The influence of hypoxia, strain and growth differentiation factors on equine adipose tissue derived mesenchymal stem cells

– a study to improve stem cell differentiation in vitro for their future application in vivo

INAUGURAL-DISSERTATION

for the acquisition of the doctoral degree at the Faculty of Veterinary Medicine of the Justus-Liebig-University Giessen

Katja Nadine Shell

From the Institute of Veterinary -Anatomy, -Histology and -Embryology
Justus-Liebig University of Giessen
Germany

Supervising tutor: Prof. Dr. Dr. Stefan Arnhold

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submitted by

Katja Nadine Shell, nee Faquet
Veterinarian from Hagen, Germany

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Dean: Prof. Dr. Dr. h.c. Martin Kramer

Assessors: Prof. Dr. Dr. Arnhold
Prof Dr. Dr. h.c. Martin Kramer

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Katja Nadine Shell, geb. Faquet

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Abbreviations

3d	three dimensional
A/A	antibiotic/antifungal agent
AP	alkaline phosphatase
ASC	adipose tissue derived stem cell
BM	bone marrow
BM-MSC	bone marrow derived mesenchymal stem cell
BMP	bone morphogenetic protein
BSA	bovine serum albumin
Ca^{2+}	calcium
CD	cluster of differentiation
CFU	colony forming unit
CG	control group
CO_2	carbon monoxide
Col	collagen
COMP	cartilage oligomeric matrix protein
Cx32	connexin 32
Cx43	connexin 43
DG	differentiation group
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
e.g.	exempli gratia (for example)
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FAC	focal adhesion complex
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorochrome “fluorescein isothiocyanate”
FTE	functional tissue engineering

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDF	growth differentiation factor
HCL	hydrogen chloride
HIF-1α	hypoxia inducible factor 1 α
Ig	immunoglobulin
IHC	immunohistochemistry
ITS	insulin-transferrin-selenium
kPa	kilo pascal
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N/A	not applicable
NC	negative control
NCws	negative control without strain
NDC	not differentiated cells
O_2	oxygen
OC	osteocalcin
Oct4	octamer-binding transcription factor 4
P/S	penicillin/streptomycin
PAA	ponceau-acidfuchsin-azophloxin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qRT	real time reverse transcribtion
rER	rough endoplasmic reticulum
RhoA	ras homolog gene family, member A
ROCK	rho kinase
RNA	ribonucleic acid
RT	reverse transcribtion
SEM	standard error of the mean
SD	standard deviation

s.	see
Scx	scleraxis
TBS	tris buffer saline
TGF-β	transforming growth factor beta

measurements:

µl	microliter
µm	micrometer
µM	micromolar
°C	degree celcius
am	ante meridiem
cm	centimeter
cm^2	square centimeter
d	days
g	gram
h	hours
kDa	kilodalton
kPa	kilopascal
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
ng	nanogram
nm	nanometer
pm	post meridiem
v/v	volume concentration
wt/vol	weight by volume concentration

1 Introduction

1.1 General facts about stem cells

Stem cells are defined as relatively undifferentiated cells with the ability to self-renew and to differentiate into different cell types. They can either divide symmetrically into two daughter cells that keep their stem cell character or they can divide asymmetrically into a stem cell and a progenitor cell, which is able to differentiate along one or more pathways. Both ways make sure that a pool of stem cells is kept to maintain the self-renewal potential of a body. It is differentiated between **hematopoietic stem cells** which provide the body with new blood cells of the lymphoid and myeloid subdivision (erythrocytes, granulocytes, platelets and lymphocytes) and **mesenchymal stem cells** (MSCs) which are able to differentiate into diverse tissues, e.g. fat, muscle, bone, cartilage, tendon and even neurons and cardiomyocytes (s. fig. 1). While the hematopoietic stem cells are only located in the bone marrow and umbilical cord blood, mesenchymal stem cells can be found in almost every tissue in the body. The application of hematopoietic stem cells in bone marrow transplantations has already been established in the 1960s to treat blood cancer patients. Mesenchymal stem cells still raise great hopes for its implementation in tissue engineering in the future. Relating to the source and the differential potential of stem cells it is distinguished between **embryonic stem cells** and **adult stem cells**. Embryonic stem cells are localised in the inner cell mass of the blastocyst; adult stem cells can be obtained from the tissue of diverse mature organs. Both stem cell types provide advantages as well as disadvantages for their application in the medical field. While embryonic stem cells are pluripotent and therefore show huge differentiation potential on the one hand, their extraction is ethically disputed and goes along with the risk of cancerous devolution on the other hand. In contrast to that, adult stem cells can be obtained from adults without ethically concerns and also they do not show cancer formation, but they are only multipotent, which means their differentiation potential is smaller than the potential of the embryonic stem cells. (Overview by Gilbert, 2010 and Rastegar et al., 2010).

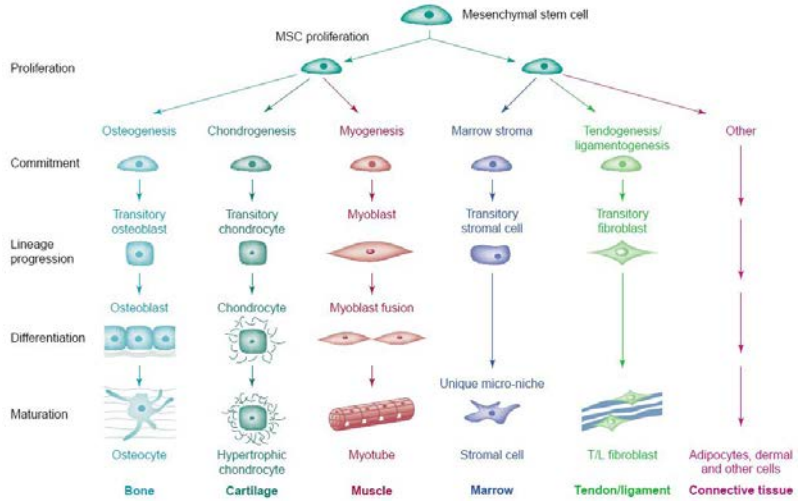


Fig. 1: The potential and process of mesenchymal stem cell differentiation (Caplan and Bruder, 2001)

1.2 Mesenchymal stem cell sources

MSCs can be obtained from almost any tissue. In the past, **bone marrow** derived MSCs (BM-MSCs) have been considered to be the main cell source. Smith and colleagues (Smith et al., 2003) described the aspiration of bone marrow from the sternum in a horse and the isolation of the stem cells. For a sternal puncture the horse has to be sedated. The procedure must be quiet painful for the donator and requires aftercare. The sternal puncture on a standing horse also includes some risks for the surgeon, since he has to work between its legs.

Therefore the discovery of other stem cell locations in the human body and other than equine species was appreciated and examined for their possible application in equine medicine. Vidal et al. (2007) and Raabe et al. (2011) explored the abilities and the possible application of equine stem cells gained from **adipose tissue** (ASCs). Vidal and colleagues compared the characteristics of growth and differentiation of equine BM-MSCs and those

obtained from adipose tissue. The source for the fat was the supragluteal subcutaneous adipose tissue. The results showed that the ASCs had the potential to self-renew and differentiate in vitro, just like BM-MSCs. Because of the easy access to this stem cell source in adults, the easy procedure that involves less pain for the horse and lower risks for the surgeon and the potential to differentiate in vitro, ASCs got in the focus of attention in equine medicine and its associated research disciplines.

Furthermore the isolation of equine MSCs from the **umbilical cord blood** has been reported (Koch et al., 2007). It was the first time that those cells were successfully differentiated into adipocytes, chondrocytes and osteocytes in vitro. Those stem cells are very interesting for the application in equine regenerative medicine and tissue engineering because of the non-invasive cell source, their proliferative potential and their high differentiation potency. To date there is already the possibility of commercial storage of those MSC for future autologous transplantations (Koch et al., 2007).

To complete the list it should be mentioned that in other than the equine species also other sources for MSCs have been described. Examples are **pancreas and salivary gland** (Gorjup, 2009), **dental pulp** (Nam et al., 2011) and the **cruciate ligament** or **endometrial polyps** (Ding et al., 2011).

1.3 The concept/definition of regenerative medicine and tissue engineering

"Regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function" (Mason and Dunnill, 2008).

The definition gives a brief explanation of what regenerative medicine is, even though the terminology is not restricted on human medicine. It applies equally for veterinary medicine, e.g. the application of stem cells in tendon lesions in horses.

"Tissue engineering is the use of cells, biological factors and biomaterials, alone or in combination, with the goal of restoring normal tissue structure and function" (Koch et al., 2009).

According to the definitions, regenerative medicine and tissue engineering belong tight together and each one is necessarily part of the other one.

As an example for tissue engineering with stem cells in the framework of regenerative medicine, review the figure below (fig. 2).

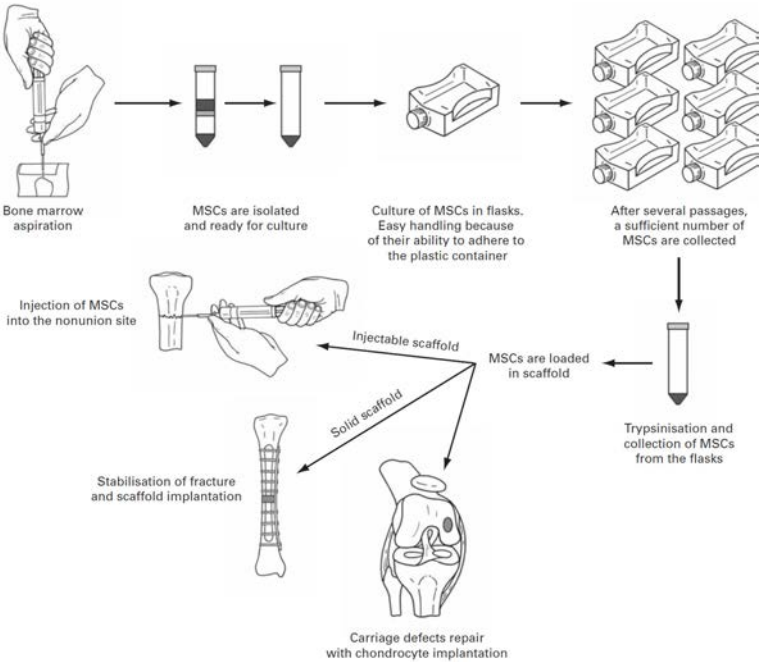


Fig. 2: Simplified scheme of the collection, cultivation and implantation of BM-MSCs as an example for tissue engineering respectively regenerative medicine. The procedure is very similar to ASC collection and application. (Pountos et al., 2006)

1.4 A closer look into the characteristics of mesenchymal stem cells and their potential for the application in the field of equine medicine

MSCs were discovered by Friedenstein and colleagues in 1968 (Friedenstein et al., 1968). He was the first one who isolated MSCs from the bone marrow and described their adherent, fibroblast-like, colony forming unit (CFU)

characteristics in vitro. In later publications he also reported the differentiation into adipocytes, osteoblasts and chondrocytes after re-transplantation in vivo.

The latest description for sound equine stem cell characterization requires **plastic-adherence**, possible in vitro differentiation into osteoblasts, adipocytes and chondroblasts (**tri-lineage differentiation**) and the **expression of the surface markers** CD73, CD90 and CD105 and lack of the expression of some other surface markers at the same time (De Schauwer et al., 2010).

Since mesenchymal stem cells have the potency to differentiate into cell types of the musculoskeletal organs, they became very interesting for tissue engineering and regenerative medicine in the horse. Cartilage lesions and cartilage degeneration, bone fractures and especially tendon lesions are extremely common in equine athletes and well known for their long healing times and therefore long periods of non-productive time for the horse which goes along with high costs and a high frustration level of the owners and trainers (Bailey et al., 1999). None of the conservative therapies have been able to provide full tissue regeneration so far; reparation at the most. Therefore it is only understandable that the application of MSCs in tissue engineering are raising hopes for faster healing and full repair, if not regeneration in the horse after musculoskeletal injuries (Koch et al., 2009).

In 2003 Smith and colleagues first reported the re-implantation of autologous BM-MSCs into the superficial digital flexor **tendon** in a horse. The BM-MSCs were expanded in vitro and re-implanted into a superficial digital flexor tendon defect of an 11 years old athlete under ultrasonography control, 5 weeks after the injury occurred. Re-examination of the horse after 10 days and 6 weeks after implantation of the MSCs revealed no lameness and no thickening in the region of the treated tendon. Although Smith`s experiment was a one-case study without a control horse, the positive outcome of the study showed the possibility of this therapy form, revealed no negative side effects and definitely opened the door for further research efforts in this field. Smiths` vision was to one day gain full regeneration of the tendon tissue by production of actual tendon matrix through MSCs, without the appearance of scar tissue.

More recent and extensive studies were performed by Burk and Brehm (2011) and Watts et al. (2011). Here the treatment of tendon lesions with stem cells was significantly more successful than the treatment without it. In a long-term

follow-up study Godwin et al. (2012) assessed the safety and the re-injury rate of racehorses with tendon injuries which were treated with BM-MSCs. Godwin and colleagues reported that the treatment is safe with no observable side effects and a reduction of the reinjury rate.

The positive influence of ASCs on tendon repair in horses has been reported by Nixon et al. (2008) in an animal trial and by Del Bue et al. (2008) in a clinical study. Both research groups reported about no adverse effects but good recovery and improved tendon healing.

Wilke and colleagues (2007) carried out a study about the influence of bone marrow derived MSC-implantation in **cartilage** healing in horses. Therefore size defined lesions were set in the cartilage of both femoropatellar joints of six healthy horses. Then one joint of each horse was treated with a cell-fibrinogen mixture which polymerized in situ while the defect of the other joint was just treated with fibrinogen. Re-examination after 30 days revealed a significantly improved arthroscopic score for MSC implanted defects and increased fibrous tissue, although assessments after 8 months did not show any significant differences between the stem cell treated group and the control group. This study showed the successful use of MSCs in cartilage lesions where they improved the early healing response.

Also the possibility of MSC-usage in **bone** repair has been explored lately. O`Rielly et al. (1998) reported the successful healing of a chronic comminuted fracture of the fibula in a horse after applying a cancellous bone graft in the fracture site.

Honnas et al. (1995) explored the application of an autologous cancellous bone grafting in the treatment of navicular bursitis and sesmoid osteomyelitis in horses with a promising outcome.

Stem cells and tissue engineering have not been interesting for **adipose tissue** regeneration yet. But fat not just serves as energy storage it also is an important gland where the production of many hormones is located. Research about adipose tissue function and its role in metabolic diseases became more and more attractive since we see the source of some of the main lifestyle diseases in the hormonal dysfunction of the fat tissue (Ferris and Crowther, 2011). In reference to the horse the metabolic syndrome is probably the most important disease in our times, caused by hormonal dysfunction of adipocytes.

The metabolic syndrome is characterized by an insulin resistance, which leads in an energy deficiency in muscle and liver while the fat tissue stores more energy. Because of the insulin deficiency the blood sugar stays high which leads in the damage of diverse tissues, especially in the hoof where it causes laminitis. In this disease a decreased expression of the hormone adiponectin can be observed, which has the capacity of increasing insulin sensitivity, glucose uptake and fat oxidation. It also acts anti-inflammatory (Berg et al., 2001, Fruebis et al., 2001, Yamauchi et al., 2001, Mohan et al., 2005, Gilardini et al., 2006). Therefore future experiments with in vitro differentiated adipose tissue and the possible application of its produced hormones (e.g. adiponectin) in vivo might be of great interest.

1.5 Future prospects of the usage of pre-differentiated stem cells in vivo

Stem cell therapy with purified and multiplied stem cells is already a well established instrument in equine regenerative medicine. As a further development of that method in vitro pre-differentiated stem cells could possibly result in faster regeneration after application in vivo.
Hopes are, that in the framework of tissue engineering, stem cells can be multiplied and differentiated in vitro into full functional tissue to be transferred into focal tissue defects in horses and gain improved repair, if not regeneration (Koch et al., 2009). To be able to do so, culture conditions, media and supplements, scaffolds and environmental conditions have to be optimized.

1.6 Special aspects of stem cell differentiation in vitro

1.6.1 The influence of oxygen tension on stem cell differentiation towards the adipogenic, osteogenic and chondrogenic lineage in vitro

Low **O_2 tension** is known to be a powerful regulator of major aspects of stem cell function including survival, proliferation, viability, differentiation and migration (Simon and Keith, 2008). However, the O_2 related potency is often overlooked in concepts developing tissue engineering approaches in vitro. Routinely, in vitro differentiation takes place under normoxic conditions (21%

O_2). As it is known that the physiological conditions in different tissues in the living organism are hypoxic, for example the O_2 concentration in the bone ranges from 1-7% (Fehrer et al., 2007), in deep zones of articular cartilage it is even less than 1% O_2 (Silver, 1975) and the mean tissue level of oxygen is about 3% O_2 (Csete, 2005), this has to be taken into account for cell culture studies. Although the effect of hypoxia on equine stem cells has not been examined yet, comparable, recent experiments with human and murine MSCs showed a variety of different results which are rather contradictory. They either reported that hypoxia decreases the differentiation potency of MSCs (D`Ippolito et al., 2006, Fehrer et al., 2007, Holzwarth, 2010, Raheja et al., 2010, Valorani et al., 2010) or that it increases the differentiation capacity of the cells (Markway et al., 2010, Meyer et al., 2010). In conclusion, species, origin of the tissue, sex, age and culture passage have a huge influence on the outcome of the results, which makes a species specific examination and a standardisation of the research conditions more than necessary.

1.6.2 Cyclic strain, growth differentiation factors and oxygen tension as stimuli for tenogenic differentiation in vitro

Tendons belong to the fibrous connective tissue and make the connection between bone and muscle. The primary function of connective tissue is supportive. Tendons are capable of tolerating enormous tension. Tendon tissue contains cells and extracellular matrix. Fibroblasts are the specific cells in this tissue type. They produce the extracellular matrix and can be described as spindle-shaped cells with irregular pattern. The cytoplasm contains the euchromatic nucleus and numerous other organelles (endoplasmic reticulum (ER), ribosomes, golgi apparatus and mitochondria), which mainly have the task to produce proteins. The extracellular components are fibres and ground substance. Tendon cells produce about 95% collagen I and 5% collagen III (Amiel et al., 1984; Riechert et a., 2001). The type I collagen contributes tensile strength by forming inelastic fibres; collagen III contributes elasticity by forming loosely organized fibrils (Benhardt and Cosgriff-Hernandez, 2009). Ribosomes and golgi apparatus produce procollagen in the cell. By exocytosis the procollagen reaches the extracellular space and is cut into smaller

tropocollagen units before they get connected to fibrils (s. fig. 3). The ground substance is mainly made out of mucoproteins and mucopolysaccharides and is able to bind water. After tissue injuries and during inflammation free fluid accumulates in this area (Rhodin, 1974).

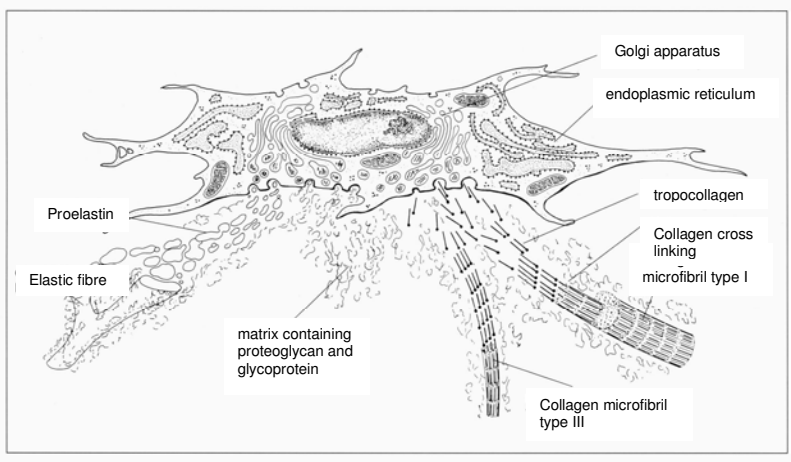


Fig. 3: Scheme of intra- and extracellular fibrogenesis and regeneration of intercellular matrix of a fibroblast (modified according to Liebich, 1999).

To create tendon tissue with full functional fibroblasts which produce extra cellular matrix that lines up in ordered direction and withstands tensile strain, some specific conditions in vitro have to be fulfilled to differentiate MSCs towards the tenogenic lineage.

Because of the high interest in tendon tissue engineering (s. above) a lot of research in this area has already been done and diverse factors that influence and stimulate tendon formation have been discovered, even though their interactions have not been examined yet in an in vitro study with equine stem cells. Consequently the search for the optimal parameters to create tendon tissue has to be continued.

Of special interest are **growth differentiation factors** (GDFs), especially GDF 5, 6 and 7, members of the TGF-β gene superfamily, also known under the term bone morphogenetic protein (BMP).

Wolfman et al. (1997) described tendon/ligament tissue formation in rats after GDF-carrier implantation in subcutaneously respectively intramuscular areas. On the one hand he showed with this in vivo experiment that progenitor cells capable of synthesizing dense connective tissue are present in these locations and on the other hand it was demonstrated that tenogenic differentiation can be induced by GDF 5, 6 or 7. Rickert et al. (2001) examined the influence of GDF 5 coated sutures in tendon healing after reconstructive surgery. The results of his study revealed that the usage of GDF 5 coated sutures compared with uncoated sutures led into thicker and stiffer tendons. Later on in vitro experiments were carried out. In 2010 Park and colleagues examined ECM synthesis, tenogenic differentiation and matrix gene expression of ASCs under the influence of different concentrations of GDF 5. A concentration of 100 ng/ ml led to enhanced ECM (amongst others collagen I) and tenogenic marker gene expression, e. g. scleraxis.

Besides the usage of differentiation factors it turned out that mechanical stimulation is necessary to direct the orientation of the ECM fibres and to gain matrix stiffness. ECM synthesis and remodelling is carried out through **mechanotransduction**, which refers to a cellular mechanism that converts a mechanical stimulus into a biomechanical signal, which leads in cell proliferation, differentiation and ECM synthesis (Giancotti, 1997).

Integrins, transmembrane ECM receptors, have a major role in the pathway of transmitting mechanical signals. The cytoplasmic portion of the integrin is mechanically coupled to the internal **actin cytoskeleton** by amongst others **vinculin**, an actin associated molecule. This construct is part of the **focal adhesion complex (FAC)**, which mediates cell-ECM and cell-cell adhesions. When cells attach to the ECM substrate, they tend to retract it (Chicurel et al. 1998).

While mitogen-activated protein kinase (MAPK) initiates gene signalling in the process of collagen generation, matrix metalloproteinases (MMP) regulate collagen degradation in relation to mechanical loading, especially collagenase (MMP-1 and MMP-8) for collagen type I and III degradation, s. fig. 4 (Kjaer, 2004).

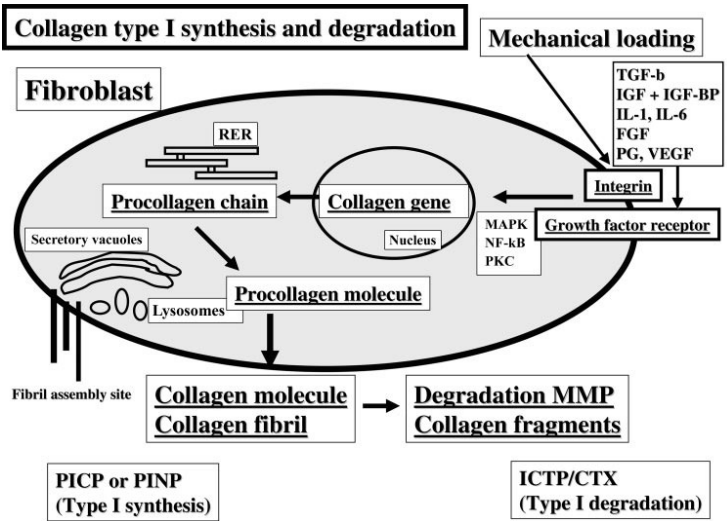


Fig. 4: Pathways involved in the collagen synthesis and degradation according to mechanical stimuli and growth factor signalling.

Here the scheme of collagen I synthesis is shown, but can be transferred on other collagen types due to high similarities. Also the growth factors shown in the scheme are just exemplary and applied amongst others for GDFs, too. (Kjaer, 2004)

It is propagated that **gap junctions**, containing diverse connexin proteins, are necessary to transfer the original mechanical signal from cell to cell in order to coordinate the collagen synthesis (McNeilly et al., 1996, Stanley et al., 2007). “Gap junctions, the only channels that allow direct exchange of small metabolites between cells, are composed of a family of integral membrane proteins, called connexins in vertebrates” (Goldberg et al., 1999).

Connexins (Cx) are referred to by their predicted molecular weight, e.g. 32kDa for Cx32 and 43kDa for Cx43. One connexon contains 6 connexin monomers. The intercellular joining of two connexons of two adjacent cells form an intercellular channel, a gap junction complex, s. fig. 5 (Goodenough and Paul, 2003).

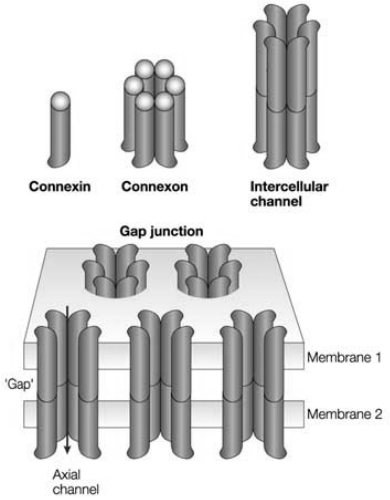


Fig. 5: Composition of connexons and gap junctions for intercellular communication. (Goodenough and Paul, 2003)

Various studies about the influence of mechanical stimulation on stem cells and fibroblasts have been carried out. Mostly the cells were seeded on collagen I sponges or on collagen I gel scaffolds and were then stretched with static or cyclic strain. All authors of the reviewed papers report consistently about enhanced linear stiffness, increased collagen production, tendon like organisation of the collagen matrix, parallel organisation of the fibres and/or higher expression of tendon specific genes (e. g. scleraxis, collagen I and III) than in mechanically non-stimulated cells (Kall et al., 2004, Juncosa-Melvin et al., 2006, Webb et al., 2006, Butler et al., 2008, Kuo and Tuan, 2008, Chokalingam et al., 2009, Nirmalanandhan et al., 2009, Scott et al., 2011).

The collagen I sponge, respectively the collagen I gel imitates the tendon like environment, gives the possibility to apply strain to the cells and also is a possible scaffold for in vivo implantation. For the realisation of the stretching experiments diverse bioreactors were used. They allow to mechanically and chemically stimulate cells and tissue-engineered constructs in culture. Common requirements are that the machine guarantees a controlled environment during the mechanical stimulation and that the stimulation of

multiple constructs with identical or individual waveforms is possible (Butler et al., 2009).

Only few research groups have reported about a combined study so far, that examines the interactions of single factors. Farng et al. (2008) put the factors GDF 5 and static respectively cyclic strain together in a study. The results showed that both factors alone increased collagen I- , collagen III- and scleraxis production, but an additive synergism between mechanical and biological stimulus could not be observed.

However, the influence of oxygen tension as one factor or in combination with others on tenogenic differentiation, especially in equine has not been examined yet. The importance of this factor on in vitro differentiation has been pointed out extensively in this chapter.

As a continuation of earlier investigations in our department, the goal of this study is to optimize the parameters oxygen tension, tensile stimulation and the application of GDFs to create full functional tendon tissue. A previous study in our institute has already shown that equine ASCs differentiate better along the tenogenic pathway under the usage of mechanical stimulation. The morphology of the cells looks more like tenocytes, the ECM is lined up in one ordered direction and the used scaffold appears to be considerably stiffer compared to the undilated control group.

2 Research aims and experimental design

2.1 Research aims

Following points shall be examined:

1. Influence of low oxygen conditions (exact 3% O_2) on the stem cell characteristics, e.g. cell morphology and stem cell marker CD90 expression.
2. Proliferation rate under the influence of hypoxic conditions (3% O_2) compared with normoxic (21% O_2) conditions.
3. Control if sufficient oxygen reduction is reached, respectively how the ASCs sense the oxygen reduction and if it puts the cells under hypoxic stress.
4. Influence of low oxygen conditions (exact 3% O2) on the differentiation potential of equine adipose tissue derived stem cells (ASCs) towards the adipogenic, osteogenic and chondrogenic lineage.
5. Influence of low oxygen conditions (exact 3% O2) on the differentiation potential of ASCs towards the tenogenic lineage.
6. Influence of tensile stimulation on the tenogenic differentiation.
7. Influence of diverse growth differentiation factors on the tenogenic differentiation.
8. Finding the best in vitro differentiation conditions towards the tenogenic lineage (combination of oxygen concentration, tensile stimulation and GDF supplement), by comparing cell morphology and for tendon tissue relevant gene expression.

2.2 Experimental design

2.2.1 Stem cell behavior and differentiation potential towards the adipogenic, osteogenic and chondrogenic lineage under the influence of 3% and 21% oxygen tension

Adipose tissue was collected from four mixed breed horses (aged, mean ± SD, 4.75 ± 1.71 years) either at the local slaughterhouse or the institute of veterinary pathology at the University of Giessen. Tissue samples were taken from the region lateral to the base of the tail.

Tab. 1: Horses in the study from which the fat tissue samples were collected

Horses in the study			
Identification number	Gender	Age	Passage
horse 12/08	mare	5 years	2
horse 05/09	gelding	3 years	1
horse 72/09	mare	7 years	2
horse 80/10	gelding	4 years	1

ASCs were extracted from the collected tissue samples and cultivated in an incubator at 37°C in a humidified atmosphere with 5% CO_2 and either with 3% O_2 or with 21% O_2. Afterwards the proliferation abilities and the stem cell characteristics of the ASCs were examined and the results of both oxygen conditions were compared. The ability and the degree of the adipogenic, osteogenic and chondrogenic differentiation were examined histological, immunohistochemical and with molecular biological techniques. For all experiments exclusively cells in passage one and two were used in order to use clinical relevant passages.

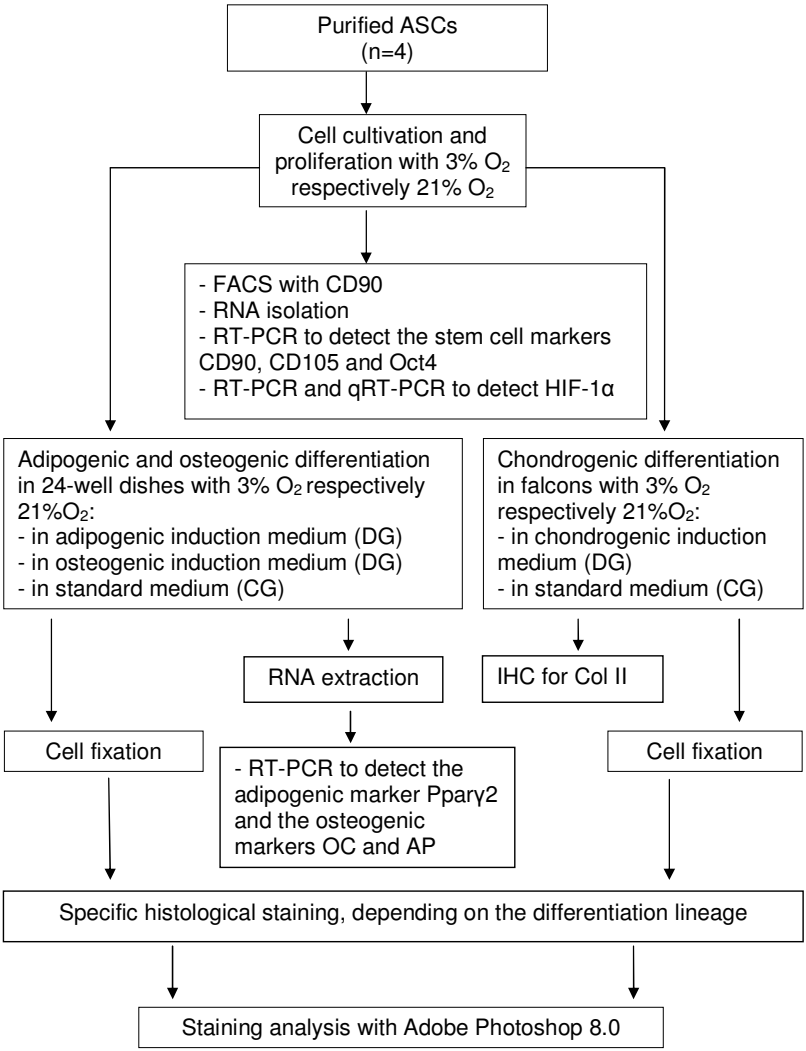


Fig. 6: Experimental setup for the differentiation experiment under different oxygen conditions.

DG= differentiation group; CG= control group; IHC= Immunohistochemistry; OC= osteocalcin; AP= alcaline phosphatase; Col II= collagen II

2.2.2 Differentiation potential towards the tenogenic lineage under the influence of applied strain, 3% and 21% oxygen tension and several growth differentiation factors in a 3d gel scaffold

The ASCs of the same four horses as in the first experiment were used for the tenogenic differentiation experiment. The cells were cultured with 3% O_2 and 21% O_2 respectively. After reaching about 80% of confluence, they were detached from the culture dish and sent to the biotechnological company Amedrix (Stuttgart, Germany), where the cells were incorporated in a collagen I gel scaffold and sent back to us.

The gel was cut into pieces and sutured into the chambers of a bioreactor, where they remained for 21 days in an incubator at 37°C in a humidified atmosphere with 5% CO_2 and 3% O_2, respectively 21% O_2. A strain of 4% of the gel length was periodically applied and in each chamber a different medium was added; one negative control (NC) with tenogenic induction medium and four chambers with tenogenic induction medium plus the growth differentiation factor GDF 5, GDF 6, GDF 7 respectively a combination of all three factors. Also one negative control without applied strain (NCws) and without any GDFs and even a negative control just covered with DMEM was performed.

After the differentiation period RNA for RT-PCR and qRT-PCR was extracted. Primers for RT-PCR/ qRT-PCR were designed to detect collagen I (Col I), collagen III (Col III), scleraxis (Scx) and cartilage oligomeric matrix protein (COMP) in order to analyse the production of extra cellular matrix typically for tendons. Immunohistochemistry (IHC) was performed to examine the structure and the formation of the cells in the gel. Using antibodies for Col III, connexion 32 (Cx32) and connexion 43 (Cx43), an analysis of tendon significant extra cellular matrix and formed cell contacts was done. To gain insight into the cell structure in detail, electron microscopy was performed.

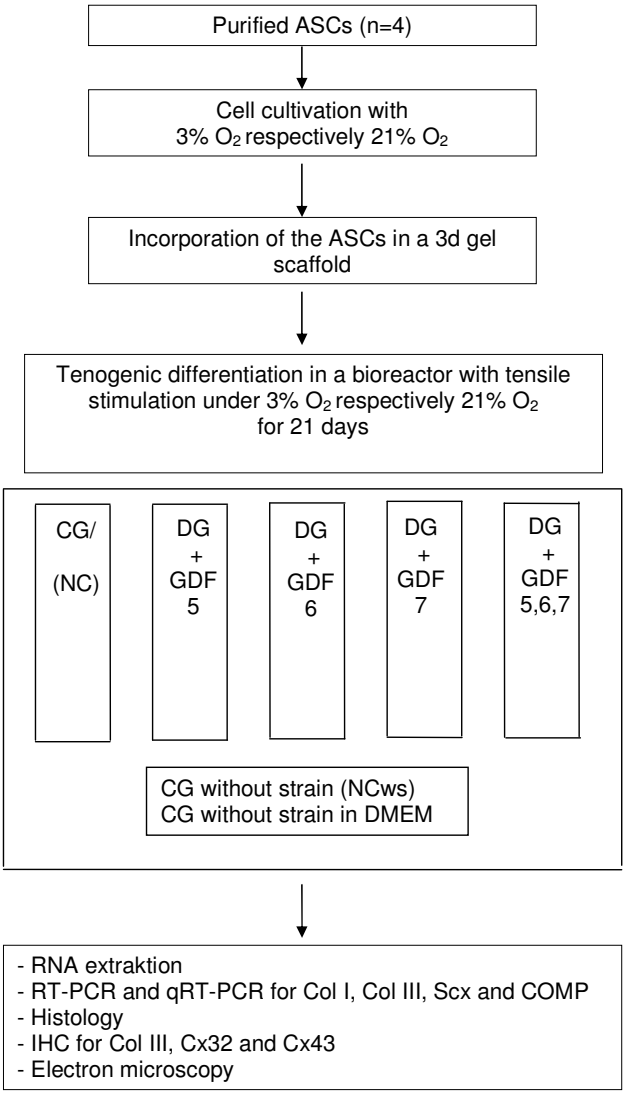


Fig. 7: Experimental setup for the tenogenic differentiation experiment.

CG= control group with standard medium; NC= negative control; DG=differentiation group with tenogenic induction medium; GDF= growth differentiation factor;

IHC= Immunohistochemistry; Col= collagen; Scx= scleraxis; COMP= cartilage oligomeric matrix protein; Cx= connexin

3 Material and methods

3.1 Materials

3.1.1 Dilutions and reagents for the cell culture

β-glycerolphosphate	Sigma, Germany
Agarose	Bioline, Germany
Alcohol	Roth, Germany
Antibiotic/Antifungal agent (A/A)	PAA, Germany
Accutase	PAA, Germany
Ascorbic acid	Sigma, Germany
Ascorbic acid-2-phosphate	Sigma, Germany
BSA (bovine serum albumin)	PAA, Germany
Collagenase I	Biochrom AG, Germany
Dexamethasone	Sigma, Germany
Dulbecco's Modified Eagle Medium (DMEM)	
low glucose	Invitrogen, Germany
DMSO (dimethyl sulfoxide/C_2H_6OS)	Sigma, Germany
FBS (foetal bovine serum)	PAA, Germany
Fish collagen (hydrolysed)	Norland Products Inc., USA
GDF 5	Biochrom, Germany
GDF 6	Abcam, UK
GDF 7	R&D Systems, Germany
Indomethacin	Sigma, Germany
3-isobutyl-1-methylxanthine	Sigma, Germany
ITS (insulin-transferrin-selenium) x 100	Sigma, Germany
PBS (phosphate buffered saline)	Invitrogen, Germany
Proline	Sigma, Germany
P/S (penicillin, streptomycin)	PAA, Germany
Sodium pyruvate	Sigma, Germany
Trypan blue 0.4%- solution	Sigma, Germany

3.1.2 Reagents used for staining, fixation and embedding

ABC complex	Biologo, Germany
Acetone	Merck, Germany
Acitic acid ($C_2H_4O_2$)	Roth, Germany
AEC substrate kit	Biologo, Germany
Alcian blue	Roth, Germany
Aluminium sulphate	Merck, Germany
Ammonium iron sulphate	Merck, Germany
Azure II	Merck, Germany
Borax	Merck, Germany
Caustic potash	
Cacodylate buffer	Merck, Germany
Citric acid	Merck, Germany
Collodion	Plano, Germany
Copper nets	Plano, Germany
Eosin	Thermo Scientific
Epon	Serva/ Plano, Germany
Ethanol 50%, 70% and 100%	Merck/ Roth, Germany
Formaldehyde 4%	Merck, Germany
Glacial acetic acid	Merck, Germany
Goat serum	Sigma, Germany
Gold chloride	
Glutaraldehyde	Merck, Germany
Haematoxylin	Thermo Scientific
Hydrochloride acid	Sigma, Germany
Kaiser Gelatine	Merck, Germany
Lead citrate	Leica- Microsystems, Germany
Methylene blue	Merck, Germany
Nuclear fast red	Merck, Germany
Oil Red O	Sigma, Germany
Osmium tetroxide	Roth, Germany
Paraffin	Vogel, Germany
Paraformaldehyde	Merck/ Roth, Germany

Potassium permanganate	Merck, Germany
Potassium disulphide	Merck, Germany
Silver nitrate	Roth, Germany
Sodium carbonate	Roth, Germany
Sodium chloride	Roth, Germany
Sodium citrate	Merck, Germany
Sodium thiosulphate	Merck, Germany
Toluidine blue	Merck, Germany
Tris Base	Roth, Germany
Uranyl acetate	Leica- Microsystems, Germany
Xylene	Merck, Germany

Used antibodies:

Primary antibody	Rabbit anti-collagen type III human (1:50), Abcam, Germany
Primary antibody	Rabbit anti-collagen type I human (1:50), Biomex, Germany
Primary antibody	Mouse anti-collagen type II human (1:100), Calbiochem, Germany
Primary antibody	Mouse anti-connexin 32 (1:100), Invitrogen, Germany
Primary antibody	Mouse anti-connexin 43 (1:100), Invitrogen, Germany
Secondary antibody	Goat anti-mouse (1:200), Dako, Germany
Secondary antibody	Goat anti-rabbit (1:200), Dako Cytomation, Germany

3.1.3 Reagents RT-PCR and qRT-PCR

50 bp DNA ladder	Biozym, Germany
Agarose	Bioline, Germany
DNase I	Roche, Germany
DNase I Recombinant buffer	Applied Biosystems, USA
EDTA (TBE)	Roth, Germany

GeneAmp Gold RNA PCR Core Kit	Applied Biosystem, Germany
IQSYBR Green Supermix	Bio-Rad, USA
Primer	Eurofins MWG, Germany
SYBR-Green	Sigma, Germany
TriFast peqGold	Peqlab, Germany
Tris boracic acid	Roth, Germany

3.1.4 Reagents for Flow Cytometry

Antibody mouse anti-human CD 90	BD, Biosciences, Belgium
Antibody anti-mouse IgG1-FITC (fluorochrome fluorescein isothioczanate)	Southern Biotech, USA
Antibody mouse IgG1	AbD Serotec, UK

3.1.5 Equipment

Autoclave “Systec DE-65”	Systec GmbH, Germany
Balance “PB 3002- S Delta Range”	Mettler, Spain
Balance “Discovery”	Ohaus, Germany
Bioreactor	work shop Giessen University, Germany
Centrifuge “Heraeus Megafuge 11R”	Thermo Scientific Inc., USA
Centrifuge “Mikro 20“	Hettrich, Germany
Centrifuge “Mikro 220R”	Hettrich, Germany
Clean bench	Thermo Scientific Inc., USA
Cytomation pen	Dako, Germany
Electron microscope “EM109”	Carl Zeiss, Germany
Electrophoresis device “Power Pac tm Basic”	Bio- Rad, USA
Embedding mashine “EG1160”	Leica, Germany
Dewatering mashine “Leica TP1050“	Leica, Germany
Flow cytometer “FACSCalibur™”	Becton Dickinson, USA
Fluorescence microscope	Carl Zeiss, Germany
Freezing container	Nalgene, USA
Fridge/freezer combination	Liebherr, Germany

Gel electrophoresis chamber „XS/S“	Biometra, Germany
Hemocytometer “NI”	Digital Bio, Korea
Humidity chamber	Kreatech Diagnostics, Netherlands
Ice bin	Magic Touch Ice Wares, USA
Incubator “Hera cell 150”	Thermo Scientific Inc., USA
Inverted phase contrast microscope	Carl Zeiss, Germany
Magnetic stirrer “IkaMAG RET”	Janke & Kunkel, Germany
Microwave Privileg 8520	Electrolux, Germany
Microtome “Leica SM 2000 R”	Leica, Germany
Nitrogen tank	Thermo Fischer Scientific, Germany
PH-electrode “SenTix 41”	WTW, Germany
Photometer	Eppendorf, Germany
Pipettor “Pipetus”	Hirschmann Laborgeräte, Germany
Real time cycler “CFX96”	Bio-Rad, USA
Spectrophotometer reader (570 nm)	Tecan, Austria
Steriliser “Heraeus”	Kendo Laboratory Products, Germany
Thermocycler “DNA Engine”	Bio-Rad, USA
Vibraxer “VF2”	IKA Werke GmbH & Co. KG, Germany
Vortex machine	IKA Werke GmbH & Co. KG, Germany
Water bath	GFL, Germany

3.1.6 Consumption items

Chamber slides	Thermo Fischer, Germany
Collagen I gel	Amedrix GmbH, Germany
Cover slips	Roth, Germany
Culture dishes 60x15 mm	Greiner Bio-One, Germany
Culture flasks 25 cm^2, 75 cm^2	Greiner Bio-One, Germany

Cuvettes	Eppendorf, Germany
Cryo tubes 1 ml	Greiner Bio-One, Germany
Suture material	Ethicon, Germany
Syringes 2 ml, 5 ml, 10 ml	Braun, Germany
PCR tubes 0.5 ml	Sarstedt, Germany
Falcons 15 ml	Greiner Bio-One, Germany
Falcons 60 ml	Sarstedt, Germany
Filters 0.2 µm, 70 µm	Sarstedt, Germany
Microscope cover glasses 13 mm	VWR, Germany
Glass slides	R.Langenbrick (RL), Germany
Pipettes (glass), 1; 2; 5; 10; 20 ml	Schuett-biotec, Germany
Pipettes (plastic), 25 ml	Sarstedt, Germany
Pipette tips 20 µl, 100 µl, 200 µl, 1000µl	Ratiolab, Germany
Scalpel blades 26er	Bayha, Germany
Water stabilizer “AquaStab”	Lauda Dr. R. Wobser GmbH & Co. KG, Germany
24-well dishes	Greiner Bio-One GmbH, Germany

3.1.7 Software

Adobe Photoshop 8.0	Adobe Systems, USA
Axiovision image analysis	Carl Zeiss, Germany
Bio-Rad CFX Manager 2.0	Bio-Rad, USA
FACS Express, version 2	De Novo, Canada
SPSS, version 19.0	IBM, Germany
UVIdoc software	Biometra, Germany

3.2 Methods

3.2.1 Adipose tissue harvest

Adipose tissue was either obtained from horses slaughtered at the abattoir in Giessen or from horses delivered at the Institute of Veterinary Pathology, Justus-Liebig University of Giessen. The region lateral to the base of the tail, above the gluteal muscle was sterile prepared with alcohol. Afterwards approximately 10 gram of fat tissue were cut out with a sterile scalpel blade. The tissue was transferred into a tube containing PBS and transported to the cell culture laboratory.

3.2.2 Isolation of the stem cells

Equine ASCs were isolated by collagenase type I digestion as described previously by Raabe et al. (2010).

Under sterile conditions the fat tissue was cut into small pieces and was washed with PBS (0.2 µM). For every washing step a centrifugation period of 5 minutes with 260 g was performed. Afterwards the PBS supernatant was sucked off using plastic pipettes and was replaced by a digestion solution. For the digestion process the mix was placed on a vibraxer located in an incubator with 37°C for up to 60 minutes. The digested fat was subsequently separated from the cell mass by centrifugation (260 g for 5 minutes). The cell pellet was filtered using a 70 µm filter and was washed with PBS. Afterwards the cells were resuspended in 1 ml standard medium and counted, using a hemocytometer.

<u>Ingredients for the digestion solution per 0.5 ml v/v fat tissue:</u>

1 mg collagenase I

10 mg BSA

1 ml PBS

3.2.3 Cell cultivation

Cells were seeded in culture flasks (75 cm^2) containing 10 ml standard medium and incubated at 37°C in a humidified atmosphere with 5% CO_2 and 21% O_2, respectively 3% O_2. Medium exchange was performed three times per week in order to remove products of cell metabolism and to supply the cells with nutrients.

Ingredients for the standard medium:
DMEM low glucose
10% FBS
1% P/S (penicillin 100 U/ml, streptomycin 0.1 mg/ml)

3.2.4 Cell passaging

When the adherent cells had reached 80% of confluence, they were detached from the culture dish in order to either be used for differentiation experiments or to be stored by cryopreservation.
Therefore the medium above the cells was sucked off and non-adherent cells and cell detritus was washed off by PBS. The adherent cells were detached by applying approximately 5 ml accutase and an incubation time of 7 minutes at 37°C. Afterwards 5 ml of standard medium were added to the accutase-cell solution in order to stop the reaction. The whole solution was transferred into a tube with another 5-8 ml standard medium. Following, the cells were separated from the accutase-medium mix by centrifugation with 100 g for 5 minutes. The supernatant was sucked off and the cell pellet was resuspended with 1 ml standard medium. The total amount of cells was estimated using a hemocytometer.

3.2.5 Cryopreservation

If cells should be preserved for a long period of time in order to be used for further experiments, the method of cryopreservation was applied.

Therefore 5 ml of cold freezing medium were added to the detached cells (see cell passaging) in cryo tubes, placed into a freezing container and stored in the fridge for 15 minutes at 4°C, followed by 1 hour at -20°C and up to 12 hours at -80°C before they were finally stored in liquid nitrogen. The process of decreasing the temperature slowly is supposed to give the cells the chance to adapt to a lower temperature and helps to reduce cell death according to environmental stress.

Ingredients for the freezing medium:
65% DMEM
30% FBS
5% DMSO

3.2.6 Thawing process

In order to thaw stem cells, the cryo tubes were taken out of the liquid nitrogen and placed into a warm water bath of 37°C for 30 seconds. Afterwards 0.5 ml of cold standard medium were added, before the cell solution was transferred into a falcon containing warm standard medium. Like in the process of freezing the cells, the thawing process aims to rise the temperature slowly in order to preserve the cells from thermal shock and therefore from cell membrane damage. Using centrifugation (100 g for 5 minutes) the cells were separated from the medium, the medium containing the freezing medium was sucked off and replaced with 1 ml standard medium, so the cells could be counted before they were used for further experiments. Also a trypan blue stain was performed to estimate the amount of vivid cells.

3.2.7 Flow Cytometry

To determine the pureness of the acquired cell population according to their surface marker expression and their identification as stem cells and to detect possible differences in the cell populations cultured under different oxygen conditions, $2x10^5$ cells of each horse cultured under both oxygen conditions, 3% O_2 and 21% O_2 respectively, were incubated with the primary mouse anti-

human CD90 antibody (1:800) and labeled with the secondary antibody anti - mouse IgG1-FITC (1:300) carrying the fluorochrome fluorescein isothiocyanate (FITC). The fluorescence emitted by FITC was induced by a laser (Argon, 488 nm) and measured by the flow cytometer. As negative control $2x10^5$ cells diluted in a solution of PBS containing 1% BSA, 0,1% sodium acid and 0,5% goat serum were used. The antibody mouse IgG1 (1:800) combined with the secondary antibody was used for isotype control. The measured results were analyzed with the software FACS Express, Version 2.

3.2.8 Cell proliferation

3.2.8.1 Colony-forming unit (CFU) assay

Passage 2 ASCs of four horses were plated at a density of 100, 500, 1000 and 2000 cells/25cm^2 bottles and cultured with standard medium at 37°C, 5% CO_2 and 3% O_2 respectively 21% O_2 for 7 days. Afterwards the cells were fixed and stained with 1% cresyl violet in 100% methanol. After drying, colonies with 50 or more cells were counted.

3.2.8.2 MTT assay

In order to compare the cell proliferation under 21% O_2 and 3% O_2 the MTT assay was performed. $1.5x10^4$ cells per well were seeded in a 24-well plate. Cell viability was evaluated using a colorimetric MTT assay measuring reduction power. Briefly, 0.5 mg/ml of 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to the cells cultivated in DMEM and incubated for 24 and 48 hours under 3% and 21% O_2 tension. 200 µl dimethyl sulfoxide (DMSO) solution was then added to dissolve the water-insoluble formazan salt. Quantification was conducted with a spectrophotometer reader at 570 nm.

3.2.9 In vitro differentiation

For in vitro differentiation only ASCs in passage one and two were used, as it would be realistic for praxis relevant application. Culture conditions were 37°C in a humidified atmosphere with 5% CO_2 and 21% O_2, respectively 3% O_2. In vitro differentiation was carried out with minor changes as described previously by Arnhold et al. (2007).

3.2.9.1 Adipogenic differentiation

Adipogenic differentiation was performed by culturing ASCs for 7, respectively 14 days in adipogenic induction medium. Therefore $3x10^3$ cells/cm^2 were seeded on microscope cover glasses in 24-well dishes and covered with 0.5 ml of adipogenic induction medium. Medium change was performed three times per week.

Ingredients for the adipogenic induction medium:
DMEM low glucose
10% FBS
1% P/S
1 µM dexamethasone
1:100 v/v Insulin-transferrin-selenium (ITS)
[1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, and 0.5 g/ml sodium selenite]
0.5 mM (IBMX) 3-isobutyl-1-methylxanthine
100 µM indomethacin

3.2.9.2 Osteogenic differentiation

Osteogenic differentiation of ASCs was taken out for 7, 14 respectively 21 days, using osteogenic induction medium. Therefore $3x10^3$ cells per cm^2 were seeded on microscope cover glasses in 24-well dishes and covered with 0.5 ml of the osteogenic induction medium. Medium change was performed three times per week.

Ingredients for the osteogenic induction medium:

DMEM low glucose
10% FBS
1% P/S
0.05 mM ascorbic acid-2-phosphate
10 mM β-glycerolphosphate
0.1 µM dexamethasone

3.2.9.3 Chondrogenic differentiation

Chondrogenic differentiation was induced by culturing ASCs for 21 days in a 3d pellet culture. Therefore $3x10^5$ cells were seeded in 15 ml tubes containing 3 ml of the chondrogenic induction medium each, followed by centrifugation at 500 g for 5 minutes.

Ingredients for the chondrogenic induction medium:

DMEM low glucose
1% P/S
1:100 v/v Insulin-transferrin-selenium (ITS)
0.1 µM dexamethasone
0.05 mM ascorbic acid
50 µM l-proline
1 mM sodium pyruvate
0.5 mg/ml hydrolyzed fish collagen

3.2.9.4 Tenogenic differentiation

For the tenogenic differentiation approximately $3x10^7$ stem cells were sent to the company Amedrix (Amedrix GmbH, Germany) where the cells were incorporated into a collagen gel, serving as a 3d scaffold. The gel contained collagen I, which was gained from the tail of rats. The gel was compacted using a pressure that was measured with 3.2 kPa, thus the elasticity of the gel construct is estimated with the same take-up. One gel was roughly 20 cm long, 2.5 cm wide and 2.5 mm thick. In the cell culture laboratory the gel was

then cut into pieces under sterile conditions. Each piece measured about 3.5 cm by 2 cm. The gel pieces were then sutured into the chambers of the bioreactor, using a simple continuous pattern, a not traumatic needle and a monofilament fibre. The gel in the chamber was covered with 10 ml tenogenic induction medium and remained in the stretching machine for 21 days. Medium change was performed three times per week. For the whole time period the stretching machine remained in the incubator at 37°C with 21% O_2 respectively 3% O_2. Every gel in each chamber was cyclical stretched 4% of its own length for the duration of two hours, followed by a six hour pause. Imitating the physiological stretching conditions for tendons and ligaments in vivo, an uniaxial stretching system was applied by the bioreactor.
Depending on the experimental design, 80 µl of GDF 5 or GDF 6 or 8 µl of GDF 7, respectively a combination of those three factors were added to the specific chamber with each medium change (see experimental design).

Ingredients for the tenogenic induction medium:
DMEM low glucose
1% (A/A) antibiotic/antifungal agent
15% FBS
0.34 mM l- proline
0.17 mM ascorbic acid
where applicable (see experimental design) GDFs were added:
80 µl GDF 5 (10 ng/µl)
80 µl GDF 6 (10 ng/µl)
8 µl GDF 7 (100 ng/µl)
The GDFs were reconstituted according to the manufacturer`s protocol.

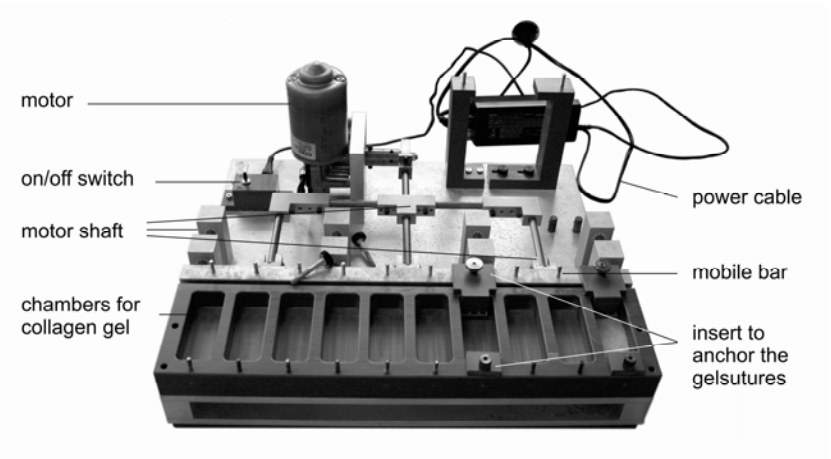


Fig. 8: Bioreactor with 10 chambers. In each chamber a collagen I gel containing ASCs was sutured, covered with medium and stretched periodically.

Stretching programme:

8 am – 10 am: stretching period

10 am – 4 pm: intermission

4 pm – 6 pm: stretching period

6 pm – 0 am: intermission

0 am – 2 am: stretching period

2 am – 8 am: intermission

3.2.10 Fixation and embedding

3.2.10.1 Fixation of the cells cultured in adipogenic and osteogenic induction medium, respectively the control group (CG)

Prior to staining the cells of the adipogenic and osteogenic differentiation lineage were fixed in 4% formaldehyde at room temperature for 30 minutes followed by several washing steps with 0.2 µM PBS and distilled water.

3.2.10.2 Fixation and embedding of the cell pellets of the chondrogenic differentiation lineage

After a culturing period of 21 days the pellets were fixed in 4% formaldehyde solution for 1 hour, dehydrated with 100% ethanol, washed with xylene and embedded in paraffin. Using a microtome, 5 µm thick cuts from the paraffin block were made, applied on glass slides and deparaffinized with xylene and ethanol.

3.2.10.3 Fixation and embedding of the gels of the tenogenic differentiation lineage

After remaining 21 days in the bioreactor with the tenogenic induction medium the gel of each chamber was taken out and cut into pieces under sterile conditions. Some of those pieces of each chamber were supposed to be used for different stains.
Therefore the gel pieces were fixed in 4% formaldehyde for 2.5 hours, washed with water for 1.5 hour and kept in PBS overnight. Afterwards they were dehydrated with 50% alcohol for 2.5 hours and kept in 70% alcohol until being embedded in paraffin, being cut and applied on slides.

3.2.11 Histological stains

3.2.11.1 Trypan blue

To estimate the amount of vital cells, the trypan blue stain was performed. Since the dye cannot penetrate intact cell membranes, only dead cells with a damaged cell membrane take up the blue colour.
To perform the staining, about 20 µl cell suspension were mixed with 80 µl trypan blue 0.4%. After an incubation time of approximately 2-5 min at 37°C the suspension was applied in a haemocytometer and the dead and the viable cells were counted under a microscope.

3.2.11.2 Oil Red O

To examine the extend of the adipogenic differentiation of the ASCs after 7 respectively 14 days in the adipogenic induction medium, the Oil Red O staining was performed, which specifically stains neutral triglyceride droplets. The fixed cells were incubated in 2% (wt/vol) Oil Red O reagent for 15 minutes at room temperature. Excess stain was removed by washing with 70% ethanol, followed by several rinsing steps with distilled water. The cells were counterstained with haematoxylin. The microscope cover glasses carrying the fixed and stained cells were taken out of the wells and applied on glass slides upside down, embedded in Kaiser Gelatine.

Recipe for Oil Red O solution:
100 ml 99% isopropyl alcohol
0.5 g Oil Red
66 ml distilled water
Warm the isopropyl alcohol up to 60°C and add the Oil Red. Let the solution sit for 24 hours and filter it afterwards. Dilute the stock solution with the distilled water, shake it and filter it one more time.

3.2.11.3 Von Kossa

After a culturing period of 7, 14 and 21 days in the osteogenic differentiation medium and after cell fixation, the von Kossa staining was performed to detect calcified extra cellular matrix. For this purpose, the cells were overlaid with a 5% (wt/vol) silver nitrate solution for 30 minutes. In order to remove surplus dye the cells were washed several times with distilled water. Afterwards some drops of 5% sodium carbonate formaldehyde solution were added for 5 minutes before being washed off by distilled water. In a next step Farmers reducer was applied for 30 seconds and then washed off again. Finally the cells were counterstained with nuclear fast red. The microscope cover glasses carrying the fixed and stained cells were taken out of the wells and applied on glass slides upside down, embedded in Kaiser Gelatine.

Recipe for sodium carbonate formaldehyde solution:

5 g sodium carbonate

25 ml formaldehyde 40%

75 ml distilled water

Dilute the sodium carbonate and the formaldehyde in the distilled water.

Recipe for Farmers reducer:

10 % sodium thiosulphate

10 % formaldehyde solution

Mix sodium thiosulphate in a relation of 20:1.

Recipe for nuclear fast red:

5 g aluminium sulphate

100 ml distilled water

0.1 g nuclear fast red

Dilute the aluminium sulphate in boiling distilled water, add the nuclear fast red and stir it, then filtrate the whole solution.

3.2.11.4 Alcian blue

To show the formation of extracellular proteoglycan after 21 days under the influence of the chondrogenic induction medium, the Alcian blue staining was applied for 30 minutes, rinsed with water and stained with nuclear fast red for 5 minutes.

Recipe for Alcian blue:

0.5 g Alcian blue

1 ml glacial acetic acid

100 ml distilled water

Sterile filtration of the solution

3.2.11.5 Hemalm and eosin staining:

With the hematoxylin colouration the cells in the gel construct were stained in order to make an announcement about the cell number in the scaffold and the location of the cells. The nuclei can be identified by blue colour uptake (hematoxylin), the cytoplasm appears red (eosin).
At first the cells were stained with hematoxylin for 5 minutes, afterwards the colour was rinsed off before the eosin was applied for about 30 seconds. The eosin was rinsed off with water, alcohol and then xylene.

Recipe for hematoxylin:
1 g haematoxylin
0.2 g sodium iodate
50 g kalinite
50 g chloral hydrate
1 g citric acid
Dilute the solids in the list above in 1000 ml demineralised water.

Recipe for eosin solution:
10 g eosin
1000 ml demineralised water
2 ml glacial acetic

3.2.11.6 Gomori silver staining:

To show the appearance and location of formed extracellular matrix in the collagen gel construct for tenogenic differentiation the Gomori silver staining was performed. With this method a specific staining of reticulin fibres/ collagen III (black) and other types of collagen fibres (reddish brown) is possible.

Recipe for the Gomori silver staining:
potassium permanganate (0.5%)
potassium disulphide solution (2%)
ammonium iron sulphate solution (2%)

ammoniac silver nitrate solution: silver nitrate solution (10%), caustic potash (10%) and ammoniac solution (25%)
formaldehyde (4%)
gold chloride (0.1%)
potassium disulphide solution (2%)
sodium thiosulphate solution (1%)
To fabricate the ammoniac silver nitrate solution, mix the silver nitrate solution and the caustic potash in the relation 5:1. Add the ammoniac solution drop by drop until the precipitate dissolves, following add the silver nitrate solution drop by drop until a slight clouding is recognizable. Add demineralised water, twice the volume of this solution.
To perform the staining, put the sections into the solutions as listed above and leave them in there for 1 min, respectively 5 min for formaldehyde and gold chloride. Before changing the solution, rinse the slides with demineralised water.

3.2.11.7 Massons trichrome staining:

The Masson`s trichrome stain was performed in order to highlight the collagen of the cartilage matrix in the pellets formed in the chondrogenic differentiation lineage. With this method the produced collagen matrix can be shown, but a specific announcement about the present collagen type cannot be made.
Furthermore this stain was utilized to visualize the cells in the gel-constructs of the tenogenic differentiation. With this stain, nuclei appear dark brown and collagen/ connective tissue appears blue-green.

Recipe for the Masson`s trichrome stain

1. Ferric haematoxylin according to Weigert

Weigert A: 10 g haematoxylin diluted in 1000 ml alcohol 96%
Weigert B: 15 g ferric chloride diluted in 1000 ml distilled water
10 ml hydrochloric acid 25%
Mix Weigert A- and Weigert B solution in equal parts.

2. Ponceau-Acidfuchsin-Azophloxin (PAA-solution)

Masson-solution: 1.5 g Ponceau de Xylidin
0.5 g acidfuchsin diluted in 200 ml distilled water
2 ml glacial acetic acid

Azophloxin-solution: 0.5 g azophloxin diluted in 1000 ml distilled water
1 ml glacial acetic acid

PAA-solution: 50 ml acetic acid 0.2%
8 ml Masson-solution
1 ml Azophloxin-solution

3. Phosphotungstic acid
40 g phosphotungstic acid
20 g Orange G
1000 ml distilled water

4. Light green
1 g light green
1000 Ml distilled water
1 ml glacial acetic acid

The sections were stained with Ferric haematoxylin for 10 minutes, rinsed with distilled water and hydrochloric acid 0.5%. Afterwards the sections remained in PAA-solution for 5 minutes before being rinsed with acetic acid 1%. Phosphotungstic acid was applied and rinsed off with acetic acid 1%. Light green was applied for 5 minutes before being rinsed off with acetic acid 1%, distilled water, alcohol and xylene.

3.2.12 Quantitative analysis of histological stains

The stained cells of the adipogenic, osteogenic and chondrogenic differentiation experiments carried out at 3% and 21% O_2 respectively were assessed under the microscope, using a light microscope and the Axiovision image analysis system.

For quantitative analysis of the pictures the software Adobe Photoshop 8.0 was used as described earlier by Hegewald et al. (2004). Briefly, particular colour of selected areas was marked and given as a number of pixels shown in the "image histogram". To estimate the success of adipogenic differentiation, the ratio of fat vacuoles to the cells was measured. In the osteogenic differentiation group the ratio of Ca^{2+} formation to the cells was evaluated and in the chondrogenic differentiation lineage the percentage of proteoglycan to cells and the pellet size was measured. From each horse (n=4), each differentiation lineage, differentiation group and control group, three pictures were evaluated. Then the mean values were determined.

3.2.13 Immunohistochemistry

IHC Collagen II

The paraffin embedded sections of the chondrogenic differentiation lineage pellets were deparaffinized and rehydrated. Tissue sections were treated with 1 mg/ml hylase for 20 min at 37°C and digested with 2 mg/ml pronase for 30 min at 37°C. Non-specific background was blocked with 3% BSA in 0.1 M Tris-HCL buffer (pH 7.4) for 30 min. The tissue sections were then incubated with the primary, monoclonal antibody anti-collagen type II human (mouse) 1:100 (Ab-I, Cat#CP18L) in 1% BSA/ Tris-HCL at 4°C overnight. After incubating the sections with the secondary antibody (biotinylated goat anti mouse, E0433) 1:100 for 30 min, avidin-ABC complex was added for 30 min. Peroxidase activity was detected using the AEC substrate kit. Sections were then mounted with Prolong®Gold and examined under the light microscope. The negative controls were performed without the primary antibody.

IHC Coll III, Cx32 and Cx43

Respectively two sections of the paraffin embedded gel-cell constructs of the tenogenic differentiation lineage were applied on a glass slide, deparaffinized with xylene and rehydrated with ethanol and being rinsed with a washing

buffer. For Cx32- and Cx43- IHC the sections were pretreated with citrate buffer for 20 min at 100 °C. Then the sections were encircled using a cytomation pen and placed in a humidity chamber. 50 µl of goat serum mix (1:4), containing goat serum and 1% BSA in tris buffer saline (TBS), were applied on each positive and negative control and incubated for 20 minutes at room temperature, before the mix was taken off again. Afterwards 50 µl of the primary antibody mix rabbit anti-collagen type III human (1:50), mouse anti-connexin 32 (1:100) or mouse anti-connexin 43 (1:100), respectively were applied on the positive control; the negative control was covered with just 1% BSA in TBS. The incubation was taken out over night with 4°C.
The next morning the slides were washed several times with washing buffer before the positive and negative controls were covered with the secondary antibody mix goat anti-rabbit (1:200), respectively goat anti-mouse (1:200). Incubation was taken out in a humidity chamber for 30 minutes at room temperature. The antibody mix was taken off and the slides were washed with washing buffer. The slides were dried before applying 1 drop of ABC solution and another incubation period in the humidity chamber for 30 minutes at room temperature. The ABC complex was washed off with washing buffer and peroxidise activating was detected using the AEC substrate kit for 20 minutes. Then the slides were washed with distilled water and were covered using Kaiser Gelatine.

Recipe for the buffer:
TBS stock solution:
60.5 g Tris-Base
90 g NaCl
900 ml distilled water
Using HCl the pH was set to 7.6 and distilled water was added to a total volume of 1 litre

Washing buffer:
100 ml stock solution
250 ml Triton-X-100
900 ml distilled water

Recipe for the citrate buffer (pH 6):

Stock solution A: 4.2 g citric acid in 200 ml distilled water

Stock solution B: 29.41 g sodium citrate in 1000 ml distilled water

Mix 7 ml of solution A with 41 ml of solution B and add 500 ml distilled water

3.2.14 Electron microscopy

For the observation of the ultramicrocellular morphology, the gel-cell constructs from the tenogenic differentiation lineage were fixed overnight at 4°C in yellow-fix (stock solution: cacodylate buffer, pH 7.2, + 2% paraformaldehyde + 0.02% picric acid; + glutaraldehyde, 2% of the stock solution). Then the gel-cell constructs were post fixed with 1% osmium tetroxide for 2 hours at room temperature in 0.1 M cacodylate buffer and contrasted with 0.5% uranyl acetate for 30 min and 0.2% lead citrate for 1 minute and 20 seconds. After dehydration the gel-cell constructs were embedded in Epon. Ultrathin sections (70 nm) were cut and examined using an electron microscope.

3.2.15 RNA isolation, DNase digestion and reverse transcription

Total RNA was extracted with TriFast peqGold® according to the manufacturer's protocol. The RNA was incubated with RNase-free DNase I (1 U/µg RNA) for 10 minutes at 37°C to digest genomic DNA. The DNase activity was stopped by incubating the sample for 5 minutes at 75°C. First-strand cDNA synthesis was synthesized from total RNA (60 ng/µl), using 8.5 µl of the RT-mix.

3.2.16 RT-PCR

PCR oligonucleotide primers are shown below. The primers were determined using established Gen Bank sequences. The amplification of the housekeeping gene GAPDH was used as a control for assessing PCR efficiency.

RT-PCR was performed using GeneAmpGold RNA PCR core Kit according to the manufacturer`s protocol. PCR conditions were: 1 x 95°C for 10 minutes, 35 x [94°C for 1 minute, annealing for 1 minute, 72°C for 1 minute] and 72°C for 10 minutes. A set of negative controls was processed in a similar manner, except that reverse transcriptase was replaced by water. The PCR products were separated on a 2% agarose gel, stained with SYBR-Green and visualized with UVIdoc software.

Table 2: **Sequences of primer pairs used**

Gene	Primer pairs	Amplicon	Accession number
GAPDH	F = 5`-GCGTGAACCACGAGAAATATGA-3` R = 5`-GGTGGTGCAGGAGGCATT-3`	62 bp	XM_001496020
CD90	F = 5`-CCCGTGGGCAGAAGGTGAC-3` R = 5`-TCAGGCTGAACTCATACTGGATGG-3`	114 bp	XM_001503225
CD105	F = 5`-CCGCCGCACTGTGGTACATCTAC-3` R = 5`-TGTGGTTGGTGCTACTGCTCTCTG-3`	108 bp	XM_001500078
Oct4	F = 5`-CGAAAGAGAAAGCGAACCAG-3` R = 5`-GCCGGTTACAGAACCACACT-3`	70 bp	XM_001490108
HIF-1α	F = 5`-AACCCATTCCTCATCCTTC-3` R = 5`-TTCTCCTGGCTCATATCCC-3`	134 bp	XM_001493206
Pparγ2	F = 5`-GTCTCATAACGCCATCAGGTTTG-3` R = 5`-GCCCTCGCCTTCGCTTTG-3`	180 bp	XM_001492411
OC	F = 5`-GAGGGCAGTGAGGTGGTGAAG-3` R = 5`-CTCCTGGAAGCCGATGTGGTC-3`	152 bp	XM_001915727
AP	F = 5`-GCTGGGAAATCCGTGGGCATTGTG-3` R = 5`-CGGCAGAGTGGGCGTAGG-3`	81 bp	XM_001504312
Col I	F = 5`-GCCTCGGAGGAAACTTTGC-3` R = 5`-GCACGGAAATTCCAGCAGAT-3`	68 bp	XM_001499586
Col III	F = 5`-ATCTGGTGCTAATGGTGCTCCTG-3` R = 5`-GTGCTCCTGGCTCTCCTTTGG-3`	82 bp	XM_001917620
COMP	F = 5`-GCAGGACTCAGACAGCGATGG-3` R = 5`-GGCACCAGGCGGCAGTTG-3`	96 bp	NM_001081856
Scl 3	F = 5`-CCCAGCCCAAACAGATC-3` R = 5`-GCATCCGCCTCTAACTCC-3	99 bp	NM_001105150

3.2.17 qRT-PCR

qRT-PCR assay was performed on a Bio-Rad real time cycler with a master mix containing 10 µl IQSYBR Green Supermix respectively 0.6 µl forward and reverse primer (10 pM) and steril aqua bidest to a final volume of 20 µl. After the activation of the enzyme at 95°C for 3 minutes, 40 cycles at 95°C for 10 seconds and 60°C for 1 minute, followed by a melt curve (95°C for 10 seconds and 65°C for 5 seconds, +0.5°C increment) were performed. The analysis of the results was performed using the software Bio-Rad CFX Manager 2.0.

3.2.18 Statistic

The statistical analyses of the qRT-PCR results were performed with the software SPSS 19.0. In order to find out if the data were normally distributed, the Kolmogorov-Smirnov-test was applied. In case of a normal distribution ($p>0.05$), a single factor ANOVA was performed in order to compare the variances, followed by an unpaired two-sample t-test, in order to compare the arithmetic mean. For not normally distributed data, the Kruskal-Wallis-test was taken out in order to compare the variances, followed by a Mann-Whitney-U-test, in order to compare the arithmetic mean. Data are shown as mean ± standard error of the mean (SEM).

4 Results

4.1 Stem cell behaviour and differentiation potential towards the adipogenic, osteogenic and chondrogenic lineage under the influence of 3% and 21% oxygen tension

4.1.1 Effect of reduced oxygen concentrations on ASCs immunophenotype as examined by flow cytometry

There were no marked differences in the percentage of cells expressing the stem cell marker CD90. While 96.37 ± 3.82 % of the ASCs cultured under 21% O_2 for 14 days were positive for the surface protein CD90, 93.62 ± 6.79 % of the cells cultured under 3% O_2 were CD90 immunopositive (fig. 9). This slight difference in the expression rate of CD90 could not be determined as significant though.

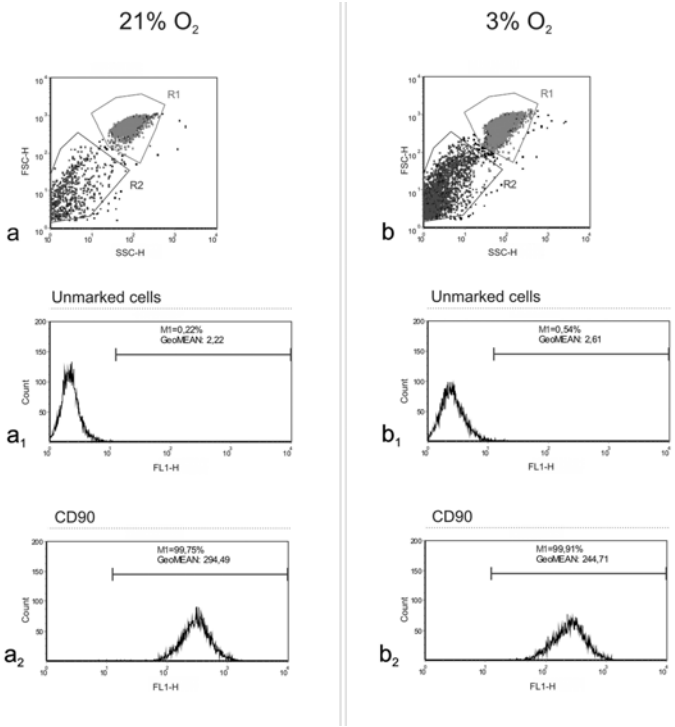


Fig. 9: FACS analysis of equine ASCs cultured under 21% O_2 respectively 3% O_2.
Values represent the mean percentage of positively stained cells with monoclonal antibody directed against CD90 and coupled to FITC. a-a2: cells cultured under 21% O_2; b-b2: cells cultured under 3% O_2.

4.1.2 Cell proliferation under the influence of 3% O_2 respectively 21% O_2

4.1.2.1 Formed colonies – CFU assay

The proliferative capacity of ASCs was assessed by the CFU assay. In all 4 assessed horses the number of formed colonies increased with the number of seeded cells. For the horses 05/09 and 80/10 there seem to be less formed colonies at a density of 2000 seeded cells per 25 cm^2 compared with the number of colonies after seeding 1000 cells per 25 cm^2. The reason for that is that some of the colonies were bigger and for that so close together, almost confluent, that less colonies could be marked-off and identified (s. fig. 11).
Besides that there is no tendency evident, that neither hypoxic conditions nor normoxic condition enhance the proliferative capacity of equine ASCs (fig. 10).

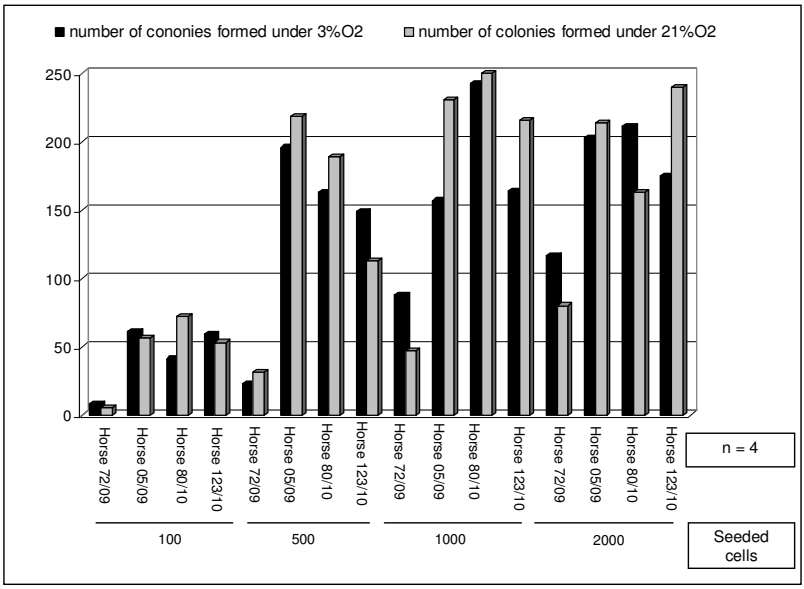


Fig. 10 : Formed colonies after 7 days under 3% O_2 and 21% O_2.
100-, 500-, 1000- and 2000 cells of four horses (n=4) were seeded in 25 cm^2 bottles and cultured with standard medium in a humidified atmosphere at 37°C.

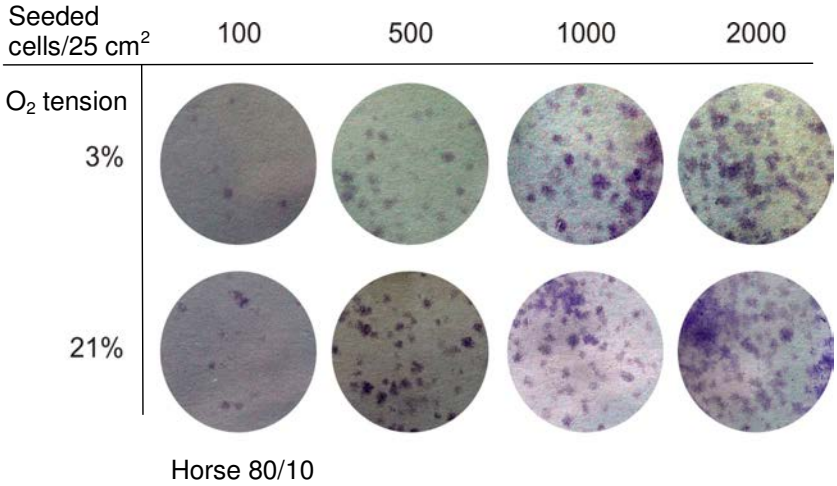


Fig. 11: CFU assay for horse 80/10 under the influence of 3% O_2 and 21% O_2. 100, 500, 100 and 2000 cells were seeded in 25cm^2 bottles and colonies were counted after 7 days.

4.1.2.2 Cell proliferation – MTT assay

The measured number of vivid cells 24h as well as 48h after cell seeding increased in both cell populations; in the group of ASCs cultured under 21% O_2 and in the group cultured under 3% O_2. At both points of estimation there were more vivid cells present in the culture with normoxic culture conditions compared with the group cultured under hypoxic conditions, with the most visible difference after 48h.

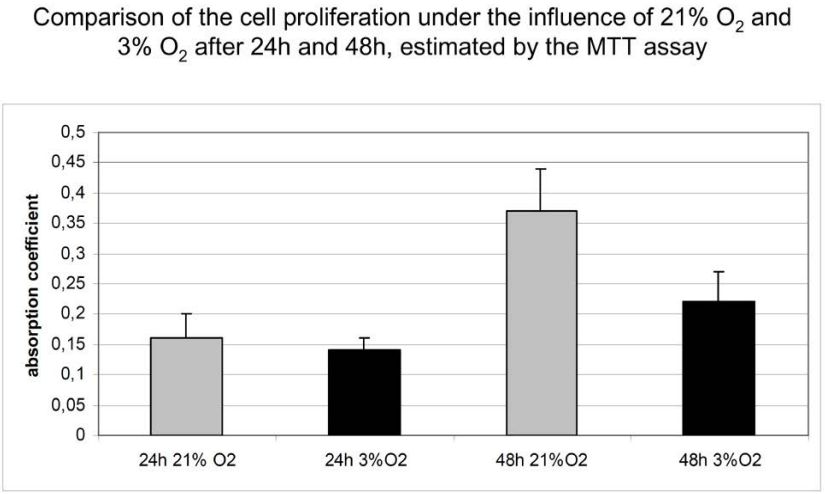


Fig.12: Cell proliferation under 21% O_2 and 3% O_2.

The data are shown as mean ± SD. The graphical display shows the increase of vivid cells 24h and 48h after cell seeding and demonstrates the higher proliferation rate of ASCs cultured under 21% oxygen tension compared with the ASCs cultured unter 3% oxygen tension.

4.1.3 Stem cell morphology under different oxygen conditions

Culturing the ASCs under hypoxic oxygen conditions (3% O_2) did not lead into a change of the morphology of the stem cells, compared with the appearance of the cells cultured under normoxic conditions.

Right after seeding the cells all showed a typical spindle-shaped appearance. After a couple of days in culture they all developed a more round shape with a grainy structured cytoplasm. Under both culture conditions the ASCs formed cell junctions and communicated with each other. Also cell division and progeny could be observed.

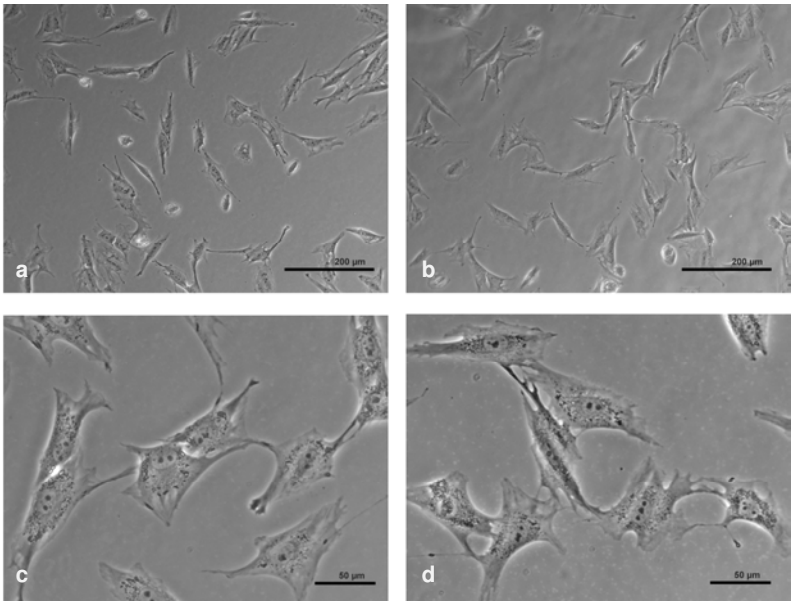


Fig. 13: Cell morphology after 2 days under 3% O_2 and 21% O_2 culture conditions. (a, c) 3% O_2; (b, d) 21% O_2.

4.1.4 Histological analysis of adipogenic differentiation

Culture of ASCs in adipogenic induction medium for 7 days under 21% O_2 as well as the culture under 3% O_2 resulted in the appearance of adipocytes, which stained positive with Oil Red O. Remarkably the number of lipid droplets was much higher in cells cultivated under hypoxic culture conditions. The same phenomenon occured after a period of 14 days, although the percentage of intracellular lipid droplets was higher in both culture conditions, compared with the first week. Still, the cells cultured with 3% O_2 produced notedly more lipid droplets than the cells under normoxic conditions. Furthermore it is conspicuous that the lipid droplets formed under hypoxic conditions are big, typical for fat tissue, while the droplets in the cells cultured under normoxic conditions remain very small (see fig. 14).
Also a very slight percentage of the ASCs cultured in the standard medium (negative control) produced lipid droplets, but distinctly less than the ASCs in the adipogenic medium (fig. 16a and fig. 17a-a2).

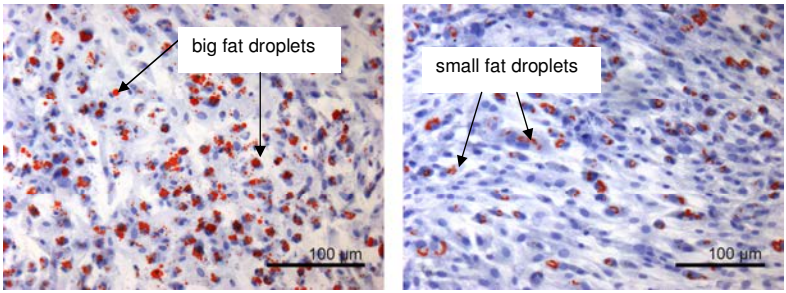


Fig. 14: Morphology of lipid droplets accumulated under hypoxia (left picture) and under normoxic culture conditions (right picture) after 14 days. Oil Red O staining.

4.1.5 Histological analysis of osteogenic differentiation

Already after 7 days in the osteogenic induction medium, the ASCs differentiated into osteoblast-like cells, as calcium deposition was detected by the von Kossa staining in both, the normoxic and the hypoxic cell culture. Although a distinct higher percentage of calcified matrix was present in the normoxic cultures after 7 and 14 days, there was a continuous increase of calcifications in both O_2 concentrations (normoxic and hypoxic) (fig. 16b and fig. 17b-b1). No calcification was detected in the negative control (fig. 16b2).

4.1.6 Histological analysis of chondrogenic differentiation

Although the fraction of mucopolysaccharides was higher in the pellets formed under normoxic O_2 conditions compared with the pellets formed under hypoxic culture conditions, the outline of the pellets under hypoxia was distinct bigger (fig. 15, fig. 16c and fig. 17c-c1). Pellets formed under 3% O_2 appeared stable and compact, while pellets formed under normoxic O_2 tension were not as densely packed and showed a more loose morphology. Furthermore the pellets formed under normoxic conditions showed more of a fibre rich structure in contrast to the pellets formed under hypoxia (fig. 15). The pellets cultured with standard medium (negative control) revealed a very loose structure, with the consequence of a complete disaggregation in some cases (fig. 17c2).
The Masson`s trichrome stain revealed collagen formation in the pellets differentiated under both oxygen conditions. Because the Masson`s trichrome stains every kind of collagen and is therefore not specific for a certain type of collagen, an announcement about the main collagen existing in the pellets cannot be made. No collagen could be seen in the negative control. (Fig. 17d-d2).

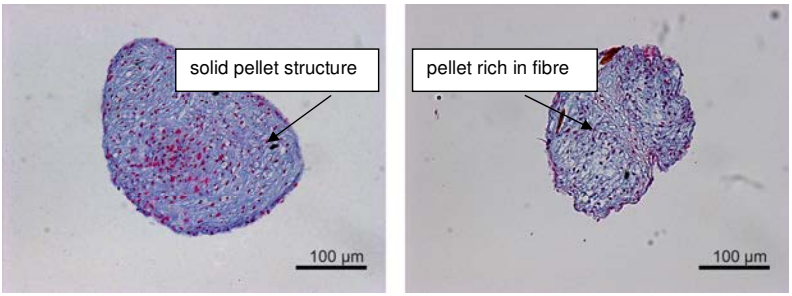


Fig. 15: Pellet formed under hypoxic oxygen conditions in chondrogenic induction medium (left) and under normoxic oxygen condition in chondrogenic induction medium (right) after 21 days in culture.

4.1.7 Collagen II immunohistochemistry

Collagen II expression was noted in the ASCs of both culture conditions (hypoxic and normoxic) after 21 days in the chondrogenic induction medium. Even though the signal was mild in both cell populations (red stain), there was a higher signal recognizable in the pellets cultured under hypoxic conditions (fig. 17e-e1). No evidence of collagen II staining could be detected in the negative control (fig. 17e2).

4.1.8 Subsumption of the differentiation results

Adipogenesis and chondrogenesis showed better results under 3% O_2; for osteogenesis an oxygen tension of 21% was more effective.

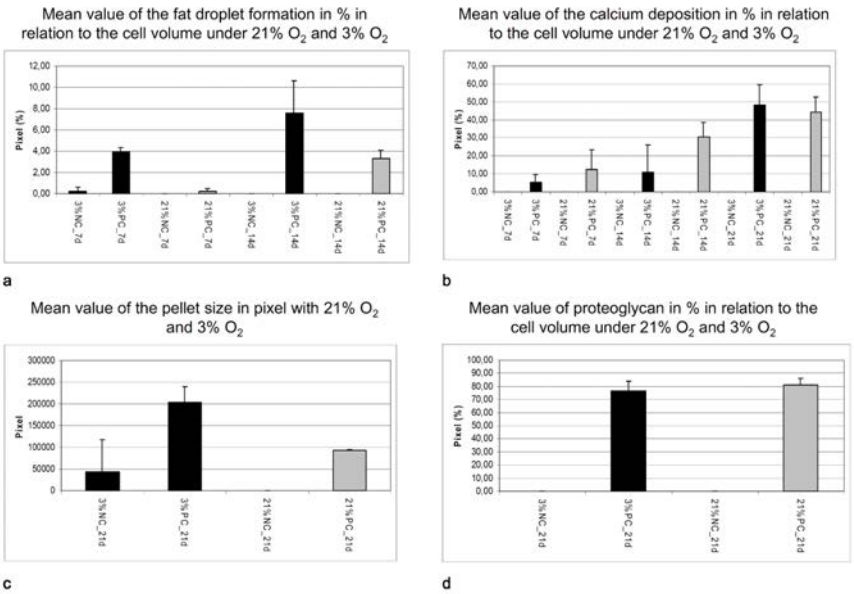


Fig. 16: Semiquantitative analysis of the adipogenic, osteogenic and chondrogenic differentiation under 3% O_2 compared to 21% O_2 (n=4).

The data are shown as mean ± SD. (a) adipogenic (Oil Red O stain); (b) osteogenic (von Kossa stain) and (c, d) chondrogenic (Alcian blue stain) differentiation under 3% O_2 compared to 21% O_2.

PC= positive control; NC= negative control; d= days.

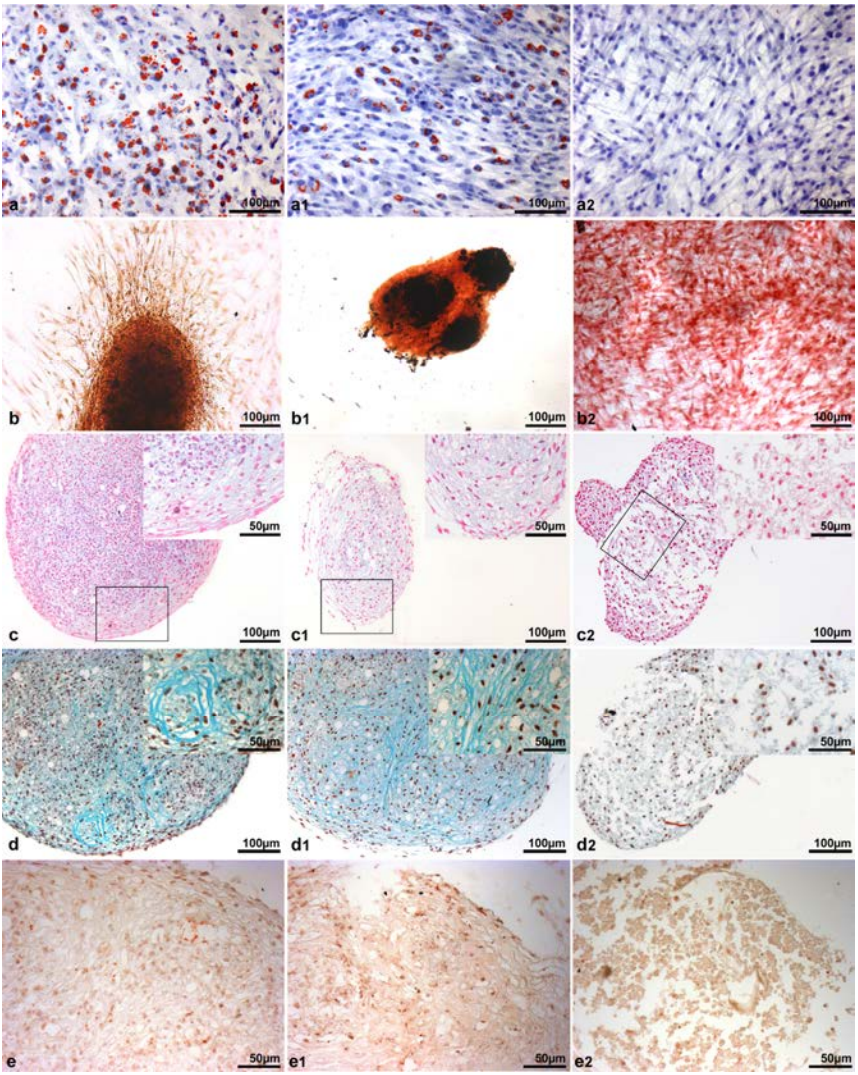


Fig. 17: Results of adipogenic, osteogenic and chondrogenic differentiation under 3% and 21% -O_2 tension.

(a) Oil Red O staining after 14 days of cultivation in the adipogenic differentiation medium in the presence of 3% O_2, (a1) with 21% O_2 and (a2) in standard medium with 21% O_2 tension. (b) Von Kossa staining of cells after 21 days in osteogenic differentiation medium under 3% O_2 , (b1) with 21% O_2 and

(b2) in standard medium with 21% O_2. (c) Alcian blue staining after 21 days in chondrogenic differentiation medium with 3% O_2, (c1) with 21% O_2 and (c2) in standard medium with 21% O_2. (d) Masson`s trichrome stain after 21 days in chondrogenic differentiation medium with 3% O_2, (d1) with 21% O_2 and (d2) in standard medium with 21% O_2. (e) Collagen II immunohistochemistry after 21 days in chondrogenic differentiation medium with 3% O_2, (e1) with 21% O_2 and (e2) in standard medium with 21% O_2. The negative controls of each differentiation lineage with 3% O_2 and 21% O_2 were very similar, that is the reason why only the negative controls with 21% O_2 are shown.

4.1.9 Expression of stem cell markers, pluripotency markers and HIF-1α

An expression of Oct4, CD90 and CD105 mRNA was observed in each cell population cultivated either under normoxic or hypoxic culture conditions (fig. 18).

The expression of Pparγ2, OC and AP was observed in the adipogenic differentiation lineage respectively the osteogenic differentiation lineage under both oxygen conditions, hypoxic and normoxic. Bands occurred in the samples treated with induction medium as well as in the samples treated with standard medium, although the bands of the cells differentiated with induction medium appear more clearly (fig. 18).

HIF-1α could be detected after cultivation of cells under 3% O_2 as well as under 21% O_2, as shown by PCR analysis (fig. 18). However, no significant difference in the degree of the mRNA expression could be revealed by qRT-PCR, neither after 7 or 14 days nor after 21 days. In contrast to our expectations, HIF-1α expression was actually higher at 21% O_2 tension (fig. 19).

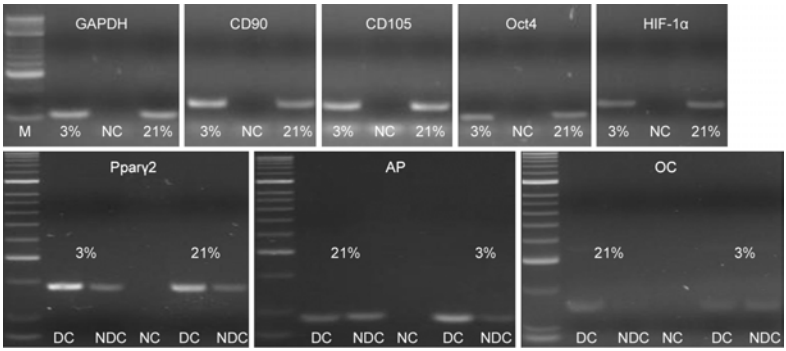


Fig. 18: mRNA expression of stem cell markers, pluripotency markers and HIF-1α.

M: 50 bp marker, for stem cell markers not differentiated ASCs with 3% O_2 and 21% O_2 were used. For pluripotency markers differentiated cells (DC) and not differentiated cells/negative control (NDC) under 3% O_2 respectively 21% O_2 were used. Pparγ2 was applied to identify adipogenic differentiation; OC and AP were used for the osteogenic differentiation lineage. NC: primer control.

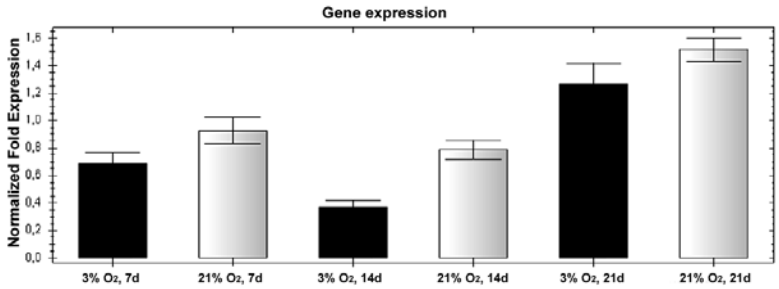


Fig. 19: HIF-1α expression measured in qRT-PCR after 7, 14 and 21 days respectively under the influence of 3% and 21% O_2.

4.2 Differentiation potential towards the tenogenic lineage under the influence of applied strain, 3% and 21% oxygen tension and several growth differentiation factors in a 3d gel scaffold

4.2.1 Alignment of the cells in the 3d gel-construct and scaffold properties under the influence of tensile strain

It could be observed, that cell morphology and cell alignment in the collagen gel as well as the scaffold properties changed during the stretching experiment. At the beginning of the experiment the cell appearance was of round shape, the nucleus was round and the cells were distributed equally in the gel construct. The collagen matrix appeared to be disordered. The collagen gel itself felt soft and was easy to tear.

After 21 days in the bioreactor the morphology and the properties changed. The cells in the gel aligned corresponding to the strain direction and were mainly located at the margins of the gel. The cells became more spindle-shaped and the nucleus developed a longish shape. The gel matrix looked totally ordered towards the tensile direction and the whole gel-construct was clearly stiffer than before the straining stimulation. Furthermore it could be observed that the gel pieces sutured in the chambers of the bioreactor started to contract after a few days in the machine under tensile strain.

However, no obvious differences in the morphology between the applied growth differentiation factors (GDF 5, GDF 6, GDF 7, GDF 567) could be identified under the light microscope. See fig. 20.

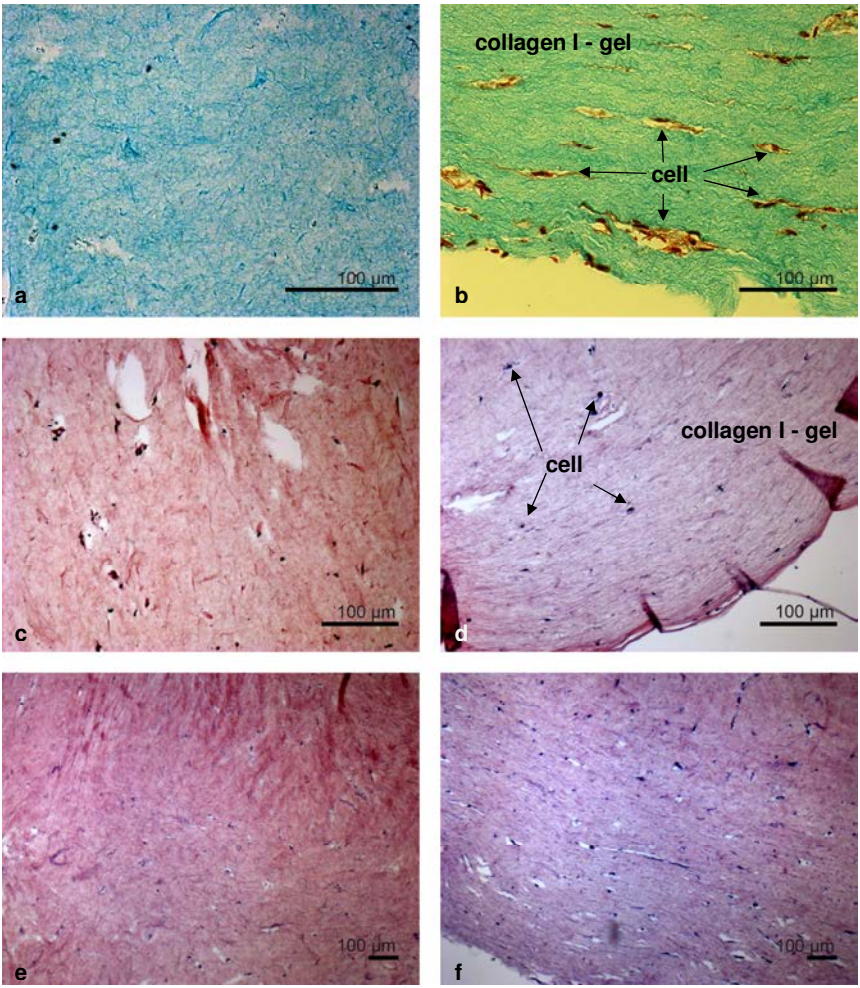


Fig. 20: Alignment of the cells and the matrix in the collagen I gel construct.

(a, e) after 3 weeks without strain, in tenogenic induction medium; (c) at day 0, at the beginning of the differentiation experiment; (b, d, f) after 3 weeks of cyclic strain in tenogenic induction medium, supplemented with GDFs; (a, b) Masson`s trichrome stain; (c-f) hematoxylin and eosin stain.

4.2.2 Ultrastructural analysis of the cell morphology formed in the gel construct under the influence of strain, different oxygen tensions and GDFs

The cell morphology was also examined by electron microscopy after 21 days in the specific differentiation culture. Comparing the results, there were obvious differences in the cell morphology noticeable.

First of all, there were a lot of vital cells detectable in the tenogenic differentiation lineage cultured with 21% O_2, but not in the culture that stood under the influence of 3% O_2. Under the influence of 3% oxygen tension almost all the cells were highly damaged and only in the sample differentiated without applied strain viable cells could be found (fig. 21).

Another distinct difference can be observed in between the samples differentiated with cyclic strain and those without strain. Independently from the oxygen concentration the cell morphology of the cells cultured without applied strain was of clearly round shape. The shape is absolutely atypical for tendon cells, as well as the round nucleus. Furthermore plenty of secretory vacuoles and vesicles, dilatated endoplasmatic reticulum with ribosomes, mitochondria and lysosomes could be observed. In addition diverse cell contacts between the cells were visible (fig. 21).

In between the varying samples cultured with 21% O_2 the differentiation results according to the morphology showed differences, too. The negative control (NC), the sample where strain was applied but no GDFs were added revealed no vivid cells. The best differentiation results according to the cell morphology were reached with strain, 21% O_2 and GDF 5 respectively a combination of all three factors (GDF 567), s. fig. 22. The cells were long and spindle-shaped and typical looking for tendon cells. The nuclei were elongated and contained as well euchromatin as heterochromatin. Cell contacts, cell appendages and vesicle transport were observable and therefor signs for cell to cell communication. Furthermore, there were cell organelles like mitochondria and endoplasmatic reticule with ribosomes visible; signs for cells in the state of synthesis activity. The cytoskeleton was clearly visible, aligns

with the tensile strain direction and marked a stable cell. Apart from that, extracellular structures in the near surrounding of the cells could be observed which are supposable pro-collagen, formed by the cells (fig. 23). This is one of the most important observations, since the generation of extracellular matrix is a main task of tendon cells and is crucial to form stable tendon tissue.

The cells treated with GDF 6 respectively GDF 7 did not show that tendon like morphology as clearly as the cells supplemented with GDF 5 respectively GDF 567 did. A lot of these cells were severely damaged.

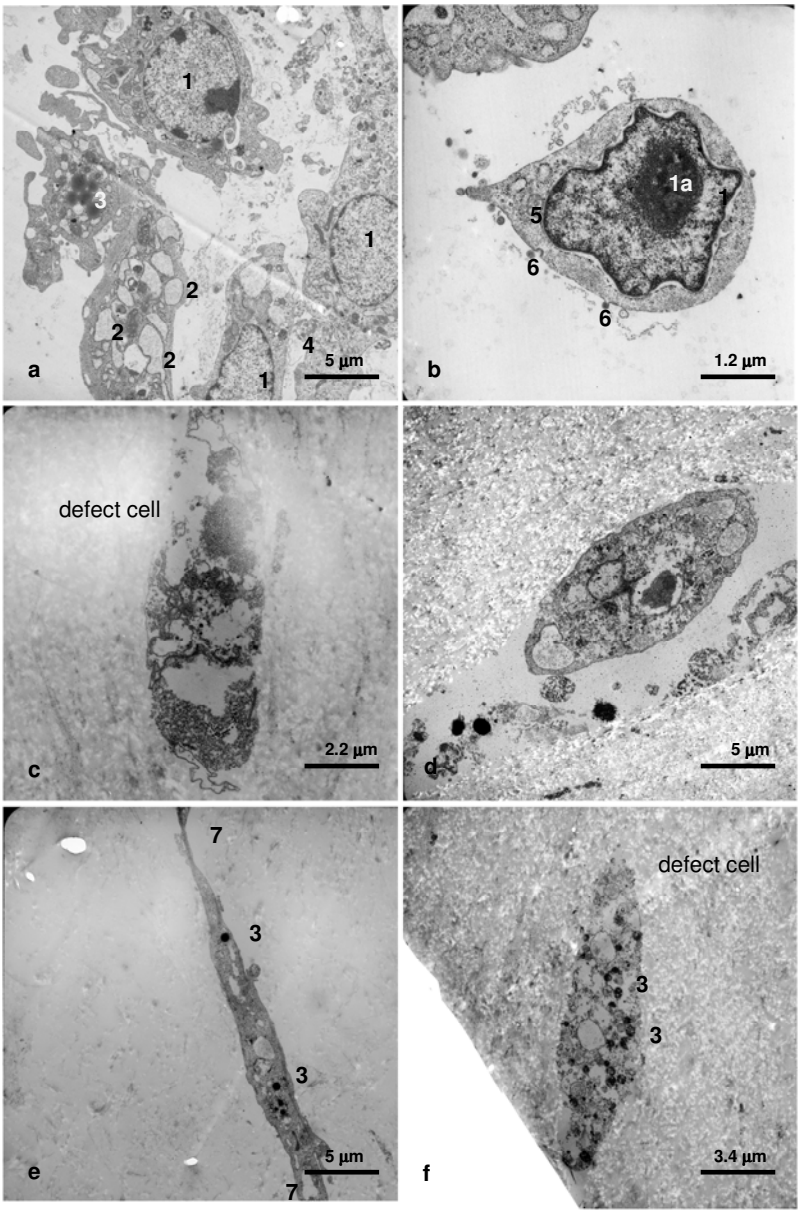
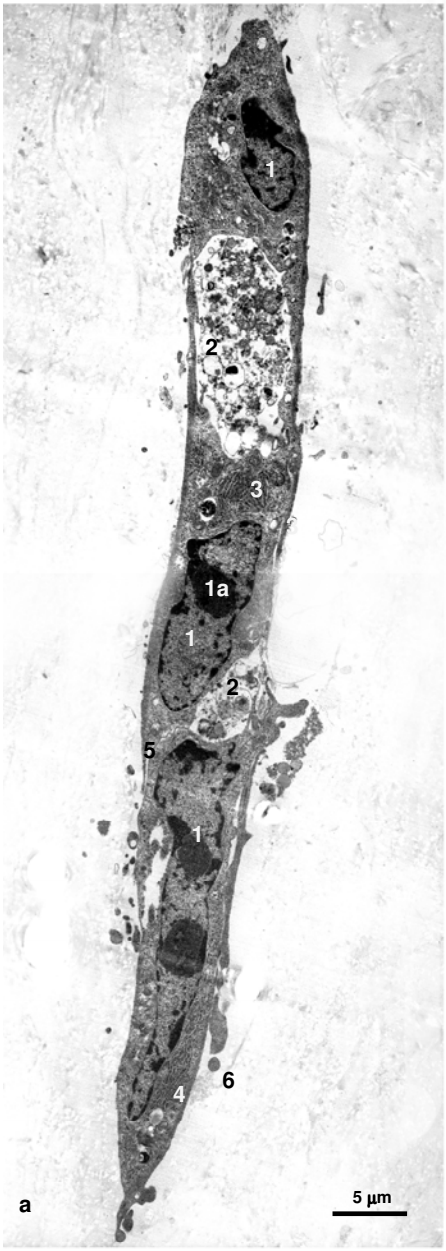


Fig. 21: Cell morphology of poorly differentiated cells, representative for the tenogenic differentiation experiment.

(a) NC without strain, 21% O_2: cells remained intact, but their appearance is untypical for tenocytes. The cells are round in shape, as well as the nucleus. The arrangement of the cells in the gel is unordered. Synthesis of extracellular matrix is not visible. (b) NC without strain, 3% O_2: the cell remained intact, but its appearance is untypical for tenocytes. The cell is round in shape, as well as the nucleus. (c) NC with strain, 21% O_2: the cell in this picture is defect. The cell membrane is not visible and the organelles cannot be clearly identified any more, but in comparison to the cells differentiated without tensile stimulation this cell is elongated. (d) NC with strain, 3% O_2: the cell is intact and shows an elongated cell form. Anyway, the cell does not look like a typical tenocyte. Furthermore, there is no sign of collagen synthesis recognizable. (e) GDF 6, 21% O_2: tenocyte-like shaped cell, nucleus not truncated. Synthesis of extracellular matrix is not recognizable. (f) GDF 5, 3% O_2: elongated but defect cell with a lot of lysosomes. The membrane is resolving.

1: nucleus, 1a: nucleolus, 2: dilatated endoplasmic reticulum, 3: lysosomes, 4: cell contact, 5: mitochondrium, 6: endo-/exocytosis, 7: cell process.



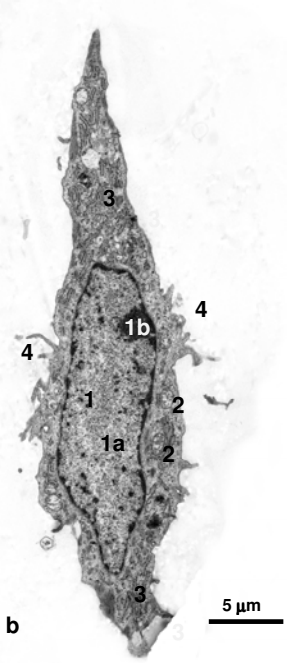


Fig. 22: Cell morphology of well differentiated cells, representative for the tenogenic differentiation experiment supported with GDF 5 and GDF 567.

The cells have a typical tendon like appearance.

(a) GDF 5, 21% O_2

1: nucleus, 1a: nucleolus, 2: phagolysosome, 3: dictyosom, 4: rough endoplasmic reticulum, 5: cell contact, 6: vesicle

(b) GDF 567, 21%O_2

1: nucleus, 1a: euchromatin, 1b: heterochromatin, 2: mitochondrium, 3: rough endoplasmic reticulum, 4: cell processes

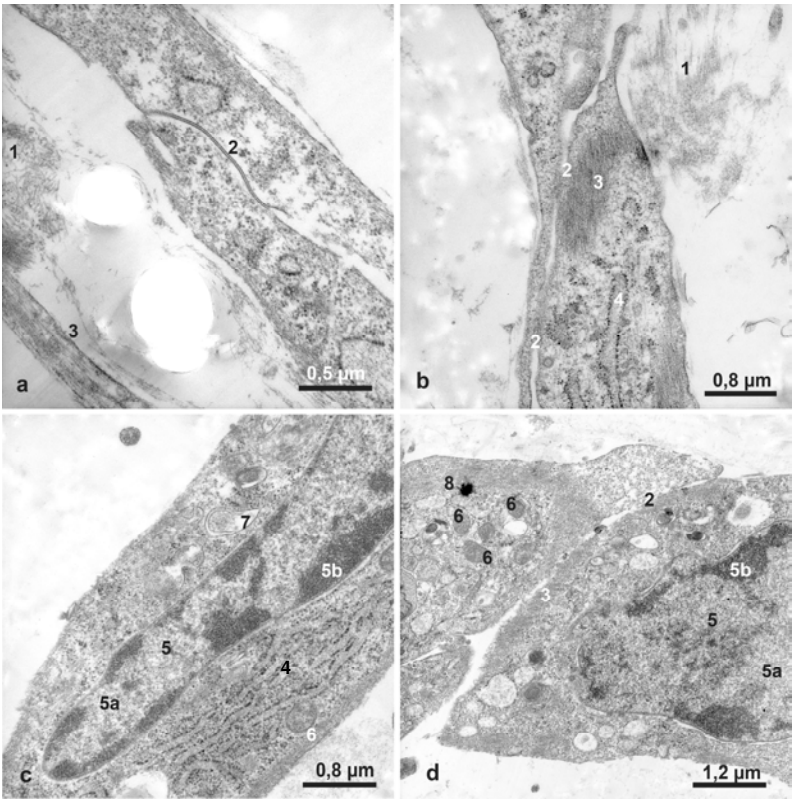


Fig. 23: Detail chart of the tenogenic differentiation with characteristic cell properties of this differentiation lineage.

(a) GDF 5, 21% O_2. 1: collagen containing ECM, 2: cell contact, 3: actin filaments of the cytoskeleton; (b) GDF 567, 21% O_2. 1: collagen containing ECM, 2: cell contact, 3: actin filaments of the cytoskeleton, 4: rough endoplasmic reticulum; (c) GDF 5, 21% O_2. 4: rough endoplasmic reticulum, 5: nucleus, 5a: euchromatin, 5b: heterochromatin, 6: mitochondrium, 7: dictyosom; (d) GDF 5, 21% O_2. 2: cell contact, 3: actin filaments of the cytoskeleton, 5: nucleus, 5a: euchromatin, 5b: heterochromatin, 6: mitochondrium, 8: lysosome.

4.2.3 Cell junction formation in the gel construct detected by immunohistochemistry

Connexins, part of gap junctions play an important role in cell contact formation and cell communication. In tendon cells they have the task of coordinating extracellular matrix generation and formation in response to mechanical stimuli.

Cx32 and Cx43 could be detected by immunohistochemistry in the 3d gel constructs, carrying the ASCs (fig. 24 and fig. 25).
Every sample (NC, NC without strain and the cells supplemented with GDF 5, GDF 6, GDF 7 respectively a combination of all three factors) was immuno-positive for both examined connexin proteins.

The positive controls, liver of a horse for Cx32 and heart of a horse for Cx43, stained with similar intensity as the cell junctions in the gel construct. Sections incubated without the primary antibody did not show any immunoreactivity (fig. 24a, 24c).

The presence of cell junctions and cell communication was also confirmed by electron microscopy in different areas of the gel constructs and in gels cultured with and without the different growth differentiation factors (see results electron microscopy).

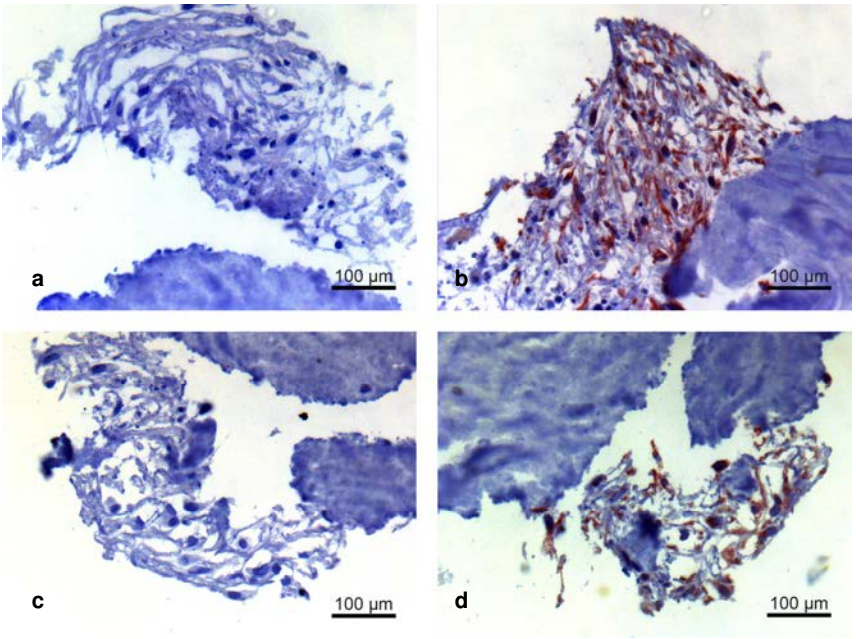


Fig. 24: Cell contact formation in the gel-cell-construct detected by immunohistochemistry for Cx32 and Cx43 and counterstained with hematoxylin and eosin.

(a-d) gel construct containing ASCs cultured without strain and without GDFs under 3% O_2

(a) negative control for Cx43, incubated without primary antibody (b) incubation with primary antibody (goat anti mouse) and secondary antibody (mouse anti-connexin 43) (c) negative control for Cx32, incubated without primary antibody (d) incubation with primary antibody (goat anti mouse) and secondary antibody (mouse anti-connexin 32).

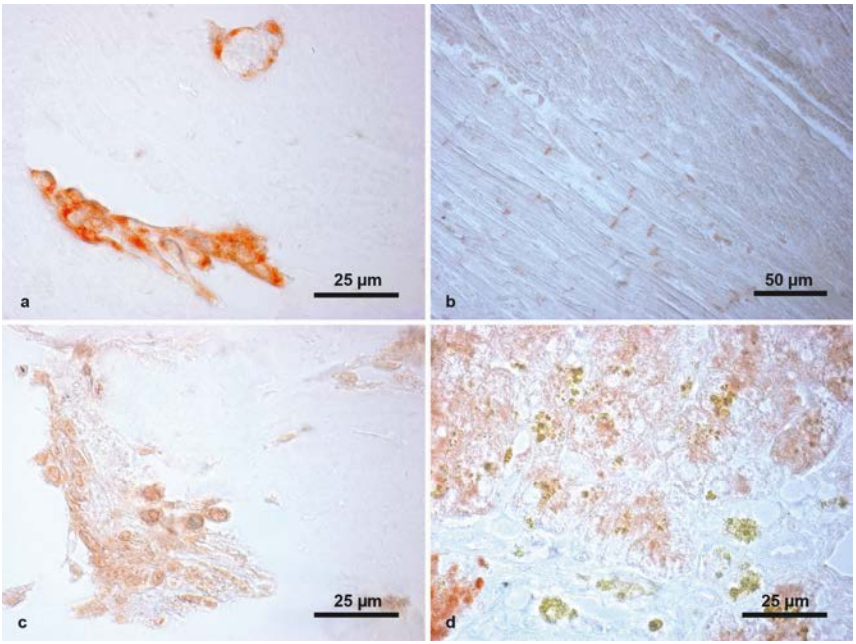


Fig. 25: Cell contact formation in the gel-cell-construct as detected by IHC for Cx43 and Cx32.

(a) ASCs in gel construct cultured with tensile strain and GDF 5 supplement under 21% O_2 – Cx43 (b) positive control for Cx43; equine heart (c) ASCs in gel construct cultured with tensile strain and GDF 5 supplement under 21% O_2 – Cx32 (d) positive control for Cx32; equine liver.

4.2.4 Collagen III formation in the gel constructs detected by silver staining and immunohistochemistry

Type III collagen as one of the typical components in tendon tissue could be detected by immunohistochemistry in various gel constructs. No evidence of collagen III staining was observed in the sections incubated without the primary antibody. The dark red staining of the type III collagen was always located at the membrane of the cells and the close surrounding of the cells in the collagen gel matrix.

Since the used gel serving as a matrix for the ASCs is basically made out of type I collagen, it stains slightly red, too, because the applied primary antibody rabbit polyclonal to Collagen III, rabbit - anti - human is polyclonal.

For that reason the Gomori silver staining was additionally applied. The silver staining after Gomori specifically stains collagen III fibres (reticulin) and shows no co-reaction with type I collagen. Here the collagen III fibres stained black in the area of the cell membrane and therefore confirmed the results of the immunohistochemistry (fig. 26).

Since tendon tissue contains about 95% type I collagen and only approximately 5% type III collagen, it would have been interesting to show the collagen I formation in the gel construct. Because of the high content of rat collagen I in the gel scaffold, it was not possible for us to block that collagen type out and specifically stain the equine collagen I, formed by the ASCs. Therefore the collagen I production of the ASCs could not be demonstrated by immunohistochemistry, but by RT- PCR and qRT- PCR (see there).

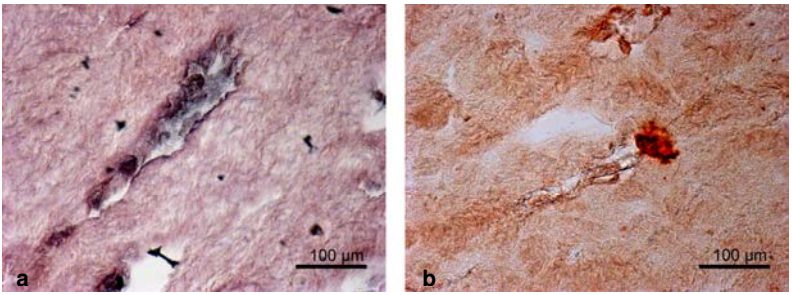


Fig. 26: Collagen III formation in the gel construct; demonstrated by (a) Gomori silver staining and (b) immunohistochemistry.

(a) collagen III stains black in the area of the cell membrane (b) collagen III stains dark red in the area of the cell membrane; the bright red staining in the matrix shows the cross reaction of the polyclonal antibody with type I collagen.

4.2.5 mRNA expression of tendon relevant markers

Collagen I, collagen III, COMP and scleraxis were chosen as tendon specific markers in order to examine the degree of tenogenic differentiation of the ASCs in the 3d gel construct under different conditions and stimuli. GAPDH served as housekeeper.

Collagen I and collagen III are important components of the extra cellular matrix in the tendon. mRNA for both genes was detected by RT-PCR in the cells cultured under both oxygen conditions, 3% O_2 and 21% O_2.
Bands emerged in every gel supplemented with GDFs (GDF 5, GDF 6, GDF 7 respectively a combination of all three factors), the gel with applied strain and the gel without tensile stimulation.

The mRNA expression of COMP and scleraxis in general was distinct lower than the collagen I and III expression. In some tested samples no bonds emerged in RT-PCR. That weak gene expression, or even non-existent gene expression, was confirmed and exact determined in the quantitative RT-PCR, see there.
COMP is a non-collagenous protein and known to be present in equine tendon tissue, particularly abundant during the growth period. It is assumed that amongst others COMP has an organisational role in the formation of collagenous matrix (Smith et al., 1997; Smith et al., 2002). Scleraxis is a distinct marker for tendon and ligament progenitor cells and differentiated cells. Analyses suggest that scleraxis function is crucial for the differentiation of force-transmitting tendon tissue (Murchison et al., 2007).

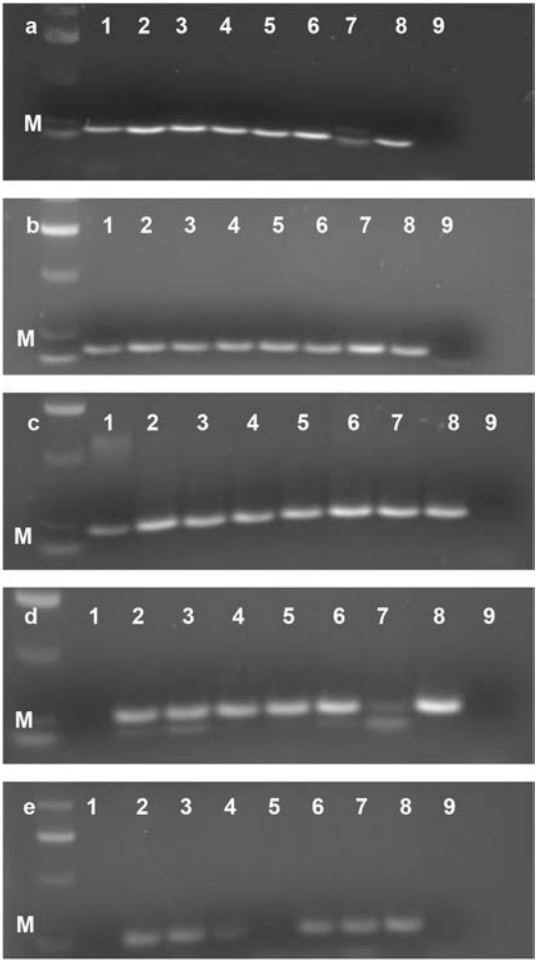


Fig. 27: mRNA expression of a: GAPDH, b: Col I, c: Col III, d: COMP and e: Scx in RT-PCR after 3 weeks of culture in the collagen I gel scaffold with 21% O_2.

M: 50 bp marker, 1: GDF 5, 2: GDF 6, 3: GDF 7, 4: GDF 567, 5: NC, 6: NCws, 7: DMEM, 8: tendon, 9: primer control.

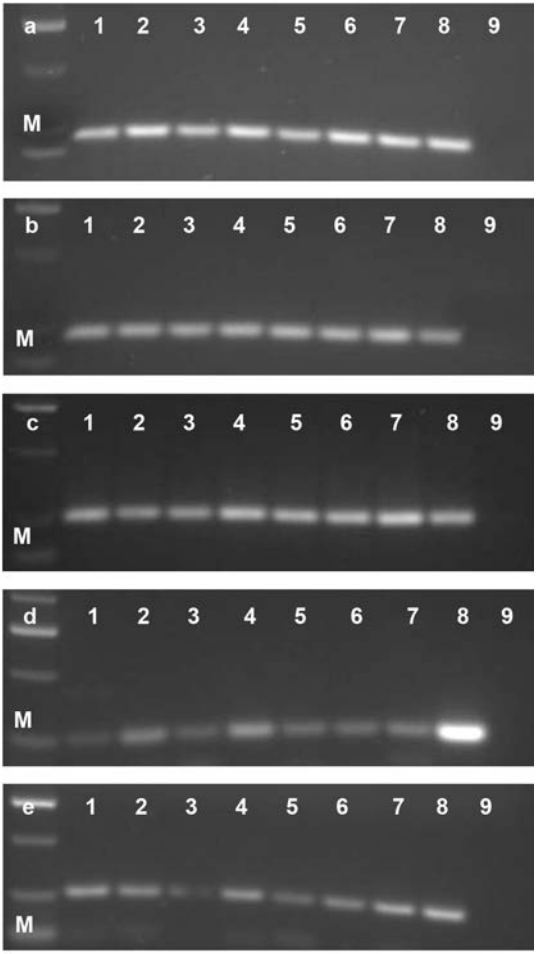


Fig. 28: mRNA expression of a: GAPDH, b: Col I, c: Col III, d: COMP and e: Scx in RT-PCR after 3 weeks of culture in the collagen I gel scaffold with 3% O_2.

M: 50 bp marker, 1: GDF 5, 2: GDF 6, 3: GDF 7, 4: GDF 567, 5: NC, 6: NCws, 7: DMEM, 8: tendon, 9: primer control.

4.2.6 Quantitative measurement of tendon relevant gene expression (qRT-PCR)

Fig. 29-fig. 32 show the data as mean ± SED.

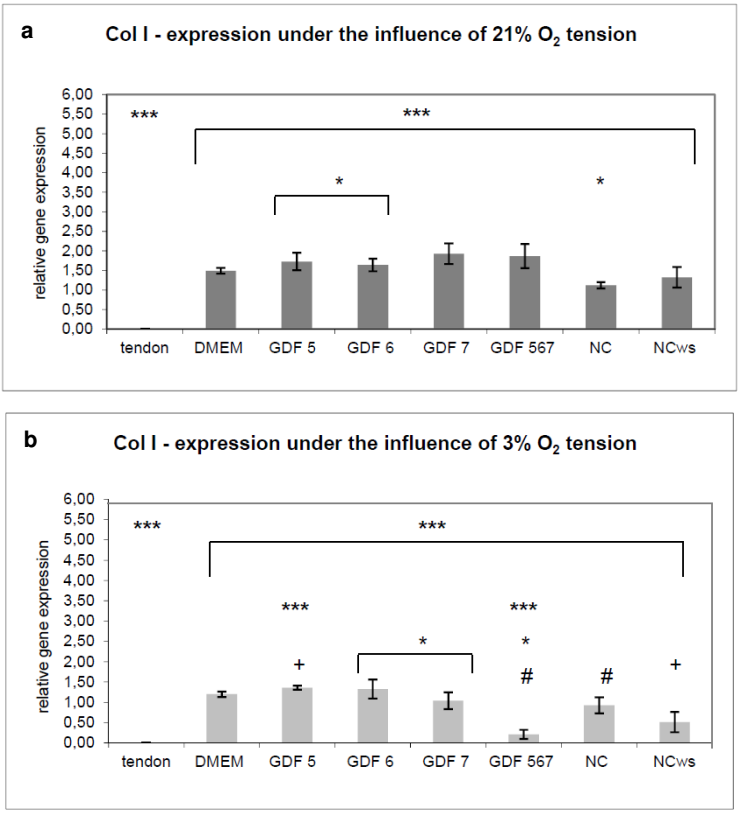


Fig. 29: Col I – expression of ASCs differentiated towards the tenogenic lineage in a collagen I gel scaffold with applied uniaxial strain under the influence of (a) 21% O_2 respectively (b) 3% O_2.

tendon: equine tendon serving as reference tissue; DMEM: ASCs in a collagen I gel scaffold cultured with DMEM and without applied strain; GDF 5, GDF 6, GDF 7, GDF 567: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium, supplemented with GDF 5, GDF 6, GDF 7 respectively a combination of the three factors; NC: negative control: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs; NCws: negative control without strain: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs and without strain.

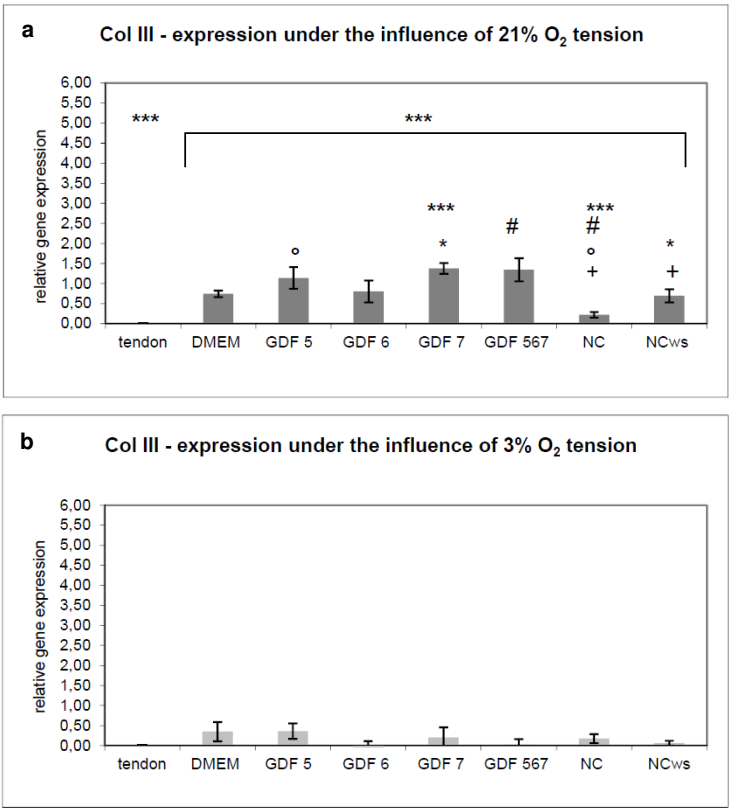


Fig. 30: Col III – expression of ASCs differentiated towards the tenogenic lineage in a collagen I gel scaffold with applied uniaxial strain under the influence of (a) 21% O_2 respectively (b) 3% O_2.

tendon: equine tendon serving as reference tissue; DMEM: ASCs in a collagen I gel scaffold cultured with DMEM and without applied strain; GDF 5, GDF 6, GDF 7, GDF 567: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium, supplemented with GDF 5, GDF 6, GDF 7 respectively a combination of the three factors; NC: negative control: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs; NCws: negative control without strain: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs and without strain.

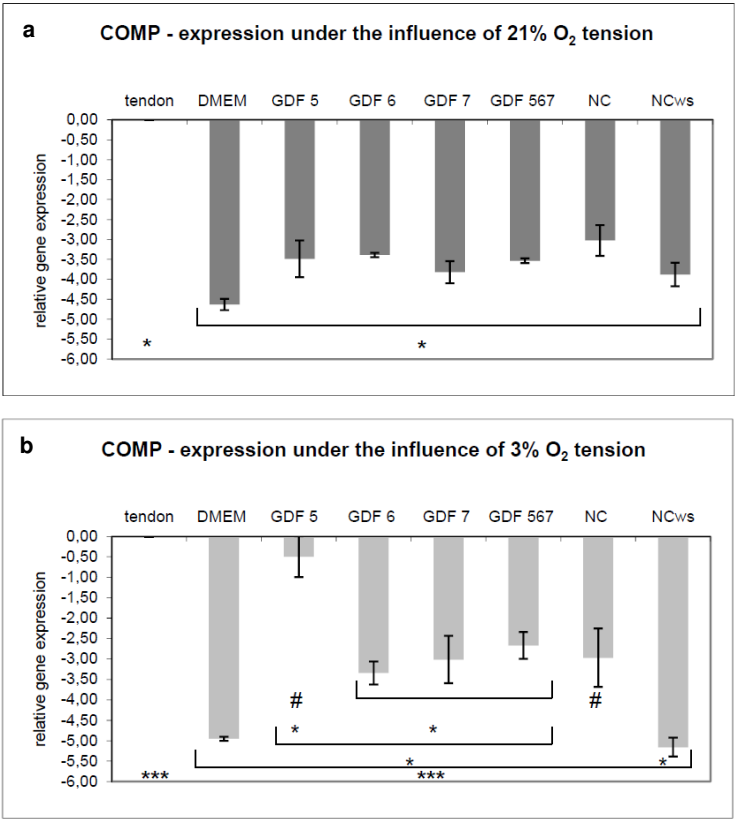


Fig. 31: COMP – expression of ASCs differentiated towards the tenogenic lineage in a collagen I gel scaffold with applied uniaxial strain under the influence of (a) 21% O_2 respectively (b) 3% O_2.

tendon: equine tendon serving as reference tissue; DMEM: ASCs in a collagen I gel scaffold cultured with DMEM and without applied strain; GDF 5, GDF 6, GDF 7, GDF 567: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium, supplemented with GDF 5, GDF 6, GDF 7 respectively a combination of the three factors; NC: negative control: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs; NCws: negative control without strain: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs and without strain.

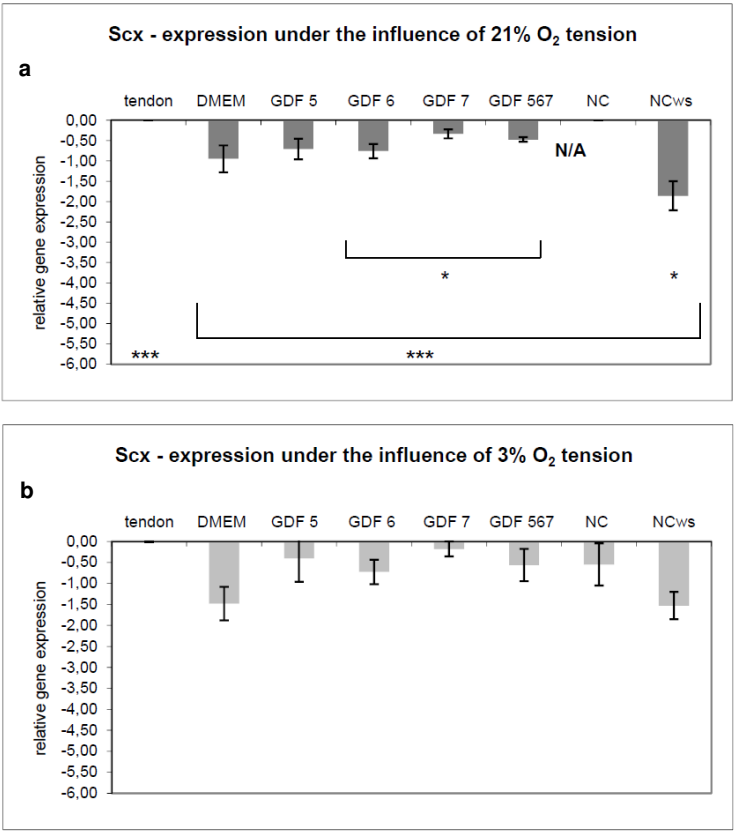


Fig. 32: Scx – expression of ASCs differentiated towards the tenogenic lineage in a collagen I gel scaffold with applied uniaxial strain under the influence of (a) 21% O_2 respectively (b) 3% O_2.

tendon: equine tendon serving as reference tissue; DMEM: ASCs in a collagen I gel scaffold cultured with DMEM and without applied strain; GDF 5, GDF 6, GDF 7, GDF 567: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium, supplemented with GDF 5, GDF 6, GDF 7 respectively a combination of the three factors; NC: negative control: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs; NCws: negative control without strain: ASCs in a collagen I gel scaffold cultured with tenogenic differentiation medium but without the supplement of GDFs and without strain.

In general, the gene expression of Col I was higher under normoxic conditions (21% O_2) than under hypoxic conditions (3% O_2). That announcement applies to all ASC – gel constructs differentiated with the different supplements. That also means that the gene expression under hypoxic conditions was closer to the gene expression of the reference tissue (equine tendon) and therefore matched the equine tendon better than the samples differentiated under normoxic conditions. For the Col I expression under both oxygen tensions the data were normally distributed and a highly significant ($p<0.001$) difference between the reference tissue and the in culture differentiated cells could be determined by the t-test. In the samples cultured under normoxic conditions, there was also a significant ($p<0.05$) difference between GDF 5 respectively GDF 6 and NC present. Under hypoxic conditions the statistical analysis revealed a highly significant difference between GDF 5 and GDF 567, a significant difference between GDF 6 respectively GDF 7 and GDF 567, between GDF 5 and NCws and between GDF 567 and NC.

The results of the Col III expression were in accordance with the results of the Col I expression. In all samples the Col III expression was higher under normoxic condition than under hypoxic conditions and therefore matched the reference value less than the samples cultured under hypoxic conditions. The data of the Col III expression under both oxygen tensions was normally distributed. In contrast to the samples from the hypoxic culture, where no significant differences could be determined, statistical analysis revealed a highly significant difference between the reference tissue and the cell culture samples for normoxic conditions. Furthermore a highly significant difference between GDF 7 and NC, a significant difference between GDF 5 and NC, between GDF 567 and NC, between GDF 7 and NCws, and finally between NC and NCws existed.

In contrast to Col I and Col III, the COMP - and Scx – gene expression in the ASC – gel constructs was lower than the gene expression of the equine tendon. Here it became clear that the gene expression of the samples differentiated without applied strain (DMEM and NCws) showed the biggest gap compared to the gene expression of the reference tissue. The data for the COMP expression under normoxic conditions was not normally distributed, therefore the Mann-Whitney-U-test was applied and revealed a significant

difference ($p<0.05$) between the reference tissue and the cultured cell samples. The data for the COMP expression under hypoxic culture conditions were normally distributed and showed a highly significant difference between the reference tissue and the cell culture samples as well as a significant difference between the cells differentiated with GDFs and NCws, between GDF 6, GDF 7 respectively GDF 567 and GDF 5 and between GDF 5 and NC.
The data for the Scx expression were normally distributed. In contrast to the samples activated under hypoxic culture conditions, the samples cultured under normoxic conditions revealed a highly significant difference between the reference tissue and the cell culture samples. Furthermore, a significant difference between GDF 6, GDF 7, respectively GDF 567 and NCws existed. NC was taken out of the analysis because no gene expression could be measured in qRT-PCR.
It can be summarized that a supplement with GDFs led to a higher expression of the four examined genes compared to the negative controls (NC, NCws), independently from the oxygen tension. Especially GDF 5 and GDF 7 bunched out. Furthermore, it became clear, that the expression of the tendon relevant markers COMP and Scx showed the lowest results, and therefore the biggest discrepancy to the reference tissue, for the samples differentiated without strain (DMEM, NCws), also independently from the oxygen tension.

4.2.7 Emigration of the cells

It could be observed that the differentiated cells emigrate from the collagen I gel scaffold, when removed from the bioreactor and placed in a dish with a chamber slide, covered with DMEM. On the chamber slide the cells were collected and further examined, here by light microscopy and electron microscopy. Judging by the cell morphology, the pre-differentiated cells remained intact and therefore could be used for further in vivo experiments.

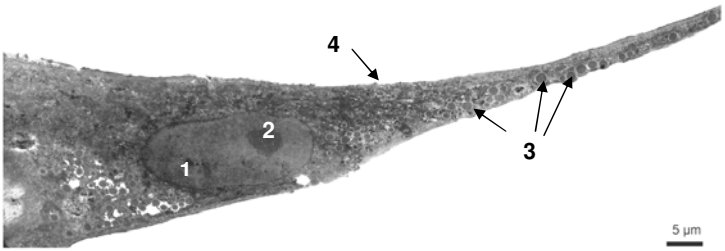


Fig. 33: electron microscopy picture of an emigrated cell from the collagen I – gel scaffold. The cell is intact and shows a typical tenocyte-like shape. 1: nucleus, 2: nucleolus, 3: vacuoles, 4: cell membrane.

5 Discussion

5.1 Stem cell behavior and differentiation potential towards the adipogenic, osteogenic and chondrogenic lineage under the influence of 3% and 21% oxygen tension

In order to prove that the cells used for our experiments were actually stem cells, we followed the latest requirements described for sound equine stem cell characterization (De Schauwer et al., 2010). Our cells were plastic-adherent, showed the capacity of tri-lineage differentiation in vitro and expressed CD90, CD105 and Oct4. The CD90 expression was even proven by flow cytometry as preferably required (De Mattos Carvalho et al., 2009, De Schauwer et al., 2010).

The results of the flow cytometry analysis showed no remarkable difference in between cells cultured with different oxygen levels, thus, hypoxia does not seem to have an effect on the immunophenotype of the ASCs, which is characterized amongst others by the cell surface expression of CD90. Similar results were shown by Holzwarth et al. (2010). They used human BM-MSCs to analyze the expression of the typical human MSC markers CD73, CD90, CD105, CD106 and CD 146 in cells cultured for 14 days at 21% O_2 and at 1% O_2 by flow cytometry. No difference was seen in the marker expression at different O_2 concentrations. Those results favor the assumption that hypoxia has no effect on the immunophenotype of stem cells.

The CFU assay allows the functional and quantitative assessment of mesenchymal progenitor cells. Our study did not reveal any significant differences, or even a tendency to a difference in the number of colonies formed under the influence of hypoxia compared with the colony number accumulated under the influence of normoxic conditions. Published results for human MSC (dos Santos et al., 2009; Pilgaard et al., 2009; Iida et al., 2010) showed a higher proliferation rate under hypoxic conditions. In contrast, equine MSCs do not seem to show the same tendency for a higher proliferation rate under hypoxia.

In contrast to the CFU assay, the MTT assay revealed a much clearer result. As shown by the MTT assay, a higher cell proliferation rate can be reached under normoxic conditions. The phenomenon that cell proliferation is reduced under hypoxic conditions has been described for human MSCs by Zeng et al. (2011) where the MTT assay was used as well. Zeng and colleagues formed the hypothesis that a

weakening of the cell-to-cell signalling may be responsible for the inhibition of the growth of the MSC cultured under hypoxic conditions. The reason for the discrepancy between the results of the CFU assay and the MTT assay may be caused by the difficulties of counting the colonies. As shown in figure 11, the colonies sometimes were very close together and it was at the discretion of the evaluator how the colonies were counted. In contrast to that, with the MTT assay the amount of viable cells is detected by photometry. Therefore the MTT assay seems to be the more objective and accurate method of cell proliferation estimation.

The potential to differentiate into various lineages seems to depend on several factors. Fehrer and colleagues (2007) reported a reduced capacity to differentiate in vitro into the adipogenic lineage of human BM-MSCs and even no capacity for an osteogenic differentiation at 3% O_2 tension, compared with 20% O_2. Interestingly, the differentiation capacity under the influence of different oxygen tensions was also passage dependant. It was demonstrated that conditions for adipogenesis were more favorable at 20% O_2 in passage 0 and 1, but from passage 2 onwards the results changed and adipogenesis results with an increased lipid droplet formation was better at 3% O_2. Since our results showed a distinct increase in adipogenesis at 3% O_2 compared with 21% O_2 in passage 1 and 2 cells, they do not contradict the results presented by Fehrer et al. (2007). An extended experiment with cells derived from different passages would have to be carried out in order to check whether the results would be different.

In contrast to Fehrer et al. (2007) we were able to show some osteogenic differentiation under hypoxic conditions (fig. 1b). However, calcification at 3% O_2 was not as prominent as at 21% O_2.

Holzwarth et al. (2010) showed a reduced adipogenic but no osteogenic differentiation potential at hypoxic conditions in higher passages of human BM-MSCs, but in their study only 1% O_2 was used. When the oxygen tension was increased from 1% to 3%, osteogenic differentiation took place in the same extent as under normoxic conditions. This indicates how sensitive stem cells react to small changes in oxygen tension at least in regard to differentiation ability. Therefore, further investigations about the exact O_2 tension in the various tissues in vivo need to be carried out, in order to better imitate the real tissue conditions in vitro. Also a standardization of the oxygen level in further experiments would be very helpful so they become more comparable.

Similar results about an inhibited osteogenic differentiation of human MSCs under hypoxia are also reported by D`Ippolito et al. (2006), Grinakovskaya et al. (2009), He et al. (2010) and Raheja et al. (2010). A possible explanation for that phenomenon might be that under physiological conditions stem cells self-renew while located in areas of low oxygen and tend to differentiate towards osteoblasts when located closer to blood vessels and exposed to higher O_2 levels (D`Ippolito et al., 2006).

The histological analysis of the chondrogenic differentiation revealed bigger and more solid pellet formation under hypoxia, while pellets formed under normoxia showed more mucopolysaccharide production as demonstrated by the Alcian blue staining (fig.1c, c1). The reason for the lower staining intensity in the pellets formed under 3% O_2 may be the bigger pellet size. This may even lead to lower O_2 concentrations, in the centre of the pellet in addition to the already low environmental oxygen tension. The occurrence of a vast amount of cell detritus within the pellet centre confirms this notion and may be a sign for an enhanced cell death in this area.

Low oxygen tension as a promoter for in vitro chondrogenesis of MSCs has already been reported by Markway et al. (2010) and Meyer et al. (2010). Since the oxygen tension in deep zones of articular cartilage is less than 1% O_2 and therefore less than in bone tissue, it is comprehensible that chondrogenesis is improved under hypoxic conditions in contrast to osteogenesis. These findings raise hope, that hypoxic culture conditions may also be helpful in other differentiation lineages, where the in vivo oxygen tension in the tissue is very low, too. This especially applies for tendons.

The transcriptional regulator HIF-1α has an important role in the cell response to hypoxia. The response involves a change in gene expressions mediated by HIF-1α. Activated target genes involve angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability and vascular remodelling (Semenza, 2000).

Our analysis revealed that HIF-1α was equally expressed at 21% O_2 as well as at 3% O_2. Actually, it was expected to detect a higher expression under hypoxic conditions. However qRT-PCR showed no significant quantitative difference in HIF-1α expression under normoxic as well as under hypoxic conditions. Possible explanations are that the O_2 reduction down to a level of 3% was not sufficient to activate the factor. Another explanation would be that the ASCs are adapted to a lower O_2 concentration in vivo anyway, so they do not recognize 3% O_2 in vitro as hypoxic. Similar results for human stem cells were reported earlier. Fehrer et al. (2007) reported an unaltered HIF-1α expression by human BM-MSCs at both oxygen

conditions, 3% and 21%. That result was taken as a proof for the assumption that 3% O_2 is sensed as normoxic by MSCs. Conforming results were also presented by Forristal et al. (2009). In their study, among others HIF-1α expression of human embryonic stem cells was measured after 3 days of culture at 5% O_2 and 20% O_2. Again, qRT-PCR revealed no significant difference in the mRNA expression of HIF-1α under different oxygen conditions. In contrast to the results above, Holzwarth et al. (2010) reported a more than 3-fold increased HIF-1α expression at 1% O_2 compared to 21% O_2. The lower oxygen tension of 1% O_2 (in our experiment we used 3% O_2) may be the explanation for the different rate of mRNA expression. However, major criticism in this context is, that only a semiquantitative and not a quantitative RT-PCR was performed, making these results less objective. The same criticism applies for the work of D`Ippolito et al. (2006) who reported about an up regulated HIF-1α expression in human bone marrow derived MSCs within 2 hours in the presence of 3% O_2 compared with 21% O_2 as analyzed by RT-PCR.

Since HIF-1α plays such an important role in gene activation under hypoxic conditions and since contrary results are presented in the literature, further research needs to be carried out to understand stem cell behavior under hypoxic culture conditions and to be able to predict the consequences of different gene expressions possibly activated by HIF-1α.

In terms of our results and the reviewed literature it becomes obvious, that a lot of different factors play a role in the outcome of in vitro stem cell differentiation. Amongst others, those factors are species-, sex-, age-, passage-, O_2 tension- and medium dependent. This multitude of factors may be responsible for the heterogeneity of data presented in the literature.

Anyway, our results may help to improve stem cell differentiation in vitro and may outline new aspects with regard to tissue engineering.

5.2 Differentiation potential towards the tenogenic lineage under the influence of applied strain, 3% and 21% oxygen tension and several growth differentiation factors in a 3d gel scaffold

Cell morphology

The analysis of the ultramicrocellular morphology displayed by electron microscopy revealed some interesting findings. Only the ASCs cultured with 21% oxygen, differentiated with applied strain and supplemented with growth differentiation factors showed a typical tendon like morphology. The ASCs treated with GDF 5, GDF 6, GDF 7, respectively a combination of all three factors developed a spindle-shaped cell morphology with irregular patterns and an elongated nucleus over the 3 week differentiation period.

The predominant fraction of euchromatin in the nucleus representing the total amount of genetically active DNS is a morphological hint for an increased metabolic activity of the whole cell, increased through nucleic acid transcription. The present big and round nucleolus, centre of RNS-formation, represents the RNS-storage and can be taken as morphological indicator for increased protein biosynthesis in the cell.

Also the other typical organelles important for protein synthesis are distinct present in the cells. The high number of visible ribosomes in the cell is expression of increased protein biosynthesis and cell proliferation. The presence of predominantly rough ER indicates distinctive protein synthesis as well, as it is the case in the cells treated with GDF 5 respectively GDF 567. If the protein synthesis through the ribosome exceeds the amount of outlet belt, the inner space of the ER dilates like a cistern. Exactly this phenomenon can be observed in some of the cells differentiated with GDFs.

In the cells differentiated with GDF 5, respectively GDF 567 an accumulation of parallel aligned microfilaments, part of the cytoskeleton, could be detected. They stabilize the cell mechanically and also have an important role in the process of endocytosis and exocytosis. After the constriction of vesicles, the actin filaments rest against the vesicles, which is a reason why endocytosis is located in the near surrounding of microfilament accumulations. The present microfilaments could also be vimentin containing filaments which are very specific for fibroblasts and typically represented in connective tissue, where they align along tension and compression forces.

Another very important task of the cytoskeleton in fibroblasts is the dissipation of tensile stress. The cells manage to do so by forming focal adhesions, cell-matrix interactions, mainly generated by the cytoskeleton proteins actin and vinculin. Vinculin basically makes the connection between actin and the cell membrane, where the interaction with the transmembrane protein integrin takes place. The integrins are the physical link to the ECM. On the one hand the focal adhesion complexes can act as mechanosensors that lead into a chemical response inside the cell and on the other hand they allow the cell to attach to the matrix and pull on the collagen fibrils and contract the matrix by cytoskeletal tension, generated within contractile microfilaments which pull inward on the membrane. The cell-matrix complex is then strained like a bow and a bowstring and allows the dissipation of tensile stress by relaxation. (Chicurel et al., 1998 and.Sarasa-Renedo and Chiquet, 2005).

The matrix contraction, in our experiment the collagen I gel contraction, was observed in all the gel samples sutured in the bioreactor and proofs the existence of cell-matrix adhesions, respectively focal adhesion complexes formed by the ASCs. Like tendon tissue in vivo, the cells in our in vitro experiment showed the ability of storing and releasing energy during motion.

When focal adhesions are formed, a remodelling of the actin mesh into stress fibres takes place (Heath and Dunn, 1978). Those stress fibres are well described by Heuser and Kirschner (1980) as demonstrated by electron microscopy. Those pictures are in accordance with our findings.

As described in the results chapter, the morphology of the cells differentiated under various conditions differs from each other. But why is it so important to reach a typical tendon like cell morphology? McBeath et al. (2004) demonstrated that cell shape as an exclusive factor regulates the differentiation of human MSCs towards a defined lineage, here the adipogenic and osteogenic lineage. In the mentioned study, it was proven that a shape-mediated signalling exists, which means that cell shape alone can alter stem cell commitment prior to exposure to differentiation factors. Cells round in shape, typical for adipocytes, tended to differentiate into adipocytes while elongated and spread cells, typical for osteocytes differentiated into osteoblasts, as shown by specific staining and gene expression. As the determinant factor in the decision process towards which lineage a MSC differentiates, the protein “Ras homologue gene family, member A”, briefly RhoA was identified. RhoA activation

leads into rho kinase (ROCK) activation, which has effects on the cytoskeletal tension. In spread cells RhoA activity was distinct higher than in round precursor cells. Further, absence of RhoA results in an adipogenic differentiation, activation of RhoA promoted osteogenesis. The thesis made by McBeath and colleagues, that cell shape as a driving factor in the differentiation process serves as a feedback control mechanism by which morphogenetic changes are tied to the programs of tissue specification.

As for adipogenesis and osteogenesis, it can be assumed that similar shape-mediated signalling pathways exist for other differentiation lineages, for example the tenogenic one. Further it can be assumed, that a tenocyte atypical shape may be recognized by the feedback control mechanism and the differentiation progress towards the tenogenic lineage might be stopped, interrupted or even reversed. Tendon typical cell morphology not only allows conclusions about cell function but also seems to be necessary for a sound tissue specific differentiation.

Gene expression

By RT-PCR and qRT-PCR the expression of the genes Col I, Col III, COMP and Scx was measured in the differently treated ASCs. Surprisingly, also the gene expression of the ASCs in the collagen scaffold which were not stretched or stimulated by GDFs was up-regulated, this applies especially for Col I and III.

How is that explainable? Engler et al. (2006) distinguished the importance of the matrix properties with regard to stem cell differentiation towards a specific lineage. Engler and colleagues even proved that stem cell lineage specification can be directed by matrix elasticity only. According to that, soft matrices with an microenvironment elasticity of about 1 kPa that mimic brain are neurogenic, while stiffer matrices with a matrix elasticity of about 10 kPa that mimic muscle are myogenic and rigid matrices with a matrix elasticity of about 100 kPa that mimic bone are osteogenic. Naive MSCs, originally small and round, seeded on a collagen I coated gel scaffold develop branched, spindle, or polygonal shapes when grown on matrices with an elasticity typical for brain, muscle or bone respectively. Besides the lineage specific morphology also the lineage specific gene expression and collagen I production increased when MSCs were grown on a matrix with the tissue typical

elasticity. Explained is the phenomenon by the actin cytoskeleton and focal adhesions, part of the above described focal-adhesion complex, which are able to sense matrix elasticity and transduce the information into morphological changes and lineage specification. Beyond that it was shown that matrix elasticity is more important for stem cell differentiation than soluble induction factors. During the first week in culture lineage reprogramming with addition of soluble factors was possible, but afterwards a reprogramming towards a lineage other than the lineage specified by matrix elasticity was not possible any more. As the coherency of matrix elasticity and neurogenic, myogenic and osteogenic differentiation respectively was shown, it can be assumed that the importance of the matrix properties also has to be taken in account for the tenogenic differentiation. It is absolutely possible that the detected gene expression, especially Col I and Col III, in the NCws (mechanically not stimulated and without substitution of GDFs) and in DMEM (mechanically not stimulated, without tenogenic induction medium and without substitution of GDFs) was induced by the collagen I gel scaffold, which has an estimated elasticity of about 3.2 kPa and therefore ranges between the estimated elasticity of neurogenic and myogenic tissue. Arda et al. (2011) examined the elasticity of diverse human soft tissues and determined the elasticity of the human Achilles tendon with 51.5 +/- 25.1 kPa. According to those results, the stiffness of the utilized collagen I gel scaffold in our experiments needed to be higher, measured in kPa, in order to better imitate the real in vivo tendon tissue properties. Although the elasticity/stiffness of equine tendon tissue has not been measured/ published yet, it can be assumed that it is at least not underneath the estimated elasticity for human tendons. Perhaps a higher gene expression of COMP and Scx could have been reached with a stiffer scaffold that imitates equine tendon tissue better.

Further scaffold properties and their influence on stem cell differentiation have been examined; for example the influence of the existence and the formation of nanofibers in scaffolds. Yin and colleagues (2010) reported that aligned nanofibers induce tendon-like tissue differentiation with a higher expression of tendon-specific genes compared with the differentiation of stem cells that were cultured on randomly orientated nanofibres. That shows on the one hand that the collagen fibres in the used collagen gels in our experiments brought the tenogenic differentiation forward, on the other hand it can be assumed that a higher expression of tendon specific

genes could have been possibly reached using aligned collagen fibres in the utilized scaffolds instead of randomly orientated fibres.

Besides the scaffold properties, the mechanical stimulation of the ASCs seems to be necessary to drive stem cell differentiation towards the tenogenic lineage. Cell morphology and gene expression, especially COMP and Scx, of the differentiated ASCs in our experiment allow the conclusion that tendon-like tissue formation cannot be reached without mechanical stimulation. Mechanically not stimulated cells presented a tendons atypical morphology and the relevant markers COMP and Scx were barely measurable and showed the biggest gap between the gene expression of the reference tissue (equine tendon) and the in cell culture differentiated ASCs.

Altman et al. (2001) demonstrated that mechanical stimulation of human BM-MSCs located in a collagen gel over 21 days induces a ligament cell lineage formation, even though no ligament-selective GDFs were used. Ligament markers like Col I and Col III were up-regulated and the mechanical stimulation led into cell alignment and collagen fiber formation.

Kall et al. (2004) examined the in vitro fabrication of tendon tissue of mechanically stimulated human MSCs in a collagen gel versus non stimulated cells. The differentiated cells in the stretched constructs clearly showed a more tendon like morphology than the not stretched constructs. In accordance with that, Chokalingam et al. (2009) reported a significantly higher collagen I gene expression in mechanically stimulated collagen constructs carrying murine stem cells than not stimulated controls.

Combining in vitro and in vivo experiments, Juncosa-Melvin and colleagues (2006) compared tendon injury healing in rabbits after implantation of mechanically stimulated versus not stimulated autogenous MSCs in a collagen construct. A peak strain of 4% was used for 2 weeks. Mechanically stimulated constructs had a 2.5 times higher stiffness compared with the not stimulated constructs. Furthermore, 3 months after surgery analyses revealed a significant improvement of tendon repair biomechanics.

Kuo and Tuan (2008) reported the up-regulation of collagen and scleraxis gene expression and matrix production of human BM-MSCs, seeded on a collagen gel, in response to cyclic tensile stimulation.

The trigger effect of the combination of MSCs, an appropriate bioscaffold and mechanical stimulation on tenogenesis was demonstrated by Butler and colleagues

(2008). The beneficial effect of MSCs in tendon therapy can be triggered using a collagen I sponge. Repair stiffness and maximum force bearing increased and matched normal in vivo properties. Mechanical stimulation of the scaffolds further increased repair biomechanics. All those published reports match our results.

As described above, the cell morphology of the ASCs treated with GDF 5, GDF 6, GDF 7, respectively a combination of these factors revealed the best differentiation results according to a tendon-like cell appearance. The results of the quantitative RT-PCR showed that the ASC-gel constructs differentiated with applied strain and supplemented with GDFs nearly almost showed the highest gene expression, especially for COMP and Scx. Even though the Col I- and Col III- gene expression in these groups was much higher than in the reference tissue and therefore matched them least, it seems normal to expect a high collagen expression in early stages of new tendon tissue formation. Diverse literature exists in which the authors report tendon-like tissue formation after utilizing GDF 5 (Rickert et al., 2001, Farng et al., 2008, Park et al., 2010), GDF 7 (Lee et al., 2011), or a combination of GDF 5, 6 and 7 (Wolfman et al., 1997).

Already in 1997 Wolfman et al. reported the induction of neotendon/-ligament formation in rats after stimulating subcutaneous respectively intramuscular progenitor cells with implantations carrying GDF 5, 6 or 7.

In vitro treatment of MSCs with GDF 5 (Farng et al., 2008 and Park et al., 2010), as well as utilization of GDF 5 coated sutures in tendon repair in vivo (Rickert et al., 2001) brought better histological and molecular results according to tendon-tissue formation compared with the control groups.

Lee and colleagues (2011) described the in vitro differentiation of rat BM-MSCs into tenocyte-like cells after GDF 7 application for a time period of only 12 hours. In vivo implantation into surgically created tendon defects led into robust tendon-like tissue formation with the tendon typical morphology and gene expression.

As described in the introduction part, cells carry growth factor receptors. Their activation leads amongst others into the collagen gene expression in the nucleus and therefor into collagen fibril production. GDF 5, 6 and 7 seem to be able to stimulate tenogenesis properly. In our experiment a supplement with GDF 5 or GDF 7 seems to bring the best results according to gene expression and cell morphology. However, some authors describe unwanted side effects, in particular the formation of cartilage-

like structures (Rickert et al., 2001). However, a differentiation other than towards the tenogenic lineage could not be observed in our experiment.
Relating to the oxygenation the results can be interpreted differently. Under the influence of 21% oxygen tension the gene expression of Col I and Col III revealed the highest results. Compared with the reference value, the equine tendon, the results for Col I- and Col III- gene expression under the influence of 3% oxygen tension matched the reference tissue best. The question is if a high gene expression or the best match with the reference value is more desirable. Since we have the situation of a neo tissue formation in the cell experiment, it can be assumed that a preferably high gene expression represents the tissue assembly best.
The results for the gene expression of COMP and Scx are very similar under both oxygen conditions, although the SEM is distinct higher under hypoxic conditions. Consequently it is more likely to reach the same results under an oxygen tension of 21%.

In summary it can be said that a differentiation of ASCs in a collagen I gel scaffold seems to be most promising with tensile stimulation, supplement of GDF 5 or 7 and an oxygen tension of 21%, mostly because the cell morphology fits tenocytes best and qRT-PCR results are more stable under normoxic conditions.

6 Summary

Oxygen tension is an important factor for stem cell culture and differentiation. Since in vivo conditions are mostly hypoxic, but in vitro conditions are generally normoxic, the goal of this study was to examine and compare equine ASCs behaviour and differentiation potential under hypoxic (3% O_2) and normoxic (21% O_2) conditions. Examined was the differentiation potential towards the adipogenic, osteogenic, chondrogenic and tenogenic lineage, with a special interest and focus on the tenogenic differentiation, since tendon injuries are one of the most common lameness reason in the horse and lack of satisfying treatment options at the same time.

While the expression of characteristic stem cell markers did not vary under normoxic or hypoxic culture conditions, the cell proliferation as determined by the MTT assay was higher under normoxic conditions. We were also able to show that adipogenesis and chondrogenesis revealed better histological differentiation results under hypoxic conditions, while osteogenesis was more effective under normoxic culture conditions.

For the tenogenic differentiation potential not only the influence of oxygen tension, but also the influence of a 3d collagen scaffold, applied uniaxial tensile strain and supplementation of growth differentiation factors, namely GDF 5, GDF 6, GDF 7, respectively a combination of those three factors was examined in a combined in vitro experiment.
Immunohistochemistry revealed that all ASCs of the differently treated collagen constructs formed cell to cell contacts and developed a 3d network in the scaffold. Furthermore it could be demonstrated that tensile strain is necessary to reach matrix stiffness, tendon-typical cell morphology and a coordinated cell alignment in the scaffold. By electron microscopy it could be shown that the examined cell morphology was more tendon-typical under an oxygen tension of 21%, while the gene expression of the tendon relevant markers Col I, Col III, COMP and Scx revealed no big differences under the compared oxygen tensions, even though the results under normoxic conditions were more stable. Compared with equine tendon, the gene expression of Col I and Col III was higher in the samples of the in vitro engineered tendon-like cells, but the gene expression of COMP and Scx was distinct

lower. A supplementation of the medium with GDFs, especially GDF 5 and GDF 7 improved the cell morphology and the gene expression. Surprisingly we could show that alone the usage of the 3d collagen scaffold helped to drive the stem cell differentiation towards the tenogenic lineage. An expression of all four examined genes could be detected, even though the gene expression of COMP and Scx was very low.

At the end of the pre-differentiation experiment the cells emigrated the collagen construct after taking the scaffold out of the bioreactor and placing it in DMEM. This way, the pre-differentiated cells can be harvested and used for in vivo application. Hopes are, that the in vivo application of in vitro pre-differentiated tenocyte-like cells lead to faster and better tendon repair, if not regeneration compared with the usage of undifferentiated ASCs.

7 Zusammenfassung

Der Sauerstoffgehalt ist ein wichtiger Faktor für die Anzucht und Differenzierung von Stammzellen. Da die in vivo Bedingungen überwiegend hypoxisch, die in vitro Bedingungen aber in der Regel normoxisch sind, war das Ziel dieser Arbeit das Verhalten und das Differenzierungspotenzial equiner, adipogener Stammzellen bei hypoxischen (3% O_2) und normoxischen (21% O_2) Bedingungen zu untersuchen und zu vergleichen. Untersucht wurde das adipogene, osteogene, chondrogene und tenogene Differenzierungspotenzial, mit besonderem Augenmerk auf die tenogene Differenzierung, da Sehnenverletzungen eine der häufigsten Lahmheitsursachen des Pferdes sind und zudem keine zufriedenstellenden Behandlungsoptionen existieren.

Während die Expression stammzellcharakteristischer Marker sich bei den unterschiedlichen Sauerstoffgehalten nicht unterschied, war die im MTT Test gemessene Zellproliferation unter normoxischen Bedingungen höher. Weiterhin konnten wir zeigen, dass die Adipogenese und die Chondrogenese bessere histologische Ergebnisse bei hypoxischen Bedingungen aufwiesen, während die Osteogenese bei normoxischen Bedingungen effektiver war.

Um das tenogene Differenzierungspotenzial zu untersuchen wurde nicht nur der Sauerstoffgehalt, sondern auch der Einfluss eines 3d Kollagengels, uniaxialer Dehnung und die Verwendung von Wachstumsfaktoren, namentlich GDF 5, GDF 6, GDF 7 und einer Kombination aus den dreien in einem kombinierten in vitro Experiment untersucht.
Die Ergebnisse der Immunhistochemie zeigten, dass alle ASCs der unterschiedlich behandelten Gel-Konstrukte Zell-zu-Zell Kontakte ausbildeten und ein 3d Netzwerk in der Matrix formten. Weiterhin konnte demonstriert werden, dass ein Dehnungsreiz notwendig ist, um eine gewisse Matrixsteifheit, eine sehnentypische Zellmorphologie und eine koordinierte Zellausrichtung im Gel zu erreichen. Mit Hilfe der Elektronenmikroskopie konnte gezeigt werden, dass eine für Sehnen typischere Zellmorphologie bei einem Sauerstoffgehalt von 21% erreicht werden kann, während die Genexpression der sehnenrelevanten Marker Kollagen I, Kollagen III, COMP und Scleraxis unter den zwei verschiedenen Sauerstoffgehalten keinen wesentlichen Unterschied aufwies. Verglichen mit der Pferdesehne war die Expression von

Kollagen I und Kollagen III in den in der Zellkultur gezüchteten Sehnenzellen höher, aber die Expression von COMP und Scleraxis war deutlich niedriger. Die Zugabe der Wachstumsfaktoren zum Medium, insbesondere von GDF 5 und GDF 7 verbesserten Zellmorphologie und Genexpression. Wir konnten zeigen, dass überraschenderweise allein die Verwendung der 3d Matrix in gewissem Masse zur Differenzierung in die tenogene Richtung führte. Die Expression aller vier untersuchten Gene konnte nachgewiesen werden, auch wenn die Expression von COMP und Scleraxis sehr gering war.

Am Ende des Differenzierungsversuches emigrierten die Zellen aus der Kollagenmatrix, nachdem sie aus dem Bioreaktor herausgenommen und in eine Schale mit DMEM gelegt wurden. Auf diese Weise können die vordifferenzierten sehnenähnlichen Zellen geerntet und für in vivo Versuche verwendet werden. Es besteht die Hoffnung, dass die in vivo Anwendung von in vitro vordifferenzierten sehnenähnlichen Zellen zu schnelleren und besseren Heilungsergebnissen, falls nicht sogar zur vollständigen Regeneration bei Sehnenverletzungen führt.

8 Outlook

After the pre-differentiation of the equine ASCs towards the tenogenic lineage, the next step would be to estimate their abilities in vivo, analogical to Butler`s functional tissue engineering (FTE) road map that requires the in vivo evaluation of in vitro engineered tissue (Butler et al., 2009). Therefor the in vitro differentiated cells shall be injected into experimentally induced tendon lesions of defined size in an animal trial with approximately twenty horses. Following, the regenerative capacity shall be examined clinically, ultrasonographically, biomechanically and histological.
In detail, the in the collagen gel mechanically stimulated and with GDFs supported, differentiated cells will be harvested after emigrating the gel. Afterwards they can be labelled in order to track them down after injection into the lesion in vivo, to determine their survival rate after implantation and to evaluate their distribution and persistence in the tissue. Possibilities of cell labelling have already been described in the horse by Sole et al. (2011) and by Smith et al. (2011). They described the application of technetium-hexamethylpropyleneamine oxime respectively technetium-99m pertechnetate to hexamethylpropyleneamine oxime for stem cell labelling, which can be identified in vivo by scintigraphy. Another option would be the usage of magnetic beads for cell labelling, which can be imaged by MRI. Tests for that method in a dog model are currently running in our institute.
Furthermore the biomechanical abilities of cell treated tendons (stem cells and pre-differentiated ASCs) compared to non-cell treated tendons will be examined in cooperation with our partner university in Siegen.

In order to reach a higher harvest of pre-differentiated cells, the application of a scaffold other than the collagen gel for in vitro differentiation would be helpful. The gel was very helpful in order to perform laboratory tests for estimation of the abilities of the differentiated cells (e.g. stains), but to gain a high cell harvest another material would be better. The solution could be a “collagen cell carrier” on which cells can be seeded, stretched and then detached again.

Now it is time to connect in vitro science with in vivo application.

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10 Attachments

10.1 Publications and poster sessions

Partial results of this dissertation have already been published:

Shell K, Raabe O, Wenisch S, Arnhold S, (2011)
Behaviour and differentiation potential under the influence of 3% and 21% oxygen tension of equine adipose tissue derived stem cells.
28th workshop oft the anatomical society in Würzburg, Germany.

Shell K, Raabe O, Freitag C, Ohrndorf A, Christ H, Wenisch S, Arnhold S, (2012)
Comparison of equine adipose tissue derived stem cell behaviour and differentiation potential under the influence of 3% and 21% oxygen tension. **Journal of Equine Veterinary Science** (accepted for publication; DOI: 10.1016/j.jevs.2012.05.002).

Shell K, Raabe O, Fietz D, Freitag C, Ohrndorf A, Christ HJ, Wenisch S, Arnhold S, (2012) Tenogenic differentiation of equine adipose tissue derived stem cells under the influence of tensile strain, growth differentiation factors and different oxygen tensions. **Stem Cell Reviews and Reports** (submitted).

Raabe O, **Shell K**, Würtz A, Reich CM, Wenisch S, Arnhold S, (2011)
Further insights into the characterization of equine adipose tissue-derived mesenchymal stem cells. **Veterinary Research Communication.**

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