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DIPLOMA THESIS

Titel der Diplomarbeit

Activity of FGF-2 in Comparison to the FGF-s3-His Fragment on Human Adipose Derived Stem Cells

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- FÜR OPA -

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Abbreviations

A	Absorbance
aa	Amino acid
Ab	Antibody
AMV	Avian Myeloblastosis Virus
as	Antisense
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
bp	Base pairs
BPB	Bromophenol blue
BrdU	5´-bromo-2´deoxyuridine
℃	Degree Celsius
Ca^{2+}	Calcium
cDNA	Complementary deoxyribonucleic acid
d	Day
Da	Dalton
dd	Double distilled
ESCs	Embryonic stem cells
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco`s modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2´-deoxynucleoside-5´-triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

EtBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescence activated cell scanner/sorter
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGF-1	(acidic) aFGF
FGF-2	(basic) bFGF
FITC	Fluorescein-iso-thiocyanate
FL	Fluorescence channel
FS	Forward scatter
g	gravity
GAPDH	Glycerinealdehyde-3-phosphate dehydrogenase
h	Hour
hASC	Human adipose derived stem cell
His	Histidine
HMW	High molecular weight
H_2SO_4	Sulfuric acid
iPSCs	Induced pluripotent stem cells
k	Kilo
L	Liter
LMW	Low molecular weight
LPS	Lipopolysaccharide
λ	Wavelength
M	Marker
M	Molar
mg	Milligram
min	Minutes
mL	Milliliter

mM	Millimolar
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
n	Number of experiments
NaAc	Sodium acetate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide hydrogen phosphate
ng	Nanogram
nm	Nanometer
OD	Optical density
o/n	Over night
P	Pellet
PE	R-phycoerythrin
PenStrep	Penicillin-streptomycin solution
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potentia Hydrogenii
PI	Propidium iodide
PS	Phosphatidylserine
rh	Recombinant human
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
s	Sense
sec	Second
SN	Supernatant
SSC	Side scatter

Tm	Melting temperature
TMB	3,3´,5,5´-Tetramethylbenzidine
Taq	Thermus acquaticus
µg	Microgram
µL	Microliter
U	Unit
V	Voltage
XCFF	Xylene cyanol FF

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1 Introduction

1.1 General introduction

Tissue engineering and cell-based therapies constitute emerging scientific areas in regenerative medicine with therapeutic fields of applications in disease management and research [1,2]. Tissue engineering is challenging, it basically implies cells, biomaterials and bioactive factors (**Figure 1**) [3].

For the engineering of surgical implants, which target the restoration of physiological tissue function and the promotion of tissue regeneration after injuries, adequate scaffolds are required [4-7]. The construction of such matrices is a major issue in tissue engineering. They contain porous structures with a large surface volume, which mechanically support cells upon *in vitro* expansion, rendering a function which is similar to the extracellular matrix *in vivo*. Cells can grow on scaffolds and are able to invade them because of the interconnected pores. Thus, 3 dimensional and tissue-like structures can be built *in vitro*. A wide variety of different permanent or biodegradable scaffolds have been described, which are either of natural origin or produced synthetically.

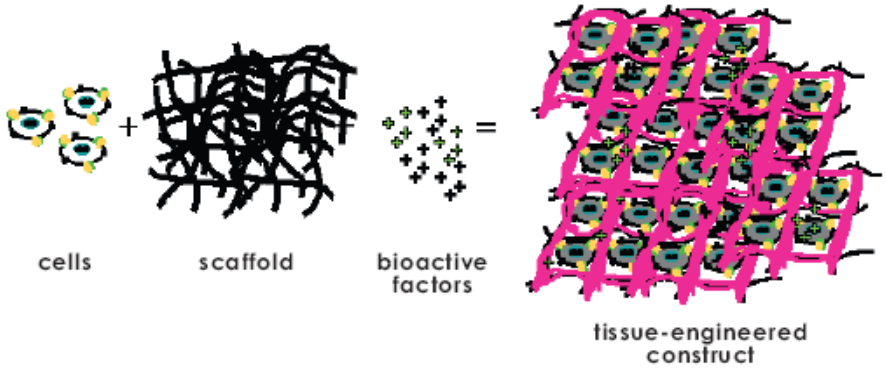

Figure 1 *Principle of tissue engineering*[1]*: Schematic overview.*

[1] source: http://www.bioeng.nus.edu.sg/research/keyrsch1.htm [online]

Much work has already been conducted with respect to sources of cells [6,8-14], including mature and differentiated cell types (*e.g.* epithelial cells, fibroblasts, chondrocytes) and undifferentiated, embryonic/adult stem cells. Many tissue engineering approaches use the benefits of stem cells, as they have a multilineage potential and can be differentiated into various cell types. Beside bone marrow derived mesenchymal stem cells (BM-MSCs), somatic stem cells isolated from adipose tissue (ASCs) have been proposed as a reliable source of autologous, multipotent cells for mesenchymal defect repair and wound healing [3,6,7,15-24,9,13,14,18]. Human adipose derived stem cells (hASCs) have a proven chondrogenic potential *in vitro* and *in vivo* [26-28]. Although, BM-MSCs and hASCs are very similar and share many properties, they are not identical and thus have to be studied separately.

Concerning the lineage commitment of stem cells, microenvironment and culture conditions seem to play an important role. Hence, additionally to matrices and adequate cell sources, specific environmental factors such as growth factors and cytokines are required for the engineering of bioartificial tissues. They provide signals which control *e.g.* cell growth and -proliferation and affect the differentiation capacity of (stem) cells in a dosage-dependent manner [6,9, 21,29]. Human fibroblast growth factor 2 (hFGF-2), a strong morphogen and mitogen with pleiotropic effects on various tissues and organs, is one of such factors [30]. It is one of the most studied growth factors with clinically proven effects in wound healing [31,32].

Engineering of tissues like cartilage, bone, ligaments and tendons would be highly beneficial for the functional restoration after chronic and acute tissue damage (*e.g.* osteoarthritis, traumatic/degenerative bone or joint defects or soft tissue reconstruction after tumor surgeries) [25]. Despite all recent progresses in stem cell research and tissue restoration, a full characterization and understanding of adult stem cell properties is missing and generalized culture conditions still have to be defined [33]. For bringing stem cell-based therapies into clinical use and, in order to be able to manipulate cells in a predictable way, it is primarily of major importance to understand the underlying molecular mechanisms of stem cell regulation [18,34].

1.2 Stem cells

Stem cells are unspecialized cells, characterized by their capacity of self-renewal (clonogenity), longevity and the potential to differentiate along multiple cell lineage pathways [35,40]. Therefore, they are able to give rise to distinct and terminally differentiated cell types and tissues, which in turn render specific functions and characteristics [1,17,36]. Stem cells are found in fetal tissue, as well as in many adult tissues. It is believed that they are persisting throughout life within the body. By replacing damaged cells and thus restoring specific tissue function, stem cells are contributing to the maintenance of the normal physiological function of an organism [16,33,37,38].

Although, the function of stem cells is not fully understood, their roles have been outlined especially

- during embryonic development, by building
  - extra-embryonic tissue (*e.g.* the placenta)
  - embryonic tissue: specification of the three embryonic germ layers (ectoderm, endoderm, mesoderm)

and

- throughout life in adulthood
  - for continuous maintenance of natural cellular homeostasis
  - for tissue remodeling
  - by replacing damaged cells and tissues they contribute to adult tissue repair and regeneration, after injuries or tissue damage [10,16,33,39].

By definition, stem cells can be classified by their origin (*e.g.* embryonic stem cells, adult stem stems), respectively by their developmental potential (**Figure 2**): totipotent stem cells show the ability of forming any cell type and tissue of the body, including extra-embryonic tissue; pluripotent stem cells are capable of building all cell types, which arise from the three germ layers and multipotent stem cells, which can give rise to terminally differentiated and mature cell types of one lineage [10,40].

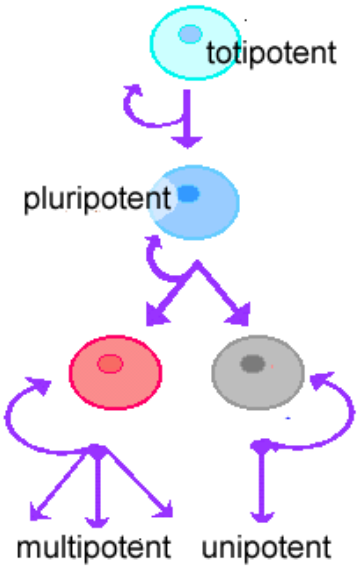

Figure 2 *Differentiation potential of stem cells*[2]

totipotent stem cells can be only isolated from the inner cell mass during early embryonic development (zygote), after gastrulation embryonic stem cells are considered to be pluripotent, multipotent stem cells give rise to different cells of one lineage, e.g. hematopoietic stem cells, which can differentiate into mature blood cells of the myeloid (monocytes, granulocytes, megacaryocytes etc.) and lymphoid (lymphocytes, natural killer cells etc.) lineage, unipotent progenitor cells, can mature into one distinct cell type (e.g. proerythroblasts).

1.2.1 Embryonic stem cells

Embryonic stem cells (ESCs) have firstly been isolated from the inner cell mass of blastocysts by Evans and Kaufman [41] and by Martins [42] in 1981. They are considered to be the least differentiated stem cells with a nearly unlimited proliferation capacity [1,36]. ESCs are totipotent, thus capable of forming a completely developed organism. However, as embryonic development progresses, their almost unlimited

[2] source: http://virtuallaboratory.colorado.edu/Biofundamentals/lectureNotes/AllGraphics/stemcellcommittment.gif [online]

proliferation potential is restricted and after gastrulation ESCs are becoming pluripotent [10,33,40].

In spite of the promising therapeutic potential of ESCs they are, mostly due to ethical issues, discussed controversially [6,7,9,16,43]. In addition, problems like contamination, the need of genetic alterations, cell regulation, the tumorigenic/teratogenic potential of transplanted cells or the procurement of sufficient cell quantities, limit their field of clinical applications [1].

1.2.2 Adult stem cells

Adult stem cells are needed for normal growth, as well as for tissue regeneration after acute or chronic tissue damage [12]. They are capable of differentiating into various distinct cell types and are responsible for tissue replacement after injuries, thereby helping to maintain tissue function and specificity [10,39,43]. Adult stem cells were originally isolated from the stroma of the bone marrow (BM) and were identified as multipotent osteoprogenitor cells [16,24,34,45-47]. Accordingly, these cells were classified as bone marrow derived mesenchymal stem cells (BM-MSCs). Many studies have demonstrated their ability to differentiate, after *in vitro* expansion and in the presence of specific differentiation- /growth factors, into mature fibroblasts, adipocytes, chondrocytes and myocytes [8-14,17]. Given that MSCs are able to transform into multiple mesenchymal cell lineages *in vitro* and *in vivo*, they appear to be an ideal population for mesodermal defect repair and tissue regeneration. However, drawbacks such as the painful and invasive isolation procedure (bone marrow aspiration), a low cell number harvest and high morbidity rates, confine their clinical use [15,17].

Since that, many reports have confirmed the presence of tissue-specific adult stem cell populations in almost all tissues of the body. So far, adult stem cells have been described for example in brain, bone marrow, peripheral blood, kidney, epithelia of the digestive system, skin, retina, muscles, pancreas, liver and adipose tissue [1,9,16,37,48].

Adipose tissue

Adipose tissue is highly complex, harboring multiple cell types, such as mature adipocytes/preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, monocytes/macrophages [49], lymphocytes [50] and, last but not least, a population of putative, multipotent stem cells [21,22]. Like BM-MSCs, stem cells isolated from adipose tissue, evolve from the embryonic mesenchyme (adipose derived mesenchymal stem cells, ASCs) [6,9,15,20,23]. They are capable of building all types of connective tissues within the body. Recently, it was shown that the differentiation potential of ASCs can be extended even across lineage boundaries, to ectodermal and endodermal tissues [7,24]. So far, ASCs were transformed already into osteogenic (bone) [3,6,9,13,17,24], adipogenic (fat) [3,6,9,13,17,21], myogenic (muscle) [3,6,9,13,17], chondrogenic (cartilage) [3,6,9,13,17,25,51], neurogenic (neurons), endothelial [6,13,18,24], hematopoietic [13,24] and cardiomyogenic [6, 24] cell lineages.

Properties of MSCs

Both, mesenchymal stem cells isolated from bone marrow and from adipose tissue, are of autologous origin and therefore immuno-compatible. They show a low tumorigenic potential and only minimal ethical restrictions accompany their use [1,6,9,10,13,52]. BM-MSCs and ASCs are not identical, but they display similar phenotypes, intrinsic-, angiogenic-, anti-inflammatory properties and differentiation capacity [6,9,15,18,19,23,53]. MSCs can be cryopreserved and are expanded easily in poor culture media [18]. They grow in clusters in monolayer-cultures, are adherent to plastic surfaces and are highly sensitive to trypsin [12,13,23,43]. In culture, MSCs progressively lose their differentiation capacity and become senescent after 15–50 doublings (variations are due to donor-to-donor differences) [12,34]. Phenotypically, BM-MSCs and ASCs exhibit a fibroblast-like, spindle-shaped morphology, although ASCs are slightly smaller (**Figure 3**) and are able to achieve higher passage numbers before becoming senescent [26].

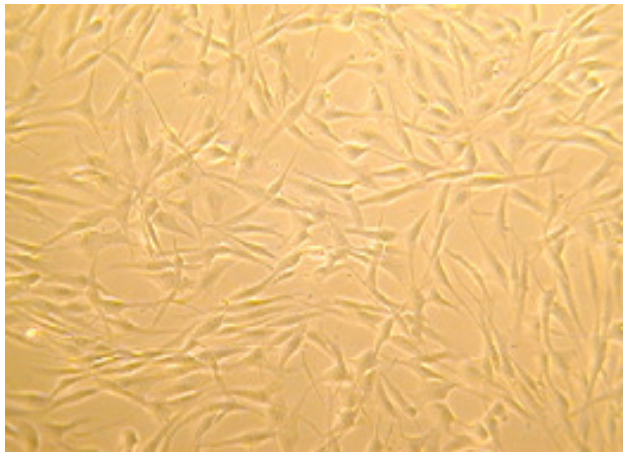

Figure 3 *Fibroblast-like morphology of hASCs*
Light microscopic picture of hASCs in passage 1, cells cultured in DMEM Ham´s F12 medium, 100x.

Human adipose derived stem cells (hASCs)

Human adipose derived stem cells are somatic stem cells of mesenchymal origin. Much effort was dedicated recently with regard to the isolation procedure, expansion and differentiation of hASCs [54]. Clinically they were applied for the first time in calvarial bone defects (the superior portion of the skull) [55].

Many advantages in the practical use of hASCs have been proposed. First of all, hASC are abundant, accessible and replenishable. The stem cell number in adipose tissue is significantly higher (2%) compared to bone marrow (0.002%) [24]. Procurement of hASCs by liposuction/lipoaspiration is surgically speaking easy, much less invasive, repeatable and brings less discomfort for the patient than bone marrow aspiration. Applications are not restricted due to low cell quantity and only minimal morbidity rates have been observed upon harvest [6,9,15,17,18,21-24].

Among many other findings which concern the functional and physiological effects of hASCs *in vitro* and *in vivo*, it was discovered that uncommitted hACS promote tissue regeneration and wound healing, by secretion of angiogenic and anti-apoptotic cytokines and growth factors [21,56]. They can undergo osteogenesis *in vitro* and it was also shown in an ectopic bone model, that hASCs are able to form bone *in vivo* [24]. Furthermore, hASCs are able to build and deliver extracellular matrix components, which are vital in engineering of structural elements and connective tissues [1,3,7,8,24, 37].

1.3 Fibroblast growth factor

Human fibroblast growth factor 2 (basic FGF, bFGF, FGF-2) belongs to the family of the heparin-binding factors. Human FGF-2 (hFGF-2) is produced by a variety of different cell types with notable differences in its tissue distribution and concentration [57]. It was one of the first family members to be identified already in 1982, along with the closely related homolog FGF-1 (acidic FGF, aFGF). Human FGF-1 and hFGF-2 are assigned as embryonic inducers with strong mitogenic activities [30,58-60].

To date, this constantly growing family of growth factors constitutes over 20 related polypeptides. Family members are between 150 and 270 amino acids (aa) long and share a conserved central region of 144 aa [30,61]. The hFGF-2 gene is located on chromosome 4. It is 56 kb long, containing 3 exons and 2 introns [59,62]. After transcription of a single mRNA, different hFGF-2 isoforms can be produced by alternative translation. There are 4 high molecular weight (HMW) hFGF-2 forms with 22, 22.5, 24, 34 kDa, and 1 low molecular weight (LMW) hFGF-2 of 18 kDa. [30,63,64]. The different hFGF-2 isoforms lead to different and specific cell phenotypes. Usually cells are able to produce all hFGF-2 forms [57,59].

High molecular weight hFGF-2 forms are residing in the nucleus, acting in a FGF-receptor (FGFR) independent manner. Their expression is directly linked to cell growth and proliferation, although cell migration is not changed upon its expression [30,59,62].

Low molecular weight hFGF-2 (18 kDa) serves as an autocrine, extracellular signaling molecule. Intracellular, LMW hFGF-2 is located in the cytoplasm. LMW hFGF-2 interacts with 4 specific, transmembrane FGF-receptor tyrosine kinases (FGFR1, FGFR2, FGFR3, FGFR4). Via FGFR activation, downstream signaling pathways linked to cell proliferation and differentiation can be altered [30,57,59,60,62,65]. LMW hFGF-2 expression leads to enhanced cell migration and mitogenesis.

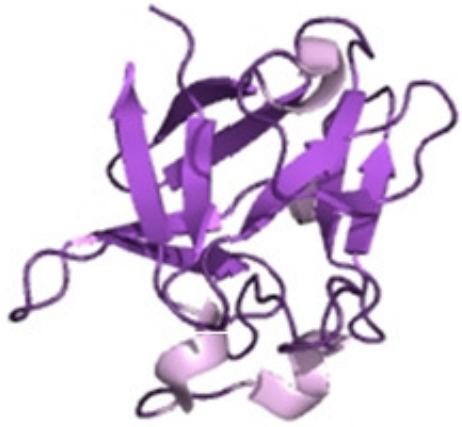

Figure 4 *Three dimensional structure of hFGF-2*[3]

Human FGF-2 is build by 3 connected groups of 11 antiparallel beta strands. Alpha helical structures are linking the residues 130 and 137, which are part of the heparin binding site.

Human FGF-2 (**Figure 4**) shows high affinity to heparin sulfate and heparin sulfate proteoglycans. Binding of heparin is important for the functional activity of hFGF-2, resulting in conformational changes, which protect hFGF-2 from proteolysis or denaturation and thus increases the biological half life of hFGF-2 [66,67].

Properties of hFGF-2

Human FGF-2 is regulating basic cellular functions in cells of mesenchymal, endo- and ectodermal origin [30,58,68]. Its strong chemotactic and mitogenic properties makes it an important molecule for both, differentiation processes during early stages of development and for tissue remodeling [51,58]. It is implicated in angiogenensis by controlling cell migration, endothelial cell proliferation and tubulogenesis [59]. Human FGF-2 was also found to be engaged in invasive cell spreading during tumorigenesis. It regulates self-renewal of embryonic-, hematopoietic- and neuronal stem cells [69]. Further observed effects of hFGF-2 are [30,57,58]:

- stimulation of cell proliferation and differentiation (*e.g.* in fibroblasts, osteoblasts, endothelial- and epithelial cells)
- morphogenic modulation of the cellular phenotype

[3] source: European Bioinformatics Institute: http://www.ebi.ac.uk/ [online]

- modulation of extracellular matrix components, organization of cytoskeleton
- delay of cell senescence
- stimulation of hematopoiesis
- impact on the function of the nervous system, eye and skeleton
- the promotion of wound healing, tissue repair and melanogenesis

MSCs are highly sensitive to hFGF-2 treatment *in vitro*. Therefore, hFGF-2 can be added as supplement to cell culture medium for optimization of the proliferation capacity [14,70]. It was shown that hFGF-2 helps to maintain the stem cell properties of MSCs and it enhances cell expansion during culture. Reports confirmed an increased chondrogenic lineage commitment of MSCs in the presence of hFGF-2 [26,34]. In hASCs, adipogenesis and chondrogenesis (induction of cartilage synthesis) was induced, whereas osteogenesis was inhibited by hFGF-2 treatment [25,51,70,71]. Other studies gave account of osteoblast and neuronal induction by hFGF-2 [43,65] *in vitro*, with some evidence of *in vivo* bone generation [34,40,70]. In animal studies, its role in healing of tendons and ligaments was outlined [8].

Contradictory data illustrate the complexity of hFGF-2 associated molecular mechanisms. Effects of hFGF-2 are strongly influenced by culture conditions, microenvironment and the applied dosage. Proliferation always seems to be up-regulated, accompanied with a change in cell size (hFGF-2 treated cells appear to be approximately 30% smaller), morphology and increased life spans of treated cells [15,34].

Recombinant, human FGF-2 constructs

Human FGF-2 constitutes a binding site for fibrin and fibrinogen near the C-terminus, which has already been described by Sahni *et al.*, 1998 [72] and Peng *et al.,* 2004 [73]. After injuries, thrombin converts fibrinogen into fibrin. Thereby a temporary, structural matrix is provided, which supports local tissue healing. Human FGF-2 is also present at sites of injuries. It is suspected that the interaction between fibrinogen/fibrin and

hFGF-2, localizes hFGF-2 to sites of injuries. By that means, endothelial cell response and vascular repair can be coordinated.

By use of commercially available fibrin sealant products, which consist of fibrinogen and thrombin, fibrin-based clots can be formed *in vitro*. Originally, these natural sealants or glues were developed for the support of homeostasis after injuries and wound care. However, fibrin-based clots can be utilized also as biodegradable scaffolds in tissue engineering [74]. A new approach in this context is the construction of truncated hFGF-2 variants, which are containing the fibrinogen binding site. Thereby drugs could be linked via hFGF-2 to fibrinogen and, after implantation, would be released slowly *in vivo* from the fibrin matrix. Ideally, truncated hFGF-2 variants should be as small as possible; containing only just the binding site. However, it was shown already that a stable interaction between hFGF-2 and fibrinogen/fibrin depends on more than only the binding site. The optimal/minimal length of such construct remains to be determined. Two such hFGF-2 variants (FGF-s3 HIS, FGF-2 HIS, **Figure 5**), which show a high binding affinity to fibrin, have been synthesized previously [61]. In this work they were tested in comparison to commercially available recombinant human FGF-2 (rhFGF-2).

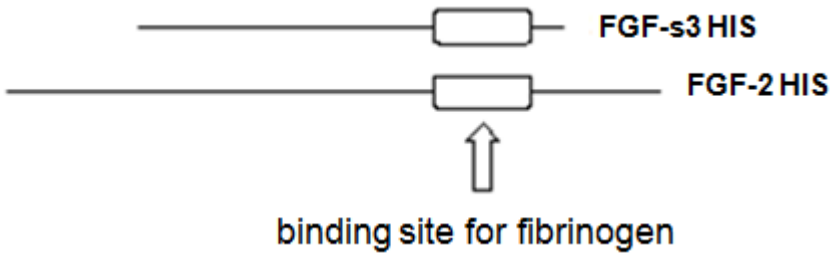

Figure 5 *Recombinant hFGF-2 constructs*

Constructs are containing the 15 aa binding domain for fibrinogen. FGF-s3 HIS truncated rhFGF-2, 156 bp; FGF-2 HIS full length rhFGF-2, 486 bp.

Aim of the study

The overall aim of this study was to examine the effects of exogenously added human recombinant fibroblast growth factor 2 (rhFGF-2) on human adipose derived stem cells *in vitro*. Following questions were to be answered:

1. Which differences in cell morphology, proliferation, apoptosis rates, cell viability and -density can be observed, when cultivating hASCs with hFGF-2 at varying concentrations?
2. Which dosage of hFGF-2 should be added to hASCs for optimal proliferation and differentiation?
3. Does the hFGF-2 concentration affect the differentiation potential, respectively lineage commitment of hASCs?

Additionally, the biological activity of two self-produced hFGF-2 constructs of varying sizes, which are containing the fibrinogen binding site, was determined: a.) FGF-2 HIS, a full length rhFGF-2 and b.) FGF-s3 HIS, a shorter truncated rhFGF-2 variant, both containing a 6x His tag tail.

4. Are the results similar to the commercially available control peptide (rhFGF-2, Peprotech, UK)?
5. What effect has the truncated FGF-s3 HIS fragment on hASCs regarding cell morphology, apoptosis rates, proliferation, cell viability and differentiation potential?

2 Materials and methods

2.1 Materials

Cells and media

hASCs	human adipose derived stem cells, Red Cross Blood Centre/Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (Linz/Vienna, Austria)
DMEM Ham`s F12	Dulbecco`s modified Eagles medium Ham`s F12, Sigma-Aldrich (Vienna, Austria), 1% PenStrep, 10% FCS, 2 mM L-glutamine

Supplements

PenStrep	penicillin-streptomycin solution, stabilized, Sigma-Aldrich (Vienna, Austria)
FCS	fetal calf serum, Cambrex (Verviers, Belgium)
L-glutamine	200 mM, Sigma-Aldrich (Vienna, Austria)

Growth factors

rhFGF-2	recombinant human FGF-2 (rhFGF-2, 462 bp), Peprotech (UK)
FGF-2 HIS	rhFGF-2 (486 bp), containing a 6x His-tag tail
FGF-s3 HIS	truncated rhFGF-2 (156 bp)

2.1.1 Solutions and chemicals

ddH_2O	double distilled water, Mayrhofer Pharmazeutika GmbH (Leonding, Austria)
10x PBS	phosphate buffered saline, BioWhittaker Europe (Belgium)
10x Trypsin	0.5% porcine trypsin, Sigma-Aldrich (Vienna, Austria)
DMSO	dimethyl sulfoxid, sterile filtered, Fluka (Buchs, Switzerland)
EDTA	ethylenediaminetetraacetic acid, VWR (Darmstadt, Germany)
Tris base	AppliChem (Darmstadt, Germany)
1 M H_2SO_4	sulfuric acid, Fluka (Buchs, Switzerland)
Trypan blue solution	0.05 g trypan-blue, add 100 mL 0.9% NaCl, Merck Chemicals (Vienna, Austria)
MTT	[3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide], Merck Chemicals (Vienna, Austria), stock solution: 5000 mg/L, working solution: cell culture medium with 650 mg/mL MTT
10x TBE Buffer	108 g Tris, 55 g boric acid, 40 mL 0.5 M EDTA, pH 8, add 1 L ddH_2O
Lipopolysaccharide	LPS from Escherichia coli serotype O26:B6, 1 mg/mL stock solution, diluted 1:1000 in ddH_2O to a final concentration of 1 µg/mL

Molecular biology

DEPC	diethyl pyrocarbonate, Fluka (Buchs, Switzerland)
0.1% DEPC-treated water	100 µL DEPC in 100 mL ddH_2O, incubated o/n at 37°C, sterilized at 121°C, 1 bar, 20 min
Agarose	Biozym (Oldendorf, Germany)

Ethidium bromide	10 mg/mL in ddH_2O, Sigma- Aldrich (Missouri, USA)
XCFF	xylene cyanol FF, DNA 10x gel-loading Dye, Sigma-Aldrich (Vienna, Austria)
6x XCFF gel loading buffer	0.025% XCFF, 30% Glycerine, in 10 mM Tris pH 8.0, for DNA fragments < 1000 bp
3 M NaAc	sodium acetate, VWR (Darmstadt, Germany), 3 M in DEPC-treated water, adjusted to pH of 5.2 with 3 M acetic acid, sterilized at 121°C, 1 bar, 20 min
Ethanol absolute p.a.	Sigma-Aldrich (Missouri, USA)
70 Vol% EtOH-DEPC	70 mL ethanol absolute, diluted with 30 mL DEPC-treated water
2-propanol	Fluka (Buchs, Switzerland)
Oligo-dT_{15}	stock solution of 1 µg/µL, MWG- Biotech AG (Ebersberg, Germany)
β-Mercaptoethanol	Sigma-Aldrich (Vienna, Austria)
dNTP mix (100mM)	deoxynucleotide mix, lyophilized primers were reconstituted with DEPC-treated water to a 100 mM Stock, PeqLab (Erlangen, Germany), stored at -20°C.
10 mM dNTP-DEPC	100 mM dNTP stock diluted 1:10 in DEPC- treated water to obtain a final concentration of 10 mM, stored at -20°C
1.5% agarose gel	0.45 g agarose in 30 mL 1 x TBE buffer, plus 1.2 µL ethidium bromide

DNA molecular weight markers:

GeneRuler™ DNA Ladder, Fermentas (St.Leon-Rot, Germany)

Low Range	1500 – 50 bp: 1500 bp, 850 bp, 400 bp, 200 bp, 50 bp
Middle Range	5000 –1000 bp: 5000 bp, 2000 bp, 850 bp, 400 bp, 100 bp

PCR primers (**Table 1**)

Primer stock MWG Biotech (Ebersberg, Germany), lyophilized primers were reconstituted with ddH_2O to a concentration of 100 µM and stored at -20°C.

Primer s/as mix 10 µm sense (forward) + 10 µm antisense (reverse) Primer in ddH_20, 10 µM

Primer	NAME	Tm [C°]		Sequence (5´to 3´)
1	COL1a2	55.3	s	GAAAACATCCCAGCCAAGAA
		59.4	as	CAGGTTGCCAGTCTCCTCAT
2	COL1a1	53,.7	s	TGACGAGACCAAGAACTG
		57.3	as	CCATCCAAACCACTGAAACC
3	COL3	61.0	s	CAGGTGAACGTGGAGCTGC
		61.4	as	TGCCACCAGTGTTTCCGTGG
4	VIM	59.4	s	CTGCCAACCGGAACAATGAC
		59.8	as	CACGAAGGTGACGAGCCATTT
5	DES	69.5	s	ATGTGGAGATTGCCACCTACCGGAAGCTG
		69.5	as	GTGTCCTGGGATGGAAGAAGGCTGGCTT
6	aSMA	59.4	s	CGACCGAATGCAGAAGGAGA
		57.3	as	TTTGCGGTGGACAATGGAAG
7	GAPDH	61.0	s	TTAGCACCCCTGGCCAAGG
		59.4	as	CTTACTCCTTGGAGGCCATG

Table 1 *DNA primers for RT PCR analysis*

Tm*: melting temperature,* ***s****: sense primer,* ***as****: anitsense primer*
1 *collagen type 1, a2 (COL1a2),* ***2*** *collagen type 1, a1 (COL1a1),* ***3*** *collagen type 3 (COL3),* ***4*** *vimentin (VIM),* ***5*** *desmin (DES),* ***6*** *alpha-smooth muscle actin (aSMA),* ***7*** *glycerinealdehyde-3-phosphate dehydrogenase (GAPDH).*

2.1.2 Biological kits and enzymes

BrdU Cell Proliferation ELISA BrdU (colorimetric) Kit, Roche (Vienna, Austria)

RNA Isolation GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich (Vienna, Austria)

Desoxyribonuclease I RQ1 RNase-Free Dnase 1 U/µL, 10x DNase reaction buffer, stop solution, Promega (Madison,

	WI, USA)
DNA Polymerase	Taq DNA Polymerase, 10x reaction buffer without $MgCl_2$, 25 mM $MgCl_2$ solution, Sigma-Aldrich (Vienna, Austria)
AMV reverse transcriptase	1000 U, plus 5x buffer, Promega (Madison, WI, USA)
Apoptose Detection Kit	Annexin-V-Fluos Staining Kit plus sample buffer, Roche (Vienna, Austria)

2.1.3 Devices and equipment

Eppendorf reaction tubes	1.5 mL, 2 mL, sterile, Greiner Bio-One (Kremsmünster, Austria)
PCR reaction tubes	Greiner Bio-One (Kremsmünster, Austria)
Pipettes	5, 10 and 25 mL, Sterilin, Dr. Bertoni (Vienna, Austria)
	2-10 µL, 20-200 µL, 200-1000 µL, Gilson (Limburg-Offheim, Germany)
Filter tips, sterile	1-100 µL and 200-1000 µL; Greiner Bio-One (Kremsmünster, Austria)
Tissue culture plates	6-, 96-well plates Corning Life Science (Schiphol-Rijk, The Netherlands)
Polypropylene tubes	15 mL, 50 mL, Greiner Bio-One (Kremsmünster, Austria)
Tissue culture flasks	150 cm^2, 75 cm^2, Greiner Bio-One Cellstar (Kremsmünster, Austria)
Neubauer haemacytometer	Marienfeld Germany (Lauda-Königshofen, Germany)
CryoS PP tubes	Greiner Bio-One (Kremsmünster, Austria)

PP-Tubes	5 mL, Greiner Bio-One (Kremsmünster, Austria)
Multichannel pipette	25-300 µL, Micronic-Systems (Lelystad, Netherlands)
Heraeus megafuge **1.OR Centrifuge**	Heraeus Holding GmbH (Hanau, Germany)
Eppendorf Centrifuge 5415R	Eppendorf AG (Hamburg, Germany)
Thermomixer	TGradient Thermoblock, Biometra (Goettingen, Germany)
Thermocycler	Tpersonal, Biometra (Goettingen, Germany)
Pipetboy	Hirschmann Laborgeräte, Roth (Karlsruhe, Germany)
Laminar flow hood	Biohazard UVUB 1200, Uniflow, KR-125 Safety, Uniequip (Munic, Germany)
Incubator	37°C, 5% CO_2, Binder (Tuttlingen, Germany)
Microscope	Zeiss Axiovert 10 (10x, 20x, 50x), Carl Zeiss (Jena, Germany)
Plate Reader	SLT Spectra, Software: BIOLISE
FACS	Fluorescence activated cell sorter Cytomics FC 500 MPL, Beckman Coulter (Fullerton, USA) Software FACS MXP Cytometer/ Analysis, Cytometer RXP Analysis
Photometer	Smart Spec™ 3000, Biorad; Hercules (CA, USA)
Electrophoresis Chamber	ComPhor L Mini, BIOzym (Hamburg, Germany)
Powersupply	Power Pac 300, Biorad (CA, USA)
ChemiImager™4400 **Fluorescence**	BIOzym (Hamburg, Germany), Software: Alpha Innotech Corporation (CA, USA)

2.2 Methods

2.2.1 Experimental design

Human adipose derived stem cells were incubated for 28 days in 6-well/96-well plates. The activity of a self-produced and purified FGF-2 HIS protein and a shorter rhFGF-2 variant (FGF-s3 HIS) was tested and compared with a commercially available rhFGF-2 (Peprotech, UK) protein. Samples were collected on days 7, 14, 21 and 28 (**Figure 6**). Growth factors were added to the media in two concentrations: low dose (3 ng/mL) and high dose (30 ng/mL). As reference, cells were cultured without hFGF-2 supplements. During the time period of 28 days, medium was changed once a week. On the selected time points (days 7, 14, 21, 28) cell viability, proliferation status, percentage of apoptotic cells, differentiation and cell density was analyzed.

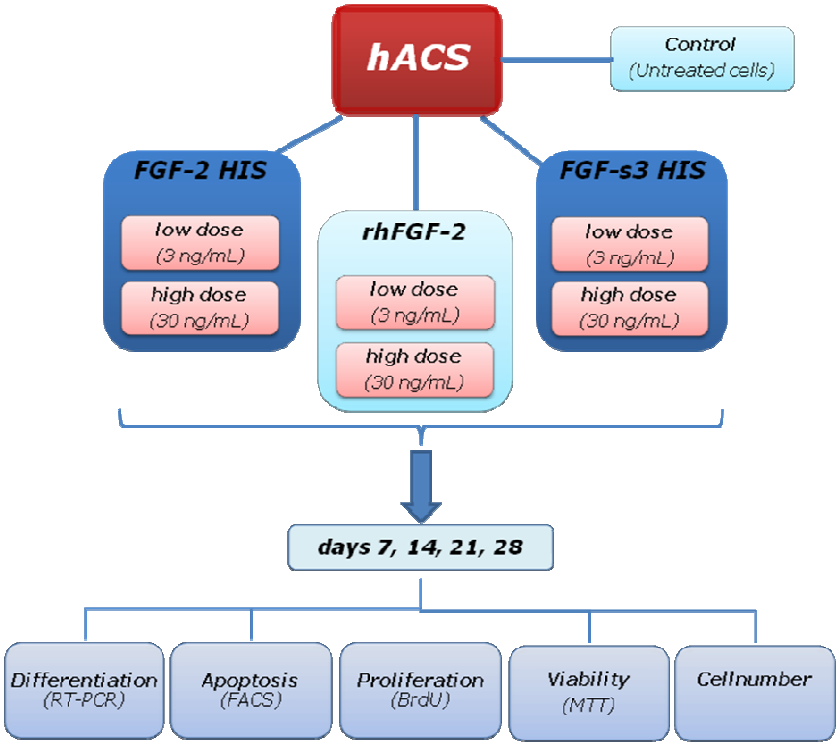

Figure 6 *Experimental design of the study*

Cell culture media supplements: rhFGF-2, FGF-2 HIS, FGF-s3 HIS.

Low-/high dose: concentration of cell culture media supplements.

2.2.2 Cell culture methods

Harvesting cells

Cells were isolated by enzymatic digestion (collagenase treatment) from human adipose tissue after liposuction at the Red Cross Blood Centre/Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (Linz/Vienna, Austria). The cells were seeded on culture dishes and subsequently harvested and cryopreserved. In the experiments hASCs isolated from 5 different, middle-aged donors were tested.

Human ASCs grow in monolayer on the plastic surface of culture flasks. In order to dissociate and harvest the isolated hASCs, they were trypsinized with 0.5% trypsin, diluted 1:4 in 1x PBS, for 5–8 minutes at 37°C. Trypsin was inactivated by adding an equal volume of medium and the cells were harvested by centrifuging at 180 g for 5 min at room temperature (RT). The cell pellet was resuspended in 2 mL cell culture medium.

Cell culture

Human adipose derived stem cells were cultivated in tissue culture flasks to a maximum confluence of 70-80%. The cells grew at 37°C and 5% CO_2 in DMEM Ham`s F12 medium, supplemented with 10% FCS, 1% PenStrep and 2 mM L-glutamine. Medium was changed twice a week. For the experiments cells were used up to passage 4 after isolation. The experiments were repeated 5 times (n = 5) with hASCs from different donors.

All steps were carried out under sterile conditions in a Laminar flow cabinet, using sterilized equipments and solutions.

Determination of cell number: Haemocytometer counting and trypan blue staining

The trypan-blue staining is a simple and commonly used method to count cells in suspension. It also allows the determination of the percentage of viable and non-viable cells in the preparation. Trypan is an acidic dye, which is absorbed by cells with damaged cell membranes (non-viable cells), though it cannot pass through intact

membranes of living cells. Hence, it accumulates in dead cells, selectively staining them blue (dye exclusion method) [76-78].

Procedure

1. Depending on the size of the cell pellet, an aliquot of the dissociated cell suspension was diluted 1:2/1:10 with 0.5% trypan-blue solution, and filled into a Neubauer haemocytometer (**Figure 7**).
2. The total cell number of the four corner primary squares was counted and the total cell concentration in the original cell suspension was calculated as follows:

$$\textbf{cells/mL = total count x } 10^4 \textbf{ x dilution factor}$$

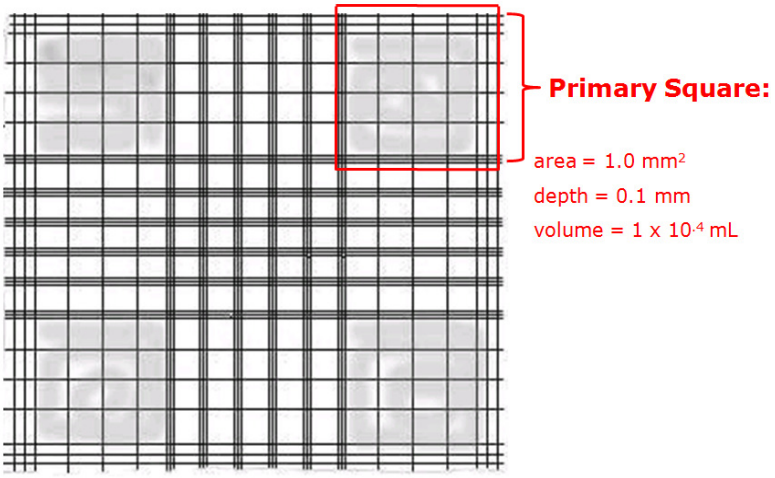

Figure 7 *Neubauer haemocytometer[4]: Schematic picture.*

Seeding

In total, for each run, 2.35 x 10^5 hASCs were required (**Table 2**). The cell suspensions were prepared separately in 50 mL polypropylene tubes (1-14, **Table 3**). Samples for cell viability (MTT) and proliferation (BrdU) tests were seeded in triplicates into 96-well plates, containing 150 cells and 100 µL medium per well. Samples for cell counts,

[4] source: http://www.laborwissen.de/old_hp/fachbereiche/hama/methoden/neubauer.php [online]

apoptosis (Flow cytometry) and differentiation (RT-PCR) analysis were seeded into 6-well plates, whereas only the wells for day 7 were seeded in triplicates, containing 5.10^3 cells and 3 mL of medium per well.

(n = 1)	96-well pates	6-well plates
cells/well	150	5000
medium/well	100 µL	3 mL
total number of wells	168	42
number of wells/preparation	24	12
required vol. cell suspension/preparation	3 mL	40 mL
cell number	25.200	210.000
total cell number	2.35 x 10^5	

Table 2 *Overview of cell numbers per experiment*

96-well plates		6-well plates	
1	medium (control)	8	medium (control)
2	medium + 3 ng/mL rhFGF-2	9	medium + 3 ng/mL rhFGF-2
3	medium + 3 ng/mL FGF-s3 HIS	10	medium + 3 ng/mL FGF-s3 HIS
4	medium + 3 ng/mL FGF-2 HIS	11	medium + 3 ng/mL FGF-2 HIS
5	medium + 30 ng/mL rhFGF-2	12	medium + 30 ng/mL rhFGF-2
6	medium + 30 ng/mL FGF-s3 HIS	13	medium + 30 ng/mL FGF-s3 HIS
7	medium + 30 ng/mL FGF-2 HIS	14	medium + 30 ng/mL FGF-2 HIS

Table 3 *Prepared cell suspensions*

2.2.3 Molecular biological methods

Total RNA isolation from cells

Cells were lysed by removing the medium and adding 250 µL of lysis buffer, containing 1% β-mercaptoethanol, to each well. Samples were incubated for 2 min on a plate shaker before proceeding with the RNA isolation, according to the instruction manual of Genelute Mammalian RNA-Miniprep-Kit (Sigma-Aldrich). RNA was eluted with 50 µL RNase free water (elution buffer). During the following steps, samples were stored on ice.

Determination of RNA concentration

For determination of the RNA concentration, 5 µL of each sample was diluted 1:20 with RNase free water (elution buffer) to measure the optical density (OD) at 260 nm/280 nm.

DNase I treatment

To the remaining 45 µL RNA, a 1/10 volume of 10x DNaseI reaction buffer (4.5 µL) and 4.5 µL DNaseI (4.5 U) was added. After 30 min incubation at 37°C the reaction was stopped by adding a 1/10 volume of stop solution (5.4 µL).

NaAc/EtOH precipitation

A 1/10 volume of 3 M NaAc, pH 5.2 and 2.5 volumes of 100% EtOH were added. The samples were incubated for at least 30 min, up to overnight (o/n), at -80°C. RNA was precipitated by centrifuging for 40 min at 14.000 g and 4°C in a pre-cooled centrifuge. The pellets were washed with 1 mL 70% EtOH (diluted in DEPC-treated water), centrifuged again for 5 min at 13.000 g and 4 °C and air dried.

Reverse transcription (RT, cDNA Synthesis)

Dry pellets were dissolved in 10 µL Oligo-dT_{15} Mix (9 µL DEPC-treated water + 1 µL 0.5 µg/mL OligodT_{15}), incubated for 5 min at 70°C and immediately cooled on ice ("snapcooling", annealing Oligo-dT primer). To each sample 15 µL AMV-mastermix was added, containing 6 µL DEPC-treated water, 5 µL 5x AMV buffer, 2.5 µL 10mM dNTP mix (diluted with DEPC-treated water) and 1.5 µL (15 U) AMV-reverse transcriptase. Samples were incubated for 1 h at 42°C, 2 min at 95°C and stored at -20°C until further procedure.

Polymerase chain reaction

The PCR-mastermixes were prepared as described in **Table 4.** The DNA Taq-polymerase was added at the end. A volume of 23 µL was transferred into PCR reaction tubes and 2 µL of the respective primer s/as mix (10 µM) were added. DNA-amplification was performed in a thermocycler. **Table 5** shows the set up of the

temperature program and **Table 6** the product sizes of DNA-amplicons and the melting temperatures (Tm) of the primers. The housekeeping gene glycerinealdehyde-3-phosphate dehydrogenase (GAPDH) was used as reference, as it is expressed continuously in cells without up- or down-regulation.

PCR-Mastermix	**1x [µL]**	**8x [µL]**
DEPC-treated water	16.8	134.4
10x Buffer	2.5	20.0
$MgCl_2$	2.0	16.0
dNTP-DEPC (10 mM)	1.0	8.0
template	0.6	4.8
Polymerase	0.1	0.8
total	23.0	184.0
Primer s/as mix (10 µM each)	2	

Table 4 *PCR-mastermix*

Temp [°C]	Time	Cycle
94	5 min	1x
94	45 sec	
60	45 sec	30x
72	45 sec	
72	5 min	1x
4	pause	

Table 5 *PCR-cycle program*

Primer	T_m [°C]	Product [bp]
Collagen type 1a2	55.3/59.4	214
Collagen type 1a1	53.7/57.3	582
Collagen type 3	61.0/61.4	640
Vimentin	59.4/59.8	223
Desmin	69.5/69.5	274
alpha-SMA	59.4/57.3	190
GAPDH	61.0/59.4	820

Table 6 *Product sizes and melting temperatures of PCR-primers*

Agarose gel electrophoresis

An amount of 0.45 g agarose was dissolved in 30 mL TBE-buffer by heating (1.5% agarose gels). Ethidium bromide was added to a final concentraion of 0.4 µg/mL (1.2 µL). After polymerization, the PCR products were mixed with 5 µL 6x XCFF gel loading buffer. 2 µL of a DNA molecular weight marker (GeneRuler™ DNA Ladder, Fermentas) and 10 µL of the samples were transferred into the gel pockets. Gel electrophoresis was run for 25 min with 120 Volt (V).

Detection and quantification

DNA bands were detected by UV-light irradiation in a ChemiImager. For semi-quantitative analysis the densitometric values of the PCR bands were determined with the ChemiImager 4400 software (Alpha Innotech Corporation). The expression levels were calculated by dividing the results by the values of GAPDH.

2.2.4 Flow cytometry

By use of flow cytometry, cells are counted and separated, in order to study specific cell types or disease condition. Specific populations and subpopulations of a given sample can be defined and even isolated, by combining this system with a dedicated cell sorter (fluorescence activated cell sorting- *FACS*). Characterization of cells can be carried out in different ways: either by measurement of cellular/physical properties of a cell, such as granulation and cell size, or by determination of specific cell surface markers, using fluorescence labeled antibodies. It is possible to measure multiple cell-surface markers simultaneously, if different fluorescence labeled antibodies are available (**Figure 8**) [79-81].

Basically a flow cytometer consists of a flow cell, which contains the cell suspension and a sheath fluid, an optical system with a light source (e.g. a laser beam) and a fixed detector off the light source (**Figure 9**). The cell suspension is transported through a capillary in a fluid stream. Single cells pass a laser beam and changes in light (scattered, absorbed or emitted light- visible or fluorescent light emissions) are measured by a detector. All light signals are converted into digital signals and

consequently displayed in histograms or dot blot diagrams. Regarding the cellular properties, it has to be noted, that forward scattered light correlates with the cell size, whereas sideward scattered light is an indicator for the granulation of a cell.

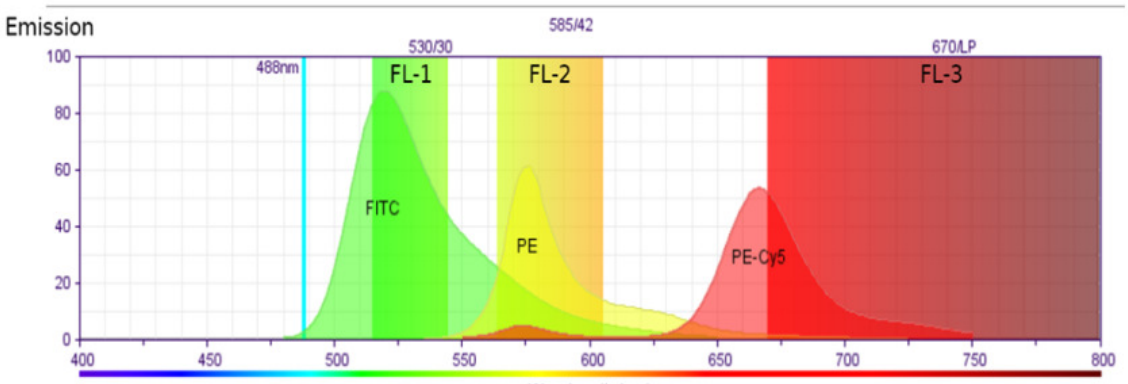

Figure 8 *Fluorescence dyes: Emission spectrum*[5]

FL *fluorescence channel, fluorescent dyes:* ***FITC*** *fluorescein-iso-thiocynanat, peak emission wavelength at 519 nm - FL1,* ***PE*** *R-phycoerythrin, peak emission wavelength at 578 nm - FL2,* ***Cy5*** *reactive fluorescent dye, belonging to the cyanin dye family* ***PE-Cy5****: PE coupled with Cy5, peak emission wavelength at 670 nm.*

[5] source: [79]

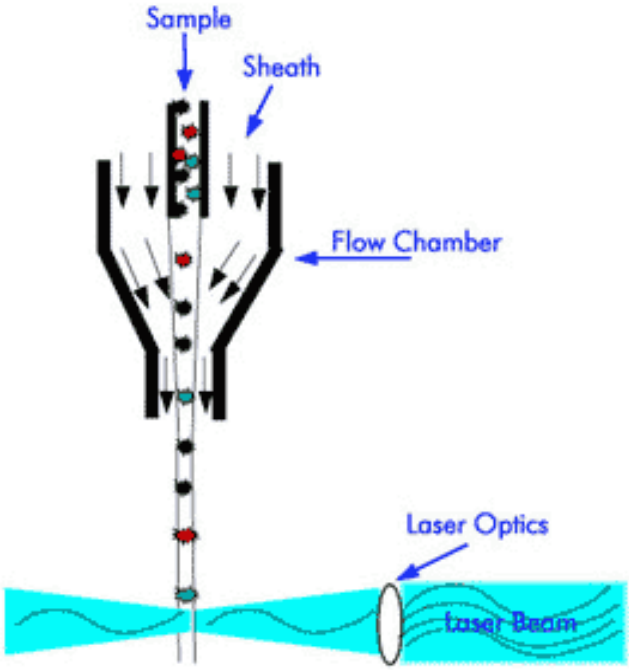

Figure 9 *Schematic buildup of a flow cytometer*[6]

A sample/cell suspension is transported in capillaries to the flow chamber. In the flow chamber cells are aligned, one after another, and presented to the laser using principles of hydrodynamic focusing.

Detection of apoptosis by ANNEXIN V/PI staining

Upon early stages of apoptosis, changes of the cell surface properties take place. Among others, they are characterized by translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the external cell surface. Annexin V is a Ca^{2+} dependent phospholipid-binding protein, which binds to PS and therefore can be used for the detection of early apoptotic cells. Necrotic cells likewise bind Annexin V, though the mechanism is different: upon necrosis, cell membranes lose their integrity, become leaky and Annexin V can bind to PS by entering the cell. Propidium iodide (PI) is a DNA intercalating, fluorescent molecule, which can only pass through defect cell membranes of necrotic or late apoptotic cells. Annexin V/PI double staining enables discrimination of necrotic and early apoptotic cells (**Table 7** and **Figure 10**) [82-85].

[6] source:[80]

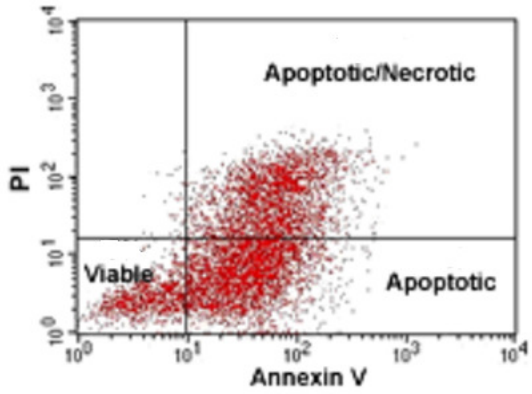

Figure 10 *A typical histogram after Annexin V/PI double staining* [7]
X-axis *Annexin V positive cells, FL1,* ***Y-axis*** *Propidium iodide positive cells, FL2.*

	Annexin	**PI**
living cells	negative	negative
apoptotic cells	positive	negative
necrotic/late apoptotic cells	positive	positive

Table 7 *Annexin V/PI staining*

Discrimination of viable, apoptotic and necrotic cells by Annexin V/PI double staining.

Procedure:

Cells were trypsinized, washed with 1x PBS and centrifuged for 5 min at 180 g and RT. The supernatant was discarded carefully and cells were resuspended in 100 µL binding buffer. As positive control, 1 µg/mL lipopolysaccharide (LPS) was added and incubated for 4 hours at 37°C. Cells were stained by adding 2 µL Annexin V-FITC and 1 µL PI and incubated for 15 min at RT in the dark. For analysis, 400 µL binding buffer was added and cells were measured with a Beckman Coulter Flow Cytometer (Cytomics FC 500 MPL), and analyzed (Software: FACS MXP Cytometer/Analysis, Cytometer RXP Analysis). Annexin was measured at 492 nm in fluorescensce channel 1 (FL1) and PI at 370/550 nm in fluorescence channel 2 (FL2).

[7] source: [83]

2.2.5 BrdU enzyme-linked immunosorbent assay (ELISA)

The BrdU ELISA is a colorimetric, cellular immunoassay, which enables quick, simple and exact analysis of cell proliferation, based on the quantification of DNA-synthesis. *Bromodeoxyuridine* (5-bromo-2-deoxyuridine, BrdU) is a thymidine analogue and thus can be incorporated in replicating cells in place of thymidine into the newly synthesized DNA strand. The incorporation of BrdU is measured by means of a peroxidase-conjugated mouse mononuclear antibody directed against BrdU (anti-BrdU-POD). The binding is achieved by denaturation of DNA, no cross-reactivities with other cellular components (*e.g.* thymidine, uracil) are observed. Only proliferating cells incorporate BrdU into DNA. For immune-detection, 3,3´,5,5´-tetramethylbenzidin (TMB) is used as substrate. It has to be noted, that *Bromodeoxyuridine* is a known carcinogen (**Figure 11**) [86,87].

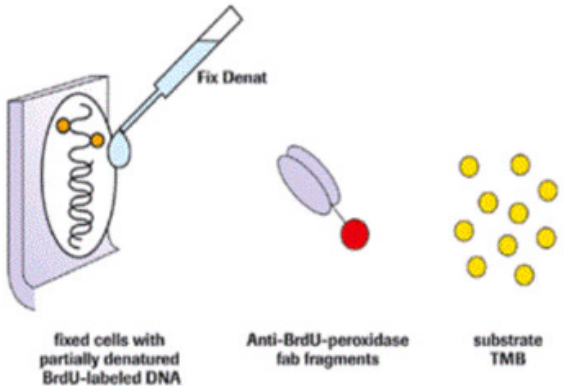

Figure 11 *Test principle of the BrdU ELISA*[8]

Colorimetric cell proliferation ELISA BrdU Assay, Roche (Vienna).
FixDenat *solution for fixation of cells and denaturation of DNA*
TMB *3,3´,5,5´-tetramethylbenzidine.*

[8] source: Roche-Applied Science (2010): http://www.roche-applied-science.com/proddata/gpip/3_5_3_21_1_8.html. [Online]

Procedure:

Cultivated hASCs (96-well plates) were labeled with BrdU by adding 10mM BrdU-labeling solution (10 µL/well) to the medium and incubated o/n at 37°C and 5% CO_2 in the incubator. In order to expose BrdU for immunodetection, cells were fixed and genomic DNA was denaturated for 30 min at RT in the dark with FixDenat-solution (100 µL/well). The BrdU label was detected with an anti-BrdU-POD antibody (100 µL/well), diluted 1:100 in cell culture medium. After an incubation of 90 min at RT in the dark, plates were washed 3x with 200 µL washing solution. 100 µL TMB-substrate-solution was added (substrate reaction) and incubated for 25 min at RT in the dark. The reaction was stopped with 25 µL 1 M H_2SO_4 per well (color change from yellow to blue). Finally, plates were incubated for 1 min on a plate shaker and the extinction was measured immediately at 450 nm (reference: 620 nm) [87].

2.2.6 MTT test

The MTT test measures cell activity by analysis of mitochondrial metabolism. MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] is a yellow, water-soluble dye, which is reduced in the mitochondria of active cells by NADH/NADPH to the blue-violet, water-insoluble Formazan. By addition of organic solvents, *e.g.* Dimethylsulfoxide (DMSO), Formazan is dissolved out of the cells. The color change in the supernatant from yellow to blue in turn can be measured spectrophotometrically at 550 nm. The height of absorption correlates with the activity status of the cells [88].

Procedure:

Cell culture medium was removed from the plates. 650 mg/mL freshly prepared MTT working solution (100 µL/well) was added and incubated for 1 h in the incubator (37°C, 5% CO_2). The supernatant was discarded and 100 µL DMSO was added to each well. Consequently the extinction was measured after 15 min incubation, at RT in the dark, at 550 nm.

2.2.7 Statistical analysis

Raw data were normalized to the control groups and results were expressed as mean ± standard error of mean (SEM). For statistical analysis the Student´s t-test was performed (Software: GraphPad Prism). P-values < 0.05 were considered to be statistically significant.

3 Results

3.1 Cell density and morphology

Human ASCs were pre-cultivated for 2 to 3 weeks to a confluence of approximately 70% (**Figure 12a,b**). During the experiment, at each selected time point (days 7, 14, 21, 28) cell morphology was observed microscopically, photographed and saved for documentation. Fibroblast-like, spindle-shaped, homogenous and compact looking cells were most abundant, especially in samples treated with rhFGF-2 and FGF-2 HIS (**Figure 13a-h** and **Figure 14a-h**). In comparison, cells treated with the FGF-s3 HIS fragment lost their spindle shape and showed a flat morphology, they appeared larger and more spread out (**Figure 15a-h**).

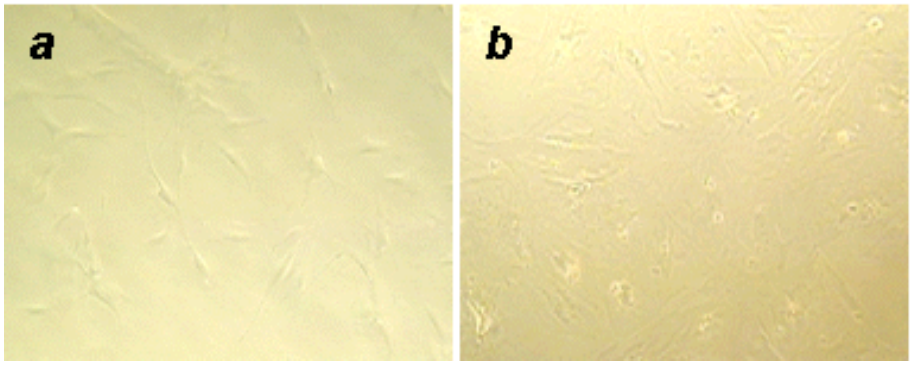

***Figure 12 a-b** Preculture**:** Light microscopic picture of hASCs, 100x. Cells cultivated in DMEM Ham`s F12 cell culture medium,* **a** *after 5 days of culture,* **b** *after 10 days of culture.*

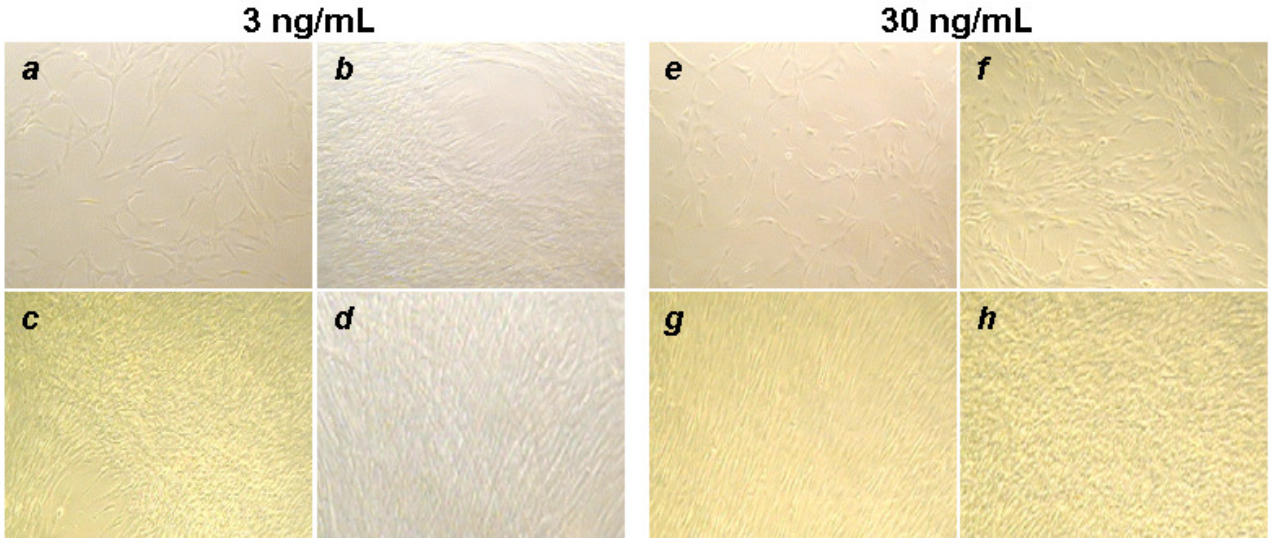

Figure 13 a-h *Light microscopic picture of rhFGF-2 treated hASCs, 100x.* ***a-d*** *3 ng/mL rhFGF-2,* ***e-h*** *30 ng/mL rhFGF-2; timepoints:* **a, e** *day 7;* **b, f** *day 14*; **c, g** *day 21*; **d, h** *day 28.*

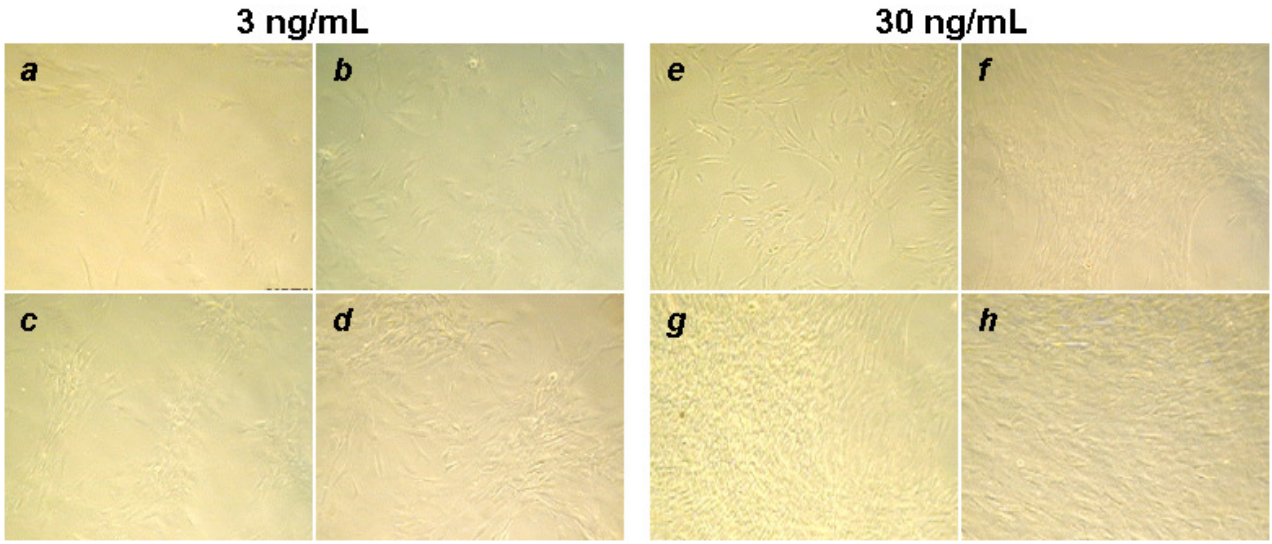

Figure 14 a-h *Light microscopic picture of FGF-2 HIS treated hASCs, 100x.* ***a-d*** *3 ng/mL rhFGF-2,* ***e-h*** *30 ng/mL rhFGF-2; timepoints:* **a, e** *day 7;* **b, f** *day 14*; **c, g** *day 21*; **d, h** *day 28.*

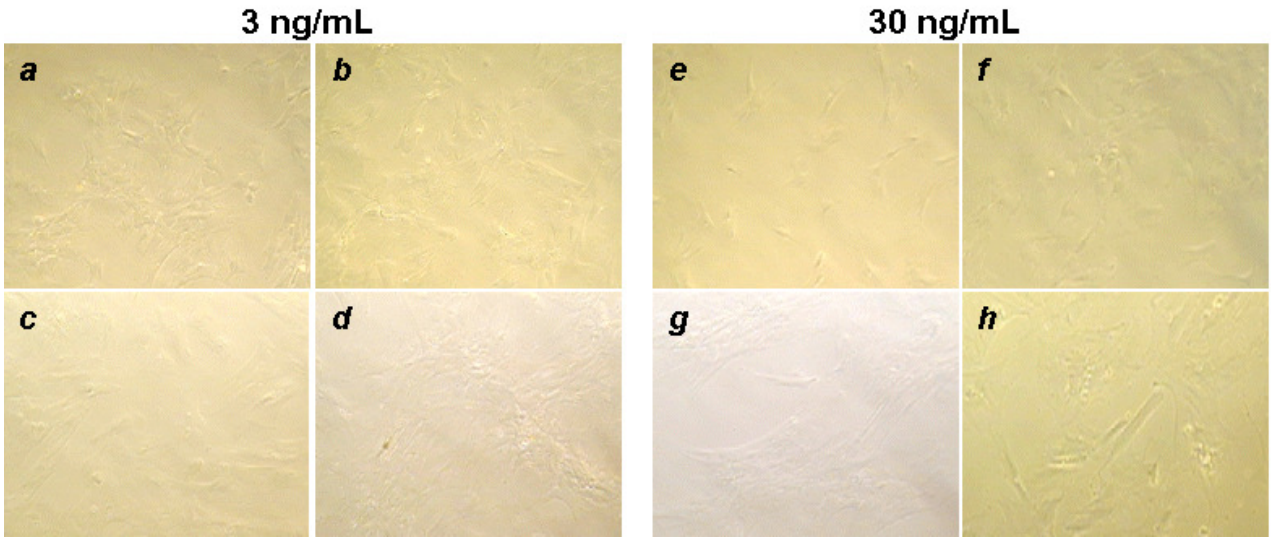

Figure 15 a-h *Microscopic picture of FGF-s3 HIS treated hASCs, 100x.* ***a-d*** *3 ng/mL rhFGF-2,* ***e-h*** *30 ng/mL rhFGF-2; timepoints:* **a, e** *day 7;* **b, f** *day 14*; **c, g** *day 21*; **d, h** *day 28.*

The results of cell counts for all groups on the corresponding days are displayed in **Figure 16** (3 ng/mL, **Figure 16a**, 30 ng/mL **Figure 16b**). Cell numbers of cultures, which were treated with FGF-2 HIS and rhFGF-2 increased until day 21 in both, the low dose and the high dose groups. Then until day, 28 cell density declined in these groups. In the FGF-s3 HIS group no cell density peak was observed, neither in the low dose, nor in the high dose samples. Cell density was significantly higher on days 14 (**$p < 0.01$), 21 (*$p < 0.05$) and 28 (*$p < 0.05$) in cultures, which were supplemented with low dose rhFGF-2 in comparison to low dose FGF-s3 HIS cultures (**Figure 16a**). In the high dose group a significant increase of cell density of rhFGF-2 treated cells was observed on days 14 (**$p < 0.01$) and 21 (*$p < 0.05$) (**Figure 16b**). Treatment with the FGF-2 HIS peptide showed significantly higher values after high dose stimulation, compared to the FGF-s3 HIS fragment, on days 7, 21 and 28 (*$p < 0.05$) (**Figure 16b**). However, no significant increase was observed in low dose FGF-2 HIS groups.

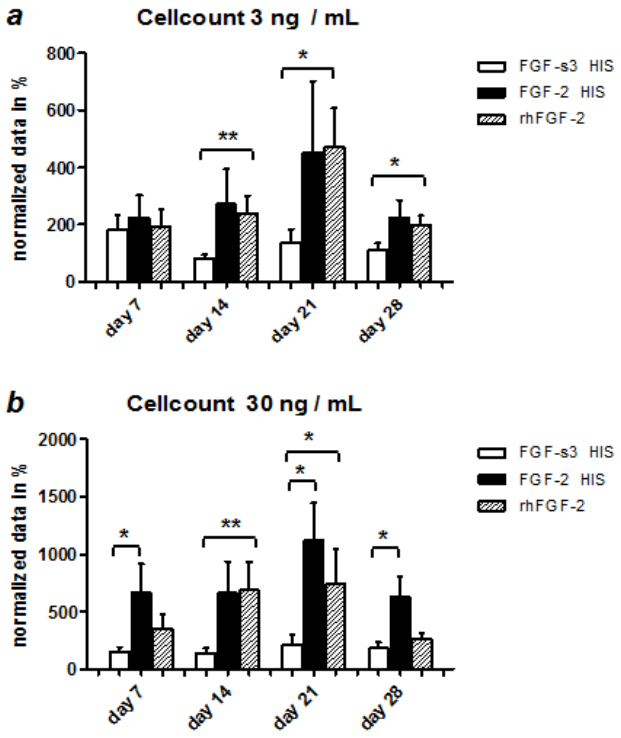

Figure 16 a,b Cell density

Concentration of supplements (FGF-s3 HIS, FGF-2 HIS, rhFGF-2): ***a*** *3 ng/mL,* ***b*** *30 ng/mL. Data in [%], normalized to unstimulated cells. Results expressed as means ± standard error of mean (SEM), n = 5, *p < 0.05, **p < 0.01.*

3.2 Expression of mRNA

Collagen type 1a2, collagen type 3 mRNA expression and the extracellular matrix protein markers (vimentin, desmin and alpha smooth muscle actin) were detectable in all groups and at all time points studied, whereas collagen type 1a1 mRNA could be detected in particular on days 28, especially after FGF-2 HIS treatment *(***Figure 17a-d** and **Figure 18c,d***).* In the rhFGF-2 and the FGF-2 HIS groups, low and high dose treatment had a similar effect regarding the collagen type 1a1 expression. FGF-2 HIS and FGF-s3 HIS treated cells displayed a similar collagen type 1a1 and collagen type 3 mRNA expression pattern between days 14 to 28. Collagen type 1a2 expression showed no significant differences throughout the experiment in all groups. Basically,

the results of the FGF-s3 HIS group resembled the results observed in FGF-2 HIS samples, though at slightly lower levels. The results of collagen type 1a1 and 3 mRNA expression in the rhFGF-2 groups were rather stagnant respectively escalating in a more fluctuating manner. All levels of gene expression were normalized to the expression of the housekeeping gene GAPDH.

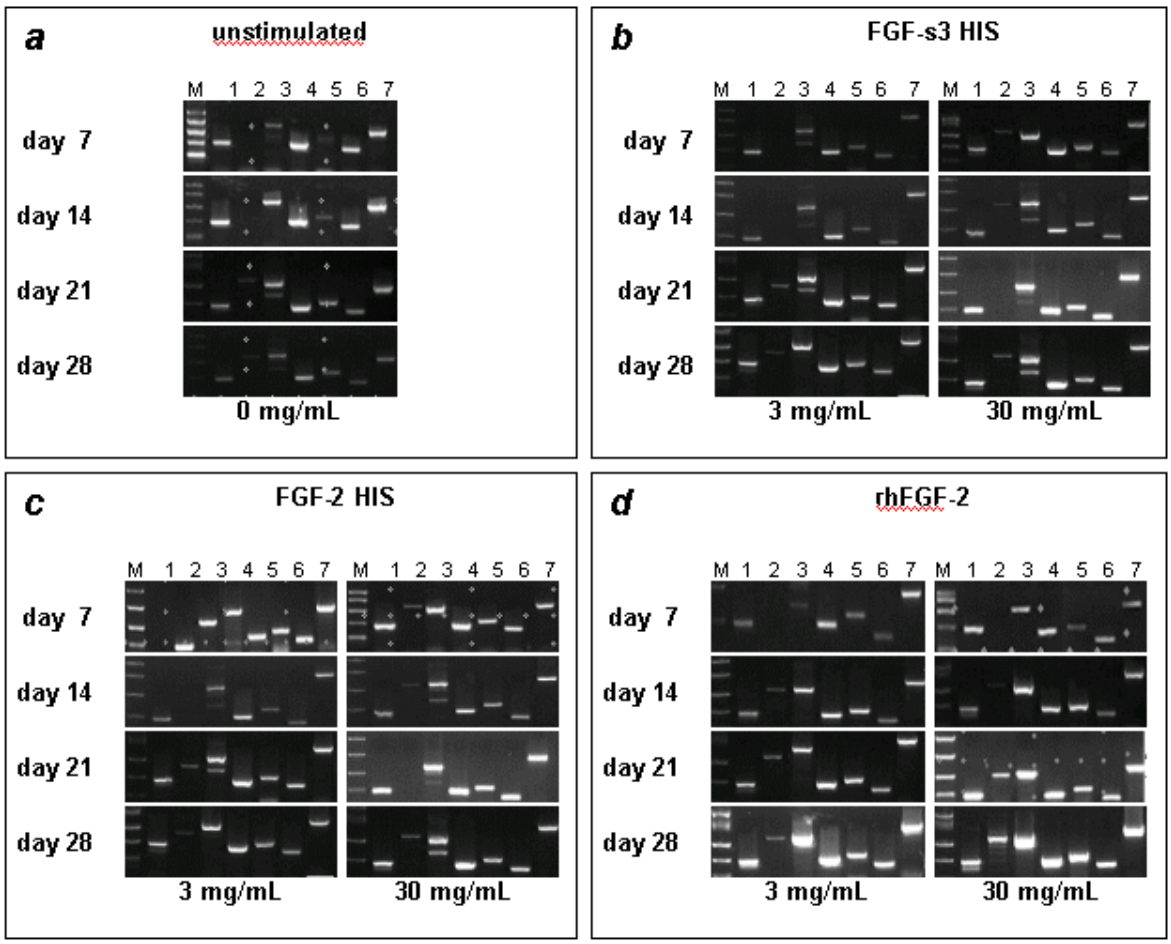

Figure 17 a-d *RT-PCR*

Representative 1.5% agarose gels after electrophoretic separation of RT-PCR amplicons: ***a*** *untreated hASCs,* ***b*** *hASCs treated with FGF-s3 HIS, left column: 3 ng/mL, right column: 30 ng/mL,* ***c*** *hASCs treated with FGF-2 HIS, left column: 3 ng/mL, right column: 30 ng/mL,* ***d*** *hASCs treated with rhFGF-2, left column: 3 ng/mL, right column: 30 ng/mL.*

M *DNA molecular weight marker,* ***1*** *Collagen type 1a2,* ***2*** *Collagen type1a1,* ***3*** *Collagen type 3,* ***4*** *Vimentin,* ***6*** *Alpha smooth muscle actin,* ***7*** *GAPDH.*

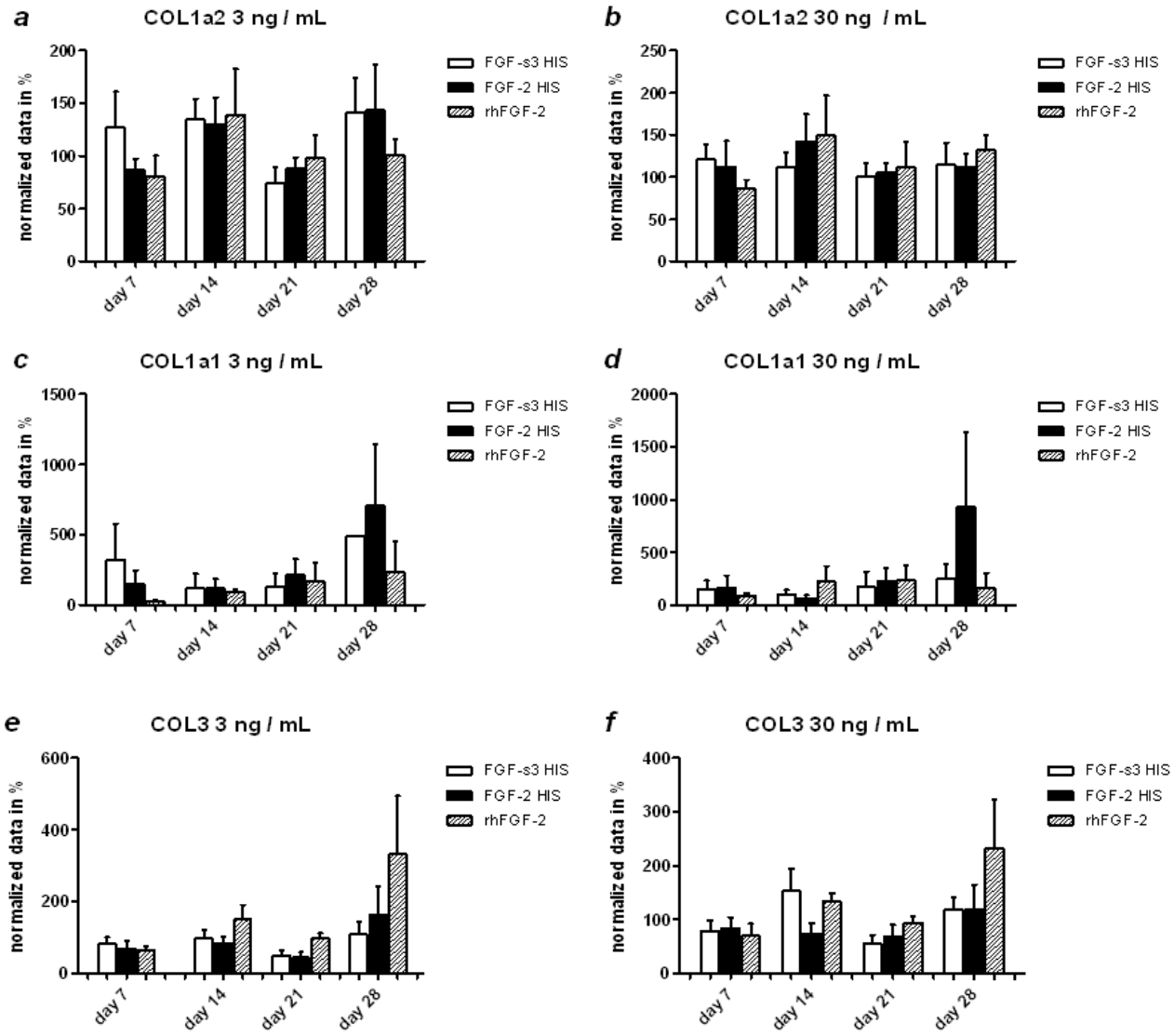

Figure 18 <u>a-f</u> *mRNA expression of collagen type 1a2, - type 1a1 and -type 3 Relative expression of collagen type 1a2 (a 3 ng/mL, b 30 ng/mL), collagen type 1a1 (c 3 ng/mL, d 30 ng/mL) and collagen type 3 (e 3 ng/mL, f 30 ng/mL). Data in [%], normalized to unstimulated cells and GAPDH mRNA levels. Results expressed as means ± standard error of mean (SEM), n = 5.*

The ratios of collagen type 1a2 and collagen type 3, respectively of collagen type 1a1 and collagen type 3 mRNA expression are displayed in **Figure 19<u>a-d</u>**. The ratio of collagen type 1a1 and collagen type 3 significantly increased on day 21 (*$p < 0.05$), in samples treated with low dose FGF-2 HIS. The ratios of collagen type 1a2 and collagen 3 showed no significant differences. It has to be noted, that the results of the

collagen type 1a2/-type 3 ratios did not reveal clear trends, indicating high and stable collagen type 1a2 mRNA reference levels.

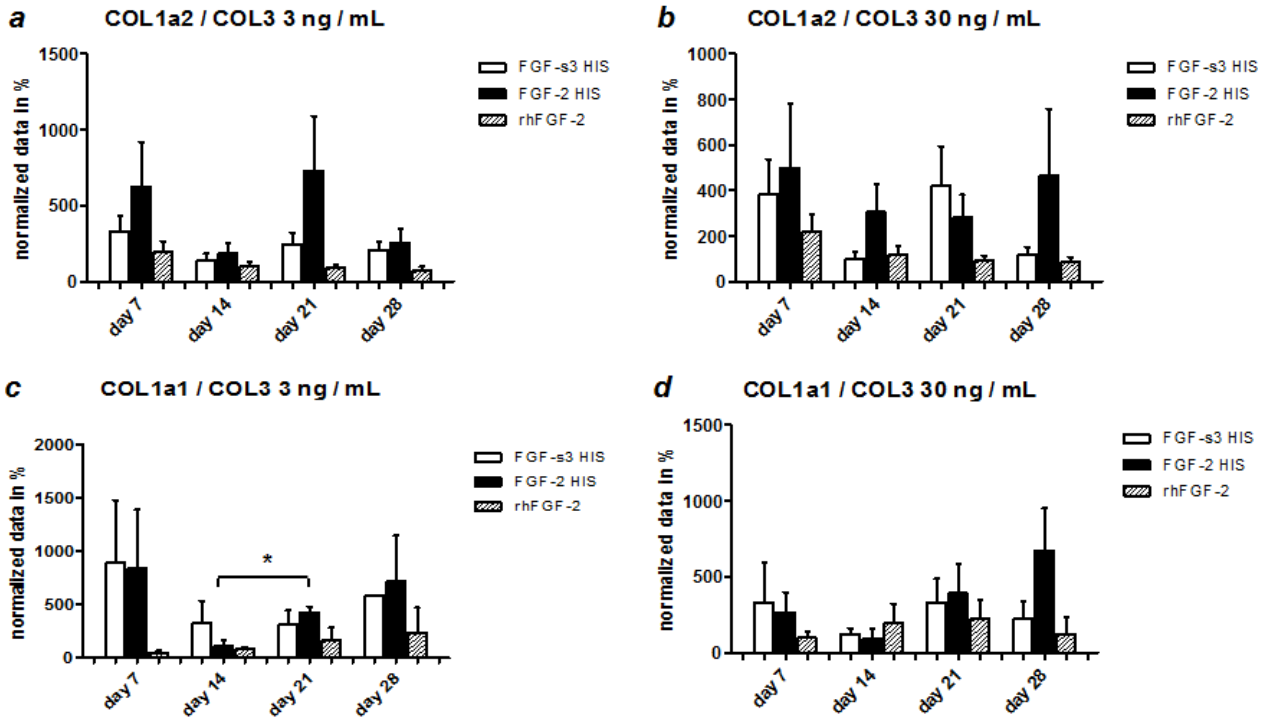

Figure 19 a-d *Ratios of collagen type 1a1/-a2 and collagen type 3 mRNA expression*
Relative collagen type 1a2/collagen type 3 (a 3 ng/mL, b 30 ng/mL) and
collagen type 1a1/collagen type 3 (c 3 ng/mL, d 30 ng/mL) mRNA expression.
Data in [%], normalized to unstimulated cells and GAPDH mRNA levels.
*Results expressed as means ± standard error of mean (SEM), n = 5, *p < 0.05.*

The highest vimentin mRNA levels were measured in the presence of high dose FGF-2 HIS on day 28 (**Figure 20a,b**). FGF-s3 HIS low- and high dose treatment had a similar effect, regarding the vimentin expression, with a peak on day 28. Expression of alpha smooth muscle actin mRNA showed again a similar trend, as observed for vimentin (**Figure 20e,f**). Its expression increased on day 28 in all groups, after both- low and high dose stimulation. Differences between high and low dose treatment were observed, regarding the desmin mRNA levels (**Figure 20c,d**). Low dose treatment with either FGF-s3 HIS, FGF-2 HIS and rhFGF-2 showed increased desmin expression levels on day 14. On days 21 and 28 the levels then decreased again. High dose

treatment did not show an increase of expression at days 7 to day 14 in the samples treated with FGF-s3 HIS and FGF-2 HIS and on day 28 the expression levels declined in all groups.

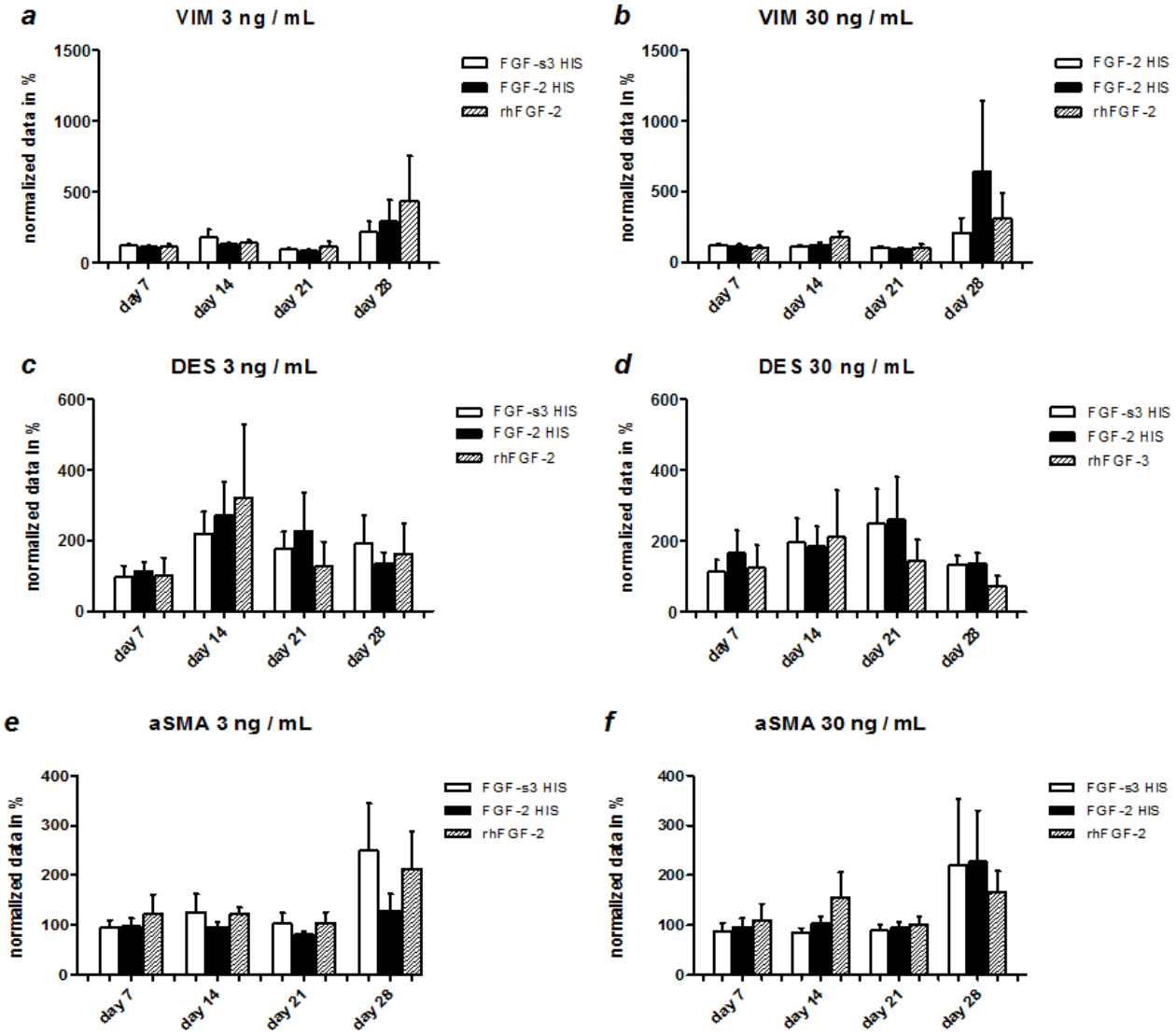

Figure 20 a-f *mRNA expression of vimentin, desmin and α-smooth muscle actin*
*Relative expression of vimentin (**a** 3 ng/mL, **b** 30 ng/mL), desmin (**c** 3 ng/mL, **d** 30 ng/mL) and α-smooth muscle actin (**e** 3 ng/mL, **f** 30 ng /mL). Data in [%], normalized to unstimulated cells and GAPDH mRNA levels. Results expressed as means ± standard error of mean (SEM), n = 5.*

3.3 Apoptosis

The apoptotic and necrotic rates did not differ significantly between the groups and studied time points (**Figure 21a-d**). FACS analysis was performed in two experiments (n = 2). Cells showed a polymorphic character and thus protocol set up had to be adjusted for each run on negative, unstained cells. It was not possible to induce apoptosis in hASCs, for positive controls, by treatment with 1 µg/mL LPS, though a marked increase in necrotic cell rates had to be noted in these preparations. Apoptosis rate was measured between 3.5 and 19.2% in low dose groups and 10.4 and 19.2% in high dose groups. Necrotic rates were measured from 1.0 to 19.4% in low dose groups and from 2.2 to 20.3% in high dose groups. The control group showed differing values, whereas apoptosis rates were ranging from 2.6 to 13.9% and necrosis rates from 0.2 to 75.2%.

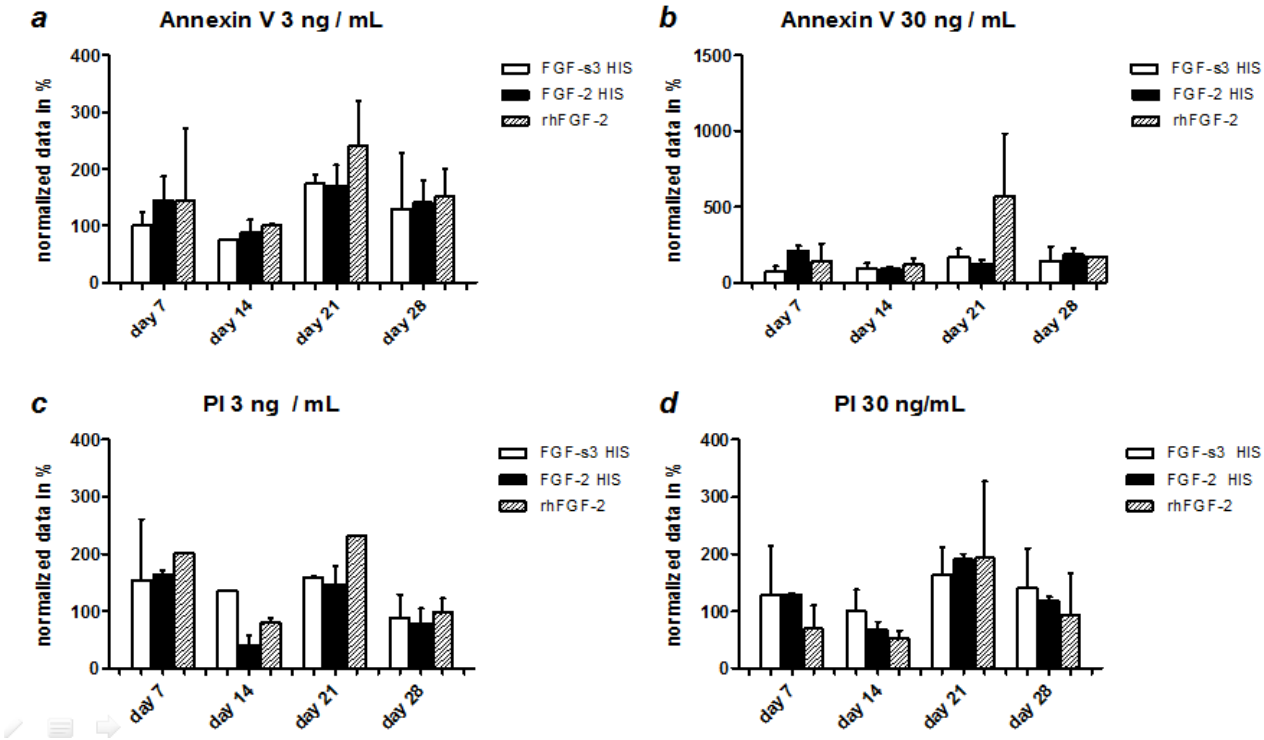

Figure 21 a-d *Results of FACS analysis upon Annexin V/PI double staining* ***a*** *Annexin V, 3 ng/mL* ***b*** *Annexin V, 30 ng/mL* ***c*** *PI, 3 ng/mL* ***d*** *PI, 30 ng/mL. Data in [%], normalized to unstimulated cells. Results expressed as means ± standard error of mean (SEM), n = 2.*

3.4 Proliferation

The results of the BrdU proliferation assay are displayed in **Figure 22a,b.** The amount of BrdU incorporation differed substantially, with highest values measured in the FGF-2 HIS treated groups. Significantly more detectable BrdU was found in FGF-2 HIS high dose samples on days 7 and 14, whereas on days 21 and 28 the levels decreased again. Low dose stimulation showed no significant differences between treatment with FGF-2 HIS, rhFGF-2 and FGF-s3 HIS.

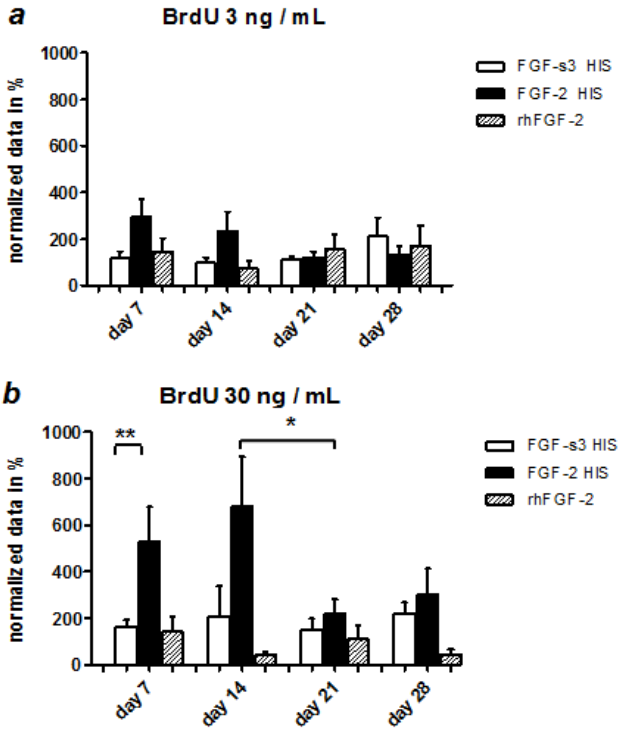

Figure 22 a,b *Results of BrdU-ELISA*

Concentration supplements (FGF-s3 HIS, FGF-2 HIS, rhFGF-2): ***a*** *3 ng/mL,* ***b*** *30 ng/mL. Data in [%], normalized to unstimulated cells. Results expressed as means ± standard error of mean (SEM), n = 5, *p < 0.05, **p < 0.01.*

3.5 Viability

Viability of cells was analyzed by determination of mitochondrial activity using MTT. Absorbance was directly proportional to the number of living cells. The results of the MTT test, which were largely associated with cell density levels, are presented in **Figure 23a,b**. Peak values were measured in low and high dose rhFGF-2 groups on days 14 and 21. Viable cell number was significantly increased in rhFGF-2 low dose samples on days 14 and 21, and on days 14, 21 and 28 in the high dose group (*p < 0.05, **p < 0.01). High dose stimulation with FGF-2 HIS showed same trends, with peak values on days 14 and 21, whereas low dose stimulation did not. After day 7, OD levels of the FGF-s3 HIS high dose groups were significantly lower in comparison to the rhFGF-2 and FGF-2 HIS high dose groups. However, after low dose stimulation with FGF-2 HIS and FGF-s3 HIS, extinction levels remained stable and low throughout the period of 28 days.

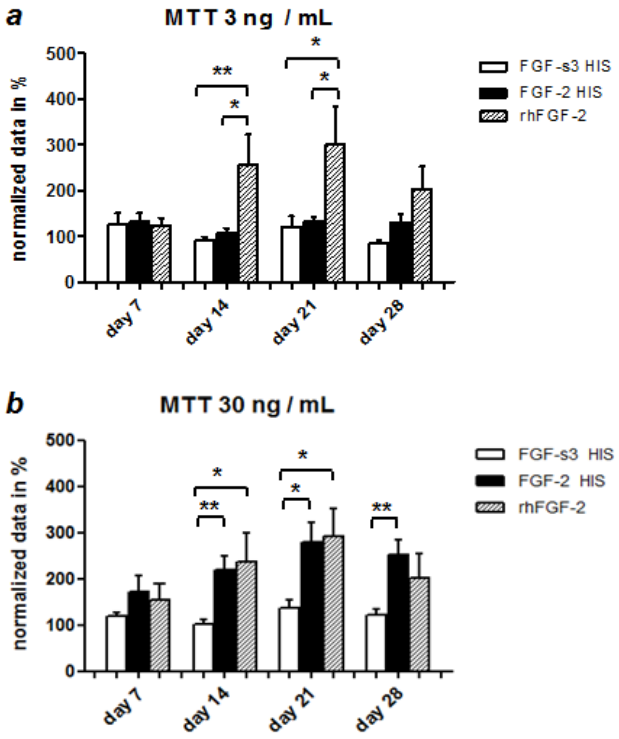

Figure 23 a,b *Results of MTT test*

Concentration supplements (FGF-s3 HIS, FGF-2 HIS, rhFGF-2): ***a*** *3 ng/mL,* ***b*** *30 ng/mL. Data in [%], normalized to unstimulated cells. Results expressed as means ± standard error of mean (SEM), n = 5, *p < 0.05, **p < 0.01.*

4 Discussion

Much work has been conducted regarding the effects of hFGF-2 on mesenchymal stem cells under various culturing conditions. It was reported that hFGF-2 may induce osteogenic differentiation in BM-MSCs in osteogenic medium containing dexamethasone [89], whereas in chondrogenic medium 1 ng/mL hFGF-2 was shown to accentuate chondrogenesis [90]. In a study by Hankemeier *et al*. [8] that analyzed the effects of low and high dose hFGF-2 treatment on BM-MSCs, without other differentiation stimuli, a tendon-like phenotype was displayed. Based on this, we chose a similar experimental set up for hASCs. Beyond that we, expanded the study by testing different rhFGF-2 variants (rhFGF-2, FGF-2 HIS, FGF-s3 HIS).

4.1 Cell proliferation, density and viability

Cell growth and proliferation are regulated by growth factors, cytokines, culture cell density, cell "age" and other environmental factors (pH value, electrolyte concentration *etc.*). Data revealed that cell growth was triggered by rhFGF-2 and FGF-2 HIS, whereas the shorter FGF-s3 HIS fragment showed only small proliferative effects on hASCs. Increased cell densities were measured after 21 days in rhFGF-2 and FGF-2 HIS stimulated samples and were then consequently followed by a slight decline in cell numbers. This course of kinetics, illustrated in **Figure 24**, is linked with cell viability rates and was observable as well in rhFGF-2, as in FGF-2 HIS groups. Furthermore, speaking in a general way, this "relative" growth course was observed with both, 3 ng/mL and the 30 ng/mL hFGF-2 stimulation.

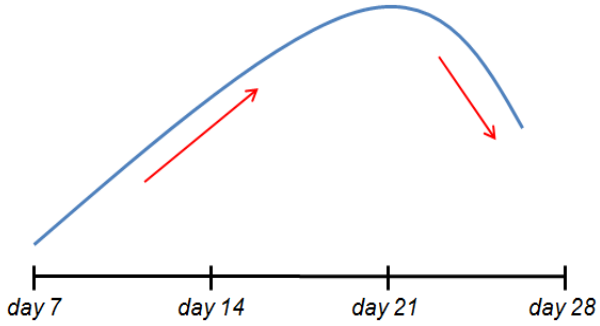

Figure 24 *In vitro effects of rhFGF-2 and FGF-2 HIS on hASCs* ***Blue line*** *course of kinetics, corresponding with results of cell counts, BrdU ELISA and MTT test.*

In BM-MSCs, adverse effects have been reported, meaning that in BM-MSCs low dose hFGF-2 led to significantly higher cell proliferation and cell density levels than high dose FGF-2 [8]. Concerning this, our data are not consistent, as absolute cell numbers and viability rates were higher in rhFGF-2 and FGF-2 HIS high dose groups. Interestingly, viable cell numbers were higher in FGF-2 HIS high dose groups than in FGF-2 HIS low dose groups. This difference between high and low dose treatment was not observed in rhFGF-2 and FGF-s3 HIS samples. Although the FGF-2 HIS construct is biological active, in comparison to the control peptide rhFGF-2, its effects are more dependent on the applied dosage of either 3 ng/mL or 30 ng/mL. Hence, hASCs might be more sensitive to different concentrations of FGF-2 HIS respectively higher concentration might be needed for achieving similar results, as observed for rhFGF-2. This finding might render a practical advantage, as cells could be influenced with FGF-2 HIS in altering manner, adaptable to the various situations and requirements. In the case of the rhFGF-2 peptide, probably, a lower concentration of 1 ng/mL would reveal similar distinct and different impacts on cell proliferation, viability and cell density. However, our findings correspond with the assumption that BM-MSCs and hASCs are responding in a different way to growth factor stimuli and environmental changes, even if both cell types are of mesenchymal origin and display similar properties.

Apart from that, we assume that the truncated FGF-s3 HIS fragment showed only little biological activities. FGF-s3 HIS does not significantly influence cell numbers,

proliferation- and viability rates, neither in low dose, nor in high dose concentration. Previously, FGF-s3 HIS was confirmed to be able to bind with high affinity to fibrin and fibrinogen [62]. Its weak biological activity might indeed be an advantage for clinical applications. The FGF-s3 HIS fragment could be used as a linking partner between fibrin and other target substances and therapeutic drugs, without altering cell behaviour *in vitro* or *in vivo*.

Morphological analyses are in concordance with the results discussed above and underline our conclusion. Cultures displayed a spindle-shaped and homogenous looking phenotype with low and high dose rhFGF-2. It has to be considered, if a lower rhFGF-2 concentration than 3 ng/mL might be more beneficial, as typical cell cluster seemed to be more abundant in rhFGF-2 low dose groups.

Phenotypic differences in the FGF-2 HIS group between low and high dose treatment were more distinct. FGF-2 HIS 30 ng/mL treatment revealed clearly a more fibroblast like phenotype as 3 ng/mL FGF-2 HIS. As expected, FGF-s3 HIS treated hASCs looked considerably flatter and did not build homogenous, spindle-shaped cell clusters, neither in 3 ng/mL, nor in 30 ng/mL preparations.

4.2 Apoptosis rates

Data of FACS analysis were not taken into consideration. It was not possible to induce apoptosis in the preparations for the positive controls and therefore the obtained data were not reliable. Evidence in the literature described that flow cytometry was already used successfully for measurement of apoptotic cell rates in mesenchymal stem cells. In BM-MSCs rather high apoptosis rates were detected in this context, although they were not changed significantly by hFGF-2 treatment [8]. However, we could not set up a fitting protocol for all measurements, but had to adjust the settings accordingly for each time point. We assume that the problems were due to low cell numbers, especially at time points 7 and 14. Therefore, we conclude that in future approaches petri dish cultures and higher cell numbers should be used in this context.

4.3 mRNA expression

Extracellular matrix (ECM) is building a natural, structural environment which influences cellular differentiation, organ morphology, -integrity and -function. Expression of mRNA encoding for collagen type 1 and vimentin, which are important elements of ECM, and of alpha smooth muscle actin, can be influenced by hFGF-2. A biphasic cellular response of consecutive reactions, characterized by an initial trigger of cell growth, followed by enhancement of osteogenic cell differentiation processes (**Figure 25**), have been described already in BM-MSCs [8,89].

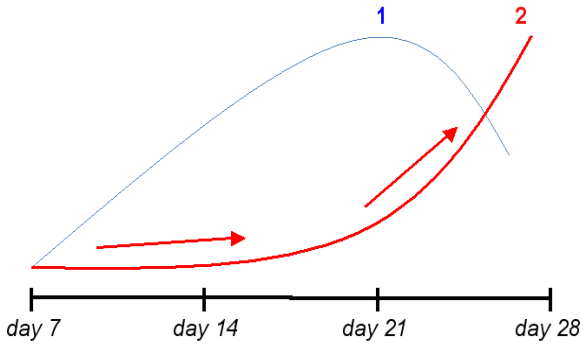

Figure 25 *Biphasic cellular respond to hFGF-2 stimulation*

1 *(blue line) first phase of proliferation and cell growth,*
2 *(red line) second phase: up-regulation of mRNA expression.*

An up-regulation of collagen type 1a1, collagen type 3, vimentin and alpha smooth actin mRNA expression was noted after the growth peak between days 21 and 28. However, the expression of desmin and collagen type 1a2 mRNA did not follow this biphasic response. The desmin gene encodes for a muscle-specific intermediate filament expressed by muscle, smooth muscle and cardiac muscle tissues. Interestingly, desmin mRNA was expressed earlier than ECM components, resembling the course of kinetics shown in **Figure 24**. Collagen type 1a2 levels were rather high from the start, without significant changes, neither at various time points, nor between rhFGF-2, FGF-2 HIS and FGF-s3 HIS. Structurally, collagen type 1 forms a

characteristic triple helix of two alpha 1 (collagen type 1a1) and one alpha 2 (collagen type 1a2) subunits, in a physiological ratio of 2 to 1 [91]. The expression of collagen type 1a1 was therefore expected to be approximately two folds higher than collagen type 1a2 expression. However, this was not confirmed after evaluation by semiquantitative RT-PCR.

By the expression of fibril forming, highly structured collagens, *e.g.* collagen type 1 and 3, tensile strength in vertebrate tissue is provided. Collagen type 1 is the major component of ECM proteins and most abundant in bone. Mature collagen type 1 is highly structured in dense boundless. Collagen type 3 is found in large vessel-, the intestine- and the uterine wall. In comparison to collagen type 1, collagen type 3 fibers are much thinner [91;92]. After injuries during early stages of wound healing, collagen type 3, which can be produced faster, leads to the characteristic scar formation [94]. Tensile strength, respectively quality of connective tissue, is influenced by the amount of deposited collagen type 1 and 3. A lowered collagen 1/3 ratio is indicative for reduced tensile tissue strength and mechanical stability, due to less cross-linkings between fibers and a change of geometrical arrangements [93,94]. Hence, not only the expression and deposition of ECM proteins is important in tissue engineering of tendons, ligaments or bone, but also its quality. We observed a lower ratio of collagen type 1a1 and collagen type 3 between days 14 – 21. Linked with the up-regulation of mRNA expression, after 28 days, the ratio increased again. This was observed in low and high dose groups, although ratios were higher after low dose stimulation. Surprisingly, FGF-2 HIS seemed to have a greater impact than rhFGF-2 on hASCs, regarding collagen type 1 expression. This could be due, as discussed above, to too high rhFGF-2 concentrations.

The FGF-s3 HIS fragment, which did not enhance cell growth, effected desmin- (with a peak on day 14, 21) and alpha smooth muscle actin mRNA expression in similar way, as FGF-2 HIS and rhFGF-2. We assume therefore, that cell growth and - differentiation might not be linked necessarily in a direct way.

However, single mRNA expression values fluctuated intensely. In order to be able to draw definite conclusions, it would be advisable in future approaches, to change the experimental settings from semiquantitative RT-PCR to quantitative Real time PCR.

In summary, it can be stated that the use of hASCs promises many perspectives for research and clinical applications in allogenic settings. In comparison to hASCs, BM-MSCs have a more restricted potential (*e.g.* low cell number harvest, invasive isolation procedure) [15,17]. However, the critical step in this context is still the lack of well-characterized adult stem cell population and the full understanding of molecular properties and mechanisms [3,33]. Human FGF-2 affects cells in a very complex way, regulating different cellular processes [34]. For the time being, based on our data, we can confirm that hFGF-2 is able to enhance the expression of important ECM proteins and cytoskeletal elements in hASCs. To prove that hFGF-2 triggers the osteogenic differentiation potential in hASCs, matrix mineralization and the expression/activity of the osteoblastic marker alkaline phosphatase (ALP) needs to be determined in future approaches. FGF-2 HIS and rhFGF-2 have different impacts on cells and optimal doses have to be adjusted accordingly. The FGF-s3 HIS fragment shows only weak biological activities. BM-MSCs and hASCs seem to react in a different way to low and high dose hFGF-2 concentrations. In conclusion, the results of our study showed that high dose hFGF-2 stimulation enhanced cell growth, whereas low dose hFGF-2 stressed the differentiation potential of hASCs. We assume that probably a combination of an initial high hFGF-2 dosage, followed by low dose hFGF-2 treatment, would be most beneficial for tissue engineering approaches.

5 Abstract

Human adipose derived stem (hASCs) cells, are adult stem cells originated from mesenchyme. Somatic stem cells are present in many tissues and organs of the body, rendering important function in tissue regeneration. Due to the ability of hASCs, to give rise to various mature cell types/tissues and the easy isolation procedure, they have been proposed as potential candidates for cell based therapies in the fields of regenerative medicine and tissue engineering.

Human fibroblast growth factor 2 (hFGF-2) is an important growth factor, which regulates, among, others cell growth, -differentiation, -proliferation and angiogenesis. Human FGF-2 is essential during early embryonic development but also throughout life in the regeneration of adult tissues. The FGF family constitutes a group of related polypeptides, which are able, upon binding to specific transmembrane receptor tyrosine kinases (FGFR), to activate signal pathways, which are associated with cell growth, migration and proliferation. Human FGF-2 is produced by various cell types *in vivo* and can be used as culture additive *in vitro*.

The aim of the project was, to examine the *in vitro* effects of different hFGF-2 doses (3 ng/mL and 30 ng/mL) on human adipose derived stem cells. Apart from commercially available recombinant hFGF-2 (rhFGF-2), additionally the biological activities of two self-produced FGF-2 constructs (FGF-2 HIS, FGF-s3 HIS) were analyzed over 28 days, regarding cell growth, morphology, viability, apoptosis rates, proliferation status and differentiation.

The results revealed only weak biological effects on cell proliferation, -growth and -viability, of the truncated FGF-s3 HIS fragment. As FGF-s3 HIS binds with high affinity to fibrin/fibrinogen, the fragment could be used as a linking partner between fibrin and drugs, rendering therapeutic issues.

FGF-2 HIS and rhFGF-2 both increased cell growth and the expression of extracellular matrix proteins (*e.g.* collagen type 1, collagen type 3, vimentin and desmin), which are essential hallmarks in engineering of bone, tendons and ligaments. Cells reacted to hFGF-2 treatment in a characteristic, biphasic respond, with an initial phase of enhanced cell growth. The second phase was marked by an increase of collagen type

1, collagen type 3 and vimentin mRNA expression. Morphologically, cells displayed a fibroblast like, homogenous looking cell morphology. Differences between low (3 ng/mL) and high (30 ng/mL) dose hFGF-2 treatment were most distinct in the FGF-2 HIS group. Highest cell numbers were obtained with 30 ng/mL rhFGF-2. Nevertheless, we suggest that low dose hFGF-2 is more suited for osteogenic differentiation. However, this hypothesis still remains to be investigated in more detail, probably by means of quantitative Real time PCR and using osteogenic medium. We conclude that both, FGF-2 HIS and rhFGF-2, are biological active, but they have to be regarded separately concerning the administered dosage.

6 Zusammenfassung

FGF-2 Aktivitäten im Vergleich zu dem FGF-s3-His Fragment in humanen Fettstammzellen

Humane Fettstammzellen sind adulte, mesenchymale Stammzellen. Prinzipiell sind in den meisten Geweben Zellen mit Stammzelleigenschaften zu finden, die dort Aufgaben in Bezug auf Geweberegeneration (*z.B.*: Knochenmark, Darm) erfüllen. Humane Fettstammzellen sind zum einen leicht zugänglich (*z.B.* plastische Chirurgie, Abfallprodukt bei Liposuktion) und haben zum anderen die Fähigkeit in verschiedene Gewebe zu differenzieren. Somit rückten sie, vor allem im Zusammenhang mit zellbasierten Therapien im Bereich der regenerativen Medizin und Geweberekonstruktion, immer mehr in den Fokus der biomedizinischen Forschung. Für den möglichen, routinemäßigen und klinischen Einsatz von humanen Fettstammzellen ist es wichtig, das Wissen der zugrunde liegenden molekularen Mechanismen und physiologischen Zusammenhänge von Wachstum und Differenzierung zu untersuchen.

Humaner Fibroblasten Wachstumsfaktor (hFGF-2) ist ein wichtiger Wachstumsfaktor durch den unter anderem Vorgänge wie Zellwachstum und -differenzierung, Gewebereparatur und -regeneration (Knorpel-, Nervengewebe) sowie Angiogenese induziert beziehungsweise gesteuert werden können. In diesem Zusammenhang ist seine Rolle vor allem in der Embryonalentwicklung, aber auch in adulten Geweben tragend. Die FGF-Familie stellt eine Gruppe von Signalproteinen dar, die spezifische Rezeptor-Tyrosin Kinasen an Zelloberflächen binden (FGF-Rezeptoren, FGFR) und durch eine Signaltransduktionskaskade ins Zellinnere FGF Zielgene aktivieren. Humaner FGF-2 beeinflusst somit die Zellphysiologie und kann als *in vitro* Zellstimulans verwendet werden.

Ziel des Projekts war es, die Auswirkungen von rekombinantem hFGF-2 auf die *in vitro* Proliferation bzw. Differenzierung von adulten Stammzellen (humanen Fettstammzellen) zu untersuchen. Es wurden zwei verschiedene hFGF-2

Konzentrationen, *niedrig* (3 ng/mL) und *hoch* (30 ng/mL), eingesetzt und die stimulierten Zellen folglich 28 Tage lang beobachtet. Weiters wurde die Aktivität von den 2 eigens produzierten und aufgereinigten FGF-(Teil)peptiden FGF-2 HIS und FGF-s3 HIS untersucht und mit dem kommerziell erhältlichen, rekombinanten hFGF-2 (rhFGF-2) verglichen.

Die Ergebnisse zeigten eine nur schwache biologische Aktivität des FGF-s3 HIS Fragments im Vergleich zu rhFGF-2 und FGF-2 HIS. FGF-s3 HIS bindet mit hoher Affinität an Fibrin/Fibrinogen, und könnte somit in Zukunft als Bindungspartner zwischen Fibrin und anderen Zielsubstanzen (*z.B.* Medikamente) für therapeutische Zwecke eingesetzt werden.

Im Gegensatz dazu, förderten FGF-2 HIS und rhFGF-2 das Zellwachstum und die die Expression von Proteinen der extrazellulären Matrix. Alle drei Punkte sind essentiell für die Gewebekonstruktion (Tissue Engineering) von Knochen, Sehnen und Bändern. Zwei Phasen der Zellantwort waren zu beobachten, ein Anstieg des Zellwachstums und der Proliferationsraten zwischen Tag 14 und 21, beziehungsweise die Erhöhung der Expression von Kollagen Type 1, Kollagen Type 3 und Vimentin ab Tag 21. Histologisch äußerte sich die hFGF-2 Behandlung in einer fibroblastischen, homogen wirkenden Zellmorphologie. Unterschiede zwischen 3 ng/mL und 30 ng/mL hFGF-2 Behandlungen wurden vor allem in der FGF-2 HIS Gruppe deutlich. Obwohl 30 ng/mL hFGF-2 höhere Zellzahlen und biologische Aktivität hervorbrachte, vermuten wir, dass sich eine Dosis von 3 ng/mL hFGF-2 besser auf die osteogene Differenzierung auswirkt. Diese Hypothese muss allerdings noch durch genauere Methoden, wie quantitative Real time PCR, die Bestimmung der alkalischen Phosphatase (ALP) bzw. durch den Nachweis mineralisierter Matrix verifiziert werden. Die Peptide rhFGF-2 und FGF-2 HIS müssen in Hinsicht der eingesetzten Konzentrationen gesondert betrachtet werden und rhFGF-2 sollte in einer noch geringeren Konzentration von 1 ng/mL getestet werden. Jedenfalls wurde die Vermutung bestätigt, dass meschenchymale Stammzellen aus humanem Fettgewebe beziehungsweise aus dem Knochenmark, unterschiedlich sensibel auf verschiedene hFGF-2 Konzentrationen reagieren.

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8 Appendix

DNA molecular weight markers (**Figure 26**)

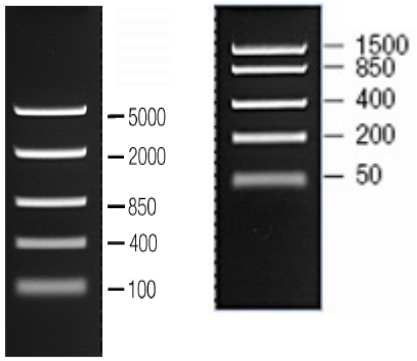

Figure 26 GeneRuler^{TM}DNA-Ladder (Fermentas)

left *middle range, 5000 – 100 bp*

right *low range, 1500 – 50 bp.*

Nucleotide blast

Collagen 1a2

Sense: Homo sapiens collagen, type 1, alpha 2, mRNA (cDNA clone MGC:57503), complete cds, length = 5060

Score = 40.1 bits (20), Expect = 0.027, Identities = 20/20 (100%), Gaps = 0/20 (0%), Strand=Plus/Plus

```
Query  1     GAAAACATCCCAGCCAAGAA  20
             ||||||||||||||||||||
```

```
Sbjct  3725  GAAAACATCCCAGCCAAGAA  3744
```

Antisense: Homo sapiens collagen, type 1, alpha 2, mRNA (cDNA clone MGC:57503), complete cds, length = 5060

Score = 40.1 bits (20), Expect = 0.027, Identities = 20/20 (100%), Gaps = 0/20 (0%), Strand=Plus/Minus

```
Query  1     CAGGTTGCCAGTCTCCTCAT  20
             ||||||||||||||||||||
Sbjct  3958  CAGGTTGCCAGTCTCCTCAT  3939
```

Collagen 1a1

Sense: Homo sapiens collagen, type 1, alpha 1, mRNA (cDNA clone MGC:33668), complete cds, length = 4752

Score = 36.2 bits (18), Expect = 0.42, Identities = 18/18 (100%), Gaps = 0/18 (0%), Strand=Plus/Plus

```
Query  1    TGACGAGACCAAGAACTG  18
            ||||||||||||||||||
Sbjct  341  TGACGAGACCAAGAACTG  358
```

Antisense: Homo sapiens collagen, type 1, alpha 1, mRNA (cDNA clone MGC:33668), complete cds, length = 4752

Score = 40.1 bits (20), Expect = 0.027, Identities = 20/20 (100%), Gaps = 0/20 (0%), Strand=Plus/Minus

```
Query  1    CCATCCAAACCACTGAAACC  20
            ||||||||||||||||||||
Sbjct  940  CCATCCAAACCACTGAAACC  921
```

Collagen 3

Sense: Homo sapiens collagen, type 3, alpha , (COL3A1), mRNA, length = 5489

Score = 38.2 bits (19), Expect = 0.11, Identities = 19/19 (100%), Gaps = 0/19 (0%), Strand=Plus/Plus

```
Query  1     CAGGTGAACGTGGAGCTGC  19
             ||||||||||||||||||
Sbjct  3407  CAGGTGAACGTGGAGCTGC  3425
```

Antisense: Homo sapiens collagen, type 3, alpha 1, mRNA, length = 5489

Score = 36.2 bits (18), Expect = 0.42, Identities = 18/18 (100%), Gaps = 0/18 (0%), Strand=Plus/Minus

```
Query  3     CCACCAGTGTTTCCGTGG  20
             ||||||||||||||||||
Sbjct  4065  CCACCAGTGTTTCCGTGG  4048
```

Vimentin

Sense: Human mRNA for vimentin, length = 1766

Score = 40.1 bits (20), Expect = 0.027, Identities = 20/20 (100%), Gaps = 0/20 (0%), Strand=Plus/Plus

```
Query  1    CTGCCAACCGGAACAATGAC  20
            ||||||||||||||||||||
Sbjct  945  CTGCCAACCGGAACAATGAC  964
```

Antisense: Human mRNA for vimentin, length = 1766

Score = 42.1 bits (21), Expect = 0.010, Identities = 21/21 (100%), Gaps = 0/21 (0%), Strand=Plus/Minus

```
Query  1     CACGAAGGTGACGAGCCATTT  21
             |||||||||||||||||||||
Sbjct  1187  CACGAAGGTGACGAGCCATTT  1167
```

Desmin

Sense: Homo sapiens desmin (DES), mRNA, length = 2268

Score = 58.0 bits (29), Expect = 6e-07, Identities = 29/29 (100%), Gaps = 0/29 (0%), Strand=Plus/Plus

```
Query  1     ATGTGGAGATTGCCACCTACCGGAAGCTG  29
             |||||||||||||||||||||||||||||
Sbjct  1282  ATGTGGAGATTGCCACCTACCGGAAGCTG  1310
```

Antisense: Homo sapiens desmin (DES), mRNA, length=2268

Score = 56.0 bits (28), Expect = 2e-06, Identities = 28/28 (100%), Gaps = 0/28 (0%), Strand=Plus/Minus

```
Query  1     GTGTCCTGGGATGGAAGAAGGCTGGCTT  28
             ||||||||||||||||||||||||||||
Sbjct  1584  GTGTCCTGGGATGGAAGAAGGCTGGCTT  1557
```

a-SMA

Sense: Homo sapiens actin, alpha 2, smooth muscle, aorta (ACTA2), mRNA, length = 1330

Score = 42.1 bits (21), Expect = 0.010, Identities = 21/21 (100%), Gaps = 0/21 (0%), Strand=Plus/Plus

```
Query  1    GAACATGGCATCATCACCAAC  21
            |||||||||||||||||||||
Sbjct  267  GAACATGGCATCATCACCAAC  287
```

Antisense: Homo sapiens actin, alpha 2, smooth muscle, aorta (ACTA2), mRNA, length = 1415

Score = 40.1 bits (20), Expect = 0.015, Identities = 20/20 (100%), Gaps = 0/20 (0%), Strand = Plus/Plus

```
Query  1     CGACCGAATGCAGAAGGAGA  20
             ||||||||||||||||||||
Sbjct  1052  CGACCGAATGCAGAAGGAGA  1071
```

GAPDH

Sense: Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA, length = 1310

Score = 38.2 bits (19), Expect = 0.038, Identities = 19/19 (100%), Gaps = 0/19 (0%), Strand=Plus/Plus

```
Query  1    TTAGCACCCCTGGCCAAGG  19
            ||||||||||||||||||||
Sbjct  571  TTAGCACCCCTGGCCAAGG  589
```

Antisense:

Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA, length = 1310

Score = 40.1 bits (20), Expect = 0.015, Identities = 20/20 (100%), Gaps = 0/20 (0%), Strand=Plus/Minus

```
Query  1     CTTACTCCTTGGAGGCCATG  20
             ||||||||||||||||||||
Sbjct  1111  CTTACTCCTTGGAGGCCATG  1092
```

9 Curriculum vitae

Personal Data

Name: Kathrin Lang

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Education

2009 – 2010	Master thesis at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna
2007 – 2010	Studies in Microbiology and Genetics at the University of Vienna, Campus Vienna BioCenter
Sep. 2004	Graduation as Medical Laboratory Technologist / Biomedical Scientist (diplomierte Biomedizinische Analytikerin)
2004	Bachelor thesis at the St. George´s Hospital, Department of Hematology, London: *PNH Platelet Activation Study*
2001 – 2004	Studies in Biomedical Science (biomedizinische Analytik) at the „Ausbildungszentrum West für Gesundheitsberufe“, Innsbruck
1998 – 2001	Studies in General Biology at the Leopold – Franzens University, Innsbruck
7. June 1998	AHS Matura
1990 – 1998	Sports High School Reithmannstrasse, Innsbruck

1886 – 1990 Elementary School, Innsbruck

Work

2009 – 2010 Bildungskarenz

2005 – 2009 Fully employed as biomedical scientist at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna

2004 – 2005 Fully employed as biomedical scientist at the Center for Anatomy and Cell Biology (Vertragsbedienstete), Medical University Vienna

1999 – 2001 Part-time employed as laboratory assistant at the histo-pathological laboratory Dr. Weiser, Hall in Tirol