with Alizarin red staining (Fig. 11).

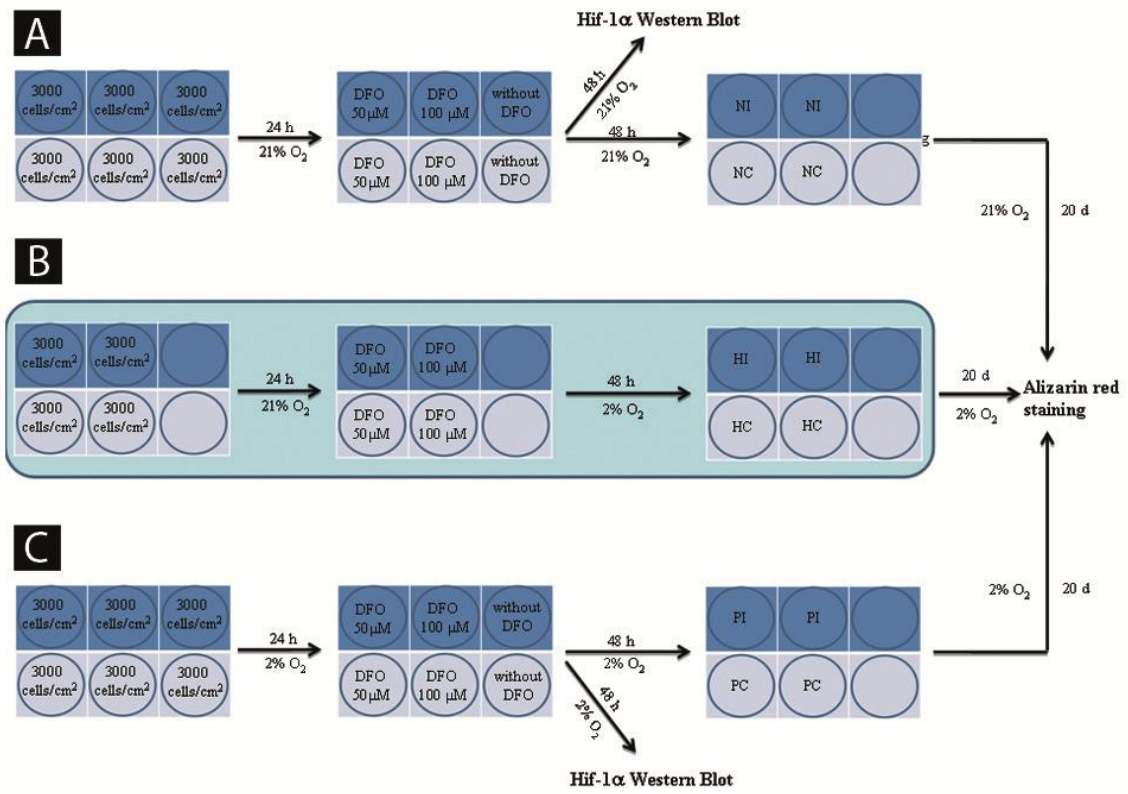


Figure 11 Schematic representation of DFO experiment. Initially 3000 cells/cm^2 were seeded out in 6 wells and cultured for 24 hours under normoxia or hypoxia. Thereafter the medium was supplemented with either 50 µM or 100 µM DFO and cultured for an additional 2 days under the same oxygen concentration. Proteins were extracted from cells that were cultured with and without DFO treatments in 21% O_2 and in 2% O_2. The osteogenic differentiation was pursued for 20 days and the success of differentiation was determined through Alizarin red staining on day 21.

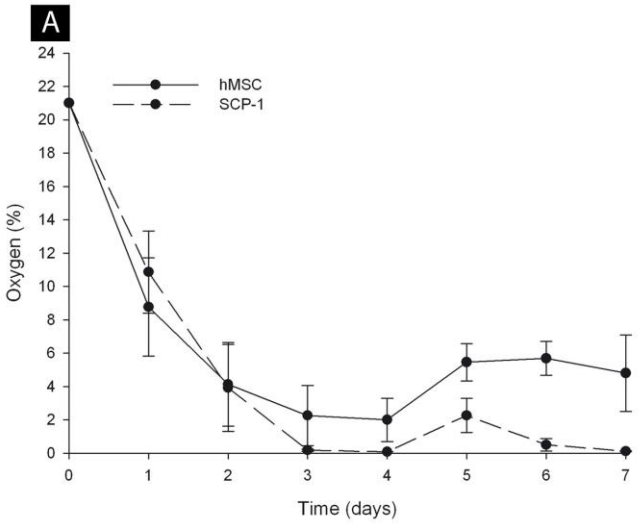
2.24 Statistical analysis

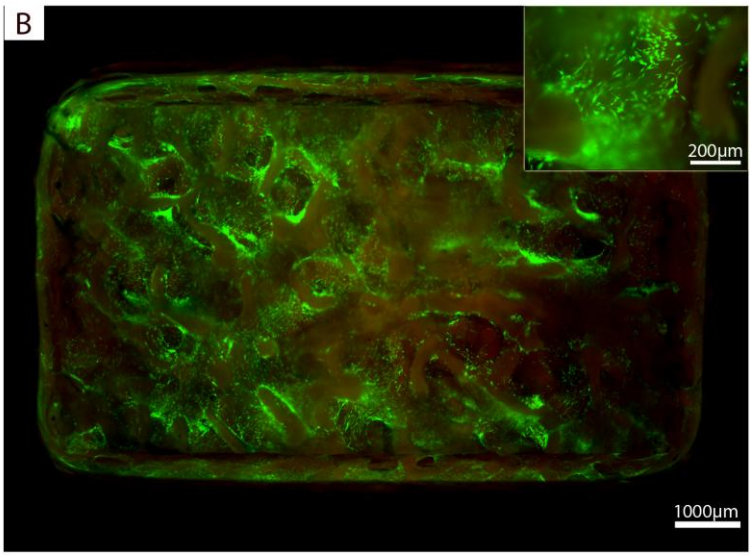
Data are expressed as means +/- standard deviations. Statistic analysis was performed using the unpaired Student's T-test. The results were taken to be significant at a probability level of $P < 0.05$. For all experiments, a minimum of 2 independent experimental runs were performed.

3 Results

3.1 3D cultures of hMSC are exposed to low oxygen *in vitro*

To assess whether hMSC in static 3D culture are exposed to hypoxia similar to murine fibroblasts (86) we measured the central oxygen concentration in scaffolds seeded with either hMSC or with hTERT-immortalized hMSC (SCP-1; 85) over a period of 7 days. The oxygen concentration in the central region of hMSC-seeded scaffolds dropped from 21% O_2 at day 1 to around 2% of oxygen at day 4 (Fig. 12A; solid line). After the medium change on day 4, the oxygen level rose to approximately 5.7% where it evened out. The well-growing SCP-1 cells in turn consumed the oxygen faster resulting in a decline to 0% at day 3 (Fig. 12A; dashed line). Upon the addition of fresh medium, the oxygen levels rose shortly to about 2% before they again dropped to 0%. A vitality assay after 7 days in static 3D culture revealed that the regular hMSC were literally all alive (green) and evenly distributed over the whole scaffold (Fig. 12B). In sharp contrast, hTERT-immortalized hMSC only survived in the periphery of the scaffold whereas in central regions of the scaffold only dead (red) cells were found (Fig. 12C).


A
hMSC
SCP-1
Oxygen (%)
Time (days)
0
2
4
6
8
10
12
14
16
18
20
22
24
0
1
2
3
4
5
6
7


B
200µm
1000µm

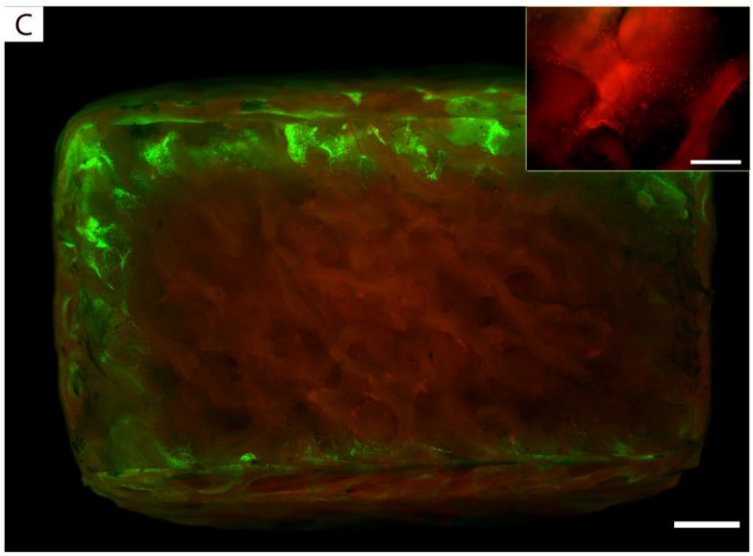

C

Figure 12 Oxygen concentration in static 3D culture of hMSC and hTERT-immortalized hMSC in correlation to cell survival after 7 days in static culture. Oxygen concentration measured in the centre of DBM scaffolds seeded with 1 million hMSC falls to ~2% after 4 days and levels out at ~5.8% upon the addition of fresh medium (A; solid line). Oxygen concentration in the centre of scaffolds seeded with 1 million hTERT-immortalized hMSC (SCP-1) falls to 0% after 3 days (A; dashed line), the addition of fresh medium after day 4 has only marginal effects. Live-dead-stain of hMSC-seeded scaffold after a 7-day-culture period reveals that virtually all cells are viable (green; B). The live-dead-assay of the hTERT-hMSC seeded scaffold reveals dead cells (red) in central and bottom areas of the scaffold and vital cells on the top and margins (C). Overview pictures were taken at 1.25 x magnification, inserts are representative pictures from the centre of the scaffold and were taken at 10 x magnification.

3.2 Detection and confirmation of cellular hypoxia

To demonstrate that the cells were exposed to hypoxic conditions in our hypoxia incubator, cells were incubated with pimonidazole, a chemical that forms adducts with cellular proteins solely under hypoxic conditions. Immunofluorescent staining was apparent only in cells exposed to hypoxia (2% O_2) and not seen in those grown under normoxic conditions (Fig. 13A vs. 13B). To confirm that cells metabolically responded to lowered oxygen conditions, we assessed whether cells exposed to 2% oxygen activated the hypoxia inducible factor 1 alpha (Hif-1α), a central regulator of the cellular response to hypoxia ubiquitously expressed in mammalian cells. As illustrated in Figure 7C, Hif-1α protein was detected in the hypoxic samples but not within the normoxic control samples (Fig. 13C).

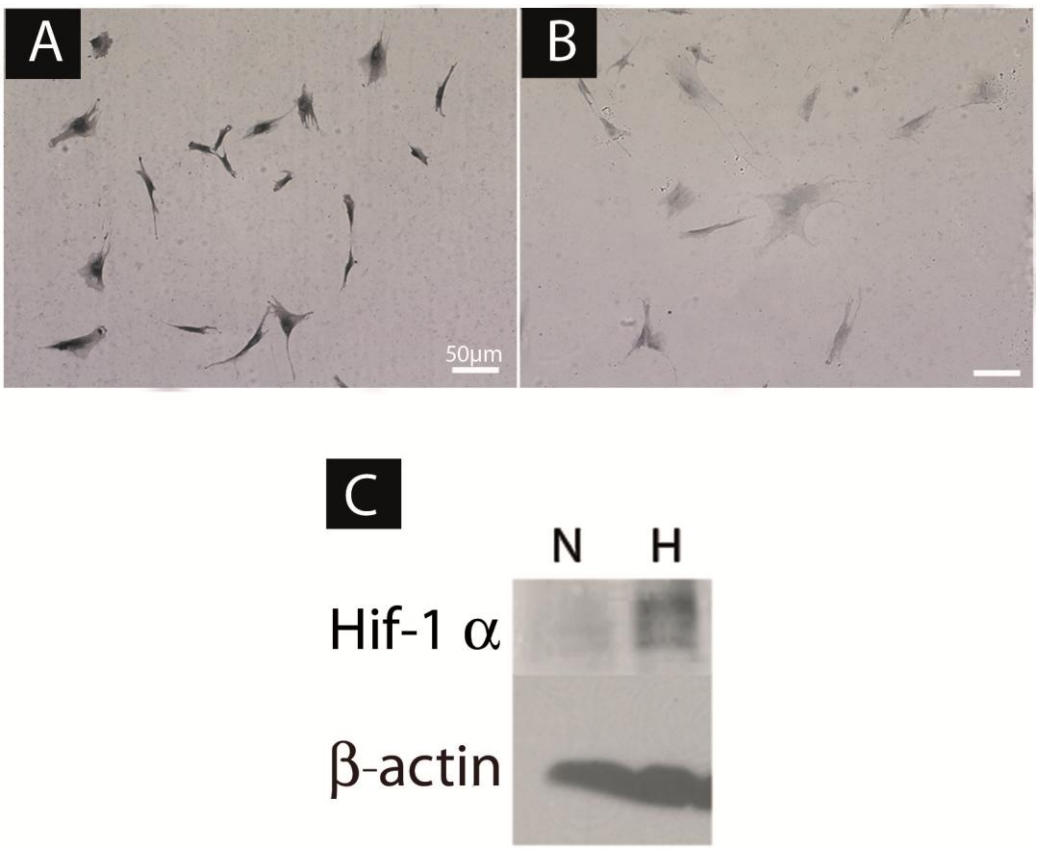


Figure 13 Confirmation of cellular hypoxia. Cells were cultured on glass slides for 48 hrs in (A) 2% or (B) 21% Oxygen, thereafter treated with pimonidazole for 2 hrs and immuno-stained. Black coloured cells indicate the presence of hypoxia-sensible pimonidazole adducts. Western blot analysis of Hif-1α reveals degradation of the protein in normoxic sample (N) and stable Hif-1α protein in the hypoxic (H) sample (C).

3.3 Hypoxia promotes proliferation of hMSC

We next asked whether hypoxia had an influence on cell proliferation and cell survival when compared to hMSC grown under normoxia. Therefore, hypoxic and normoxic cell samples were incubated with the WST reagent, a tetrazolium salt that is cleaved by the mitochondrial dehydrogenase of metabolically active cells into

formazan. Spectrophotometric quantification of formazan revealed that prolonged exposure to hypoxic conditions did not negatively affect cell survival and proliferation (Fig. 14A). To the contrary, after a lag phase of 5 days, during which hypoxia-exposed cells produced less formazan, they began to metabolize more WST reagent than their normoxic counterparts until formazan production evened out at day 21 (Fig. 14A). These differences were statistically significant at days 5, 13 and 18.

To verify the WST results, cell numbers were determined over time in hypoxic samples compared to samples cultured under normoxia. Cells grew equally well under both conditions with a slight tendency towards a better growth under hypoxia (Fig. 14B). This difference, however, was statistically significant only at day 8.

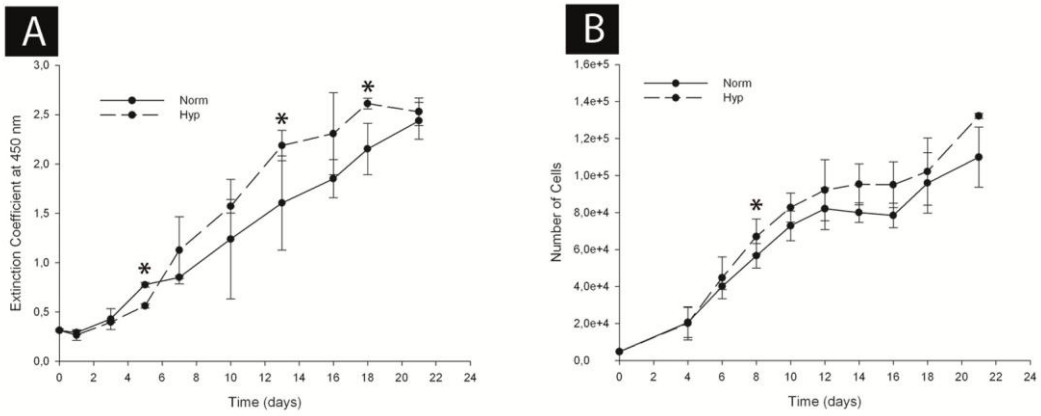


Figure 14 Cell viability and growth kinetic profile of hMSC grown under normoxia (21% O_2) and hypoxia (2% O_2). Cell viability was assessed by measuring the metabolic activity of normoxic cells (Norm; solid line) and hypoxic cells (Hyp; dashed line) using the WST reagent (A). After a lag phase of ~5 days, cells grew better under hypoxic conditions. These differences were statistically significant at days 5, 13 and 18. Data were obtained from 2 independent experiments done in duplicates and are presented as mean +/- standard deviation. The growth kinetic profile of hMSC grown under normoxia (21% O_2) and hypoxia (2% O_2) showed a

similar trend towards a better proliferation under hypoxia with statistical significance on day 8 (B). Data were obtained from 3 independent experiments done in duplicates and are presented as mean +/- standard deviation. Asterisks indicate the statistically significant difference between normoxia and hypoxia (* P < 0.05).

3.4 Hypoxia increases DNA synthesis in hMSC

We next screened whether the BrdU incorporation differed in hMSC grown under hypoxia (2%) to those grown under normoxia (21%). BrdU incorporation accounts for cells that are in their S phase, as these cells actively synthesize their DNA. BrdU is an anolog to thymine and gets incorporated into the sythesizing DNA strands in place of thymine. hMSC were grown either under normoxia or hypoxia for various time intervals and just 24 hours prior the evaluation BrdU was supplemented into the culture medium. At all time points the BrdU incorporation was higher in cells exposed to hypoxia than cells exposed to normoxia (Fig. 15). These differences were statistically highly significant at day 3, 7, 10 and 21 and significant at day 4.

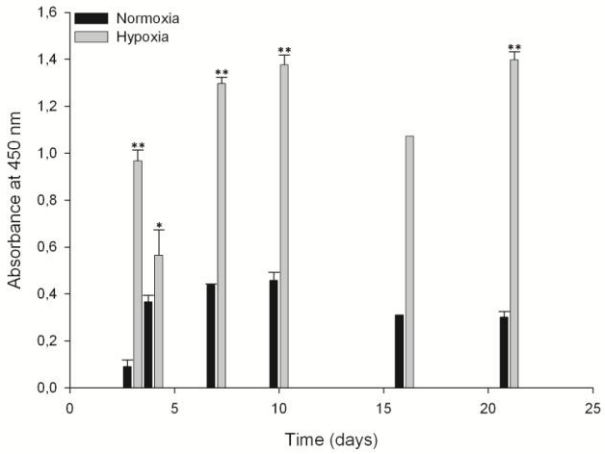


Figure 15 Impact of oxygen on DNA synthesis of hMSC. The BrdU incorporation was measured in hMSC that were either cultured under normoxia (21%) or under hypoxia (2%) for 3, 4, 7, 10, 16 and 21 days respectively. At any given point of time

the BrdU incorporation was significantly higher in cells that were exposed to hypoxia. Asterisks indicate the statistically significant difference between normoxia and hypoxia (* $P < 0.05$; ** $P < 0.005$).

3.5 Hypoxia inhibits osteogenic differentiation of hMSC

To assess whether constant exposure to hypoxia has an impact on the capacity of hMSC to differentiate along the osteogenic cascade, we osteogenically differentiated hMSC in the presence of 21% oxygen compared to a 2% oxygen atmosphere. It is important to note that in contrast to several other studies assessing the osteogenic potential of cells under hypoxia, we exerted constant exposure to 2% oxygen over the full period of differentiation. While normoxic samples differentiated well as confirmed by a strong Alizarin red staining, samples differentiated poorly under hypoxia (Fig. 16A), suggesting a decrease or delay in osteogenic differentiation under low oxygen conditions.

As a control, we monitored osteogenic differentiation by measuring the temporal RNA expression patterns of the representative osteogenic markers ALP and OPN (Fig. 16B). The RT-PCR results revealed that the expression level of OPN under normoxia constantly increases from day 5 to 16 over day 21. The expression of ALP was equally high across the three time points indicating robust upregulation of osteogenic markers under normoxic osteogenic differentiation (Fig. 16B). In contrast, at day 16 and 21, the expression of the two markers was clearly reduced when cells were differentiated under hypoxia. On day 5, in turn, upregulation of ALP was almost equal under hypoxia, whereas OPN expression was even higher in the hypoxic setting as compared to normoxic samples.

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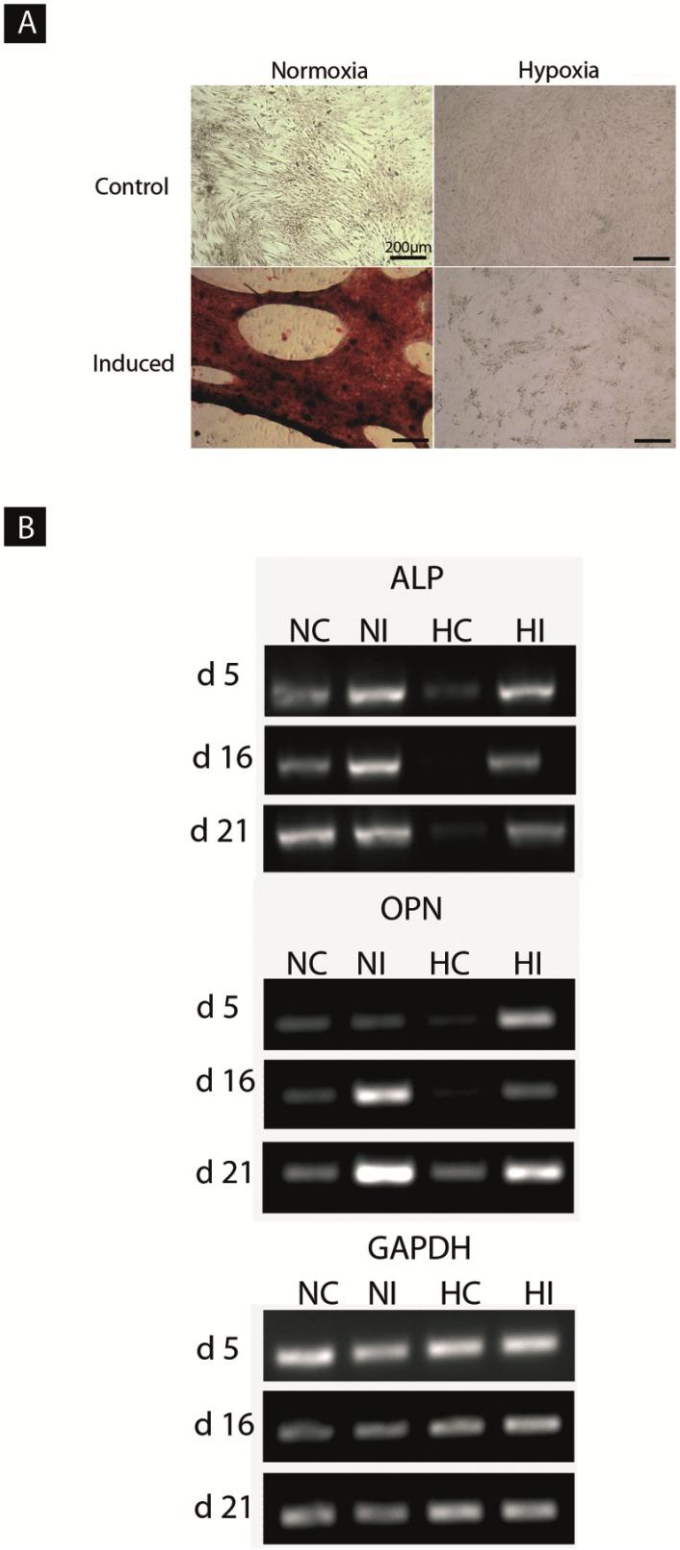


Figure 16 Osteogenic differentiation under hypoxia. hMSC that have been osteogenically induced under normoxia display strong Alizarin red staining whereas cells induced under hypoxia did not stain with Alizarin red (A). ALP and OPN RT-PCR analysis was performed on RNA extracted from hMSC, which were

osteogenically induced under 2% oxygen or under normoxia for 5, 16 and 21 days. The expression level of OPN and ALP in the induced samples is remarkably lower in the hypoxic (HI) sample compared to normoxia (NI) except for OPN on day 5, where it is upregulated in the hypoxic induced sample. Abbreviations: ALP, alkaline phosphatase; OPN, osteopontin; NC, normoxia control; NI, normoxia induced; HC, hypoxia control; HI, hypoxia induced.

3.6 Hypoxia favours stemness over differentiation

Ma et al. described that the physiological niche of hMSC is hypoxic (70). We therefore speculated that the delay in osteogenic differentiation of hMSC seen in hypoxia is due to the fact that under reduced oxygen tension hMSC retain their stem cell characteristics. To assess the role of oxygen tension on the maintenance of stemness of hMSC in culture, we performed a clonogenic assay. The CFU-F assay revealed that cells exposed to hypoxia generated more colonies compared to those hMSC that were exposed to normoxia (Fig. 17A). The difference was statistically significant on days 16 and 21 respectively (Fig. 17B). It was also noticed that the colony numbers were maintained by the cells that were exposed to hypoxia over the time periods of 5, 10, 16 and 21 days. In contrast the cells exposed to normoxia showed a decreasing number of colonies over time, with day 5 and day 21 having the highest and least number of colonies respectively.

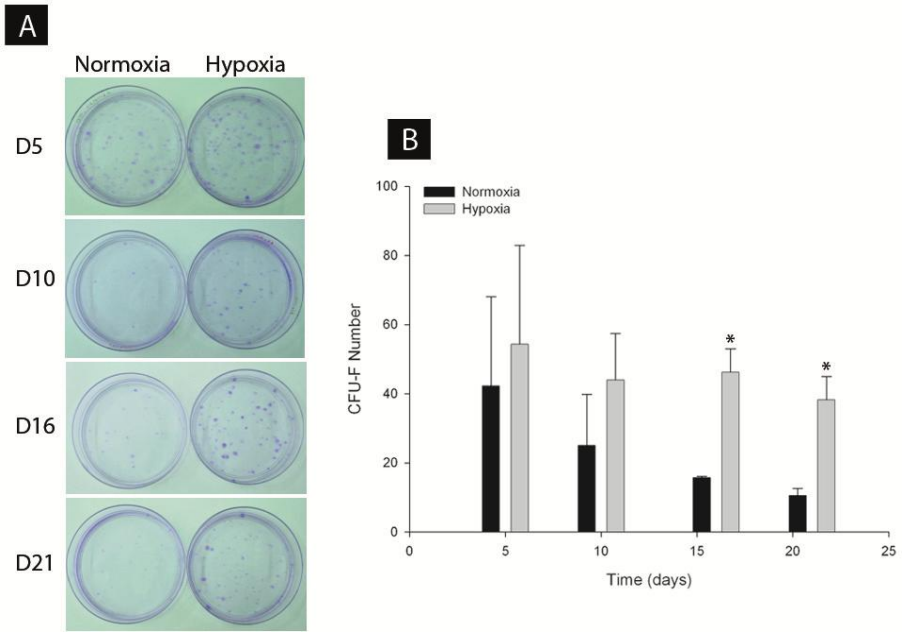


Figure 17 Clonogenic assay of hMSC. Cells were exposed to normoxia or hypoxia for 5, 10, 16 or 21 days and thereafter trypsinised and seeded into new 10 cm petri-dishes at a density of 14 cells/cm^2. The cells were then allowed to incubate at 37˚C for 12 days prior to crystal violet staining. The staining revealed that at any given time point, hMSC that were exposed to hypoxia formed more colonies (A). Quantification of the CFU-F assay revealed that the difference in colony numbers was significant at day 16 and 21 respectively (B). Asterisks indicate the statistically significant difference between normoxia and hypoxia (* $P < 0.05$).

To further validate these findings we screened for the expression of embryonic stem cell markers NANOG and OCT 4. The embryonic transcription factor OCT 4 is one of the earliest marker expressed during embryogenesis in mammals (69). NANOG is another important transcription factor that is believed to be involved in the self-renewal of undifferentiated embryonic stem cells. The semi quantitative RT-PCR showed a subtle upregulation of OCT 4 and a robust upregulation of NANOG in the hypoxic samples when compared to normoxia (Fig. 18).

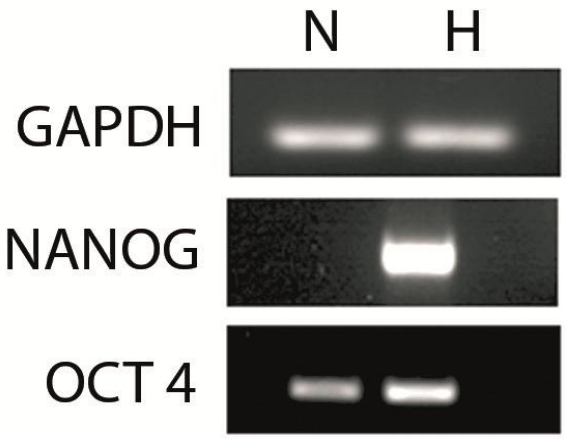


Figure 18 Semi quantitative RT-PCR of embryonic stem cell marker. Semi quantitative RT-PCR of NANOG and OCT 4 was performed on RNA extracts from hMSC, which were cultured under 2% oxygen (H) or under 21% oxygen (N) for 21 days. There was a clear cut upregulation in the hypoxic samples for NANOG, and a subtle upregulation for OCT 4 compared to their normoxic counterparts.

3.7 Hypoxic preconditioning restores hypoxia-induced delay in osteogenic differentiation

Recently Rosova et al. showed that preconditioning hMSC under hypoxic conditions prior to transplantation would enhance their therapeutic potential (117). Another study revealed that pre-treating hMSC in a non-lethal hypoxic environment would prevent or delay the development of apoptosis when cells were subsequently exposed to lethal hypoxic conditions (118). Because osteogenic differentiation is a crucial quality of cells which are aimed at regenerating bone tissue, we wondered whether preconditioning hMSC in hypoxia would improve their potential to differentiate along the osteoblastic lineage under constant hypoxia. To evaluate this hypothesis we cultured cells under hypoxia (2% O_2) for three days prior to osteogenic differentiation at 2% O_2. This time frame was selected as the cells needed around 3

days to reach about 70-80% confluency, which was in turn a precondition to start osteogenic differentiation. We termed this setup “hypoxic preconditioning” in distinction to the protocol of hypoxic differentiation described above, where cells were first grown to 70-80% confluency in normoxia and thereafter osteogenically challenged under hypoxia (Tab. 2).

Placing the cells into the hypoxia incubator for 3 days prior to adding the osteoinductive medium resulted in restoration of the differentiation capacity as substantiated by a positive Alizarin red staining (Fig. 19A). Quantification of the Alizarin red staining showed a decreased but sizeable extent of staining within the preconditioned samples compared to normoxic samples, whereas there was literally no detectable Alizarin red staining in samples from the hypoxic setup without preconditioning (Fig. 20A). The RT-PCR results disclosed that hypoxic preconditioning restored both OPN and ALP upregulation upon osteogenic induction at days 16 and 21 (Fig. 19B). Quantification of the PCR-bands normalized to GAPDH expression showed increased levels of OPN and ALP on day 16 within the preconditioned samples when compared to the hypoxic samples, however without statistical significance (Fig. 20B).

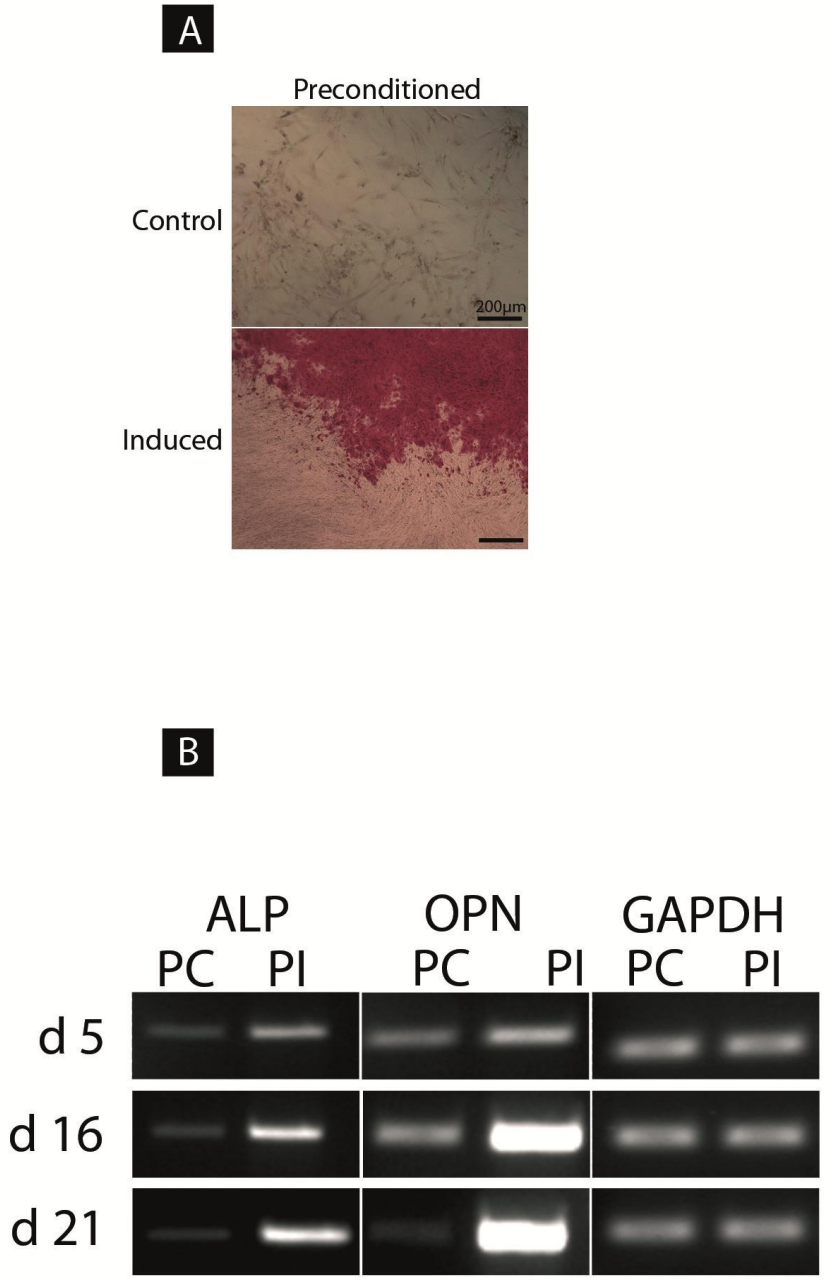


Figure 19 Hypoxic preconditioning restores hypoxia-induced inhibition of osteogenic differentiation of hMSC. Cells were cultured under hypoxia (2%) for 3 days prior to osteogenic induction and thereafter maintained at 2% oxygen (preconditioned samples; PI). Cells were induced for 20 days and then stained with Alizarin red (A). Preconditioned samples did differentiate under hypoxic conditions indicating that hypoxic preconditioning restores hypoxia-induced inhibition of osteogenic differentiation. RT-PCR of preconditioned samples documents robust upregulation of osteogenic markers OPN and ALP. Abbreviations: ALP, alkaline

phosphatase; OPN, osteopontin; PC, preconditioned control; PI, preconditioned induced.

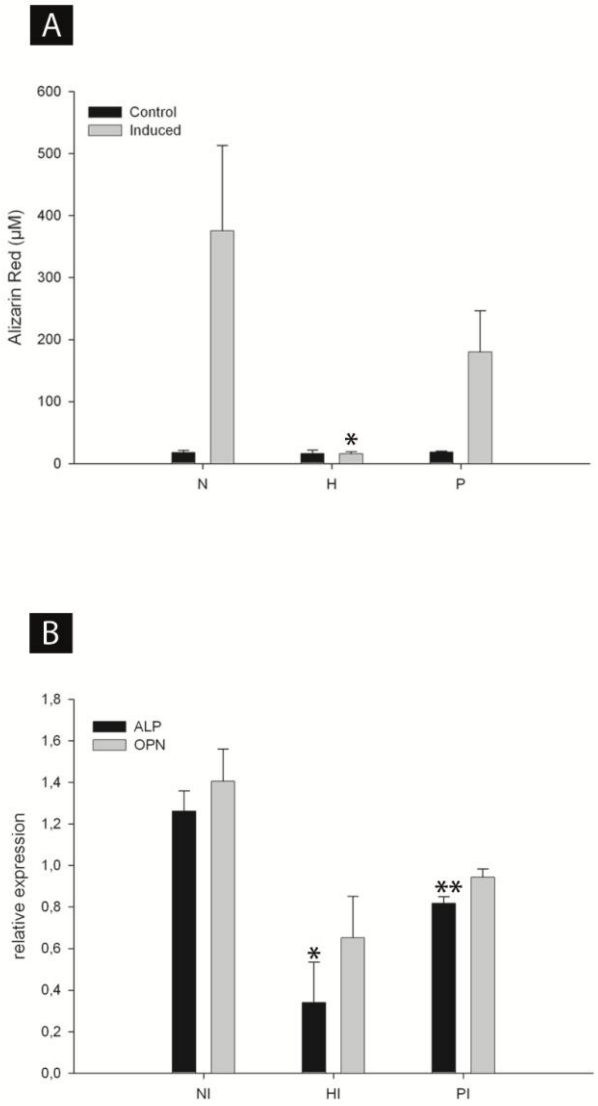


Figure 20 Quantification of Alizarin red staining and of RT-PCR results. Data from normoxic, preconditioned, and non-preconditioned samples substantiates that hypoxic preconditioning restores hypoxia-induced inhibition of osteogenic differentiation. Quantification of Alizarin red staining of normoxic (N), preconditioned (P), and hypoxic (H) samples showed a significantly enhanced mineralization of induced normoxic and induced preconditioned samples when compared to induced hypoxic samples (A). Quantification of RT-PCR bands normalized to GAPDH using the image J software revealed a trend towards a better differentiation after

preconditioning (PI), with differentiation being best under normoxic conditions (NI; B). Asterisks indicate the statistically significant difference ($P < 0.05$) between normoxic induced and hypoxic induced (*) as well as between normoxic induced and preconditioned induced (**) samples. Abbreviations: ALP, alkaline phosphatase; OPN, osteopontin; NI, normoxia induced; HI, hypoxia induced; PI, preconditioned induced.

3.8 The effect of hypoxic preconditioning on osteogenic differentiation is not donor dependent

In order to verify that the effect of hypoxic preconditioning of hMSC on osteogenic differentiation at low oxygen tension is not donor dependent we carried out the osteogenic differentiation experiment using the 3 different experimental setups, in either normoxic, hypoxic or hypoxic preconditioning with 2 more donors. The Alizarin red staining after 20 days of induction clearly showed strong staining in the normoxia, hardly any in hypoxic induced samples and reduced staining in the preconditioned induced samples (Fig. 21A and B).

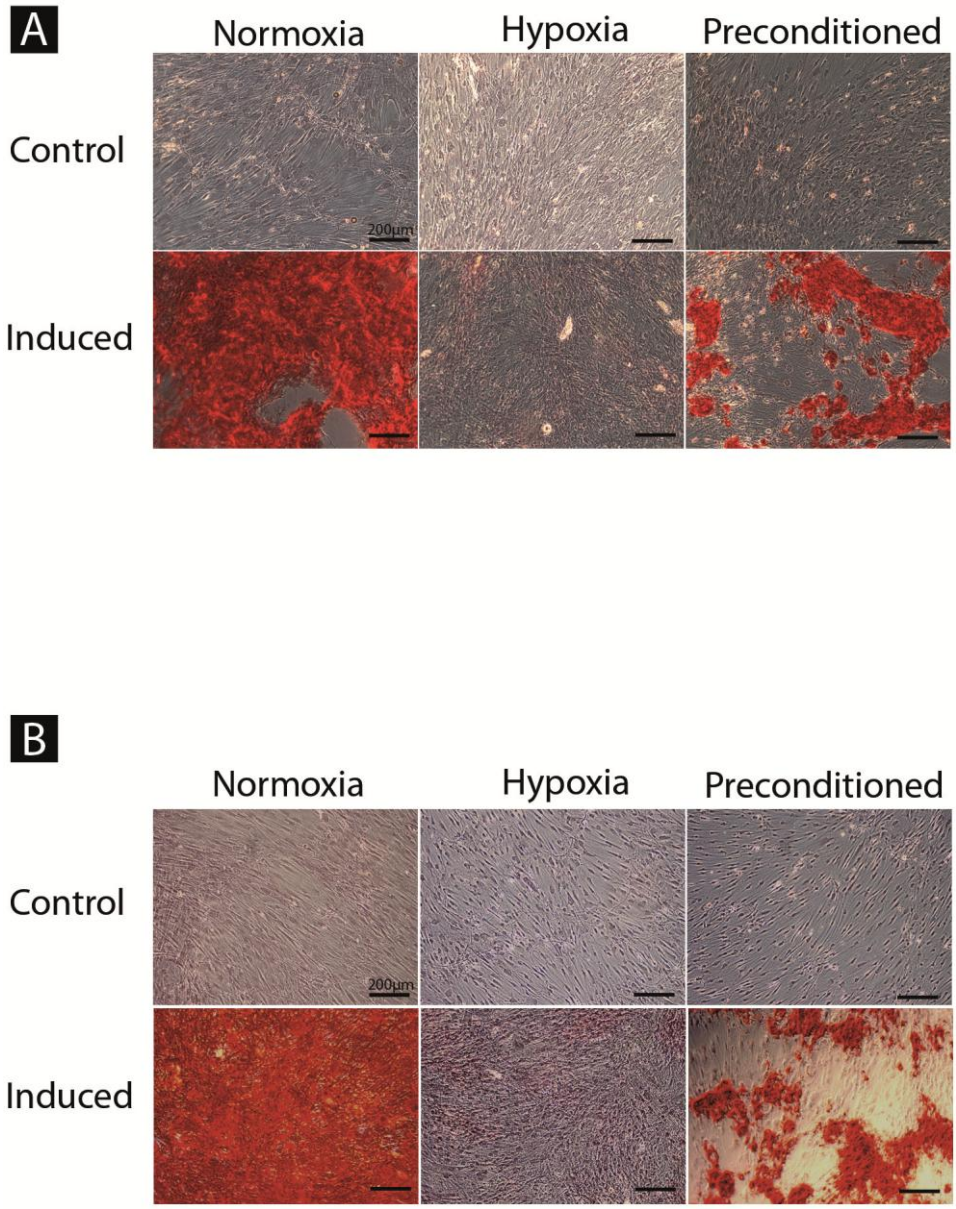


Figure 21 Osteogenic differentiation of two additional donors under normoxia, hypoxia and under hypoxia after hypoxic preconditioning. P6 hMSC of two further donors, a 24-year old male (A) and a 32-year old male (B), that have been induced osteogenically under normoxia for 21 days display strong Alizarin red staining (left panels). Cells induced under 2% of oxygen (hypoxia) under otherwise identical circumstances do not stain with Alizarin red (middle panels). After a phase of hypoxic preconditioning, where cells were cultured under 2% hypoxia for 3 days prior to osteogenic induction, hMSC from both additional donors (A and B) differentiated towards the osteogenic lineage under hypoxic conditions (2% of

oxygen) as evidenced by re-established Alizarin red staining (right panels), indicating that hypoxic preconditioning restores hypoxia-induced inhibition of osteogenic differentiation.

3.9 HIF-1α does not play a major role in osteogenic differentiation of hMSC

Wang et al. reported that HIF-1α is important in the development of the skeleton system as it couples angiogenesis to osteogenesis (94). Another group has shown that the HIF-1α pathway accelerates bone regeneration (118). We therefore asked whether HIF-1α had a direct link to osteogenic differentiation. In order to determine whether HIF-1α plays a major role in the osteogenic differentiation of hMSC under low oxygen tension, we transiently knocked down HIF-1α using silencing RNA during the expansion culture of preconditioned samples (Fig. 8). The transfection efficiency of siRNA against HIF-1α as determined through semi quantitative RT-PCR revealed a good efficiency: The expression of HIF-1α from cells cultured for 3 days under hypoxia showed a strong band compared to those cells that were cultured under hypoxia and transfected with siRNA against HIF-1α, which showed a very faint band (Fig. 22C). Transiently knocking down of HIF-1α had no apparent effect on the osteogenic differentiation ability of hMSC under hypoxia as evidently seen with Alizarin red staining (Fig. 22A). As a control hMSC under normoxia were transfected with siRNA against HIF-1α. Preconditioned hMSC transiently transfected with siRNA against HIF-1α differentiated as well as cells without transfection as seen via the Alizarin red quantification (Fig. 22B).

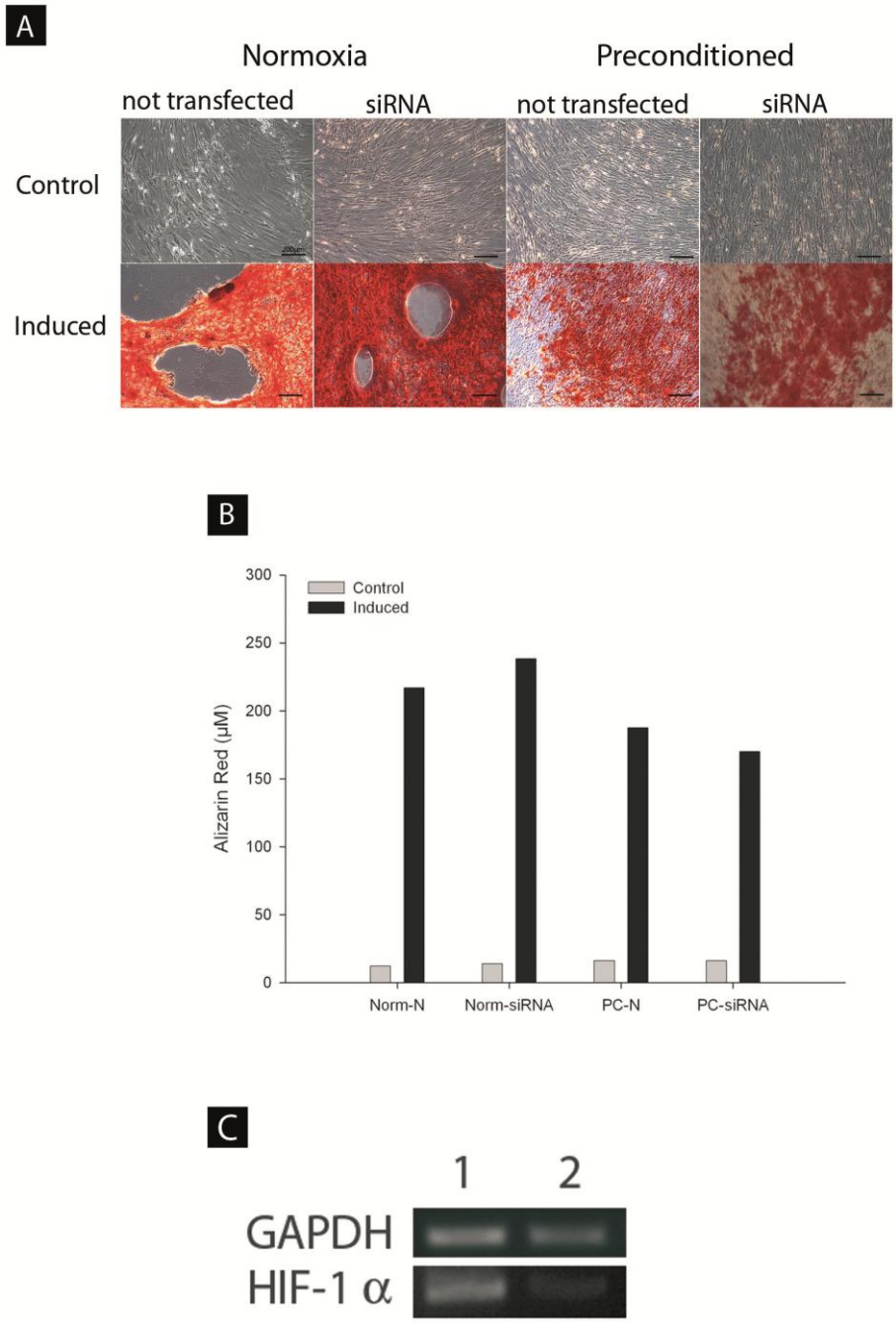


Figure 22 The effect of transiently knocking down of HIF-1α during expansion culture of hypoxic preconditioned sample on osteogenic differentiation. hMSC were seeded for osteogenic differentiation into 6 wells in normoxia and hypoxia respectively. Twenty four hours after plating they were transiently transfected with silencing RNA against HIF-1α. After 3 days they were osteogenically induced. hMSC that were either transfected or not transfected in normoxia or hypoxia were stained with Alizarin red after 20 days of induction. (A) Alizarin red quantification showed no

significant differences between the transfected and not transfected samples in either oxygen conditions (B). After 3 days the transfection efficiency was determined through RT-PCR of HIF-1α. There was a strong band of HIF-1α in the preconditioned not transfected sample (lane 1; C) and a very weak band was seen in the transfected sample (lane 2; C). Abbreviations: Norm-N: Normoxia not transfected; Norm-siRNA: Normoxia with silencing RNA against HIF-1α; PC-N: Preconditioned not transfected; PC-siRNA: Preconditioned with silencing RNA against HIF-1α.

To further clarify the role of HIF-1α during osteogenic differentiation, desferrioxamine (DFO) was given to the cells during the expansion culture of hypoxic samples, stabilizing HIF-1α under normoxic conditions. This was done in order to see whether early activation of HIF-1α prior to osteogenic differentiation was the reason for the improved differentiation of hMSC under hypoxia as seen with hypoxic preconditioned samples compared to the hypoxic samples. As DFO, a well known hypoxia-mimetic agent, is known to induce apoptosis in a HIF-1α independent mechanism, we conducted the experiment with 2 low concentrations of DFO (124). The concentrations were chosen in such a way that the effect of DFO induced apoptosis was kept low but the DFO concentration was sufficient enough to stabilise Hif-1α protein under normoxia. Twenty four hours after plating the cells were incubated for 2 days with either 50 µM or 100 µM of DFO and thereafter induced into the osteogenic lineage without DFO treatments. Hypoxic differentiation did not result in any mineralisation as indicated through the Alizarin red staining for both the 50 and 100 µM DFO treated cells (Fig. 23A and 23B). As a control normoxic and preconditioned samples were treated with 50 µM or 100 µM of DFO during their expansion culture, in order to see whether DFO itself had an effect on osteogenic differentiation. In either condition cells differentiated indicating that DFO itself did not

have an impact on the osteogenic differentiation potential of hMSC. Even though the cells were only incubated for 2 days with either 50 µM or 100 µM of DFO during the expansion culture one observed that the cells did not tolerate the treatment with DFO well. This effect was dose dependent as with 100 µM of DFO lesser number of cells survived and differentiated into the osteogenic lineage compared to cells treated with 50 µM of DFO, as evidently seen particularly in the normoxic samples (Fig. 23A and 23B). On treatment with either 50 or 100 µM DFO the cells expressed stably the Hif-1α protein even under normoxia as indicated through the western blot (Fig. 23C).

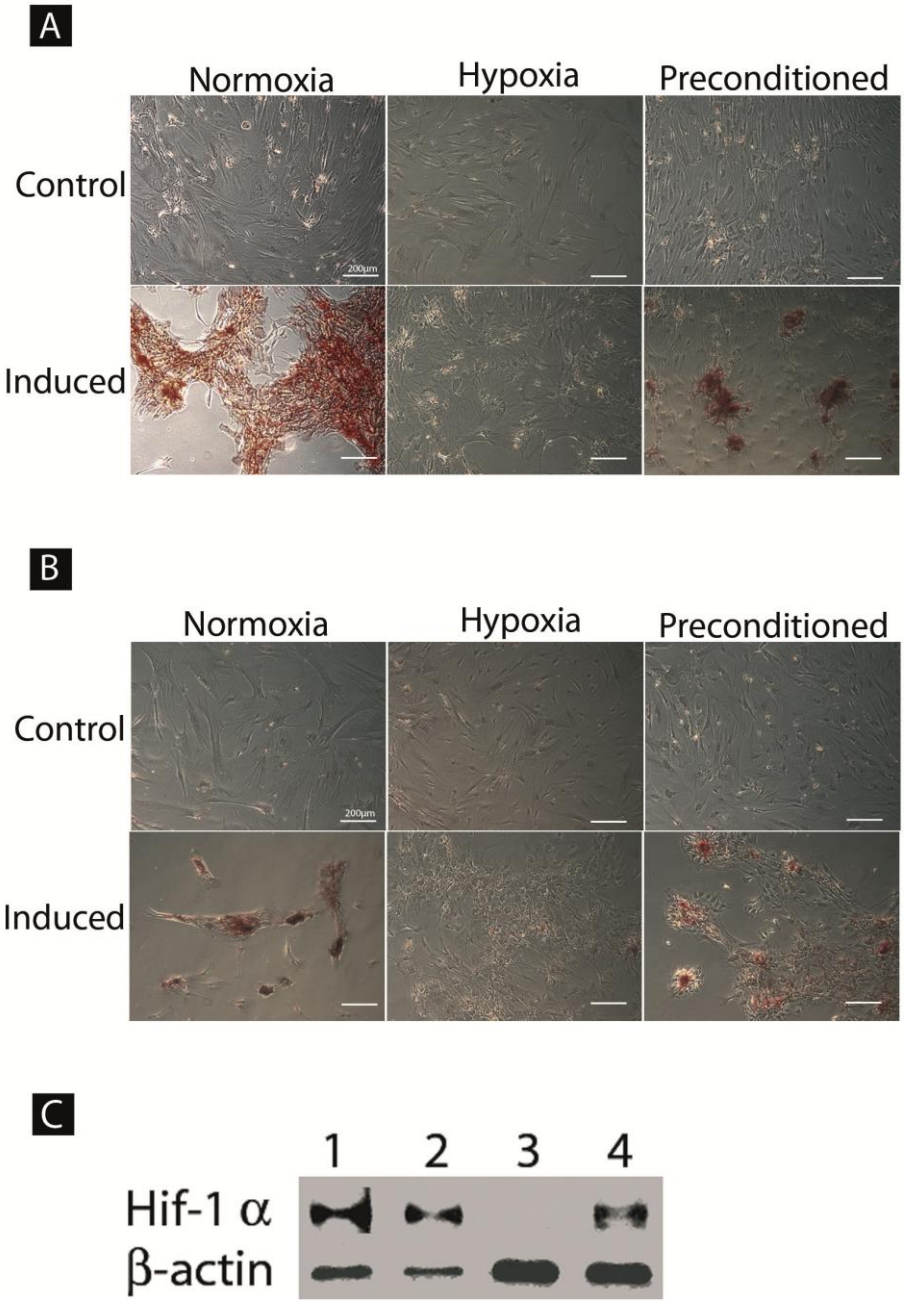


Figure 23 The effect of stabilising Hif-1α on osteogenic differentiation under hypoxia. hMSC were seeded for osteogenic differentiation into 6 wells for hypoxia, normoxia and preconditioned samples. After 24 hours the medium was supplemented with 50 µM DFO (A) or 100 µM DFO (B) and incubated for additional 2 days. After 3 days proteins were isolated from hMSC in normoxia that were treated with 50 µM (lane 1), 100 µM (lane 2) and 0 µM (lane 3) of DFO (C). In addition to that protein was isolated from hMSC in hypoxia (lane 4). Western blot analysis against Hif-1α was performed with the protein samples. After 3 days cells were then

differentiated into the osteogenic lineage without DFO supplements. Following 20 days of induction hMSC were stained with Alizarin red. hMSC exposed to 50 µM and 100 µM DFO in normoxia and preconditioned samples stained with Alizarin red, whereas hypoxic sample did not, suggesting that HIF-1α does not play a crucial role in the osteogenic differentiation of hMSC under hypoxia. Note that DFO had a dose dependent effect on cells.

3.10 Impact of low oxygen tension on SCP-1

After having established that the osteogenic differentiation potential of hMSC is delayed under hypoxia, and that this in turn could be overcome through hypoxic preconditioning, we now aimed at transferring this knowledge to SCP-1, an immortalised hMSC cell line that was created in our lab. Böcker et al. have characterized this cell line and compared it to hMSC. They were able to show that the SCP-1, despite of their immortalization still possessed all the characteristics of a mesenchymal stem cell (85). Apart from being immortalised the other advantage of this cell line is that it is a fast growing cell line. This cell line represents an ideal model cell line to test various hypotheses as it is easy to handle, inexpensive and can be expanded to a desired cell number in a very short period of time compared to hMSC. We therefore initially characterised the cell line with respect to hTERT expression, growth kinetics, by clonogenic assay and regarding embryonic stem cell marker expression.

Analysis of the growth kinetics showed that SCP-1 grew equally well under normoxia and hypoxia until they reached 100% confluency on day 14 (Fig. 24A). Once they reached 100% confluency, cells cultured under hypoxic condition tended to attain a plateau phase whereas cells cultured under normoxia continued proliferation. After

21 days in culture the SCP-1 that were cultured under normoxia reached a cell number of 2 million cells, whereas the cells grown under hypoxia merely a number of 900 thousand cells. Low oxygen tension had no apparent effect on DNA synthesis at all time points when compared to their normoxic counterparts (Fig. 24B).

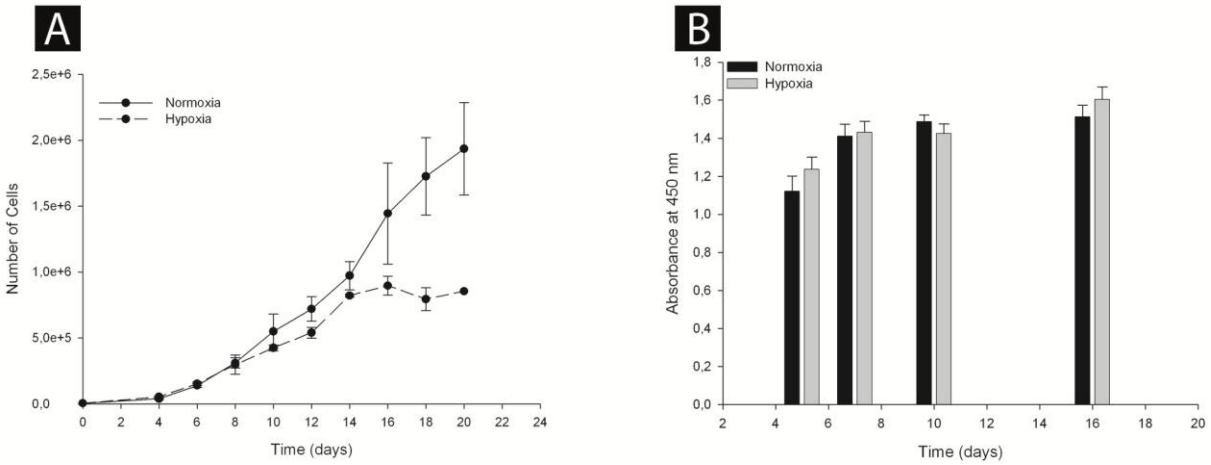


Figure 24 Growth kinetic profile and impact of low oxygen on DNA synthesis of SCP-1. The growth curves of SCP-1 grown under normoxia (21%) and hypoxia (2%) show a similar trend until day 14 where they become 100% confluent. Following day 14 cells under hypoxia reach plateau phase whereas cells under normoxia still proliferate at an exponential rate (A). The BrdU incorporation was measured in SCP-1 that were either cultured under normoxia (21%) or under hypoxia (2%) for 5, 7, 10 and 16 days respectively. The BrdU incorporation in either oxygen concentration was the same independent of time (B).

Nishi et al. reported that hTERT expression in human placenta was upregulated under hypoxia (125). In order to verify whether hypoxia had an impact on hTERT expression we performed an immuno staining against hTERT. In addition we also screened for hTERT expression at the mRNA level via RT-PCR. The hTERT staining revealed an equally positive signal for both normoxic and hypoxic samples. The hTERT staining (green; Fig. 25B) was primarily focused in the nucleus as evidently

seen with the counter stain DAPI (blue; Fig. 25A) which stains the nucleus. To further emphasize that hTERT resides in the nucleus, the green and blue channels were overlapped, resulting in a 100% overlap, giving rise to a turquoise blue nucleus (Fig. 25C).

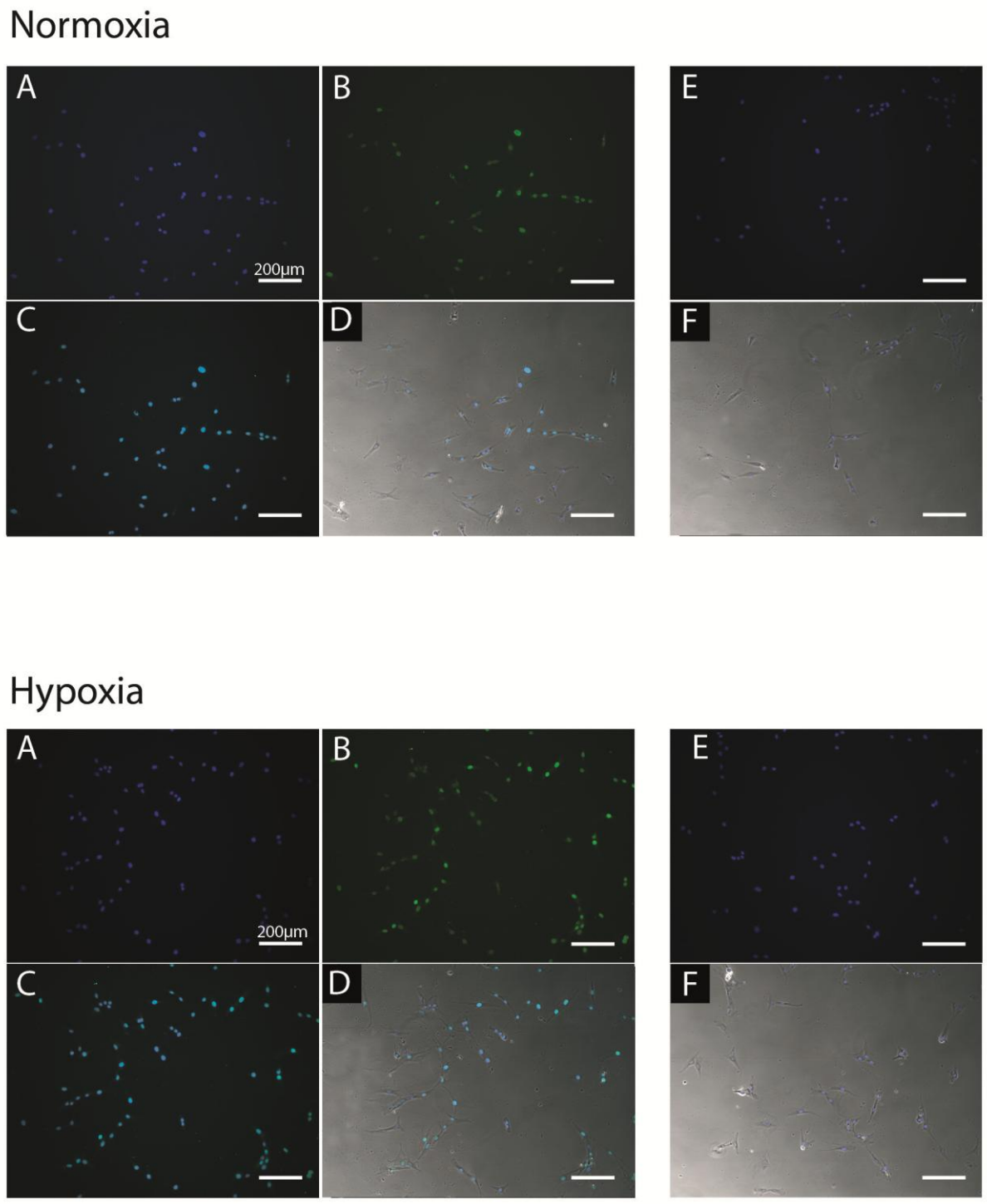


Figure 25 hTERT immuno staining. Cells were cultured on glass slides for 48 hours either in normoxia or hypoxia respectively and thereafter immunostained against hTERT protein using an anti-hTERT rabbit polyclonal antibody. (A) shows cells with DAPI staining, (B) shows cells with hTERT staining, (C) shows (A) + (B) overlapped (D) is (C) under phase contrast. (E) shows cells with the omission of primary antibody and stained with DAPI. (F) shows (E) under phase contrast.

To assess the role of oxygen tension on the maintenance of stemness of SCP-1 in culture, we performed a clonogenic assay. In addition to this we also screened for embryonic stem cell markers at the mRNA level via RT-PCR. Clonogenic assay showed that cells exposed in normoxia generated more colonies than those cells that were exposed to hypoxia. The difference was statistically significant at day 16 (Fig. 26).

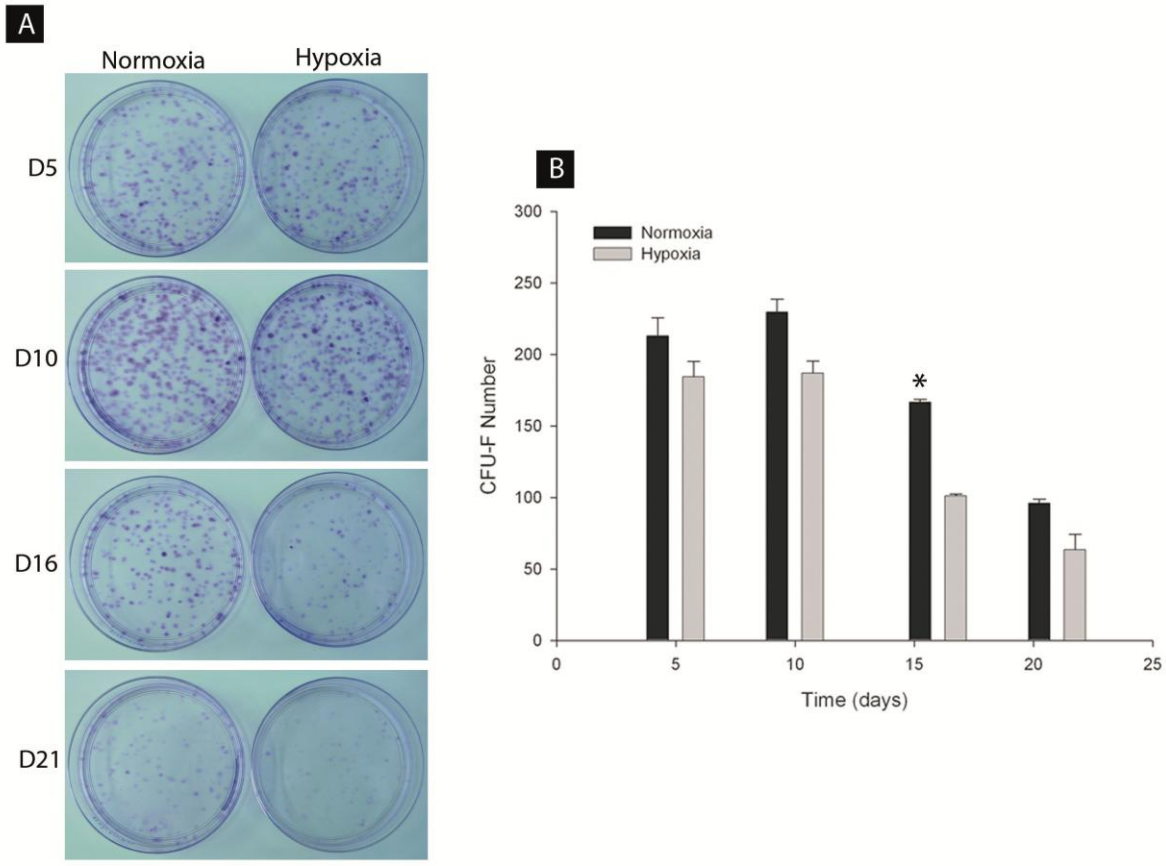


Figure 26 Clonogenic assay of SCP-1. Cells were exposed to normoxia or hypoxia for 5, 10, 16 or 21 days, and thereafter trypsinized and seeded into new 10 cm petri-dishes at a density of 14 cells/cm^2. The cells were then allowed to incubate at 37˚C for 12 days prior to crystal violet staining. The staining revealed that at any time period, SCP-1 that were exposed to normoxia formed more colonies (A). The

difference in colony number was significant at day 16 (B). Asterisks indicate the statistically significant difference between normoxia and hypoxia (* $P < 0.05$).

The expression of NANOG, OCT 4 and hTERT was slightly upregulated in the hypoxic samples compared to normoxia (Fig. 27).

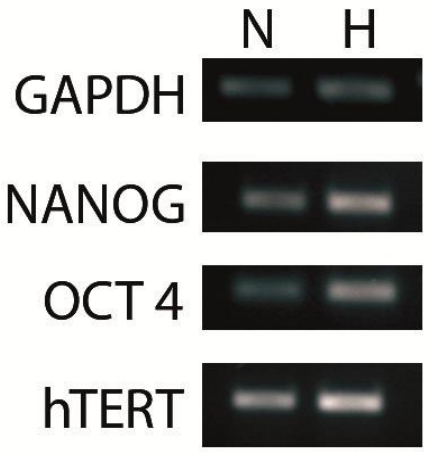


Figure 27 Semi quantitative RT-PCR of embryonic stem cell markers and hTERT. RT-PCR against NANOG, OCT 4 and hTERT was performed on RNA extracts obtained from SCP-1 that were cultured either in normoxia (21%) or hypoxia (2%) for 21 days. The embryonic stem cell markers and hTERT were slightly upregulated under hypoxic condition.

3.11 Oxygen measurements of SCP-1 in 3D versus 2D culture

In order to assess whether hypoxic preconditioning had an effect on cell survival in SCP-1-seeded DBM scaffolds, we determined the oxygen concentration at the core of the scaffolds. The oxygen concentration at the core of the scaffolds dropped down to zero after 2-3 days of initial seeding independent of the hypoxic preconditioning. After medium change on the 4th day the oxygen concentration rose slightly but dropped down to zero again during day 6 and 7 for normoxia and preconditioned

cells respectively (Fig. 28A and 28B). Overall there were no major differences noticed in the central oxygen measurements within the preconditioned and non-preconditioned scaffolds.

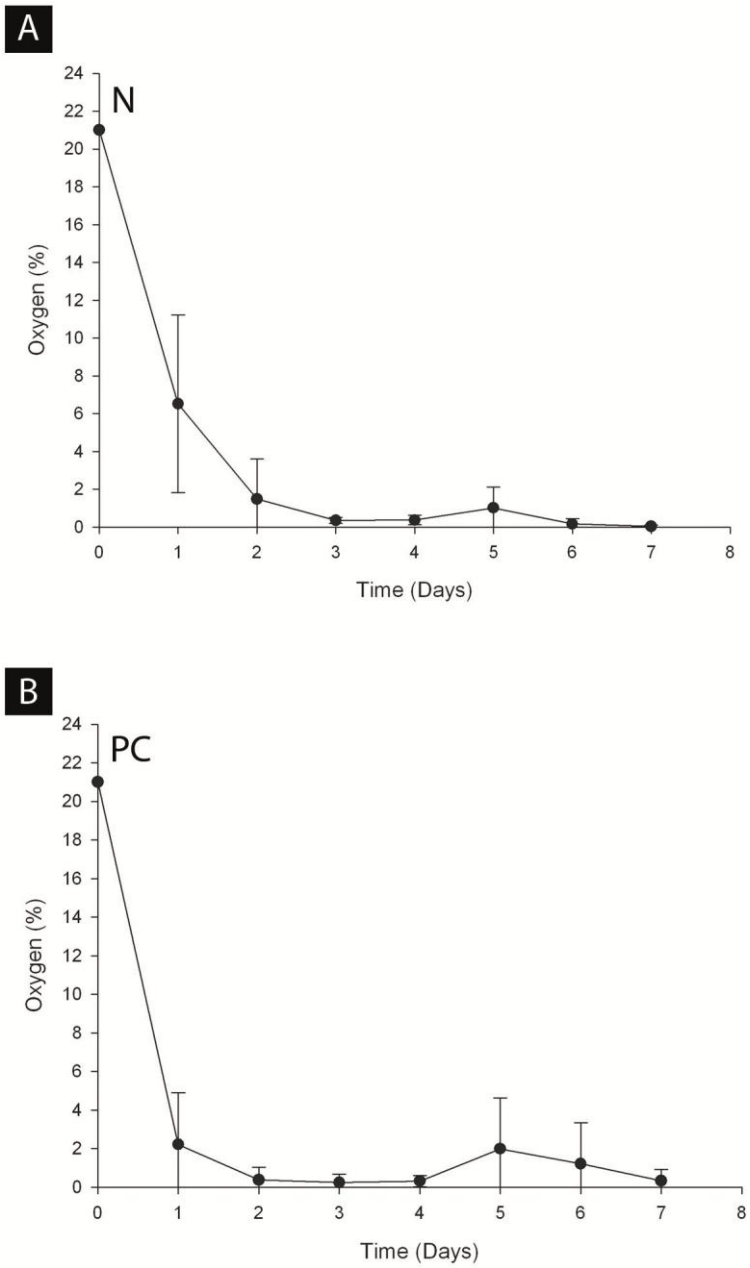


Figure 28 Central oxygen measurements within static DBM scaffolds seeded with SCP-1 Oxygen concentrations were measured at the centre of scaffolds that were either seeded with 1 million normoxic SCP-1 (N; A) or preconditioned SCP-1 (PC; B). The central oxygen concentration in the scaffolds dropped from 21% to 0%

after 2-3 days and showed a small rise in the oxygen concentration at day 5 after medium was changed on day 4. The oxygen then dropped down to zero again.

We already found out that SCP-1 are exposed to 0% oxygen after day 3 of initial seeding in static 3D culture (Fig 6A). We concluded that due to the depletion of oxygen within the centre the cells would simply die off. Wang et al. have shown that preconditioning cells under low oxygen would make them more resistant to a subsequent exposure to lethal oxygen concentration (118). We wanted to see whether this statement holds good for 3D culture as well, and speculated that by culturing the cells under hypoxia before seeding them on the scaffold they would have an improved life expectancy under such adverse oxgen concentrations. We therefore seeded 1 million SCP-1 that were either cultured for 3 days under normoxia or hypoxia on DBM scaffolds and cultured them for 7 days under static condition, in an humidified incubator having 5% CO_2 and 21% O_2. 3 days were chosen for hypoxic preconditioning as we wanted it to be comparable to our previous finding that preculturing hMSC for 3 days under hypoxia would improve their osteogenic differentiation potential at low oxygen tension (126).

Live-dead staining after 7 days showed no significant differences between scaffolds that were seeded with preconditioned or cells grown under normoxia. In both conditions cells preferentially inhabited the periphery of the scaffold. The larger magnification (insert) showed dead cells within the core of the scaffold (Fig. 30A). The area quantification of green area versus red area further assured that there was no statistical significance between cell survivals of normoxic to preconditioned cells (Fig. 30B).

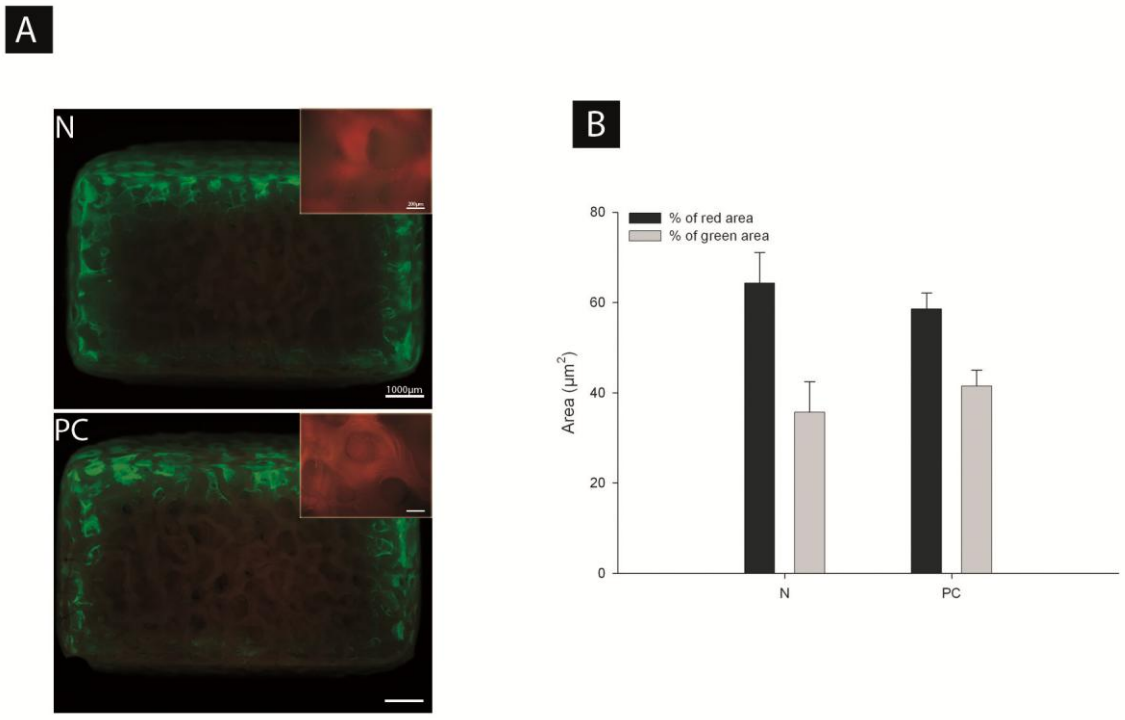


Figure 29 Live-dead assay of SCP-1 cultured on DBM scaffolds. 1 million of SCP-1 cells that were either cultured in normoxia (N) or preconditioned in hypoxia (PC) for 3 days were seeded on DBM scaffolds and cultured for 7 days in a static culture under normoxia. Live-dead-assay after 7 days revealed that almost all vital cells (green) reside at the periphery of the scaffold. Higher magnification (insert) showed dead cells (red) at the centre of the scaffold (A). Anyalysis of the % of green area to % of red area of the scaffolds did not show a significant difference between the 2 groups (B).

In order to assess whether the oxygen consumption seen in the 3D culture of preconditioned and normoxic cells would be similar to 2D culture, we cultured SCP-1 either in hypoxia or normoxia for 3 days and thereafter seeded equal numbers of preconditioned or normoxic cells on 24 well oxodishes and measured the oxygen concentration within the medium over a period of 7 days in static culture in an humidified incubator having a 5 % CO_2 and 21 % O_2. The oxygen measurements

performed in the 2D setting showed a gradual drop in the oxygen concentration for preconditioned and normoxic cells. Cells that were cultured in normoxia prior to oxygen measurements (normoxic) showed a drop in their oxygen concentration from 15.7 to 12.8% (Fig. 29B solid line), whereas the cells that were cultured prior the oxygen measurement in hypoxia (preconditioned) showed a drop in their oxygen concentration from 16.1 to 13.4% (Fig. 29B dashed line). Although the oxygen consumption in 2D culture seemed to be generally less in those cells that were preconditioned compared to their normoxic counterpart, there was no statistical significance observed (Fig. 29B). Both preconditioned and normoxic cells reached 100% confluency after 7 days in culture as shown in figure 29A.

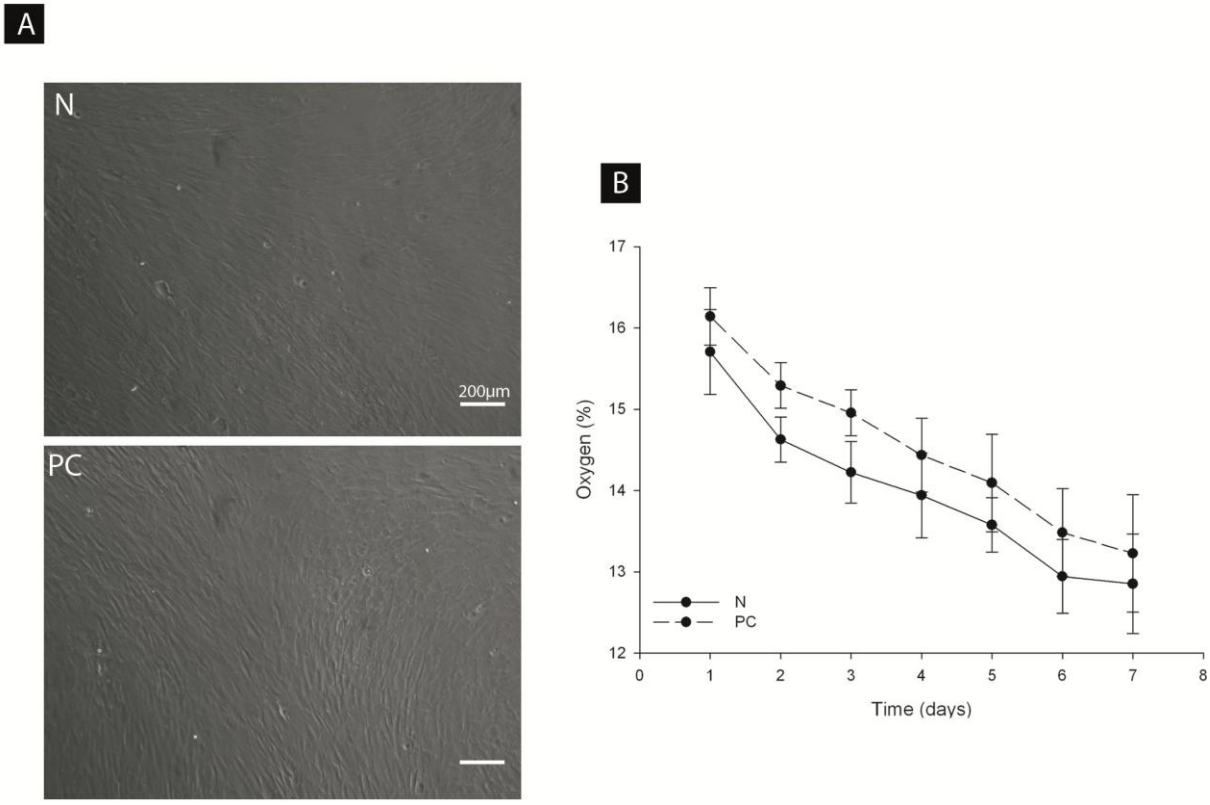


Figure 30 Oxygen measurement of SCP-1 grown in 2D culture. One hundred thousand SCP-1 cells that were either precultured in normoxia or hypoxia for 3 days were seeded in oxodishes and cultured for a period of 7 days under static culture in

normoxia. After 7 days both normoxic and preconditioned samples became 100% confluent (A). The oxygen concentration within the medium was measured and dropped gradually from 15,7 to 12,8% and 16,1 to 13,4% for normoxic and preconditioned samples respectively (B).

3.12 The effect of hypoxic preconditioning on the differentiation capability of SCP-1

To see whether the knowledge of hypoxic preconditioning and its effect on the osteogenic differentiation of hMSC is also transferrable to SCP-1, we cultured SCP-1 under hypoxia (2% O_2) until the cells reached a confluency of 70-80% and then induced them into the osteogenic lineage at 2% O_2. Hypoxic preconditioning did indeed lead to osteogenic differentiation as evidenced through Alizarin red staining (Fig. 31B). We further asked whether this approach of hypoxic preconditioning could also be used to differentiate SCP-1 cells into other lineages such as into adipocytes. We therefore cultivated SCP-1 under hypoxic condition prior to start of adipogenic induction under the same oxygen tension. The Oil red O staining revealed that SCP-1 even with hypoxic preconditioning did differentiate into the adipogenic lineage. The amount of vacuole formation was fewer in the hypoxic preconditioned samples than compared to their normoxic counterparts (Fig. 31A).

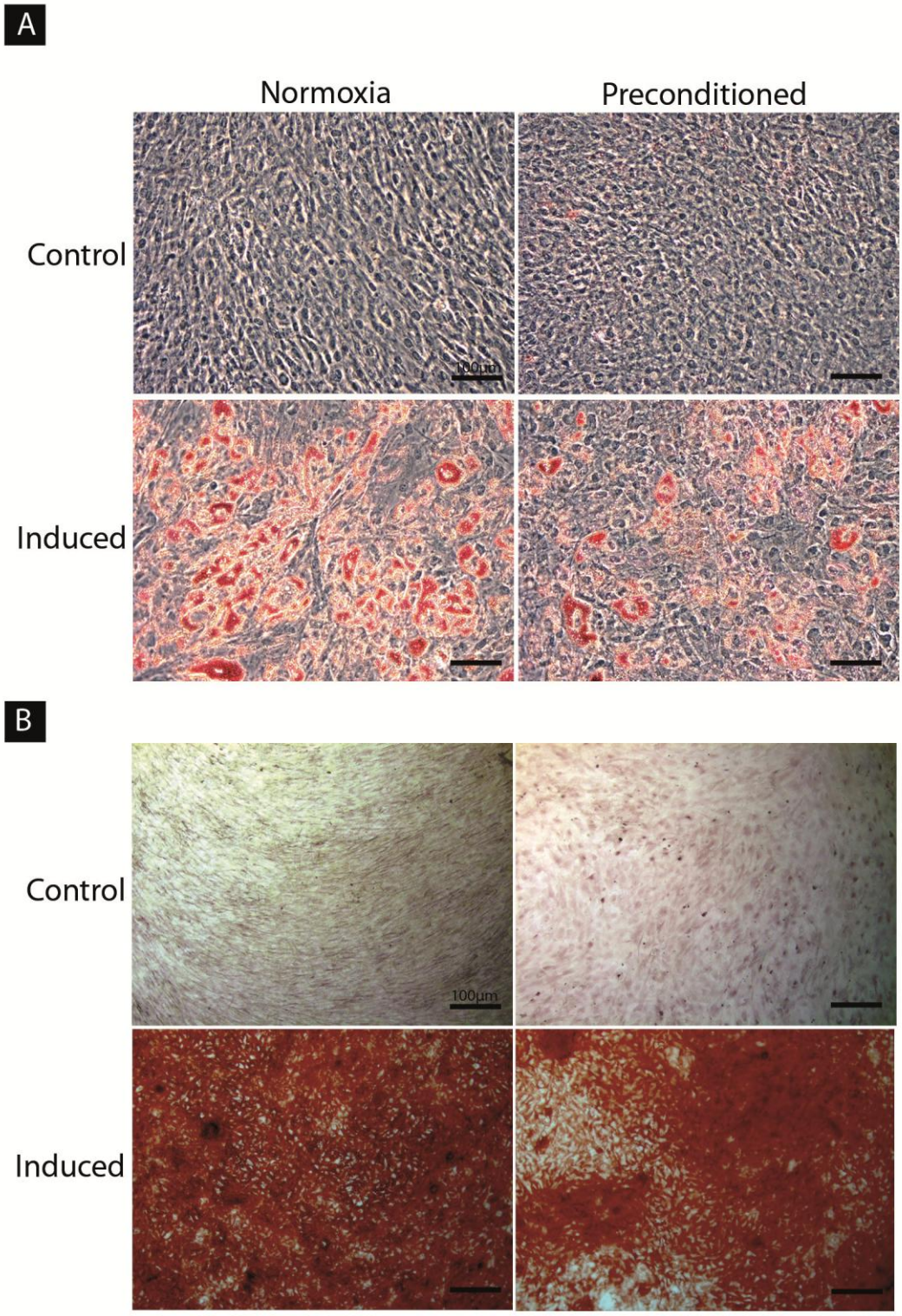


Figure 31 The effect of hypoxic preconditioning on adipogenic and osteogenic differentiation of SCP-1. Cells were cultured in hypoxia (2%) or normoxia (21%) for 3 days prior adipogenic (A) or osteogenic induction (B) at 2% oxygen (preconditioned) or at 21% oxygen (normoxia). In both setups the differentiation

towards adipogenic and osteogenic lineage worked. In the adipogenic differentiation the vacuole formation was somewhat weaker in the preconditioned sample compared to normoxia. In the osteogenic differentiation there was no difference observed with Alizarin red staining.

4 Discussion

4.1 Hypoxia as a limiting factor in cell-based tissue engineering

The introduction of multipotent, proliferative hMSC from adult origin into regenerative medicine has driven speculations that the repair of large tissue defects will soon be within our reach (23, 127-130). However, several hurdles remain to be taken before adult progenitor cells will finally restore organ functions *in vivo*, one of which is adequate oxygen supply of cells in 3D constructs (86, 131, 132). This is especially true in the early phase after transplantation of cells when oxygen supply has yet to be established by the ingrowth of blood vessels (86, 133, 134). As cells are exposed to a reduced oxygen environment in literally any kind of regenerative cell therapy, a better understanding of the impact of hypoxia on cellular processes such as proliferation and differentiation is now warranted.

4.1.1 Cells used for tissue engineering applications are exposed to hypoxia

We have previously shown that mouse fibroblasts are exposed to significant oxygen gradients in 3D culture *in vitro*, even if dynamic culture systems are used (86). In the present study, we demonstrated that considerable oxygen gradients exist in static 3D cultures of hMSC as well. Similar to mouse preosteoblasts, the hTERT-immortalized hMSC (SCP-1) grew fast and therefore consumed the oxygen more rapidly, which resulted in cell death in central regions of the scaffold. The oxygen concentration declined to 0% in central areas of the scaffold and was virtually unaffected by the addition of fresh medium. When normal hMSC were seeded on the scaffolds, the oxygen dropped to only 2% and then levelled out at about 5.7% after one medium change. These findings suggest that normal hMSC adapt to the low oxygen

atmosphere better than hTERT-immortalized hMSC. One explanation may be that hMSC simply grow slower and thus have more time to slow down proliferation when oxygen levels decrease. In line with our findings, a recent study revealed that rat MSC (rMSC) tolerate low oxygen atmospheres better than rat cardiomyocytes because they utilised oxygen dependent metabolism more effectively (135). This trait was consequently termed metabolic flexibility, and we believe that, while hMSC preserved their metabolic flexibility, the transduction of hMSC with hTERT may have decrease their appropriate adaptive response to low oxygen tensions. Nonetheless hMSC were exposed to oxygen concentrations as low as 2% further emphasizing the importance of studying the cell behaviour and cell performance under reduced oxygen atmospheres.

It is of paramount importance to define the oxygen conditions and their effect on the cells properly prior to analyzing further details of cell metabolism under hypoxia. In our study, cells were exposed to 2% of oxygen (hypoxia) in a hypoxia incubator, which guaranteed a constant experimental oxygen condition. We confirmed that exposure of cells to hypoxia resulted in true intracellular hypoxia by a positive pimonidazole staining, a chemical that forms intracellular adducts under roughly 1.5-2% of oxygen. We further substantiated that cells were exposed to hypoxia by the detection of Hif-1α protein, a ubiquitously expressed mediator of the cellular response to hypoxia in mammalian cells, which plays a vital role in the activation of anaerobic metabolism, angiogenesis, erythropoiesis and vasodilation. The Hif-1α protein is degraded when exposed to oxygen through its oxygen-dependent degradation domain. Under hypoxic conditions the Hif-1α protein is translocated into the nucleus where it binds to Hif-1β to form a stable heterodimer (91-94). It is important to note that Hif-1α is detectable on the mRNA level under both normoxic

and hypoxic conditions and that only on the protein level they are distinguishable (94). In our study, the Hif-1α protein was abundantly seen in the western blot analysis within the hypoxic samples, whereas no signal was found in the normoxic samples, further supporting that cells were undeniably exposed to hypoxia.

4.1.2 Hypoxia promotes cell proliferation

When performing hypoxia experiments, it is vital to assess the proliferation behaviour of the cells under normoxic as well as under hypoxic conditions. We showed that a prolonged exposure to 2% oxygen over a period of 21 days was neither cytotoxic nor did it negatively affect cell proliferation. Contrariwise, cells tended to grow better under hypoxia, and this trend was even statistically significant on days 5, 13 and 18 when looking at the WST assays, and on day 8 when looking at the cell counts. Similar results were obtained by Grayson et al. who observed that hMSC exposed to 2% hypoxia proliferated more rapidly than the normoxic cells (103). The same group showed that this trend persisted over several passages, and that hMSC maintained their growth rates even after confluency was reached. In parallel, a group that focused on the optimization of hMSC expansion cultures *in vitro* also found improved cell growth under hypoxia and therefore called for expansion cultures to be performed at low oxygen concentrations (114). Consistent with these results, it was shown that constant hypoxia promotes the proliferation of human bone marrow stromal cells (136), of human neural progenitor cells (137), and of human embryonic stem cells (138), suggesting that hypoxia promotes stem cell proliferation. On the contrary, culturing hMSC under hypoxia brought about a decrease in the proliferation and colony forming capacity of hMSC in two studies wherein hypoxia was applied

intermittently (106, 139), indicating that sudden falls of oxygen tension negatively affect cell proliferation.

4.1.3 Hypoxia inhibits osteogenic differentiation

Differentiation processes are an inevitable step from multipotent precursor cells to bone tissue. As the hMSC in 3D culture were exposed to as little as 2% oxygen we aimed at clarifying the effect of constant hypoxia of 2% oxygen on the process of osteogenic differentiation of hMSC. We found a decreased extent of osteogenic differentiation under hypoxic conditions. This is in agreement with many studies which found a negative effect of hypoxia on osteogenic differentiation of mouse (104, 107, 136, 140), rat (110), and human (69, 106, 107) bone precursor cells. Interestingly, however, on day 5 we discovered OPN to be more abundantly expressed under hypoxia than under normoxia when analysed by RT-PCR. Similar observations were made previously in rat vascular smooth muscle cells (141), in mouse osteocytes (142), in human renal proximal tubular epithelial cells (143), and in hMSC exposed to anoxia (144). Osteopontin is an extracellular matrix protein that binds to cells via an RGD sequence that recognizes the $\alpha_v\beta_3$ integrin (145). It has been attributed to regulate bone cell adhesion and matrix mineralization (145, 146). An additional intriguing feature of osteopontin is to promote macrophage infiltration and osteoclast recruitment (146). It is tempting to speculate that the physiological significance of increased osteopontin expression of cells exposed to hypoxia may be to attract macrophages and osteoclasts to the site of fractured bone thus setting off or maintaining the bone repair process. However, future studies will have to clarify this issue in more detail.

4.1.4 Hypoxia promotes stemness

As hMSC showed a reduced capability to differentiate towards the osteogenic lineage under hypoxia, we aimed to find out whether this was partly due to a varying stemness potential in normoxia compared to hypoxia. It was already reported that bone marrow derived mesenchymal stem cells reside within stem cell niche under low oxygen tension that would keep them multipotent and prevent them to be committed towards a particular lineage (69).

One of the easiest and relatively inexpensive approaches to compare the stemness potential between two groups of cells is by means of a clonogenic assay. The CFU-F number was higher for cells exposed to hypoxia compared to normoxia, indicating that hypoxic cells possess more stemness than normoxic cells. This finding is in accordance with previous studies done by Grayson et al. who determined that hMSC grown under hypoxia produced a higher CFU-F number compared to the CFU-F numbers produced by hMSC exposed to normoxia (103). Ezashi et al. showed that it would be better to culture human embryonic stem cells under hypoxic conditions rather than normoxia as it would prevent spontaneous differentiation as seen in normoxia (138).

In support of these findings we could show that the embryonic stem cell markers OCT-4 and NANOG were upregulated under hypoxic conditions, further indicating that hypoxia favours stemness over differentiation. It is believed that the pluripotency of embryonic stem cells relies on a set of transcription factors that are centred around NANOG, OCT-4 and SOX-2 (55). OCT-4 also known as POU5F1 (POU class homeobox 1) is a homeodomain transcription factor which belongs to the class of

POU (Pit, Oct, Unc) class of transcription factors. It governs in part the pluripotency of ES, and it is often used as a marker for undifferentiated cells. NANOG is a transcription factor that is required for the self-renewal of undifferentiated embryonic stem cell markers (147). In mouse embryos NANOG expression is limited to the founder cells of the inner cell mass (ICM; 148). The ablation of NANOG expression within the founder cells results in failure to generate epiblasts (147). Its expression is high in ES and embryonic carcinoma cells (149), but gets downregulated upon differentiation (150).

D'ippolito et al. have shown that marrow isolated adult multilineage inducible (MIAMI) cells have an enhanced cell proliferation and an increase in DNA synthesis when grown under 3% O_2. In addition to that they were able to show that low oxygen tension promotes stemness by elevated expression of embryogenic stem cell markers such as OCT4 and REX-1. Furthermore they could show enhanced expression of stage-specific embryogenic antigen 4 (SSEA-4) surface marker in cells that were cultured under hypoxic conditions through cytometric analysis. Above all they were able to show that low oxygen tension suppressed the osteogenic differentiation of human MIAMI cells (69). Taking the results from proliferation and differentiation under low oxygen tension together, it suggests that hypoxia is a key condition for promoting stem cell self-renewal rather than stem cell differentiation.

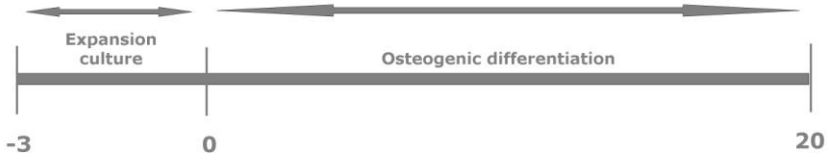
4.2 Hypoxic preconditioning as a treatment for cells in tissue engineering applications

The fact that human mesenchymal stem cells exposed to hypoxia stop to differentiate along the osteoblastic lineage does impose substantial difficulties when aiming at regenerating bone tissues *in vivo*. While maintaining adequate oxygen supply *in vitro*

seems to be a difficult but feasible task, it appears almost impossible to guarantee constant oxygen supply to cells after implantation *in vivo*. Within the last year, a series of studies provided evidence that hypoxic preconditioning of hMSC brought about enhanced cell survival, reduced apoptosis rates, an increased cell engraftment and cell motility as well as an enhanced therapeutic potential (117, 118, 139, 151). We therefore reasoned that hypoxic preconditioning of hMSC may also improve their potential to differentiate towards the osteoblastic lineage under constant low oxygen conditions. Our findings do support this theory as preconditioning hMSC in hypoxia restored their capacity to differentiate osteogenically under constant hypoxia (for a summary of the differentiation experiments see table 2). It was reported that rat cells cultured under low oxygen tension prior implantation lead to more bone formation compared to cultures grown at 21 % O_2 (112). Oxygen is a potent regulator of multiple cell functions including cell metabolism and differentiation (94, 103, 137, 152-154). We therefore assume that the process of osteogenic differentiation is disturbed by changes in oxygen tension, whereas constant conditions support the process of differentiation. This would explain why hMSC either differentiated under constant normoxia or if cells were grown in hypoxia for three days before they were differentiated under hypoxic conditions. Ezashi and co-workers reported that a reduced oxygen concentration maintained the pluripotent state of embryonic stem cells and reduced the amount of spontaneous differentiation seen under normoxia (138). Accordingly, several researchers have postulated that low oxygen concentrations more accurately resemble the stem cell niche and therefore preserve an increased stemness of hMSC (104, 112, 117, 155, 156). These discoveries may explain why the preconditioned samples did not differentiate as well as the normoxic samples. Taken these findings together, we assume that an important pretreatment

of hMSC prior to transplantation is to grow them under a low oxygen atmosphere in order to preserve their stemness and their differentiation capacity.

Table 2 Overview of the experimental setup of osteogenic differentiation and their outcome.



Expansion culture	O_2 (%) for Osteogenic differentiation	Terminology	Success of differentiation
Normoxia	Normoxia (21%)	Normoxic	+++
Normoxia	Hypoxia (2%)	Hypoxic	+/-
Hypoxia	Hypoxia (2%)	Hypoxic Preconditioning	++

Collectively, our data suggest that prolonged exposure to oxygen levels of 2% enhances hMSC proliferation. We have provided evidence that transducing hMSC with hTERT may decrease their metabolic flexibility, a fact that has to be kept in mind if genetically modified stem cells are generated for regenerative medicine. We demonstrated that preconditioning under 2% of oxygen prior to osteogenic stimulation enhances the differentiation potential of hMSC if differentiation is carried out under constant low oxygen tensions. We believe that hypoxic preconditioning is a helpful tool for successful regenerative cell-based therapies.

4.3 HIF-1α has no direct effect on osteogenic differentiation

As preculturing cells for 3 days under hypoxic conditions prior to osteogenic induction resulted in an improved osteogenic differentiation as compared to the normoxia-hypoxia (hypoxic) samples, we speculated that the activation of HIF-1α during the 3 days of preconditioning may play a role in the process of osteogenic differentiation of preconditioned samples. However, a transient knock-down of HIF-1α through the application of silencing RNA during the hypoxic preconditioning period resulted in no visible differences in the osteogenic differentiation behaviour, suggesting that HIF-1α activation was not the reason why hypoxic preconditioned samples differentiated into the osteogenic lineage. In this experiment the transfection efficiency was high. However, even with very good transfection efficiency it is estimated that only about 70% of HIF-1α function is eliminated. In order to rule out that the remaining 30% would be enough to bring about an adequate osteogenic differentiation, we performed an experiment where we treated hypoxic cells with DFO during their expansion culture.

Desferrioxamine (DFO) is an iron chelator that is commonly used in clinical applications to treat iron overload diseases. The DFO treatment prevented the degradation of Hif-1α in normoxia. Zhou et al. showed that hypoxia and its mimetic DFO upregulated Hif-1α compared to normoxia (157). Woo et al. showed that DFO enhanced Hif-1α accumulation via a cyclooxygenase-2 signalling pathway (158). Hydroxylation of HIF complexes via a family of oxygen sensitive prolyl hydroxylases (PHD 1,2,3) brings about E3 ligation and this in turn triggers the proteosomal degradation of the protein complex. Several compounds have been now identified

which can inhibit prolyl hydroxylase activity (159-161). DFO, a pharmacological compound inhibits PHD activity by taking away iron, which is required as a cofactor for its proper enzymatic function (119). In this particular experiment Hif-1α of normoxia-hypoxia (hypoxic) samples was stabilized during the entire differentiation period, either during the expansion culture in normoxia with the help of DFO, and during the induction period under hypoxia. This setup therefore mimicked the hypoxic preconditioned sample with respect to Hif-1α stability, and hence one expected to see some extent of differentiation in the normoxia-hypoxia (hypoxic) sample, if one assumed that HIF-1α played a central role in bringing about osteogenic differentiation of hypoxic preconditioned samples. But despite of Hif-1α stabilisation there was hardly any difference observed, when analyzed by Alizarin red staining. This further strengthens the notion that HIF-1α activation does not influence the ostegenic differentiation of hMSC under hypoxia. As a control we treated normoxic and preconditioned samples with DFO during the expansion culture to show that DFO itself would not interfere with osteogenic differentiation. In either case there was differentiation, indicating that even with DFO treatment cells were still able to differentiate. This is in line with previous publications which reported that DFO enhances alkaline phosphatase activity and stimulates osteocalcin secretion in MG-63 bone cells (162, 163). Furthermore it was shown that moderate concentration of DFO was able to promote osteogenic differentiation in hMSC, and that β-catenin signalling pathway might be responsible for DFO induced osteogenesis (164).

The first experiment revealing the requirement of vascular supply for skeletogenesis was shown by Trueta and colleagues, who demonstrated that an interruption of blood supply to the growth plate of bone resulted in decreased bone mineralization and in the expansion of the hypertrophic zone in the growth plate (165, 166). Wang et al.

demonstrated through *in vivo* models that HIF-1α plays an important role in coupling angiogenesis to osteogenesis during skeletal development (94). They demonstrated that mice lacking VhL (Von hippel-lindau) protein in osteoblasts and therefore constitutively over expressed the HIF α complex, display an increased bone formation phenotype. This phenotype was also associated with an increased angiogenesis and vascular endothelial growth factor (VEGF) production. In further support of this, they were able to show that by inactivating HIF-1α in osteoblast resulted in a decreased bone volume and vascularity. In their study they were not able to demonstrate whether HIF-1α had a direct influence on osteogenisis, they have merely shown that HIF-1α drives the formation of bone via angiogenesis. It is now known that the HIF system plays an important role in the regulation of angiogenesis by hypoxia (167). In another paper it was noted that HIF-1 α plays a key role in hypoxic preconditioning (168).

Collectively previous studies have shown that HIF-1α is required for bone development *in vivo*, through the activation of angiogenesis. Taken together our data obtained from the experiments with silencing RNA where we transiently knocked-down HIF-1α during the expansion culture of hypoxic preconditioned samples and the experiments with DFO, where we stabilised HIF-1α during the expansion culture of normoxia-hypoxia samples, show that HIF-1α does not play a central role in directing hMSC into the osteogenic lineage *in vitro*.

4.4 SCP-1 as a model system

Progenitor cells like MSC are readily used in the field of cell based tissue engineering and gene therapy because of their ease of isolation and their multilineage

differentiation ability. However during long term *in vitro* culture of hMSC they undergo alteration in their physiological properties, and the mechanism underlying this still remains elusive (169). But their senescence-associated growth arrest under normal *in vitro* culture conditions restricts their clinical use. A reduced activity to undergo cell division, increased actin stress fiber formation, an increase in cell size and increased β-glactosidase activity *in vitro* are signs of senescence (73). Numerous research groups have successfully overcome the problem of senescence-associated growth arrest by immortalizing hMSC through the introduction of the hTERT transgene.

SCP-1 being an immortalised hMSC cell line has the advantage to be cultured to a high cell number within a relatively short period of time. The growth kinetics revealed that SCP-1 proliferated at a much higher rate than hMSC under normoxia. This was in line with previous publications who have reported that immortalised adult stem cells showed an enhanced proliferation (170-172). We believe that immortalization of hMSC apart from prolonging their life span would also bring about the selection of a subset of the hetrogenous hMSC population that are faster growing. The fact that SCP-1 tend to reach plateau phase in hypoxia once they reach 100% confluency while under normoxia they keep growing might be explained in that hypoxia in general helps to retain stem cell characteristics. It has been previously shown that stem cells gown to a high confluency display higher rate of spontaneous differentiation (138). Therefore, the fact that SCP-1 grown in hypoxia reached a plateau phase earlier than the SCP-1 grown in normoxia might be a sign that hypoxia lowers the chance of spontaneous differentiation.

Previous research has shown that hTERT is upregulated under low oxygen tension (125). In order to validate this we screened for hTERT at mRNA and protein level to

see any differences between SCP-1 that were either exposed to normoxia or hypoxia. Immuno staining against hTERT did reveal that in both oxygen conditions they expressed equally well the hTERT protein. On mRNA level though one could see a subtle upregulation in samples that were cultured in hypoxia in comparison to normoxia.

From the RT-PCR results one could also deduce that embryonic stem cell markers, NANOG and OCT-4 were upregulated under hypoxic condition. This goes in line with the growth kinetic analysis that hypoxia preserves the stem cell characteristics of the cells. To our surprise the clonogenic assay showed a higher CFU number for cells that were exposed to normoxia which is in direct contradiction to the results obtained with the growth kinetic and RT-PCR of embryonic stem cell markers both indicating that cells exposed to hypoxia are more stem like than those cells exposed to normoxia. The gradual decrease in the CFU numbers over time could be explained due to the fact that as cells tend to stay longer in culture become more confluent and hence start to differentiate spontaneously towards a particular lineage, and hence they might loose their ability to form colonies.

From our experiments with hMSC we realised that preconditioning hMSC in hypoxia before inducing them into the osteogenic lineage under hypoxia would result in an improved osteogenic differentiation. We tried now to transfer our knowledge with preconditioning onto SCP-1. We were able to show that upon preconditioning SCP-1, the cell line differentiated into the osteogenic and adipogenic lineages. This was in accordance with a previous publication showing that immortalised hMSC maintained their differentiation potential (173). Furthermore it was shown that hMSC overexpressing hTERT showed enhanced osteogenic differentiation ability (82)

whereas hMSC deficient in hTERT expression showed an impaired differentiation potential (174). The adipogenic differentiation results were in line with previous reports wherein it was shown that hypoxia suppresses adipogenic differentiation (107).

In summary, SCP-1 seems to be a suitable candidate to be used as a model system for hMSC *in vitro*. It would be recommended to culture the cells under hypoxia as the low oxygen tension favours the stemness and prevents spontaneous differentiation. Hypoxic preconditioning prior to tissue engineering application would also be favourable as this would acclimatise the cells for their upcoming oxygen milieu at a site of ischemia where they would be used to trigger the regeneration of the injured tissue.

4.5 Hypoxic preconditioning of SCP-1 does not improve cell survival in DBM scaffolds

It has been shown that a large number of transplanted cells die within the initial days after transplantation. Several factors account for the cell death such as hypoxia, oxidative stress, inflammatory response and trophic factor deficiency. Research is ongoing to improve the life span of transplanted cells especially in the initial stages where viable vasculature and direct trophic support are absent. Various strategies have been followed up such as genetic manipulation of cells by overexpressing anti-apoptotic and growth factor genes or by knocking down of pro-apoptotic genes in order to keep the cells alive during the critical initial stages after implantation where they are exposed to reduced oxygen tension (105, 175, 176). Even though these approaches seem to be quite promising, there are several issues to be addressed

before clinical use. The primary concern with genetically modified cells is their safety issues associated with permanent gene alteration and their chance for inducing cancer in the patient. However recent investigations have shown a harmless new approach that would make the cells more resistant to hypoxic exposure without undergoing any genetic modifications, namely hypoxic preconditioning. In a recent study it was shown that preconditioning cells in low oxygen tension would make cells more resistant towards a subsequent exposure to low lethal oxygen tension (118). Wang et al. showed that hypoxic preconditioning of hMSC prior implantation would make them more resistant towards hypoxia/reoxygenation-induced apoptosis. They were able to show that this protective mechanism was due to the stabilization of mitochondrial membrane potential, upregulation of BCL-2 and VEGF, and promoting extracellular signal-regulated kinase (ERK) and AKT phosphorylation. Theus et al. showed that transplantation of hypoxic preconditioned embryonic stem cell derived neural progenitor cells into ischemic rat brain lead to increased early survival of transplanted cells and also accelerated behavioural recovery following stroke (177). Several researchers have shown that hypoxic preconditioning is neuroprotective against ischemic brain injury (178, 179). With this idea in mind we have grown preconditioned and non-preconditioned SCP-1 on DBM scaffolds and grew them over a period of 7 days under static culture. We had already shown that SCP-1 are subjected to lethal oxygen concentrations of 0% oxygen and would result in death of cells within the core of the scaffold. We now assumed that if we would precondition SCP-1 in hypoxia prior to seeding them onto the scaffold might result in a higher number of viable cells at the end of 7 days, due to the assumption that preconditioning makes them more resistant to subsequent low oxygen concentration.

We initially analyzed the central oxygen measurements within the scaffolds. Central oxygen measurements within the scaffolds over a period of 7 days in static culture did not show any significant differences. To our surprise, preconditioned scaffolds did not differ from non-preconditioned scaffolds concerning cell survival characteristics. We thus have to conclude that in our model system in vitro hypoxic preconditioning did not improve cell survival in scaffolds exhibiting hypoxia. At last we decided to see whether there would be any differences in the oxygen measurements in 2D, and henceforth we seeded equal number of preconditioned and normoxic cells in oxodishes and measured the oxygen consumption over a period of 7 days. The data showed that preconditioned cells consumed less oxygen compared to normoxic cells at all different time points of oxygen measurements. There were though no significant differences in the oxygen measurements between the two cell groups. It is thus likely that preconditioning does protect cells from hypoxia-induced cell death by down-regulating oxygen dependent metabolism. However our model system was unable to pick up this preconditioning induced benefit.

4.6 Conclusion

Taking the data together, we confirmed that constant exposure to low O_2 does indeed reduce osteogenic differentiation of hMSC. We believe that hypoxia favours stemness over differentiation, but the differentiation under hypoxia can be restored via hypoxic preconditioning. Hypoxic preconditioning may help to restore the otherwise reduced osteogenic potential of hMSC, either within a hypoxic fracture environment or at the site of implantation of tissue engineered bone constructs. We therefore believe that hypoxic preconditioning is a helpful tool for successful regenerative cell-based therapies in bone tissue engineering. HIF-1α seems not to play a significant role in osteogenic differentiation of hMSC under hypoxia. SCP-1 might be used as a model system for hMSC but constant monitoring of neoplastic transformation is mandatory.

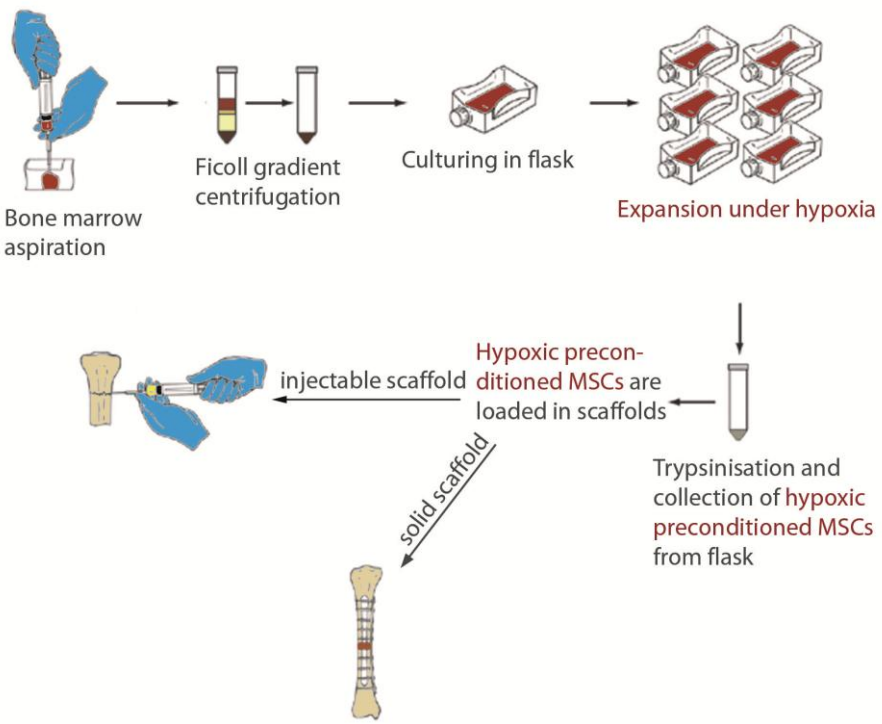


Figure 32 Hypoxic preconditioning as an improved method for cell based tissue engineering. Autlogous stem cells are obtained via bone marrow aspiration. Mesenchymal stem cells are isolated via Ficoll gradient centrifugation and their plasticity nature on tissue culture flasks. They are expanded *in vitro* under hypoxia to the desired cell number and thereafter put into the site of fracture either via injection or seeded on biocompatible scaffolds and implanted at the site of fracture where they will aid in regenerating new healthy tissue.

4.7 Outlook

As hMSC used in tissue engineering are subjected to low oxygen concentrations either in the 3D construct or at the site of defect, it is of critical importance to investigate how cells respond to hypoxia and how their regenerative and differentiation potential can be maintained despite exposure to hypoxia. We believe that hypoxia-preconditioned hMSC represent a more suitable candidate for tissue engineering applications than hMSC grown under normoxia because of their enhanced osteogenic differentiation potential under low oxygen tension and because of a putatively increased survival under hypoxia.

In the future more studies are warranted in order to substantiate the potential of hypoxically preconditioned hMSC. Further experiments need to be conducted in order to answer questions like what hypoxic preconditioning does to hMSC and why it improves the osteogenic differentiation potential under hypoxia. One has also to figure out how long one needs to preculture hMSC and under which degree of hypoxia, in order to achieve optimal osteogenic differentiation potential and survival under reduced oxygen tension. The effect of hypoxic preconditioning as seen *in vitro* should also be looked at *in vivo*.

Summary

Osteogenic differentiation of hMSC into osteoblasts is a prerequisite for subsequent bone formation. Numerous studies have explored osteogenic differentiation under standard tissue culture conditions, which usually employ 21% of oxygen. However, bone precursor cells such as hMSC reside in stem cell niches of low oxygen atmospheres. Furthermore, they are subjected to low oxygen concentrations when cultured on three dimensional scaffolds *in vitro* for bone tissue engineering purposes, and even more so after transplantation when vascularisation has yet to be established. Similarly, hMSC are exposed to low oxygen in the fracture microenvironment following bony injury. Recent studies revealed that hypoxic preconditioning improves cellular engraftment and survival in low oxygen atmospheres.

In the present study we therefore investigated the osteogenic differentiation potential of hMSC under 2% O_2 (hypoxia) in comparison to a standard tissue culture oxygen atmosphere of 21% (normoxia). The success of differentiation was validated through Alizarin red staining and RT-PCR analysis of osteoblast markers ALP and OPN. We assessed osteogenic differentiation of hMSC following hypoxic preconditioning to address whether this pretreatment is beneficial for subsequent differentiation under low oxygen tension. To validate our findings we carefully characterised the extent of hypoxia exerted on cells with respect to cell survival (WST assay) and proliferation (growth curve). Furthermore we also tried to elucidate the role of HIF-1α with respect to osteogenic differentiation under hypoxia via silencing RNA and DFO, a pharmacological agent. Finally we tested whether an immortalized hMSC-line (SCP-1) would serve as a model system for hMSC.

We found that hMSC proliferate better if cultured under 2% of oxygen. We confirmed that osteogenic differentiation of hMSC is indeed inhibited under hypoxia. We showed for the first time that hypoxic preconditioning of hMSC prior to osteogenic induction restores osteogenic differentiation of hMSC under hypoxia. HIF-1α seemed not to play a significant role in osteogenic differentiation under hypoxia, as transiently knocking down of HIF-1α in preconditioned samples did not show any differences in their osteogenic differentiation. Moreover stabilising Hif-1α in hypoxic samples did not yield any osteogenic differentiation either substantiating the notion that HIF-1α does not have a direct role in the osteogenic differentiation of hMSC under hypoxia.

Together our data suggest that hypoxia favours stemness over differentiation by upregulating embryonic stem cell markers like OCT-4 and NANOG. Hypoxic preconditioning may help to restore the otherwise reduced osteogenic potential of hMSC, either within a hypoxic fracture environment or at the site of implantation of tissue engineered bone constructs. We therefore believe that hypoxic preconditioning is a helpful tool for successful regenerative cell-based therapies in bone tissue engineering. SCP-1 cells might be used as a model system for hMSC as they are easy to handle, can be cultured to a desired cell number within a very short period of time, are relatively inexpensive and above all do not go into senescence as seen with hMSC after approximately 20 passages. Apart from their distinct advantages SCP-1 cells still maintain the specific CD markers characteristic for hMSC and are able to differentiate into adipogenic, osteogenic and chondrogenic lineages. However for *in vivo* experiments in animals a constant monitoring of neoplastic transformation is mandatory.

Zusammenfassung

Eines der größten Probleme des zellbasierten „Tissue Engineering“ von Knochen ist das Vorkommen von Sauerstoffgradienten in dreidimensionalen Zell-Leitschienen-Konstrukten sowohl *in vitro* als auch *in vivo*. Aber auch nach einer Knochenverletzung sind humane mesenchymale Stammzellen (hMSC), die die Knochenheilung unterstützen, einer reduzierten Sauerstoffkonzentration ausgesetzt. Es ist deshalb wichtig, die osteogene Differenzierung von hMSC unter erniedrigter Sauerstoffkonzentration zu untersuchen. In der vorliegenden Arbeit wurde zunächst untersucht, ob die osteogene Differenzierung von hMSC durch eine Kultivierung unter Hypoxia (2% Sauerstoff) beeinträchtigt wird. Da einige aktuelle Studien nun Hinweise dafür geliefert haben, dass eine hypoxische Präkonditionierung sowohl für das überleben von hMSC unter hypoxischen Bedingungen als auch für deren "Engraftment" nach Transplantation vorteilhaft ist, haben wir untersucht, ob eine hypoxische Präkonditionierung von hMSC zu eine Verbesserung der osteogenen Differenzierung unter konstant erniedrigter Sauerstoffkonzentration führt.

In den vorliegenden Untersuchungen wurde die osteogene Differenzierung von hMSC unter 2% O_2 (Hypoxie) im Vergleich zu 21% O_2 (Normoxie) durch Alizarin-Rot-Färbung sowie durch RT-PCR osteoblastärer Marker (ALP, OPN) untersucht. Anschließend wurden hMSC hypoxisch präkonditioniert um zu klären, ob diese Vorbehandlung die anschließende osteogene Differenzierung unter hypoxischen Bedingungen verbessert. Um die gewonnenen Ergebnisse zu validieren, wurden die Sauerstoff-Bedingungen sorgfältig charakterisiert, insbesondere wurde die Expression eines Hypoxie-Markers (Hif-1α) mittels Western-Blot-Analyse sowie das Proliferationsverhalten von hMSC unter Hypoxie vs. Normoxie mittels WST-Assay bestimmt. Außerdem wurde die Rolle von HIF-1α durch Modulation mittels silencing

RNA beziehungsweise eines pharmakologischen Wirkstoffes (DFO) auf die osteogene Differenzierung von hMSC unter Hypoxie untersucht. Im weiterem wurde getestet ob die immortalisierte Stammzelllinie SCP-1 als Modellsystem für hMSC geeignet ist.

Unsere Untersuchungen haben ergeben, dass hMSC unter 2% Sauerstoff mindestens genauso gut prolifieren wie unter Normoxie. Ferner konnten wir bestätigen, dass die osteogene Differenzierung tatsächlich unterdrückt wird, wenn sie unter 2% Sauerstoff durchgeführt wird. Wir konnten zum ersten Mal zeigen, dass die osteogene Differenzierung unter 2% O_2 wiederhergestellt werden kann, wenn die hMSC zuvor im hypoxischen Milieu vorkultiviert werden. HIF-1α scheint allerdings keine größere Rolle in der osteogene Differenzierung von hMSC unter Hypoxie zu spielen, da weder die herauf-Regulation noch die herunter-Regulation von HIF-1α einen messbaren Effekt auf die osteogene Differenzierung hat. SCP-1 eignet sich als ein Modellsystem für hMSC, jedoch muss *in vivo* eine konstante Überwachung von tumorigenen Veränderungen gewährleistet sein.

Das osteogene Differenzierungspotential von hMSC unter hypoxischen Bedingungen kann erhöht werden wenn sie zuvor hypoxisch präkultiviert werden.
Dies ist von herausragender Bedeutung für regenerative zellbasierte Therapien, die Knochenregeneration nach Trauma oder bei Knochendefekten zum Ziel haben.

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Figure 23 The effect of stabilising Hif-1α on osteogenic differentiation under hypoxia. hMSC were seeded for osteogenic differentiation into 6 wells for hypoxia, normoxia and preconditioned samples. After 24 hours the medium was supplemented with 50 µM DFO (A) or 100 µM DFO (B) and incubated for additional 2 days. After 3 days proteins were isolated from hMSC in normoxia that were treated with 50 µM (lane 1), 100 µM (lane 2) and 0 µM (lane 3) of DFO.(C) In addition to that protein was isolated from hMSC in hypoxia (lane 4). Western blot analysis against Hif-1α was performed with the protein samples. After 3 days cells were then induced into the osteogenic lineage without DFO supplements. Following 20 days of induction hMSC were stained with Alizarin red. hMSC exposed to 50 µM and 100 µM DFO in normoxia and preconditioned samples stained with Alizarin red, whereas hypoxic sample did not, suggesting that HIF-1α does not paly a crucial role in the osteogenic differentiation of hMSC under hypoxia. 71

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a similar trend untill day 14 where they become 100% confluent. Following day 14 cells under hypoxia reach plateau phase whereas cells under normoxia still proliferate at an exponential rate. (A) The BrdU incorporation was measured in SCP-1 that were either cultured under normoxia (21%) or under hypoxia (2%) for 5, 7, 10 and 16 days respectively. The BrdU incorporation in either oxygen concentration was the same independent of time. (B)..... 73

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Figure 26 Clonogenic assay of SCP-1. Cells were exposed to normoxia or hypoxia for 5, 10, 16 or 21 days, and thereafter trypsinized and seeded into new 10 cm petri-dishes at a density of 14 cells/cm^2. The cells were then allowed to incubate at 37˚C for 12 days prior to crystal violet staining. The staining revealed that at any time period, SCP-1 that were exposed to normoxia formed more colonies (A). The difference in colony number was significant at day 16 (B). 76

Figure 27 Semi quantitative RT-PCR of embryonic stem cell markers and hTERT. RT-PCR against NANOG, OCT 4 and hTERT was performed on RNA extracts obtained from SCP-1 that were cultured either in normoxia (21%) or hypoxia (2%) for 21 days. The embryonic stem cell markers and hTERT were slightly upregulated under hypoxic condition. 77

Figure 28 Central oxygen measurements within static DBM scaffolds seeded with SCP-1 Oxygen concentrations were measured at the centre of scaffolds that were either seeded with 1 million normoxic SCP-1 or preconditioned SCP-1. The central

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Figure 32 Hypoxic preconditioning as an improved method for cell based tissue engineering. Autlogous stem cells are obtained via bone marrow aspiration. Mesenchymal stem cells are isolated via Ficoll gradient centrifugation and their plasticity nature on tissue culture flasks. They are expanded *in vitro* under hypoxia to the desired cell number and thereafter put into the site of fracture either via injection or seeded on biocompatible scaffolds and implanted at the site of fracture where they will aid in regenerating new healthy tissue. 102

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Abbreviations

µl	Microliter
µm	Micrometer
µM	Micromolar
Fig.	Figure
dNTP	Deoxynucleoside triphosphate
g	Gram
h	Hour
HRP	Horseradish peroxidase
ISCT	International society for cellular therapy
Kb	Kilobase
mg	milligram
MSC	Mesenchymal stem cell
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-PCR
s	Seconds
siRNA	Silencing RNA
HIF-1α	Hypoxia inducible factor-1α
DFO	Desferrioxamine
G	Earth gravitational force
ml	Millilitre
mM	Millimolar
hMSC	Human mesenchymal stem cell

NI	Normoxia induced
NC	Normoxia control
HI	Hypoxia induced
HC	Hypoxia Control
PI	Preconditioned induced
PC	Preconditioned control
mRNA	Messenger ribonucleic acid
cDNA	Complimentary deoxyribonucleic acid
CFU-f	Colony forming unit-fibroblast
SCP-1	Single cell pick- 1
BSA	Bovine serum albumin
PBS	Phosphate buffered saline
hTERT	Human telomerase reverse transcriptase
P	Passage
ALP	Alkaline phosphatase
OPN	Osteopontin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
OCT-4	Octamer 4
nm	Nanometer
TBST	Tris-buffered saline tween 20
PVDF	Polyvinyldenefluoride
FITC	Fluorescein isothiocyanate
3D	Three dimensional
SDR	Sensor dish reader

DMSO	Dimethysulfoxide
DBM	Demineralised bone matrix

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Acknowledgement

The compilation of this doctoral thesis has been one of my highest triumphs in my academic life. During the course of my doctoral work several people were instrumental in shaping up my academic career. Without their support, patience and guidance, this study would not have been completed. It is to them that I owe my deepest gratitude.

First and foremost I would like to thank my parents Joseph Cherian Kallukalam and Yoobrad Cherian Kallukalam and my brother Rudy Cherian Kallukalam, for their unflagging love and support throughout my life; this dissertation would have been simply impossible without them.

My deepest gratitude goes to my supervisor, Dr. med Elias Volkmer. I have been fortunate to have a supervisor who gave me the freedom to explore on my own and at the same time the guidance to recover when my steps faltered.

I would like to thank Professor Dr. med Wolf Mutschler and Prof. Dr med Matthias Schieker for providing me the opportunity to work on this intriguing project.

Many friends have helped me stay sane through these difficult years. Their support and care helped me overcome setbacks and stay focused on my graduate study. I greatly value their friendship and I deeply appreciate their belief in me.

I acknowledge the financial support from the Bavarian research foundation.

Declaration

I, the undersigned, hereby declare that this dissertation entitled, “Investigation of the effect of low oxygen tension on the osteogenic differentiation of human mesenchymal stem cells” is my own work, and that all the sources I have used or quoted have been indicated or acknowledged by means of completed references.

_____ _____

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POSTER PRESENTATION

2009	Kallukallam et al., (2009) **Hypoxic preconditioning overcomes hypoxia induced delay in osteogenic differentiation of human mesenchymal stem cells**. PhD Symposium, Interact, Munich
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