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**Characterization of human mesenchymal stem cells by the appearance of  
integrins and functional analysis of collagen I-binding integrins**

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## 1. Introduction

### 1.1. Human mesenchymal stem cells (hMSC)

Human mesenchymal stem cells (hMSC) or mesenchymal stromal cells (MSC) are undifferentiated multi-potent cells [1-3] which reside primarily in the bone marrow (BM) and have the multilineage potential. In the human body, they could be regarded as readily available reservoirs of reparative cells able to mobilize, proliferate, and differentiate in to the appropriate cell type in response to specific signals [4-6]. Depending on the place of isolation or the group of people which isolates them, hMSC are known in literature also as marrow stromal cells, colony-forming unit fibroblasts (CFU-Fs), bone marrow stromal (stem) cells (BMSSCs), stromal precursor cells (SPCs), skeletal stem cells (SSCs) and multi-potent adult progenitor cells (MAPCs).

#### 1.1.1. Definition and sources

hMSC were firstly described by the group of *Till* [7] and *Friedenstein* [8] as a plastic-adherent cells isolated from BM which possess a self-renewing potential and multipotent differentiation capacity *in vitro* [9].

The self-renewing potential depends highly on the cell division properties by which they can be classified as a symmetric or asymmetric type. Symmetrically dividing cells (cell lines or cancer cells) are characterized by geometric progression (one cell divides to two new daughter cells which divide and give four new cells). Asymmetric divisions often give rise to only one novel cell type in addition to a new copy of the mother cell. Such divisions are called self-renewing and are characteristic for stem cells populations. Unfortunately, such cells have limited lifespan restricted by cell division capability, differentiation or apoptosis. hMSC are restricted to maximum 44 weeks [10] or 50 population doublings [11] in culture. Moreover, self-renewing potential depends not only on the culture time but as well as on the cell density. *Sekiya et al.* [12] found that when propagated in lower density, hMSC could improve proliferation capacity and preserve “stemness”.

The ability of stem cells to differentiate into a limited number of cell types or closely related family cells is known as multipotency [13]. hMSC from BM aspirates have been shown to differentiate towards osteocytes, chondrocytes, adipocytes [2], muscle cells [14], endothelial [15] and tenogenic like cells [16] *in vitro* (Fig. 1). MSC from rat and human have also been

reported to differentiate into neurons [15,17]. However, endothelial and neuronal differentiations are still contradictory.

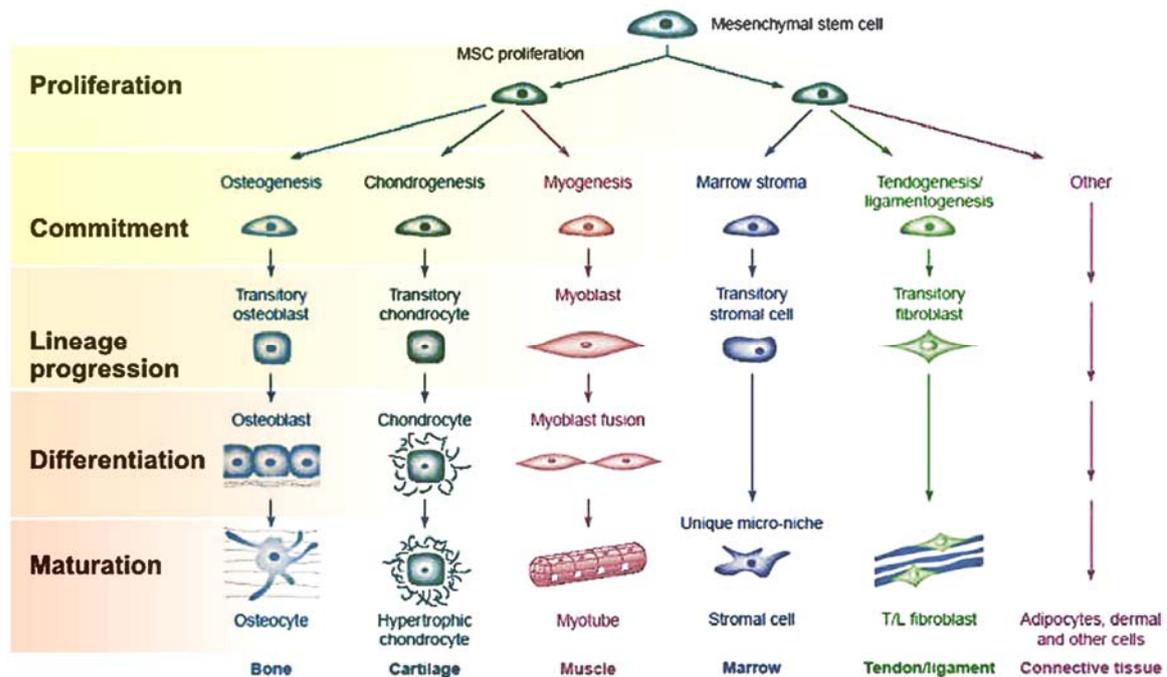


Fig.1. HMSC multilineage potential - ability of hMSC to differentiate to numbers of different end-terminal cells when maintain in proper culture conditions [4].

HMSC sources are constantly increasing. Now they can be isolated from various tissue such as adipose tissue, periostium, synovial membrane, skeletal muscle, dermis, pericytes, blood, trabecular bone, human umbilical cord, lung, dental pulp and periodontal ligament [18]. These cells still possess the general hMSC ability described by *Friedenstein et al* [8], but depending of the isolated tissue they posses some differences in term of predisposition of differentiation to the source-specific cells.

### 1.1.2. Criteria and markers

Besides the fact that the knowledge and usage of hMSC have increased dramatically over the last decades, there are still a lot of questions waiting to be answered. The general problem in the field of regenerative medicine is the lack of a unique marker or group of markers allowing an easier and faster discrimination and isolation of the stem cells from the mixture of other cells in their locality. Since the field of MSC increases very rapidly the establishment of criteria for defining and validating the MSC populations was needed. In 2005, the International Society for Cellular Therapy postulated the three minimal criteria for MSC. First, the cells

must be adherent to plastic, when maintained in culture. Second, MSC populations must be positive more than 95% for at least several antigens such as CD73 (5'-nucleotidase), CD90 (Thy-1) and CD105 (endoglin). CD73 is a dimer of two identical subunits glycosylphosphatidylinositol anchored adhesion protein that catalyzes the dephosphorylation of extracellular purine and pyrimidine nucleotides and also mediates co-stimulatory signals in T cell activation. CD90 is glycosylphosphatidylinositol anchored conserved cell surface protein with a single V-like immunoglobulin domain used as a marker for a variety of stem cells. It participate in axon grow regulation, adhesion, migration and apoptosis. CD105 is a type I membrane glycoprotein located on cell surfaces and is part of the TGF beta receptor complex. HMSC are found positive for some additional receptor, like STRO-1, CD146 (melanoma cell adhesion molecule), CD166 (activated leukocyte cell adhesion molecule), CD271 (low affinity nerve growth factor receptor) and SSEA-4 (thiosulfate sulfurtransferase) but the information about their expression is still quite contradictive. STRO-1 is an antibody created against unknown receptor, shown to identifies non-hematopoietic stromal cell in BM cell populations [19]. *Bianco's* group [20] showed that CD146 expression distinguishes BM-derived MSC from other osteogenic and non-osteogenic cell strains. CD166 was shown to be present on undifferentiated MSC and to disappear following their differentiation to an osteogenic lineage [21,22]. CD271 was also used for the enrichment of MSC, particularly from BM [23]. SSEA-4 expressing BM cells appeared to represent a MSC population devoid of haematopoietic cells [24]. Additionally, these cells must lack the expression ( $\leq 2\%$ ) of haematopoietic antigens like CD34 (primitive hematopoietic progenitor), CD45 (protein tyrosine phosphatase receptor C) and markers for monocytes macrophages and B cells such as CD11b (integrin  $\alpha$ M), CD14 (monocyte differentiation antigen), CD19 (B-lymphocyte antigen), CD31 (platelet/endothelial cell adhesion molecule 1) and HLA-DR [2,25]. Finally, the cells must be able to differentiate at least to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions [25].

### 1.1.3. BM cell niche

#### Cell types

In the BM, hMSC share space with hematopoietic stem cells (HSC), osteoblasts, stromal cells, adipocytes, vascular elements and sympathetic nerve cells. All together, those cells and the extracellular matrixes (ECM) in which they are embedded form an environment, termed as “bone marrow cells niche” (fig. 2) [26,27].

Still, little is known about the cell crosstalk inside this niche. Recent studies showed that hMSC and osteoblasts can influence the fate of HSC by intracellular signals, cytokine and matrix production. These processes appear to be initiated by cell-to-cell adhesive interaction of HSC with special spindle-shaped N-cadherin<sup>+</sup>CD45<sup>-</sup> osteoblasts that line the bone surface [28]. These osteoblasts are characterized with increased expression of bone morphogenic protein receptor type IA (BMPRIA), parathyroid hormone (PTH) and PTH-related protein (PTHrP) receptor and their number was suggested to be the limiting factor to control the quantity of HSC in BM [29]. Besides supporting the expansion of HSC, MSC can prevent apoptosis in T- and B-cell [30] and can inhibit dendritic cell differentiation by inducing cell cycle arrest [31].

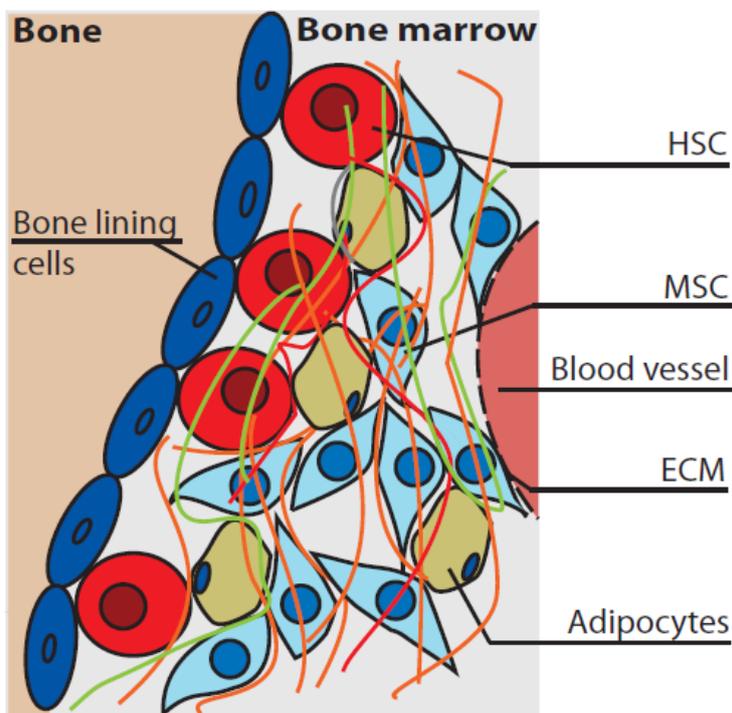


Fig. 2. Bone marrow cell niche composed of cells and ECM adopted from [32].

Another important factor in the BM niche, besides cell-to-cell contact, is the ECM complex produced by the stromal cells. Analysis of native BM and of cultured MSC has shown presence of collagens (I, III, IV, V, and VI), fibronectin, laminins, large molecular weight proteoglycans (syndecan and perlecan), small leucine-rich proteoglycans (biglycan and decorin) and hyaluronan [33].

## **ECM proteins**

### **Collagens**

The name “collagen” is used as a generic term for proteins forming a characteristic triple helix of three polypeptide chains. All members of the collagen family form these supramolecular structures in the ECM although their size, function and tissue distribution vary considerably. The collagen molecules consist of three alpha polypeptide chains (α-chains) each of which is coiled into a left-handed helix and the three chains are twisted around each other into a right-handed super-helix forming a rod-like molecule. Each α-chain consists of three separate domains – amino-terminal 7S, middle triple-helical and carboxy-terminal globular non-collagenous (NC) 1 which participating in collagen assembling and functions. Each polypeptide chain has repetitive Gly-X-Y-sequences located in the middle triple-helical domain containing proline and hydroxyproline residues in the X and Y position, respectively. Hydroxyproline makes up about 12% of the mass of a fibrillar collagen molecule and can be used as a general measure of the collagen content of a tissue. So far, 26 genetically distinct collagen types have been described [34,35].

The different collagen types are characterized by considerable complexity and diversity in their structure, their assembly and their function. The most abundant type of collagens (about 90% of the total collagen) is represented by the fibril-forming collagens.

The type I collagen (ColI) triple helix is formed as a heterotrimer by two identical α1-chains and one α2-chain. ColI is the most abundant and best studied collagen comprising about 70% of the total collagen in the human body. It forms more than 90% of the organic mass of the bone and is the major collagen of skin, tendons, ligaments, cornea and many interstitial connective tissues with the exception of very few tissues such as hyaline cartilage, brain and vitreous body. Its torsional stability and tensile strength leads to the stability and integrity of these tissues [36]. The triple helical fibers of ColI are mostly incorporated into composite containing either type III collagen (in skin and reticular fibers) [37] or type V collagen (in bone, tendon, cornea) [38]. In most organs and notably in tendons and fascia, ColI provide tensile stiffness. In bone, it defines considerable biomechanical properties concerning load bearing, tensile strength and torsional stiffness. On the cellular level ColI function as an enhancer of cell adhesion, migration and differentiation [39]. Despite of the positive effect, mutations in the ColI gene are found to cause several disease such as osteogenesis imperfecta (OI), Ehlers-Danlos syndrome (EDS) and Caffey’s diseases [40].

Type III collagen is a homotrimer of three α1-chains and is widely distributed in ColI-containing tissues with the exception of bone. It is the second most abundantly expressed col-

lagen in the human organism. It is found in tissues exhibiting elastic properties such as the skin, blood vessels, internal organs such as lungs, liver and spleen, and often is associated with type I collagen [34]. This is the collagen of granulation tissue and is produced quickly by young fibroblasts before the ColII is synthesized [41]. Mutations in the collagen III gene lead to EDS [40].

Type IV collagen have more flexible triple helix assembly as a combination of 6 different  $\alpha$ -chains are combined into a meshwork that is restricted to basement membranes. The microfibrillar type IV collagen is highly disulfide cross-linked and contributes to a network of beaded filaments interwoven with other collagen fibrils. Collagen IV is an ECM component essential during the differentiation of neuronal cells [42]. Lack of collagen IV expression leads to Alport syndrome [40].

Type V collagen is formed as heterotrimer of three different  $\alpha$ -chains ( $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ). This collagen forms heterofibrils with types I and III collagens, and contributes to the organic bone matrix, corneal stroma and the interstitial matrix of muscles, liver, lungs, and placenta [36]. Collagen V is highly expressed in developing and remodeling tissues and is believed to play an important role in processes such as tissue formation and wound healing. On cell level collagen V can affect fibroblast morphology, attachment, focal adhesion formation and actin organization [43]. Lack of collagen V expression leads to EDS [40].

### Fibronectin

Another ECM component is fibronectin (FN) - a dimer composed of two nearly identical subunits linked covalently near their C-termini by a pair of disulfide bonds. Each monomer consists of three types of repeating units (termed FN repeats): type I, II and III. FN contains 12 type I repeats, two type II repeats and 15-17 type III repeats, which account together for approximately 90% of the FN sequence (fig. 3) [44].

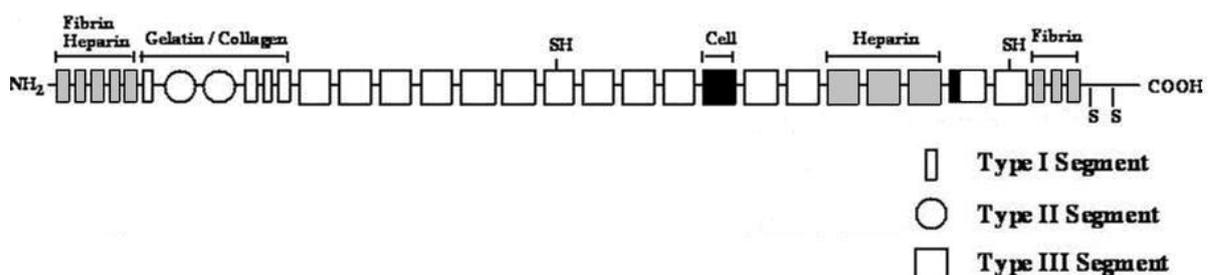


Fig. 3. FN monomer composition, adopted from [44].

FN contains two major heparin-binding domains that interact with heparan sulfate proteoglycans. The collagen-binding part of FN was found to be far more effective when it binds denatured (gelatin) than native collagen which is related to clearance of collagenous materials rather than mediating cell adhesion to collagen [45]. FN also contains two fibrin-binding sites which are localized in the both end of the molecule. The interaction of FN with fibrin is thought to be important for cell adhesion or cell migration into fibrin clots [45].

FN mediates a wide variety of cellular interactions with the ECM and plays important roles in cell adhesion, migration, growth and differentiation. The major function of FN is binding cell surfaces through integrins but it also binds to a number of important molecules like heparin, collagen/gelatin and fibrin which additionally supports cell adhesion [45]. FN plays a crucial role in wound healing as it participates in the formation of the blood clot. FN is expressed by multiple cell types and is indispensable in vertebrate development, as demonstrated by the early embryonic lethality of mice with targeted inactivation of the FN gene [46]. FN is important for guiding cell attachment and migration during embryonic development. In mammalian development, the absence of FN leads to defects in mesodermal, neural tube and vascular development. Similarly, the absence of a normal FN matrix in developing amphibians causes defects in mesodermal patterning and inhibits gastrulation.

### **Laminins**

Laminins are also found in BM cell niche. Twelve different laminin heterotrimers have been identified so far in mammals. Laminin heterotrimers are quite large molecules, ranging from under a half of a million to nearly a million daltons in molecular mass. As the distinct laminin subunits have been identified and characterized, a common structural feature has been the tandem distribution of globular, rod-like and coiled-coil domains, the last serving to join the three required chains in register. So far, five a, three b and three g-chains have been described in mammals [47].

Members of the laminin family of glycoproteins are major constituents of basement membrane (basal lamina) found in intimate contact with individual cells and cell layers [48]. Laminins critically contribute to cell attachment and differentiation, cell shape and movement, maintenance of tissue phenotype, and promotion of tissue survival [48]. Dysfunctional structure of laminin-2 cause congenital muscular dystrophy [49].

## **Proteoglycans**

Proteoglycans (PGs) are a ubiquitous family of biomolecules that are composed of a core protein and one or more covalently attached sulfated glycosaminoglycan (GAG) chains. The GAGs are linear polymers of repeated disaccharide units of hexosamine and hexuronic acid, except for keratan sulfate in which hexuronic acid is replaced by galactose. The presence of either two hexosamine isomers, D-glucosamine or D-galactosamine divides the GAGs into two groups: glucosaminoglycans [heparin/heparan sulfate and keratan sulphate] and the galactosaminoglycans [chondroitin/dermatan sulfate]. Hexuronic acid is also present as two 50 epimers: D-glucuronic acid and L-iduronic acid. Hyaluronic acid is a non-sulfated, non-attached to protein GAG composed of D-glucuronic acid and D-glucosamine. The degree and position of sulfate as well as the degree and position of 50 epimerisation are extremely variable in sulfated GAG depending on the tissue, cellular and metabolic context, ensuring structural variability of these polysaccharides [50].

PGs have been found associated with intracellular compartments, the cell surface, ECM and basement membranes in almost all tissues in adults. The importance of the ECM in skeletal development has been overlooked until the development of improved extraction procedures and recombinant DNA technology. It is now well known that bone ECM is a dynamic network of molecules secreted by cells. PGs together with collagens are the major constituents of the organic matrix of bone, constituting about 5–7% of the non-collagenous matrix components [51]. GAGs form an important component of connective tissues as a component in the synovial fluid, cartilage and tendons. An inability to break down PGs is characteristic of a group of genetic disorders, called mucopolysaccharidoses which leads to the accumulation of PGs within cells causing variety of disease symptoms [52].

## **1.2. Cell surface receptors**

Living cells, in order to maintain general biological processes, need to exchange information with the surrounding environment. This process is mediated by special surface molecules, termed receptors. Depending of the interactions they can be grouped as cell-to-cell or cell-matrix receptors.

### **1.2.1. Cell-cell receptors**

HMSC are engaged in cell-cell contacts via several types of receptors which allowed them to exchange information or support HSC differentiation and by this to contribute functionally to

BM niche. Notch, a single-pass transmembrane receptor and their ligands have been shown to be expressed in hMSC by *Oldershaw et al.* [53]. The Notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult stage as in hMSC are reported only Notch 1, 2 and 3 receptors and their ligand Jagged 1. Moreover, *Rangappa et al.* [54] showed that hMSC expressed also gap junction proteins such as connexin 32, 40, 43 and 45 which directly connects the cytoplasm of two cells and allows various molecules and ions to pass freely between cells. Other types of cell-to-cell receptors are the members of the immunoglobulin super-family. Flow-cytometry analysis of hMSC determined the expression of ALCAM, ICAM-1 and -2, and VCAM-1 [55]. Among them VCAM-1 – integrin  $\alpha 4$  binding between hMSC and T lymphocytes seems to be very critical for the hematopoietic development as blocking integrin  $\alpha 4$  by antibody impairs the T cell attachment to hMSC [56].

### 1.2.2. Cell-matrix receptors

Despite of the cell-to-cell interactions, hMSC also mediate an adhesion to growth factors, chemokines and ECM by corresponding receptors to the binding matrix.

#### **Growth factors**

Growth factors are expressed in the matrix by number of different cells in response to the environment. It is known that hMSC express various growth factor receptors as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor 1 (bFGFR), insulin-like growth factor 1 receptor (IGFR), transforming growth factor beta receptor I and II (TGF $\beta$ RI/ TGF $\beta$ RII) which, with the exception of TGF- $\beta$  receptors (serine/threonine kinases), are belonging to the family of tyrosine kinase receptors.

#### **Chemokine receptors**

The chemokine receptors are classified as G-protein-coupled receptors that bind to CXC, CC, C or CX3C chemokines [57]. One characteristic feature of chemokines is that several chemokines bind to more than one receptor and the majority of chemokine receptors have multiple possible ligands. To date, hMSC are known to express CCR1, CCR2, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR1, CXCR2, CXCR4, CXCR5, CXCR6 and CX3CR1 receptors as their expression is inconsistent maybe due to the heterogenic nature of the population. Nonetheless, some chemokines and chemo-attractants, for example CCL5 (RANTES), CCL22 (MDC), CXCL12 (also known as SDF-1) [58] and CXCR4 [59] have been demon-

strated to be more persistently expressed. In addition, inflammatory cytokines such as TNF $\alpha$  are able to increase the sensitivity of hMSC to chemokines.

### **Other matrix receptors**

The complex interactions of cells with ECM play crucial roles in mediating and regulating many processes, including cell adhesion, migration and signaling during morphogenesis, tissue homeostasis, wound healing and tumorigenesis [60]. CD44 is an important non-integrin receptor that is involved in cell-matrix interactions and is responsible for binding to hyaluronan. *Zhu et al.* [61] studied the role of CD44 – hyaluronan interactions for MSC migration. They have found that upon PDGF stimulation, the cells elevated CD44 expression and that their adhesion and migration on hyaluronan was indeed dependent on CD44, since it can be blocked by either CD44 antibody or small interfering RNA.

The cell-surface receptors that mediate cell–matrix adhesion are primarily members of two gene families — the syndecans and the integrins. Intriguingly, nearly all ECM molecules contain binding sites for both types of receptors and there is substantial evidence that a full cell-adhesion response requires engagement of both receptor types. Syndecans and integrins are required for generating a physical link to the cytoskeleton, force transduction, spatial control of the assembly of the adhesion signaling complex and regulation of cytoskeletal dynamics. The syndecans are a four-member family of transmembrane cell surface PGs that bear heparan sulfate GAG chains. The syndecans are expressed on virtually all cell types throughout development and adulthood. There are four members of the syndecan family in mammals of which three (syndecan-1, -2 and -3) have a restricted tissue distribution whereas the fourth (syndecan-4) is expressed ubiquitously. Their heparan sulfate chains endow these receptors with the ability to bind numerous "heparin" – binding growth factors, FN, vitronectin, laminins and the fibrillar collagens [62]. Thus, the syndecans can have roles in cell adhesion and signaling, possibly as co-receptors with integrins and other cell-cell adhesion molecules [63].

### **1.2.3. Integrins**

Integrins are matrix-responsive signaling receptors, comprised of non-covalently bound heterodimers. The  $\alpha$  and  $\beta$  integrin subunits collaborate in "inside-out" signaling that leads to activation of the receptor, classically defined as an increase in affinity for the ligand resulting from a change in the conformation of the integrin extracellular domain. However, integrins are also subject to "outside-in" signaling in which the ligand-bound receptor initiates intracel-

lular signaling through association of the  $\beta$  subunit cytoplasmic domain with a myriad of intracellular effectors including focal adhesion and Src family kinases, cytoskeletal components such as  $\alpha$ -actinin and vinculin, and the Rho family of cytoskeletal regulatory G-proteins [63]. Integrins mediate cell-matrix and cell-to-cell adhesion and affect many cellular processes like cell attachment, spreading, motility, proliferation, differentiation and apoptosis. Mammals contain 18  $\alpha$  and 8  $\beta$  integrin subunits that in combined manner can produce at least 24 different heterodimers, each of which can bind to a specific repertoire of cell (surface), ECM or soluble protein ligands [18,64,65].

Depending on their preferable matrix and cell specific expression, integrins can be classified as a FN- (RGD-), laminin- and collagen-binding, and leukocyte-specific receptors (Fig. 4).

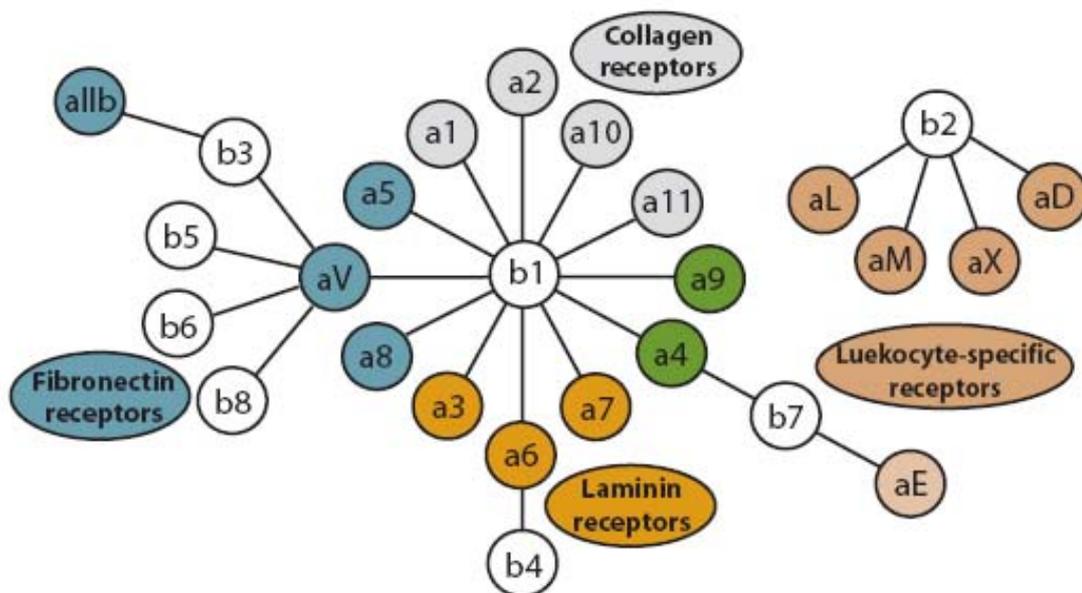


Fig. 4. Integrin family. Organization and grouping of the integrin subunits in mammalian cells based on their matrix affinity or cell specific expression [64].

However, the integrin grouping is a fictional taking in account that one integrin can successfully bind to more than one matrix molecules. A typical example for this is an integrin  $\alpha V$  which can bind collagens, FN, vitronectin, thrombospondin, bone sialoprotein, osteopontin and cartilage oligomeric matrix protein. There is as well a way to categorize integrin based on their major subunit. In this respect, integrins form the families of  $\beta 1$ ,  $\alpha V$ ,  $\beta 2$ ,  $\beta 3$  and  $\beta 7$ .

The  $\beta 1$ -subunit group is the biggest containing 12 integrin combinations. This family is one of the most studied and seems to be the most important for cell survival and development. Knockout mice lacking integrin  $\beta 1$  die at embryonic day 5,5 due to defective endodermal morphogenesis and migration causing the blastocoele collapse [66,67]. The integrins belong-

ing to b1 family have the ability to bind many ECM proteins, as within the family are presented the integrins known to have the higher affinity towards fibronectin -  $\alpha 5\beta 1$  and  $\alpha 4\beta 1$ ; collagens -  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 10\beta 1$  and  $\alpha 11\beta 1$  or laminins -  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 7\beta 1$ .

The next group containing 5 members is the one of  $\alpha V$ -subunit. The knockout model shows placenta defects that cause about 80% of the generation to be lost. Placentas from these mice show defects, and the mice have abnormalities in central nervous system and gastrointestinal blood vessels. Cleft palate is also a frequent abnormality. The  $\alpha V$  integrin subunit associates with  $\beta 1$ ,  $\beta 3$ ,  $\beta 5$ ,  $\beta 6$  and  $\beta 8$  integrin subunits.

Last is the group of  $\beta 2$  or leukocytes-specific integrins acting as traffic signal molecules to regulate leukocyte extravagation from the bloodstream during inflammation and lymphocyte homing [68]. Lack of  $\beta 2$  causes severe defect in T cell proliferation [69]. Integrins formed from the  $\beta 2$  subunit interact majorly with the family of intercellular adhesion molecules (ICAMs) and FN matrix.

### **1.2.3.1. Focal adhesions and integrin signaling**

Adherent cells form specialized structures, termed focal adhesions or focal contacts, at sites of close contact between the plasma membrane and the underlying ECM where integrins, signaling and cytoskeleton molecules co-localized. The signaling mechanisms of integrins involve a numbers of signals transduced bi-directionally through “outside-in” and “inside-out” pathways, as well by reciprocal crosstalk between integrins and other receptors regulating cell behavior [70].

#### **Focal adhesion components**

Since 1992, the number of signaling proteins linked to integrin activation (by association in focal adhesions or the regulation of activity) expanded and now includes an overwhelming collection of molecules. These include enzymes such as the Src family kinases, Abl, Syk/ZAP, Csk, Ras, Raf, Mek, Erk, phosphatidylinositol-3-OH kinase (PI(3)K), PKC, Jnk, Cbl, Pyk2 (a homologue of FAK), protein kinase A, Etk, as well as adaptor proteins like Crk, Nck, Grb-2 and many others [71-74]. The mechanism by which these proteins are activated, how they couple with each other, and how their activation by integrins affects different cell functions are still under investigation.

Recently, it was shown that plating fibroblasts on three-dimensional matrices results in the formation of novel focal adhesion structures that had not been detected when cells are grown on immobilized matrix proteins [75]. The differences between the two- and three-dimensional

structures were attributed to differences in the pliability and rigidity of the matrices under the two different conditions. These results, together with other studies using flexible matrices, indicate that differences in mechanical tension can regulate cell adhesion complexes, cell shape, polarity and the expression of differentiated cell functions [71,76-78].

### **“Outside-in” signaling and focal adhesion organization**

The firmly binding of integrin receptors to the matrix leads to grouping of the receptors localized nearby and to formation of an integrin cluster. Together by an actin polymerization, the cluster activates focal adhesion kinase (FAK). This results in auto-phosphorylation at a docking site for the recruitment of SH2-containing proteins such as Src, Fyn, PI(3)K or PLC $\gamma$  [79-81]. Src mediates phosphorylation at the other sites of FAK, creating additional SH2-domain binding sites [82]. Protein binding to the phosphorylated FAK-sites result in cascades of protein interactions that can transduce the signal throughout many downstream pathways, such as Ras/ Erk, PI(3)K/ Akt, and Crk/ Dock180/ Rac [83-85]. Current evidence suggests that multiple pathways were utilized by integrins to activate specific signaling proteins. This was the best illustrated by the Erk-activation [86]. It is known that FAK can activate Erk mitogen-activated protein kinase (MAPK) through the recruitment of Grb2 [87], Shc or Src [88].

A significant breakthrough in the understanding of the focal adhesion comes with the discovering of the guanine triphosphatases (GTPases) families, converting the guanine triphosphate to diphosphate and thus transporting an active phosphate. These molecules are involved in the induction of the actin polymerization and the formation of focal complexes, lamellipodia and filopodia [89,90]. Moreover, members of Rho family are found to directly participate in integrin signaling mechanism [91] and activation of these GTPases is now regarded as a critical event in integrin-mediated regulation of cell adhesion, cell spreading and cell motility [92].

### **“Inside-out” signaling**

Information can also be directed from integrins to the ECM ligand-binding domain. This regulation is not dependent on the recruitment of receptors to the cell surface but rather on an increase in the binding activity of the receptor [93,94]. The cytoskeleton can also regulate inside-out signaling events. The activation of  $\beta$ 2 integrins for example, involves changes in ECM through the integrin clustering [95,96]. These clustering is regulated by signaling enzymes like PI(3)K, PKCs and Ras or Rap GTPases [97,98], also adaptor proteins like SLAP 130/Fyb [99,100] which are involved in the actin reorganization. In conclusion, changes in ac-

tin cytoskeletal structures can effect the lateral movement of integrins and affect the ECM outside the cells [97,101,102].

### **1.2.3.2. Importance of integrin signaling**

#### **Integrin control of cell proliferation**

Since nearly a decade, investigators have attempted to define which steps in cell cycle progression are dependent on attachment to the ECM. It was demonstrated that there is not a single “checkpoint” that monitors cell adhesion status, but rather there are multiple steps in cell cycle progression that require matrix attachment [103,104]. It was first shown that the induction of cyclin A production is blocked in suspended cells treated with growth factor [105]. Later, the activation of several cyclin-dependent kinases (Cdks) involved in G1 phase progression and S phase initiation, were found to be controlled through multiple integrin-dependent events [106]. Integrins control these events through several mechanisms, including the enhancement of growth factor signals, the recruitment of proteins to membrane/cytoskeletal complexes or the enhancement of nuclear translocation. Many of these regulatory events involve both transcriptional and post-transcriptional control.

Although most of the studies infer that signals from integrins regulate proliferation in a dose-dependent fashion, experiments addressing the relationship between the number of adhesive contacts and the regulation of signaling and cell behavior suggest a non-linear relationship and additional levels of control. Varying the extent of cell spreading, without changing the adhesive contact area of cells, was found to govern the proliferative capacity of cells and cell survival [107]. Erk activation by growth factors does not vary with spreading. However, cyclin D expression and downregulation of p27<sup>kip</sup> is defective in poorly-spread cells. These studies suggest that certain signaling events are regulated by cell shape and provide a molecular explanation for previous reports indicating that cell shape and surface area are critical determinants for cell proliferation [108]. In addition, it is also suggested that cell-shape sensors have an important function in regulation of signal transduction through the integrins.

#### **Integrin control of cell survival**

Studies from several groups showed that cell attachment is required for the survival of normal cells [109-111]. Complete loss of cell contact with the substratum (e.g., suspension culture) or adhesion to a nonspecific substratum such as poly-L-lysine (PLL) induces apoptosis of primary cells such as fibroblasts [109], endothelial cells [111,112] and epithelial cells [110,113]. Apoptosis that is induced by cell detachment has been referred to as “anoikis” the Greek word

for homelessness [114]. Integrins regulate cell survival through the inhibition of pro-apoptotic and increased expression of anti-apoptotic proteins of Bcl-2 family [115]. Conversely, detachment from the ECM results in the activation of pro-apoptotic proteins, such as Bax [116], caspases [117,118] or the death ligands Fas and Trail [119], or inhibition of anti-apoptotic proteins.

Recent studies of Bim and Bmf, two pro-apoptotic Bcl-2 family proteins that contain only BH3 domains, indicate that they may function as intracellular sensors of the cytoskeleton and the state of ECM attachment. These proteins are bound to isoforms of the dynein light chain which associates with either microtubules or microfilaments [120,121]. Detachment from the ECM or inhibition of actin polymerization results in the dissociation of Bmf from dynein light chain and its relocation to the mitochondria where it functions as a pro-apoptotic protein. Bim expression is induced by downregulation of Akt, so induction of Bim expression may also be involved in anoikis [122].

Cell death can also occur when a subset of integrins in a cell fail to bind their ECM ligands [110,112]. For example, expression of  $\alpha$ V $\beta$ 3 or  $\alpha$ 5 $\beta$ 1 can inhibit cell survival in cells attached to the matrix through other integrins [110,112]. The expression of  $\alpha$ V $\beta$ 3 inhibits cell survival in cells attached to native collagen through integrin  $\alpha$ 2 $\beta$ 1 [110]. As integrin  $\alpha$ V $\beta$ 3 does not bind native collagen, these results indicate that the none-ligated integrin  $\alpha$ V $\beta$ 3 induces cell death. In a similar manner, antibody inhibition of integrin  $\alpha$ 5 $\beta$ 1 activity induces apoptosis of endothelial cells that are attached to vitronectin through  $\alpha$ V- integrins [112]. In addition, expression of dominant negative integrins (e.g., Tac-b3, the IL-2 receptor fused with the integrin  $\beta$ 3 subunit cytoplasmic tail) also inhibits survival by impairing normal integrin-mediated survival signaling [110]. Integrin ligation suppresses caspase 8 activation, while none-ligated integrins facilitate caspase 8 activation in a stress response and death receptor-independent manner [110,112]. Additional studies suggest that none-ligated integrins activate membrane-associated protein kinase A (PKA), which itself can activate caspase 8 in endothelial cells [112]. Thus, in normal cells some integrins can promote cells survival when ligated and induce apoptosis when none-ligated.

The ability of integrins to protect cells from apoptosis is both, integrin- and cell-specific. For example, primary mammary epithelial cells treated with insulin are protected from cell death when plated on laminin, tenascin C or collagen IV, but not on ColII [123,124]. The protective effects of insulin are dependent on the ability of the integrin to promote activation of Akt/PKB through the insulin receptors. In CHO cells  $\alpha$ 5 $\beta$ 1,  $\alpha$ V $\beta$ 3 and  $\alpha$ 1 $\beta$ 1 integrins protect

cells from anoikis, whereas  $\alpha$ V $\beta$ 1 does not provide this protection [115]. Protection from death correlates with the ability of the integrin to induce Bcl-2 expression [125].

Lastly, recent studies indicate that non-ligated integrins can also induce cell death distinct from anoikis under certain conditions where other of the cell integrins are ligated [110].

### **Integrin role in cell migration**

While integrin ligation to the ECM positively regulates migration, inhibiting integrins prevent cell migration. Although blocking integrin ligation can prevent cell attachment to the ECM and thus inhibit migration, recent studies show that antagonized integrins actively inhibit signal transduction leading to cell migration [126]. For example, the inhibition of integrin  $\alpha$ 5 $\beta$ 1 negatively regulates fibroblast, endothelial cell and tumour cell migration even when other integrin receptors for available matrix proteins are ligated. Antagonists of integrin  $\alpha$ 5 $\beta$ 1 suppress cell migration on vitronectin but not cell attachment to vitronectin, indicating that these antagonists affect the migration machinery rather than integrin receptors for vitronectin [126]. In fact,  $\alpha$ 5 $\beta$ 1 antagonists activate PKA which then inhibits cell migration by disrupting the formation of stress fibers [126]. Direct activation of PKA by forskolin or by overexpression of the catalytic active subunit of PKA also inhibits cell migration [111,126]. Thus, integrins regulate cell migration by making contact with the substratum and by promoting signal transduction cascades that support migration.

### **Integrin crosstalk with other receptors**

One of the most striking examples of receptor crosstalk is the integrin activation of growth factor receptors. EGFR, PDGFR, vascular endothelial growth factor receptor (VEGFR) and hepatocyte growth factor receptor (HGFR; Met) are all activated after the engagement of integrins [127,128]. Integrin-activated growth factor receptors are capable of amplifying integrin signals. Shc/Erk activation in several cell types is dependent on integrin-induced EGFR activation. Furthermore, adhesion-induced cell survival, mediated through PI(3)K, also requires ECM activation of EGFR via integrins [127]. The ability of cell adhesion to activate the HGFR is crucial for tumour metastasis in a hepatocyte tumour model [129]. As discussed above, growth factors and other agonists can activate integrins through changes in integrin affinity and avidity.

A second example is the receptor coordination. In this type of crosstalk, two or more receptors components contribute to the activation of an intracellular event. Coordination is observed between integrins and growth factors or integrins and syndecans. For example, signals from

syndecans can influence integrin-mediated focal adhesion to FN as this is done through PKC, Rho and syndesmos (a paxillin binding protein) [130].

Third is a receptor pathway modulation. In this type of crosstalk, a signal from one receptor provides a costimulatory or inhibitory signal to another receptor pathway. There are numerous examples of such regulations related to Erk activation and cell cycle progression [103,104]. It has been shown that integrin signals are required for growth factor activation of Erk throughout the MAPK-pathway. Although the initial signaling by Ras is integrin independent, its downstream targets - Raf or MEK required integrin adhesion [131].

Fourth, the modulation of receptor expression - this mechanism involves the induction or repression of receptor expression by another receptor. Growth factor receptor enhancement of motility in several cell types results from the upregulation of integrin receptor expression [132]. Manipulations that lower either EGFR or b1 integrin expression in three-dimensional gels, but not in two-dimensional culture, were found to cause downregulation of the other receptor [75]. These results suggest that integrins and growth factors couple in distinct ways, depending on the context in which the cells are cultured.

### **1.2.3.3. *In vivo* functions of integrins**

Integrins have been implicated in many cellular functions through the *in vitro* studies. However, recent analyses of integrin mutants in worms, flies and mice have provided important information on integrin function *in vivo*. In *Drosophila melanogaster* and *Caenorhabditis elegans*, integrin mutations cause defects in multiple developmental events, including the expected alterations in the attachment of cells within and between tissues [133]. The effects of integrins are mainly studied in knockout mouse models (tab. 1).

Gene	Phenotype
a1 V, F	Increased collagen synthesis, reduced tumor vascularization
a2 V, F	Platelets phenotype
a3 L, birth	Defects in kidneys, lungs, and cerebral cortex; skin blistering
a4 L, E11-E14	Defects in chorioallantois fusion and cardiac development
a5 L, E10	Defects in extraembryonic and embryonic vascular development
a6 L, birth	Defects in cerebral cortex and retina; skin blistering
a7 V, F	Muscular dystrophy
a8 L+V/F	Small or absent kidneys; inner ear defects
a9 L, perinatal	Bilateral chylothorax
a10 V, F	No apparent phenotype
a11 V, F	Periodontal ligament phenotype
aV L, E12-birth	Defects in placenta and in CNS and GI blood vessels; cleft palate
aD .....	.....
aL V, F	Impaired leukocyte recruitment and tumor rejection
aM V, F	Impaired phagocytosis and PMN apoptosis; obesity; mast cell development
aX .....	.....
aE V, F	Inflammatory skin lesions
aIIb V, F	Defective platelet aggregation
b1 L, E5,5	Inner cell mass deterioration
b2 V, F	Impaired leukocyte recruitment; skin infections
b3 V, F	Defective platelet aggregation; osteosclerosis
b4 L, prenatal	Skin blistering
b5 V, F	No apparent phenotype
b6 V, F	Skin and lung inflammation and impaired lung fibrosis
b7 V, F	Abnormal Peyer's patches; decreased No. of intraepithelial lymphocytes
b8 L, E12-birth	Defects in placenta and in CNS and GI blood vessels; cleft palate

Tab. 1. Phenotype of integrins knockout mice adopted from [134]. Abbreviations: V- viable; F- fertile; L- lethal; L+V/F- mutations that disrupt development but also allow survival in a fraction of mice; CNS- central nervous system; GI- gastrointestinal; and PMN- polymorphonuclear neutrophil.

The disruption of b1 integrin, which is a subunit of at least 12 integrins, causes peri-implantation lethality [135]. Analysis of chimaeric mice that lack b1 integrin in a subset of cells or tissues and mice lacking other a or b subunits, have identified more specific defects in many processes, including haematopoiesis, haemostasis, immune defenses and the migration of several cell types, neural organization, organ development, the formation and maintenance of vasculature, and the integrity of skeletal and cardiac muscle, skin, bone and cartilage [133]. It is difficult to establish whether processes regulated by integrins *in vivo* require intracellular signal transduction or merely extracellular adhesive functions; however, genetic ablation of integrin genes in mice (but not in flies) suggest a requirement for integrins in regulating proliferation of certain cell populations, including keratinocytes, dermal fibroblasts, mammary and intestinal epithelial cells [134]. Cell survival defects have also been observed in some integrin-null mice [134].

In humans, a lack of the platelet integrin  $\alpha$ IIb $\beta$ 3 or the  $\beta$ 2 leukocyte integrin subunit result in diseases associated with bleeding and recurrent infections, respectively [136]. Mutations in the  $\beta$ 4 subunit cause a severe skin blistering disease, epidermolysis bullosa, caused by defects in attachment to laminin.

### 1.3. Integrin expression in hMSC

As a member of the cell population within the BM, hMSC express receptors for all presented ECM. Using fluorescence activated cell sorting (FACS) method in hMSC was detected the expression of  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 5,  $\alpha$ 6,  $\alpha$ V,  $\beta$ 1,  $\beta$ 3 and  $\beta$ 4 integrin subunits [55]. *Gronthos et al.* [137] showed that hMSC bind to collagen I, III and IV predominantly with integrin  $\alpha$ 1 $\beta$ 1 and  $\alpha$ 2 $\beta$ 1, to laminin by  $\alpha$ 6 $\beta$ 1, to FN through  $\alpha$ 5 $\beta$ 1 and vitronectin by  $\alpha$ V $\beta$ 3 as those data was obtained from analyzing the adhesion and cell growth in presence of blocking antibodies. Moreover, *Salasznyk et al.* [39] observed a tremendous effect of ColII and vitronectin on protein osteogenic differentiation, suggesting the importance of corresponding matrix integrins.

#### The collagen binding integrins in hMSC

Collagen-rich ECMs are not only critically important for the biomechanical properties of tissues but are also intimately involved in cell adhesion and migration during growth, cell differentiation, morphogenesis and wound healing. In humans, numerous diseases are caused by mutations in collagen genes and cell–collagen interactions are perturbed in many other pathological situations [138].

In general, four  $\beta$ 1 integrins ( $\alpha$ 1 $\beta$ 1,  $\alpha$ 2 $\beta$ 1,  $\alpha$ 10 $\beta$ 1 and  $\alpha$ 11 $\beta$ 1) are functioning as collagen receptors. The most widely distributed collagen-binding integrins  $\alpha$ 1 $\beta$ 1 (predominantly in mesenchymal cells) and  $\alpha$ 2 $\beta$ 1 (predominantly in epithelial cells, as well as platelets) were detected also in hMSC [139]. The importance of these integrins was validated by investigation of the corresponding knockout mouse models. Interestingly, integrin  $\alpha$ 1- and  $\alpha$ 2-knockout mice develop normally and are fertile [140]. However, more detailed studies within  $\alpha$ 1-deficient mouse have uncovered specific effect on fibroblast proliferation [141], tumour vascularisation [142] and renal injury [143]. Integrin  $\alpha$ 2-deficient mice had a mild defects in branching morphogenesis of the mammary gland and in platelet adhesion to ColII [144,145]. More recently discovered integrin  $\alpha$ 10 $\beta$ 1 and  $\alpha$ 11 $\beta$ 1 integrins are localized in specific areas such as chondrocytes for  $\alpha$ 10 $\beta$ 1 and mesenchymal cells for  $\alpha$ 11 $\beta$ 1. An integrin  $\alpha$ 10-deficient mice show a cartilage growth plate defect [146] whereas integrin  $\alpha$ 11-deficient mice show pronounced tooth

defect, combine with reduced body length and deduced attachment, spreading and migration of embryonic fibroblasts on Coll I [147]. It is worth to notice that individual ablation of other matrix-binding integrins such as integrin subunits  $\alpha 3-8$  and  $\alpha V$  results in more severe defects than loss of any single collagen-binding integrin [64]. Thus, it seems likely that there is functional redundancy between the collagen-binding integrins.

The collagen specificity of  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 10\beta 1$  and  $\alpha 11\beta 1$  integrins has been studied extensively [148,149]. Two important discoveries have greatly aided structure–function studies of collagen-binding integrins. First, it was found that isolated I domains, which are relatively easy to crystallise for structural analysis, retain the specificity and high affinity to collagen [150,151]. Second, several groups have identified specific integrin-binding sequences within the triple-helical region of collagens. Using synthetic triple-helical peptides, *Knight et al.* [152,153] showed that the collagen motif - GFOGER is a high-affinity-binding site for  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrin. The GFOGER sequence was also identified in another study which reported also two additional  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrin-binding sites in collagen - GLOGER and GASGER [154]. *Sweeney et al.* [155] found that both GFOGER and GLOGER were recognized by  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrin but only the former sequence appeared to be involved in  $\alpha 2\beta 1$  integrin-mediated endothelial tube formation. Finally, GFOGER is also a binding site for  $\alpha 11\beta 1$  integrin [156,157].

#### **1.4. Methods of studying gene function in human cells**

Primary hMSC have a limited lifespan. Moreover, these cells are slow dividing and have very low rate of DNA transfection and integration. Therefore, in order to study a gene function in this cell type, a new approach, allowing for a stable downregulation of the gene of interest, was needed. In the past decade, a new technique of gene regulation has emerged, termed RNA interference or RNAi. This technique utilizes a double-stranded RNA (dsRNA) which effectively inhibits a specific gene expression by interfering with its complementary messenger RNA (mRNA). The RNAi response can be triggered by the introduction of short-interfering RNA (siRNA) strands into cells expressing a homologous gene target. The dsRNA fragments engage an endogenous complex of cellular proteins known as the RNA-induced silencing complex (RISC) which pairs with and then degrades the corresponding mRNA and thus blocks the protein translation. The discovery of this technique has re-awakened interest in nucleic acid-based approaches for gene suppression for scientific and therapeutic purposes [158].

### 1.4.1. History of RNAi

First evidence for the existence of RNAi control system was observed in late 1980s. The molecular mechanism remained unclear till late 1990s when the breakthrough articles of *Fire et al.* [159] and *Reinhart et al.* [160] were published. They showed in *Caenorhabditis elegans* that RNAi is an evolutionary conserved gene-silencing mechanism related to a normal defence against viruses and mobilisation of transposable genetic elements (transposons) [161]. Subsequently, the phenomenon was demonstrated in worms, flies and vertebrates [162-164]. The initial experiments with RNAi in mammals were unsuccessful. The usage of long dsRNA (>30 nucleotide) sequences, as in the earlier studies with plants and worms, was found to induce an interferon response resulting in a global inhibition of mRNA translation and cell death. This problem was overcome by *Tuschl et al.* who demonstrated that chemically synthesized, shorter siRNA sequences could successfully and efficiently silence endogenous genes in mammalian cells [165,166]. Recently, a vector-based siRNA expression system was developed. It allows for stable and longer period of gene silencing [167,168]. For delivery were used virus-based vectors including adenovirus, adeno-associated virus (AAV), retrovirus and lentivirus systems [169,170].

### 1.4.2. Mechanism of RNA interference

The mechanism of RNAi is a simple system of interacting complementary RNA sequences. The process starts with transcription of dsRNA in the nucleus, modification and complex formation with intracellular proteins, and ends with sequence-specific degradation of a target mRNA. The long dsRNA segments are first processed by an RNase III enzyme, termed Dicer, into small dsRNA duplexes of 21–23 nucleotides, termed siRNA. The siRNA duplex strands (synthetically or cell produced) are then shuttled into a multi-protein complex consisting of the RISC and the cleaving enzyme Argonaute 2 (Ago2). The RISC discards one strand leaving a “processed” strand of siRNA incorporated into the protein complex. This single strand of siRNA serves as the guiding sequence for recognition of a target mRNA. When a complementary base pairing of a single-stranded guide siRNA interacts with the target mRNA, the RISC/Ago2 complex cleaves the target mRNA. This results in a post-transcriptional gene silencing and gene translational blocking (fig. 5) [171]. In addition to post-transcriptional RNA silencing, components of the RNAi pathway are postulated to be involved in transcriptional gene silencing through RNA-dependent DNA methylation [172].

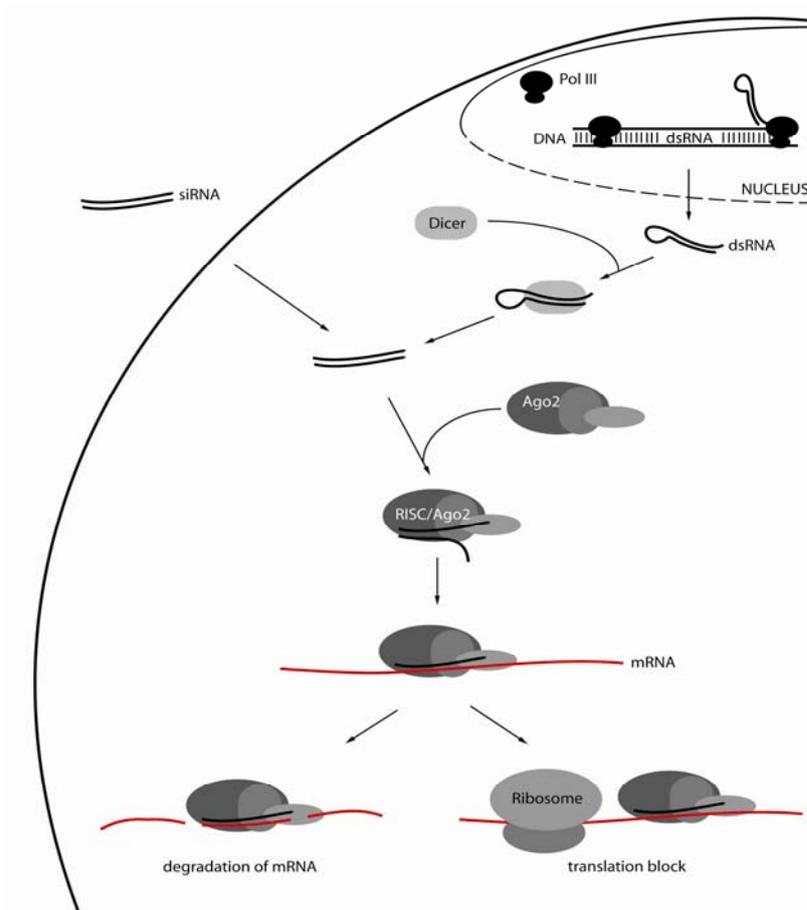


Fig. 5. Overview of RNA interference (RNAi) mechanism - expression of dsRNA and formation of the RISC complex by ds- or siRNA inhibiting the protein production on mRNA level.

### Small interfering RNA (siRNA)

This technology allows examination of the gene silencing in plants and animals within a short timeframe. Compare to the plants, it was found that dsRNAs bigger than 30 bp trigger the  $\gamma$ -interferon (IFN) pathway in mammals. Nevertheless, dsRNAs consisting of 21–23 bp, generated by chemical synthesis [166], enzymatic cleavage [173] or expression systems [174] were found to be optimal for gene silencing by mimicking Dicer products. Moreover, the siRNA duplexes have to be delivered into mammalian cells with transfection either by lipid-based formulations [175], electroporation [176] or by linking to peptides [177] as the half-life of the molecule do not exceed 3 days.

### Short hairpin RNA (shRNA)

In contrast to *C. elegans*, where RNAi effects are stable, long lasting and are passed onto the offspring [178], gene silencing by transfected siRNA duplexes in mammalian cells is transient. This is because mammalian cells lack the RNA-dependent RNA polymerases that amplify siRNAs in *C. elegans*. As a result, gene silencing is dependent on the number of siRNA

molecules transfected into the cells and the duplexes become progressively diluted as cells divide. The persistence of siRNA activity in mammalian cells varies with the proliferative status of the cells, such that siRNA activity lasts for 3–7 days in proliferating cells, but can persist for 3 weeks or more in terminally differentiated cells, such as neurons [179]. To overcome this problem, vector-based systems for introduction and stable expression of siRNA in target cells have been developed [165]. These vectors contain RNA polymerase III promoters that either express sense and antisense strands from separate promoters (tandem type) or express short hairpin RNA (shRNA) that are cleaved by the Dicer to produce siRNA. Stably transfected cell lines can be generated by selecting for a drug resistance marker expressed either by the vector or with a co-transfected plasmid. Such vector systems have been successfully used to obtain efficient and stable knockdown of target genes in mammalian cells [180]. The limitations of using plasmid vectors in terms of efficiency and difficulty in transfecting primary cells [181] have resulted in developing retroviral [182], adenoviral [183] and lentiviral [184] vector systems for shRNA delivery. Viral vectors permit the efficient delivery and stable expression of shRNA constructs in a range of mammalian cells (including primary cells) and a variety of animal species. Retroviral vectors are based on Moloney-murine leukemia viruses as they permit a stable introduction of shRNA into the dividing cells genome as they have been already shown to successfully suppress a gene expression in stem cells [185]. Adenoviral vectors based on adeno-associated viruses (AAV) can infect both, dividing and nondividing cells and they are successfully used for genes silencing *in vitro* and *in vivo* [186-188]. Lentiviral vectors are derived from human immunodeficiency virus (HIV-1) and can also infect both dividing and non-dividing postmitotic cells (e.g., neurons), and have been used to generate transgenic animals that display loss-of-function phenotypes and vector transmission to offspring [184,189].

### **1.4.3. Advantages and disadvantages of the RNAi technology**

In mammalian cells, induction of RNA silencing is usually achieved with the use of a shRNA system, since the short hairpins are considered too small to induce the interferon response. Short hairpin systems are also a method of choice for large-scale and long-term experiments because of stable expression of larger number of shRNAs and because of the advantage of antibiotic selection which allows working with a homogeneous cell population. Unfortunately, the antisense molecules have been plagued by a lack of target specificity, poor binding to target mRNA and susceptibility to nuclease degradation. Despite modifications to the oligonu-

cleotide molecules, the inefficient gene inhibition and a risk of unintended consequences, has largely sidelined this technology in the face of siRNA breakthrough [190].

### **Off-targeting RISC complex**

As siRNAs have become more widely used, the basic structure of effective siRNAs has been defined, including the need for a 19-bp RNA duplex with a 2-nucleotide overhang on the 3'-ends. It has also become clear that the effectiveness of siRNA silencing is sequence specific, hence, rules for siRNA design have been developed [180,191]. An siRNA duplex may target more than one mRNA molecule because of sequence homologies. It is now widely observed that most siRNAs can tolerate one mismatch to the mRNA target and at the same time retain good silencing capacity [192]. In some cases, siRNAs can tolerate several mismatches [193] or even tolerate mismatches while acting as a single-stranded antisense siRNA [194]. In addition, some domains of the siRNA sequence can tolerate more mismatches than others do [195]. *Saxena et al.* [193] also demonstrated tolerance for G/U wobble pairing between the RNA oligo and the target RNA. The efficiency with which a mismatched RISC can mediate transcript cleavage is probably significantly reduced as compared to when there is total complementarity [196]. Moreover, microarray analysis has revealed that the expression of a non-targeted transcript with more than 11 consecutive nucleotide matches with the siRNA sequence can be also downregulated [197]. These observations have led to concerns that anything less than optimal RISC-transcript interactions could permit RNAi against an unintended target or an "off-target" interaction, which may limit the ability to interpret a specific functional effect of RNAi. Currently, it is unclear to what extent off-target translational repression interactions occur as microarray analysis can only detect downstream changes in RNA levels after repression of protein translation. Nevertheless, studies of RNAi transgenic animals [184,198,199] suggest that off-target effects may be minimal on a whole organism basis and that in cell culture, minimizing the concentration of a particular RNAi effectors may reduce the likelihood of an off-target effect.

### **Non-specific responses to dsRNA**

The use of a minimal amount and shorter sequences of the RNAi effectors reduce the possibility of nonspecific dsRNA responses exhibited by the most mammalian cells. Mammalian cells have a number of non-sequence specific responses triggered by dsRNA which form the viral host defense system and activate the programmed cells death. Key-effector proteins of these responses are the family of 2'-5' oligoadenylate synthetase or AOS (fig. 6).

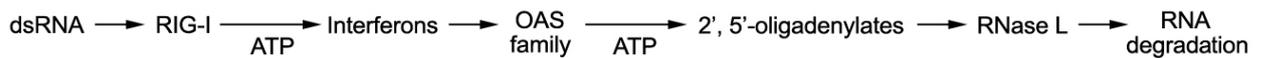


Fig. 6. Activation of cell defence IFN pathway by virus dsRNA (adopted from [200,201]).

*Yoneyama et al.* [200] showed in two independent cell lines that a presence of dsRNA in the cells results in activation of immune response and induction of IFN type I genes, such as interferon stimulated genes 20 and 56 (ISG20 and ISG56), through binding to retinoic acid inducible gene I or RIG-I. Increased expression of interferon induced genes leads to an activation of OAS family members which thereon transmit the dsRNA signal and cause activation of the RNaseL enzyme resulting in total RNA degradation. On the basis of the few studies available, the degree of activation depends of the size and concentration of the dsRNA [202]. In addition to the IFN response, it has been reported that si/shRNA initiate immune activation in macrophages and dendritic cells through toll-like receptor 3 [203]. Currently, it is still unclear how often si/shRNA triggers such effects in cells and what conditions lead to such response. Data addressing the degree to which siRNA and shRNA can interact with and activate non-RNAi-associated dsRNA binding proteins is still limited. However, the studies that have been performed so far suggest that a it needs to be paid attention to the transcription and intracellular processing of shRNA so that the siRNA generated does not trigger nonspecific responses and that directly administered siRNA should consist of a high-quality, size-homogenous population [204,205].

#### 1.4.4. Usefulness of RNAi technology

Since RNAi is the latest technique widely used to down-regulate the mRNA level of any gene of interest, RNAi therapy is therefore speeding up on the determination of gene function related to certain clinical diseases [206]. To date, exciting impacts have been acquired by RNAi on many fields of human diseases, including cancers, virus infection, neuroscience and etc. [206]. Recent study of *Owen et al.* [207] showed that silencing of EWS/FLI protein by siRNA successfully inhibited propagation and restored its osteoblastic differentiation potential in Ewing's sarcoma cells. Another study by *Lin et al.* [208] found that knockdown of Runx2 markedly attenuated osteoblast differentiation in cultured primary mouse osteoblasts by downregulation of osteoblastic markers such as Col I, osteopontin, bone sialoprotein and osteocalcin gene expression, decreased alkaline phosphatase activity and reduced matrix mineralization which can be used as a potential tool to prevent or treat heterotopic ossification in humans. At

present, *in vivo* studies were also performed in collagen-induced arthritis rats which were transfected with NF- $\kappa$ B-specific siRNA by intraarticular injection which resulted in improvement of joint destruction [209]. All together, these studies provide sufficient information for the possible application of RNAi technique in the investigation of the physiological function of bone-related diseases; discover the new modulators and development of therapy of bone-related diseases.

## 2. Aim and milestones of the thesis

The main aim of this doctoral thesis is:

Investigation of the basal integrin expression of hMSC and functional analysis of the role of a single collagen I-binding integrin receptor on hMSC behavior by knockdown studies *in vitro*.

In order to accomplish the main aim, the following project milestones were defined:

1. Evaluate the matrix affinity of hMSC and characterize their integrin expression.
2. Establish a stable knockdown for  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 11$  collagen I-binding integrins.
3. Investigate the effect of integrin knockdowns on cell attachment, spreading and migration.
4. Investigate the effect of integrin knockdowns on osteogenic differentiation.
5. Investigate the compensatory mechanism between collagen I-binding integrins.
6. Investigate the collagen I-binding integrins expression in human healthy and osteoporotic patients.

### 3. Material and Methods

#### 3.1. Human primary cells and cell lines

##### **hMSC (Cambrex – Lonza GmbH, Germany)**

hMSC were isolated from human BM. They have fibroblast-like cell morphology and are growing in a monolayer. These cells are tested for the positive expression of CD73, CD90 and CD105, and negative for CD14, CD34 and CD45 genes by FACS analysis. Cells were also differentiated towards osteogenic, adipogenic and chondrogenic lineages. In this work we use three different hMSC donors (tab. 2):

<b>Abbreviation</b>	<b>Lot number</b>	<b>Sex</b>	<b>Age</b>
hMSC donor XI (hMSC XI)	1F2155	M	24
hMSC donor XIII (hMSC XIII)	4F0591	M	32
hMSC donor XV (hMSC XV)	6F3837	F	34

Tab. 2. hMSC donors used for experiments. The cells were isolated from young donors.

In our experiments we predominantly use hMSC XI. This donor was used in all types of experiments as some of the critical experiments, such as cell differentiation, integrin downregulation, cell adhesion, spreading and migration and cell apoptosis have been confirmed also with the other two donors.

##### **hOB (Cat.Nr.: C-12720, Promo cell, Germany)**

hOB cells were isolated from normal femoral bone tissue. They stain positive for osteocalcin and alkaline phosphatase. hOB are large mononuclear cells of mesenchymal origin and growing in monolayer.

##### **293FT cell line (Cat. Nr.: R700-07; Invitrogen, Germany)**

293FT cell line is derived from primary embryonal human kidney 293 cells transformed with human adenovirus type 5 DNA [210] and expresses the SV40 large T antigen. They have epithelial-like cell morphology and are growing in a monolayer. The 293FT cells are suitable host for generating lentivirus constructs. In addition, these cells have a neomycin resistance.

**HeLa cell line (Cat. Nr.: ACC 57; DSMZ, Germany)**

HeLa cell line is established from epitheloid cervix carcinoma (adenocarcinoma) cells. The cell line has epithelial-like morphology and grows in a monolayer.

**3.2. Culture conditions****Cell culture dishes**

The dishes and flasks used for *in vitro* cells culture were purchased from Nunc (Nunc GmbH & Co KG, Germany), BD Biosciences (BD Biosciences, Germany) and Sarstedt (Sarstedt, Germany). In this study were use T-25, T-75 and T-225 cell culture flasks; 6-well, 12-well, 96-well and 10 cm culture dishes.

**Complete cell culture media**

HMSC and HeLa cells were cultured in aMEM-glutamax culture media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma, Germany) and 1% penicillin/ streptomycin (Pen/Strep) antibiotic (PAA, Germany).

For 293FT cell line was used D-MEM (high glucose) culture media (Invitrogen) supplemented with 10% FBS, 1%Pen/Strep, 0.1mM non essential amino acids (NEAA) (Invitrogen) and 1mM Sodium pyruvate (Sigma).

For hOB cells was used Osteoblasts growth media (Promo cell, Germany).

**Cell culture conditions**

The human cell culture was performed according to the manufacturer's recommendations. Cells were grown in monolayer in a cell culture incubator (Ser. Nr.: 39709355, Jouan, France) with constant conditions of 37°C and 5% CO<sub>2</sub>.

HMSC were cultivated maximum until passage 10 and for the most of the experiments hMSC in passage 7-9 were used. Cell culture was maintained in low confluence (max 50-60%). The culture media was exchanged every third day.

293FT cells were used for virus production and were cultured to maximum 20<sup>th</sup> passage. The confluence of the monolayer was approx 90-95%. The media was exchange every second day.

HOB and HeLa cell line was maintained in approx. 80% confluence. Media was exchanged three times per week.

### 3.2.1. Passaging and counting

For splitting or transferring cells, the cultured monolayer was first washed with PBS (PAA). Then the cells were trypsinated by using 1x Trypsin/EDTA (PAA) for 5 min at 37°C/5%CO<sub>2</sub>. The detached cells were washed and resuspend in culture media. A small portion of the cell suspension was taken for cell counting. In an eppendorf tube 10 µl of the cell suspension and 5 µl of trypan blue stain solution (Invitrogen) were mixed. The tripan blue staining was used for counterstaining of dead cells. The counting was performed in a Neubauer cell counting chamber (Brand, Germany). Cells in four outer quadrants were counted and the blue, death cells were excluded. The total number of cells was calculated by the formula:

$$\text{cells / ml} = \frac{A + B + C + D}{4} \times 1.5 \times 10000$$

where A-D are the counted cells in four quadrants and 1.5x is the dilution factor. After the counting, a define number of cells were re-plated or cryo-preserved.

### 3.2.2. Cryopreservation

For cryo-preservation, a specific freezing media was prepared for each cell type. HMSC freezing media contained 70% normal culture media, 20% FBS and 10% Dimethylsulfoxid (DMSO) (Merck, Germany). HeLa and 293FT freezing media composition was 90% normal culture media supplemented with 10% DMSO. After cell tripsinization and counting, the cells were pelleted by centrifugation for 5min at 500 rpm. The supernatant was completely aspirated and the cell pellet was resuspend in pre-cooled at 4°C freezing media. Next, the cell suspensions were aliquoted in pre-labeled cryovials which were then placed on dry ice and finally stored at -80°C freezer or liquid nitrogen.

HOB cells were frozen in Cryo-SFM media (Promo cell) according to the manufacturer instructions.

## 3.3. ShRNA sequences

### Design

The design of the shRNA oligonucleotides (oligos) was performed by using the Invitrogen's BLOCK-iT RNAi Designer (tab. 3) against published gene molecules as sense and antisense sequence was separated with AACG loop. The designed oligos were then purchased from Invitrogen.

Gene	Accession number	Sequence
LacZ DNA oligo	manufacturer	5'-GCTACAAATCAGCGATTT-3'
Integrin a1 (ITGA1)	NM_181501	5'-GCTCCTCACTGTTGTTCTACG-3'
Integrin a2 (ITGA2)	NM_002203	5'-GCAAACCTCAACAAGCATTC-3'
Integrin a11 (ITGA11)	NM_001004439	5'-GGATGTGTTCAAGAGTCAACT-3'

Tab. 3. ShRNA sequences against the LacZ, ITGA1, ITGA2 and ITGA11 genes designed against published molecules

### Assembling of the double-stranded Oligos (dsOligos)

The assembling of the dsOligos was performed according to the Invitrogen's instructions. Briefly, the oligos were diluted to 200  $\mu$ M in DNase/RNase free water and annealing reaction was performed. The reaction products were then diluted to final concentration of 5 nM dsOligos and were checked on ethidium bromide gel.

## 3.4. Bacterial cloning

### 3.4.1. Bacterial strains

For plasmid cloning and propagation we used:

#### **One Shot TOP10 Competent Cells (Cat.Nr.: C4040-03, Invitrogen)**

These cells are derivatives from DH10B *E. coli* strain which provides high cloning efficiency and plasmid propagation. They are suitable for high-copy number plasmids amplification and are sensitive to temperature variations and mechanical disruption.

#### **One Shot Stbl3 Chemically Competent Cells (Cat.Nr.: C7373-03, Invitrogen)**

DH10B *E. coli* strain derivative, recommended for use with unstable inserts such as lentiviral DNA. They are also highly sensitive to temperature variations and mechanical disruption.

### 3.4.2. Bacterial culture media

The LB (Luria-Bertani) bacterial cultivation media (10% trypton, 10% NaCl and 5% yeast extract) was adapted from *Sambrook et al* [211]. All components of the media were mixed, autoclaved and immediately used or preserved at 4°C.

For preparation of hard selective media, 15% agar was added to the LB media. After heat sterilization, the media was cooled to RT and depending on the plasmid resistance a selective an-

tibiotics, ampicillin or kanamycin, were added. Media was then mixed by gentle shaking and poured into 10 cm bacterial petri dishes. The obtained LB-agar plates were stored at 4°C.

### 3.4.3. Plasmids

#### **BLOCK-iT U6 RNAi Entry Vector or pENTR/U6 (Cat.Nr.: C4945-00; Invitrogen)**

A PUC-based plasmid modified for expression of shRNA and containing a kanamycin resistant gene for bacterial selection. The plasmid provides a rapid and efficient way for cloning of the desired shRNA sequences. The vector contains RNA Polymerase III (Pol III)-driven eukaryotic expression cassette (*i.e.* U6 RNAi cassette) which makes it very useful for earlier testing of the shRNA sequences in mammalian cells.

#### **pLenti4/BLOCK-iT-DEST or pLenti4-BlockIT (Cat.Nr.: C4925-00; Invitrogen)**

A PUC-based plasmid used for generation of replication-incompetent lentivirus for effective transduction of dividing and non-dividing mammalian cells. This plasmid contains also an ampicillin resistant gene for bacterial selection. The vector provides a stable and long-term expression of shRNA sequence into the host cells. It is used for production of pseudotyped virus which has a broadened host range. It include a multiple biosafety features such as psi packaging signal, Rev response and  $\Delta$ U3 elements. The vector also contains zeocin as a resistance marker for selection of the transduced mammalian cells.

### 3.4.4. Ligation and recombination reactions

Ligation and recombination reactions were performed according to the manufacturer description. Briefly, 20 $\mu$ l of the ligation reaction containing the dsOligos and linearized pENTR/U6 vector was mixed and incubated for 2 hours at RT, followed by cooling of the reaction mixtures on ice. A 10nM of pENTR/U6-shRNA ligated plasmid was used for transformation of TOP10 competent bacterial cells. The transformation was performed by bacterial heat-shock for 30 second at 42°C and subsequent propagation at 37°C with 200rpm constant shaking for 1 hour in 250 $\mu$ l Invitrogen's S.O.C. media (LB media supplemented with 2.5mM KCl, 10nM MgCl<sub>2</sub>, 10nM MgSO<sub>4</sub> and 20mM glucose). Next, the transformed cells were grown for 12 hours at 37°C on kanamycin LB agar plates to obtain resistant bacterial clones.

For the recombination reaction, a mixture of 100ng of pENTR/U6-shRNA and 50ng pLenti4/BlockIT-DEST with 2 $\mu$ l from LR Clonase II (Invitrogen) were incubated for 6 hours at RT. The reaction was stopped by addition of 1 $\mu$ l Proteinase K and incubation for 10 min at

37°C. Stbl3 competent cells were mixed with 3µl of the recombination reaction, heat-shocked and incubated at 37°C with 225rpm constant shaking for 1 hour in 250µl S.O.C. media. The cells were grown on ampicillin LB agar plates for 12 hours at 37°C to obtain resistant bacterial clones.

#### **3.4.5. Isolation of plasmid DNA (pDNA)**

TOP10 or Stbl3 clones were picked and expanded in 5ml LB media, supplemented with kanamycin or ampicillin, correspondingly. The bacterial culture was performed at 37°C with 225rpm constant shaking for 12 hours.

Plasmid DNA (pDNA) was isolated by using GenElute Plasmid Miniprep Kit (Cat.Nr.: PLN350-1KT, Sigma) and following the manufacturer's instructions. Briefly, the cells were pelleted, resuspend and lysed for 5 min at RT. The lysis was stopped with the neutralization solution and cell debris was pelleted by centrifugation. The supernatant was loaded on previously equilibrated column and washed twice with washing buffer. The pDNA was eluted in elution buffer (5mM Tris-HCl buffer pH8.0) and measured spectrophotometrically in order to estimate the pDNA yield. The pDNA was digested with restriction enzymes and then sequenced for confirmation of the plasmid size, orientation and quality of the shRNA insert.

From the correct shRNA clones, a bigger amount of pDNA was isolated by HiSpeed Plasmid Maxi Kit (Cat.Nr.: 12663, Qiagen) for the pENTR/U6-shRNA clones or EndoFree Plasmid Maxi Kit (Cat.Nr.: 12362, Qiagen) for pLenti4-BlockIT-U6-shRNA clones. The kits were used according to the manufacturer's instructions. Briefly, the bacteria were pelleted and lysed, and then the cell debris was precipitate and filtrated. The bacterial lysate, after the filtration, was loaded on a column, washed, eluted and the pDNA was precipitated with isopropanol and pelleted by centrifugation. The pDNA pellet was washed with 70% ethanol, air-dried and resuspend in elution buffer (5mM Tris-HCl buffer pH8.0). The pDNA concentration was measured and the quality of pDNA and the shRNA insert was analyzed by digestion and sequencing.

### **3.5. Virus production**

For production of the viruses an established protocol based on the manufacturer's recommendations were used. Briefly,  $1.2 \times 10^6$  cells 293FT cells were resuspend in Opti-MEM media (Invitrogen). Three plasmids pLP1, pLP2 and pLP/VSVG from ViraPower lentiviral packaging mix (Cat.Nr.: K4975-00, Invitrogen) responsible for amplification and packaging of the

viral particles were mixed in ratio 3:1 with pLenti4-U6-shRNA plasmids in Opti-MEM media and Lipofectamine 2000 (Cat.Nr.: 11668-027, Invitrogen). The cells were resuspended in the media containing plasmids–Lipofectamine 2000 complex and incubated in cell culture incubator at 37°C/5%CO<sub>2</sub> for 6 hours. After that media was replaced with complete culture media and the cells were further cultured for 48 hours. Virus-containing media were harvested, filtered through 0.22µm filter and stored, in aliquots, at -80°C. All viral material was produced in S2-laboratory and stored at Max-von-Pettenkofer-Institute, Virology Section, University of Munich led by Prof. Dr.med. Ulrich Koszinowski.

### **Viral infection of HMSC**

HMSC media was half replaced with medium supplemented with 16 µg/ml polybrene (Sigma) and the cells were incubated in cell culture incubator for minimum of 30 min. Then, a virus-containing media was added to the flask in ratio 1:1 and the cells were incubated for another 24 hours at 37°C/5%CO<sub>2</sub>. After this period the media was exchanged with a fresh complete growth media. Cell selection started 48 hours after the infection as the media was replaced with selective medium containing 50 µg/ml zeocin (Cat.Nr.: R250-01, Invitrogen) for a period of 8 days [212]. Finally, the cells were tested for a presence of viral particles by using HIV-1 p24 ELISA kit (Cat.Nr.: NEK050, PerkinElmer, USA). Only virus-free cells were transferred to S1-laboratory and used in the following experiments.

## **3.6. RNA and copy DNA (cDNA) preparation**

### **Total RNA isolation**

Isolation of total RNA was performed with Qiagen RNeasy Mini kit (Cat.Nr.: 74106, Qiagen, USA). Briefly, the cells were washed with PBS and scraped in presence of RLT buffer and 1% β-mercaptoethanol. The cell lysates were then filtrated through QIAshredder spin column mixed with 70% ethanol in ratio 1:1 and loaded on RNeasy spin columns. Contaminants from genomic DNA were digested with 10U of DNase for 15 min at RT. Columns were washed twice with washing solution and dried by centrifugation at maximum speed. High-quality total RNA was eluted in RNase-free water and measured spectrophotometrically for evaluation of the RNA concentration and purity.

### **cDNA synthesis**

The cDNA synthesis was performed with Cloned AMV First-Strand cDNA Synthesis Kit (Cat.Nr.: 12328-040, Invitrogen) following the manufacturer's protocol. Briefly, 1 µg of total RNA, random primers and 10mM dNTPs were heated for 5 min at 65°C. The denatured RNA was added to a mixture of PCR buffer, 15U of reverse transcriptase, 40U of RNase inhibitor and 0.1M DTT and the mixture was incubated for 1 hour at 50°C. The newly synthesized cDNA was tested for expression of a "housekeeping" gene – GAPDH in order to evaluate the synthesis quality and to normalize different probes.

### **3.7. RT-PCR**

A specific amount of cDNA (determined by the expression of GAPDH) was added to a master mix containing PCR buffer, 1.5mM MgCl<sub>2</sub>, 0.2mM dNTPs, 0.5µM Primers and 1U Taq DNA polymerase (Cat.Nr.: 10342-020, Invitrogen). The tubes containing the PCR reactions were placed on MG Research PCR machine (BioRad, USA). Normally, the PCR programs had 30 to 37 cycles of amplification as each cycle consisted of denaturation (94°C for 30 sec), annealing (45-65°C for 30 sec) and elongation (72°C for 60 sec) steps. The annealing temperatures of the primers varied according to their GC content (supplementary tab. 1). The amplified products were analyzed on 2% agarose gels and visualized by ethidium bromide. As a reference for the correct size of the amplified product a 100bp molecular weight standard (Invitrogen) was used. Pictures with different exposure times were taken by using a gel imaging system (Vilber Lourmat, Germany).

### **3.8. Light Cycler (LC)-PCR**

The LC-PCR kits for ITGA1, ITGA2, ITGA11 and GAPDH genes were all purchased from Search-LC GmbH (Germany). The LC-PCR procedure was performed as recommended by the manufacturer. For each PCR reaction 10µl of 1:10 diluted cDNA sample was used. The PCR reaction was performed on Light Cycler 1.5 instrument (Roche, Germany). Standard curve build from 3 standard dilutions was used to determine the amount of cDNA copies. The results of the LC-PCR were analyzed by using Light Cycler 1.3 software and the relative gene expression was calculated as a ratio to GAPDH.

### 3.9. Cytochemistry

#### Protein coating

For coating of cell culture dishes and glass slides were used 20µg/ml rat tail collagen type I (Chemicon, USA), 10µg/ml of bovine FN, Cultrex PLL or mouse laminin I (R&D systems, USA). The coated dishes were incubated at 37°C for 40 min, blocked for 30 min with blocking solution consisting of 5% skim milk/PBS (Merck) or 3%BSA/PBS (Sigma) and washed three times with PBS. The coated dishes were used immediately or were stored for 3 to 5 days at 4°C.

#### Cell plating and fixation

HMSC ( $2 \times 10^5$ ) cells were plated on plastic or coated glass slides and were cultured in complete media for minimum of 24 hours. After this the media was removed, cells were rinsed with PBS and fixed with 4% PFA (Merck) for 20 min at RT or with methanol (Merck) for 10 min at -20°C. After the fixation the slides were washed with PBS, air-dried and immediately used or stored at -80°C.

#### Cell staining

The fixed cells were rehydrated in PBS (3x5 min at RT) and permeabilized with 0.2% Triton X-100/PBS for 15 min. Image enhancer solution (Invitrogen), which reduce the unspecific binding of secondary antibodies, was applied for 30 min. Blocking was perform with 3% BSA/PBS for 3 hours and it followed by addition of primary antibodies (supplementary tab. 2) for 12 hours at 4°C. After PBS washing (3x5 min at RT), secondary antibodies were added for 1 hour at 37°C. F-actin staining was usually performed in parallel by using pre-labeled phalloidin in dilution of 1:13 (Invitrogen). Finally, the slides were washed with PBS (3x5 min) and nuclear contrastaining was performed with a 4',6-diamidino-2-phenylindole (DAPI) in dilution of 1:10000 in H<sub>2</sub>O. Following a PBS washing (3x 5 min) the slides were mounted with Mowiol anti-fading media (6g glycerol, 2.4g Mowiol, 12ml 0,2M Tris-HCl pH8.5, 0.024g DABCO and 6ml H<sub>2</sub>O). The stained slides were stored overnight (ON) at 4°C. Pictures with different magnification were taken on Axioskope2 microscope (Carl Zeiss MicroImaging GmbH, Germany).

### **3.10. Western blotting**

#### **Protein extraction**

For protein extraction was used Urea extraction buffer composed of 8M Urea, 50mM Tris-HCl pH8.0, 1mM EDTA and 1mM DTT. Cultured cells were lysed in the above buffer, mixed and centrifuged at 12 000 rpm and 4°C for 15 min in order to separate the cellular debris. The supernatant was aliquoted and stored at -80°C. The protein concentration was measured by using the bicinchoninic acid (BCA) protein assay kit (Cat.Nr.: 23225; Thermo scientific, USA) by ELISA. Briefly, the protein concentration was determined based on a chemical reaction where BCA interact with the cuprous cations that are reduced by the proteins in an alkaline media. As a result an intense purple-coloured reaction is observed and the intensity of the color correlates with the protein concentration. The protein amount was calculated using a standard curve that is build by serial BSA dilutions. The measurements are performed at 450nm on microtitre-plate reader (Microtek Laborsysteme GmbH, Germany).

An other method used for protein extraction was direct lysis in which the cells were lysed in 1x Laemmli buffer consisting of 200mM Tris-HCl pH6.8, 40% glycerol, 10% SDS, 30% 2-mercaptoethanol, 0.02% bromphenolblue and 0.2M DTT. After washing with cold PBS, cells were lysed in the above lysis buffer for 2 min at RT. Next, the cell lysis were homogenized by sonication, denatured by heating to 99°C for 5 min and centrifuge at 4°C/10000rpm for 10 min. The protein lysates were aliquoted and immediately used or stored at -80°C.

#### **SDS-PAGE**

Urea isolated Protein extracts were mixed with 4x Laemmli buffer described above and were boiled for 5 min at 98°C. The protein mixtures were spined down and loaded on 8 or 15% acrylamide gels (Bio-rad, USA). The electrophoresis was performed in 1x running buffer formulated from 0.25M Tris-base pH8.3, 1% SDS and 1.92M glycine. For a molecular weight standard, Seebule plus 2 protein marker (Invitrogen) was used. The electrophoresis was run at 60mA for 1 gel or 100mA for 2 gels. The procedure continued with Coomassie staining or protein transfer to PVDF membrane.

#### **Coomassie staining**

The gel was first incubated in isopropanol fixation solution consisting of 10% acetic acid and 25% isopropanol (Merck), and then in coomassie staining solution formulated from 7% acetic acid, 40% methanol (Merck) and 0.025% coomassie brilliant blue (Roth, Germany). In this solution the gel was boiled for 30 sec and gently shaken for 20 min at RT. Destaining solution

consisting of 7% acetic acid and 40% methanol was applied to the gel for 12 hours with gentle shaking. This staining was used to analyze the quality loading and transfer of the protein extracts.

### **Protein transfer**

The protein transfer was performed by using the vertical “wet” transfer method. In brief, gel loaded with proteins was equilibrated for 15 to 20 min in 1x blotting solution containing 250mM Tris-base and 1.92mM glycine. During this equilibration time, the PVDF membrane was incubated in methanol, rinsed in water and soaked in 1x blotting buffer. The Western blot setup was then assembled. The protein transfer was performed ON with 30V at 4°C. After the transfer, the membrane was stored ON at 4°C in a TBS-T20 washing solution consisting of 1mM Tris-base pH7.4, 150mM NaCl and 0.05% Tween20 or was blocked with blocking solution.

### **Protein immunodetection**

The membrane was incubated with blocking solution consisting of 5% skim milk/TBS-T20 for 1 hour with gentle shaking. Then, primary antibody (supplementary tab. 2), diluted in the blocking solution, was applied with vigorous shaking ON at 4°C. The membrane was washed 4x7 min with TBS-T20 washing solution and secondary antibody, diluted in the blocking solution, was applied to the membrane for 1 hour with vigorous shaking. Finally, the membrane was again washed 4x7 min with TBS-T20 and proteins were visualized by using chemiluminescent ECL solution (GE Healthcare, USA) and detection film (Lumi-film chemiluminescent, Roche) in developing instrument Scopix LR5200 (Agfa, Belgium).

### **3.11. Cell adhesion assay**

Cell adhesion assays were performed on protein pre-coated 96-well plates (Nunc), procedure described in 3.9. HMSC cells were plated in triplicates ( $3 \times 10^3$  cells/well) and incubated for various time periods (from 30 to 180 min) in cell culture incubator. Non-adherent cells were removed by washing with PBS. Cell adhesion was estimated by NPAG (4-Nitrophenyl 2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside, Sigma)-protein staining which can be calorimetrically measured. The adherent cells were incubating ON at 37°C with a substrate buffer consisting of 7.5mM NPAG, 0.1M sodium citrate, pH5.0 and 0.5% Triton X-100. Prior measurement, a stopping buffer (50mM glycine, pH10.4 and 5mM EDTA) was added to the wells

and then the optical density was measured at 405 nm on a microtitre-plate reader. The amount of adherent cells was finally calculated as a percentage of the maximum value cell reference ( $3 \times 10^3$  cells, directly lysed with the substrate buffer).

### 3.12. Proliferation assays

#### Population doublings

In order to calculate the population doubling (PD) and population doubling time (PDT) the following formulas were used respectively:

$$PD = 3.33 \times \log_{10} (N/N_0);$$

$$PDT = D/PD,$$

where N is the cell number at the end of the experiment,  $N_0$  is the cell number at the beginning and D is the number of days (or hours) of the experimental period (from  $N_0$  to N). Growth curves were built by using cumulative population doublings which is a sum of individual population doublings.

#### Bromodeoxyuridine (BrdU) assay

The Cell Proliferation ELISA, BrdU (colorimetric) (Roche) assay was performed according to the manufacturer's description. Cells triplicates of  $3 \times 10^3$  cells/well were plated and grown for 12 hours on protein pre-coated 96-well dishes in cell culture incubator. Next, the culture media was replaced with 10  $\mu$ M BrdU-containing media and incubated for another 24 hours. The cells were then fixed, stained with anti-BrdU-POD antibody for 30 min, washed and incubated in substrate solution for 30 min. Then, stopping solution was added and measurements were performed using 450 nm wavelength and reference filter of 620 nm on a microtitre-plate reader.

### 3.13. Time lapse experiments

The time lapse system consist of automated inverted microscope Axiovert100, additionally modified with a isolation chamber, which allows maintaining of constant 37°C/5%CO<sub>2</sub> with proper humidity conditions for a long period of time. Cells ( $2 \times 10^4$  cells/well) were plated on protein pre-coated 6-well dishes (Nunc). Cells spreading and migration were analyzed by series of consecutive pictures from initially defined cells areas. For spreading analysis the cells were imaged immediately after plating. The series of pictures were produced with speed of 20

frames per hour as for the analyses were used minimum three different cell areas containing 20-30 cells.

For migration analysis, the cells were incubated for 2 hours prior picturing. The series of pictures were produced with speed of 4 frames per hour as for the analyses were used minimum three different cell areas with 20-30 cells.

The data from the movies was extracted by using AxioVision LE software (Carl Zeiss Micro-Imaging GmbH) and analyzed by using ImageJ program (<http://rsb.info.nih.gov/ij/>).

### **3.14. Osteogenic differentiation**

Osteogenic differentiation was performed as described in [213]. Briefly, cells were plated in 6-well dishes with concentration of  $3.5 \times 10^3$  cells/cm<sup>2</sup>. When the cell density reached approximately 80-90% confluence the media was changed to osteogenic media consisting of DMEM-high glucose media supplemented with 10%FBS, 1%Pen/Strep, 100nM Dexamethasone, 10mM Glycerol 2-phosphate and 50 $\mu$ M L-Ascorbic acid 2-phosphate (all Sigma) for 21 days. In the control wells, the cells were incubated in the standard culture media. The medium for all wells was exchanged twice per week. Phase-contrast pictures of stimulated and unstimulated cells were taken with different magnifications on Axiovert100 microscope using AxioCam ICc3 colour camera (Carl Zeiss MicroImaging GmbH).

The osteogenic differentiation was evaluated by Alizarin red (AR) staining. AR red is an anthraquinone derivative which binds to calcium deposits and therefore is used to quantify the extent of matrix mineralization. After 21 days of osteogenic stimulation, stimulated and unstimulated cells were washed PBS, fixed with 4% formaldehyde and washed again with excessive amount of water. Then AR solution was added to the wells and it was gently rocked for 20 min. Dishes were washed with water (4x 5 min) and pictures with few different magnifications were taken on Axiovert100 microscope using AxioCam ICc3 colour camera.

For quantification of the extent of osteogenic differentiation, AR staining was measured with Osteogenic Quantification kit (Cat.Nr.: ECM815; Chemicon, USA). AR was extracted from the ECM by scrapping with 10% acetic acid, followed by heating to 85°C for 10 min and centrifugation for 15 min. Then, the supernatant was neutralized with 10% ammonium hydroxide and the absorption was measured at 405 nm on microtitre-plate reader. The amount of AR ( $\mu$ M) was calculated against a standard curve prepared from AR serial dilutions.

### 3.15. Apoptosis analysis

For apoptosis analysis, first, was evaluated the percentage of lost cells during a prolong cultivation period after the viral infection (death curve analysis). Cells ( $2 \times 10^4$ ) from each shRNA type were plated in 12-well dishes (Nunc). Pictures were taken daily for a period of 14 days on Axiovert100 microscope using the mosaic option provided by Axiovision 1.5 software (Carl Zeiss MicroImaging GmbH). Each of the mosaic pictures consisted of 4x4 picture frames which covered approximately 60% of each well. Data were extracted and analyzed by using ImageJ and Microsoft Excel (Microsoft, USA) programs.

For determination of mitochondrial damage, a hallmark of apoptosis, was used JC-1 staining (Cat.Nr.: T3168, Invitrogen). JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl-carbocyanine iodide) is a fluorescent cationic dye which exists as a monomer in the cytosol (green) and also accumulates as aggregates in the mitochondria which stains them red. Therefore, non-apoptotic cells in which the mitochondria is intact have green stained cytoplasm and red stained mitochondria whereas the apoptotic cells which mitochondria are depolymerized and JC-1 leaks entirely in the cytoplasm have stained only green. Cells ( $2 \times 10^3$  cells/cm<sup>2</sup>), 10 days after viral infection, were plated on pre-coated with Coll glass slides and cultured for 24 hours in cell culture incubator. In parallel, a portion of hMSC ( $3 \times 10^3$  cells) were used as a negative and positive control as apoptosis was induced with 1 µg/ml FAS antibody in serum free culture media. Afterwards, the cells were incubated with 3 µg/ml JC-1 and 1 µg/ml Hoechst 33342 (Invitrogen) diluted in complete cell culture media for 30 min at 37°C/5%CO<sub>2</sub>, washed with PBS (3x 5 min) and pictured on Axiovert100 microscope using AxioCam MRm camera (Carl Zeiss MicroImaging GmbH).

### 3.16. Interferon stimulation

For analysis of the interferon (IFN) pathway, was used RT-PCR for a number of genes known to be upregulated by the presence of dsRNA or virus infection (supplementary tab. 1). For positive control, we used hMSC cells stimulated with 2000U/ml IFN-beta (Cat.N.: CYT-26766; Dainova, Germany) for 72 hours at 37°C/5%CO<sub>2</sub> [214].

### 3.17. Microscopy

The microscopes and cameras used in this thesis are purchased from Carl Zeiss MicroImaging GmbH, Germany. Axioskope2 microscope was used for taking immunofluorescent micropho-

tographs. Pictures were taken with 40x, 63x and 100x objectives and using AxioCam MRm black-white camera (Ser. Nr.: 118051871).

Axiovert100 was used for imaging of live and osteogenic differentiated cells, and for performing time lapse experiments. We used objectives having 5x, 10x and 20x magnifications. For taking pictures was used AxioCam ICc3 colour camera (Ser. Nr.: 13-218537987).

### **3.18. Computer programs and web links**

In this doctoral thesis was used a number of specialized programs for processing and analyzing the obtained data. The charts were created by using Microsoft office 2003 (Microsoft, USA) and SigmaPlot 8.0 (Systat Software, USA). Photomicrographs were processed with using AxioVision LE software (Carl Zeiss MicroImaging GmbH) and Adobe Photoshop CS2 program (Adobe Systems Incorporated, USA). The time lapse evaluations and cell counting for the death curves analysis were performed by using ImageJ software. The figures were created using Adobe Photoshop CS2 program (Adobe Systems Incorporated).

We used the followed links:

<http://www.ncbi.nlm.nih.gov/sites/entrez/> - major source of publications.

<http://rnaidesigner.invitrogen.com/rnaiexpress/> - for design of the shRNA sequences.

<http://rsb.info.nih.gov/ij/> - ImageJ program

### **3.19. Statistics**

The statistical relevance was measured by using the Student t-test on SigmaPlot 8.0 (Systat Software). The ELISA enzymatic and colorimetric experiments, such as cell adhesion assay, BrdU cell proliferation and osteogenic quantification consisted of minimum 3 independent repeats, each performed in triplicates. The quantification of the ColI-binding integrin basal expression, integrin knockdown efficiency and integrin compensation by LC-PCR consisted of three hMSC donors as the runs were repeated at least three independent times. Spreading and migration experiment were repeated three times as in each experiments the samples were in triplicates. The figure bar charts represent mean and standard deviation of minimum three independent experiments.

## 4. Results

### 4.1. Characterization of hMSC

#### 4.1.1. Morphological appearance and growth capacity of hMSC

In this doctoral thesis we used three different hMSC donors – hMSC IX, XIII and XV. All experiments were initially performed with hMSC XI (our “master” donor) as the most important founding we reproduced with the other two hMSC XIII and XV donors. In order to be able to compare the obtained data from the all three hMSC donors, we briefly characterized them based on their morphological appearance and growth capacity. The hMSC donors demonstrated a similar cell phenotypic appearance as all of them had the characteristic spindle-shape cell morphology [215] (fig. 7A). Moreover, we also observed no difference in their growth capability and PDT (fig. 7B) as all of the hMSC donors had a PDT of approx. 55 hours.

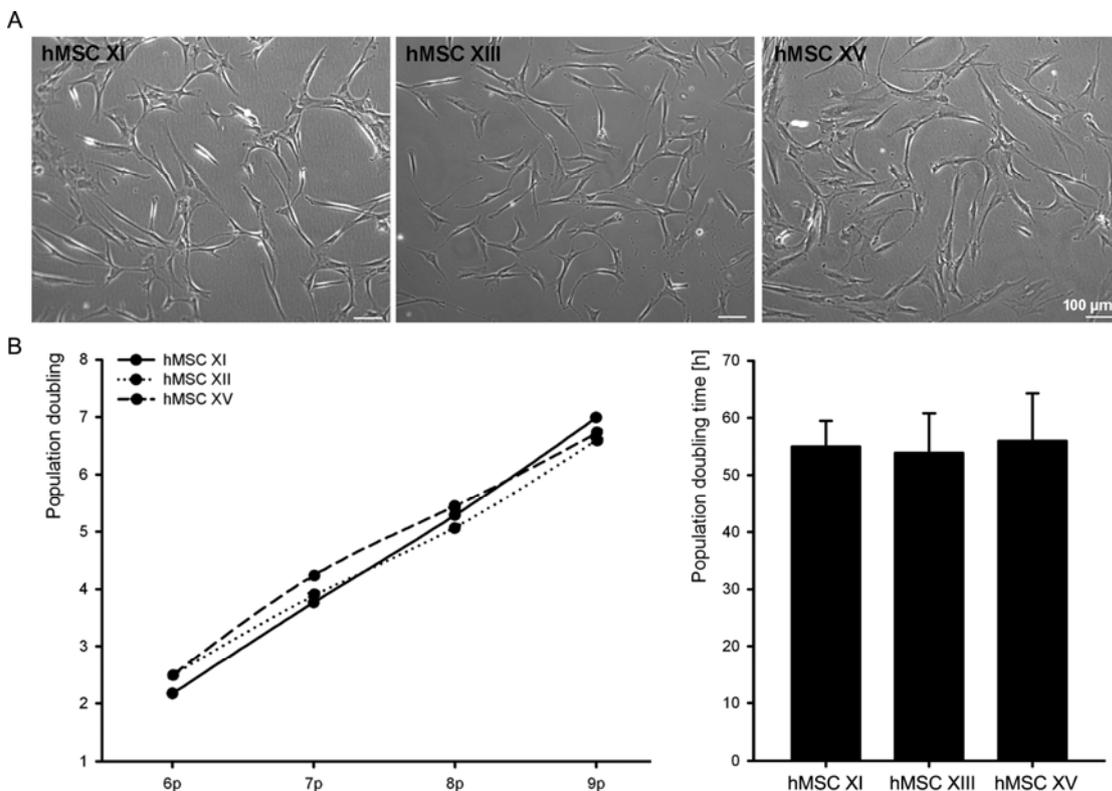


Fig. 7. Morphological appearances and growth characteristic of hMSC donors. A) Phase contrast pictures of hMSC XI, XIII and XV when cultured on polystyrene; bar 100 μm. B) Cumulative PD and PDT.

#### 4.1.2. HMSC affinity towards different ECM proteins

In order to characterize the bone marrow derived hMSC, we first analyzed their ECM preferences by cell adhesion assay (fig. 8). For this purposes, we compared their adhesion capability to four different ECM proteins – CollI, FN, laminin I, PLL and plastic at four different time points. The initial hMSC adhesion to CollI, FN and laminin I was greater than on plastic. In comparison, hMSC exhibit very low adhesion affinity towards PLL as this fact was true throughout the all experiment. After 45 minutes, the cell adhesion to CollI and FN reached a plateau as  $95\pm 5\%$  of the cells were attached. In comparison to them, hMSC attachment to laminin I, plastic and PLL continue to increase with time. At 60 min, the cells attached to laminin I and plastic reached approx.  $90\pm 5\%$  whereas on PLL hMSC were able to adhere only with  $77\pm 2\%$  from the initial cells.

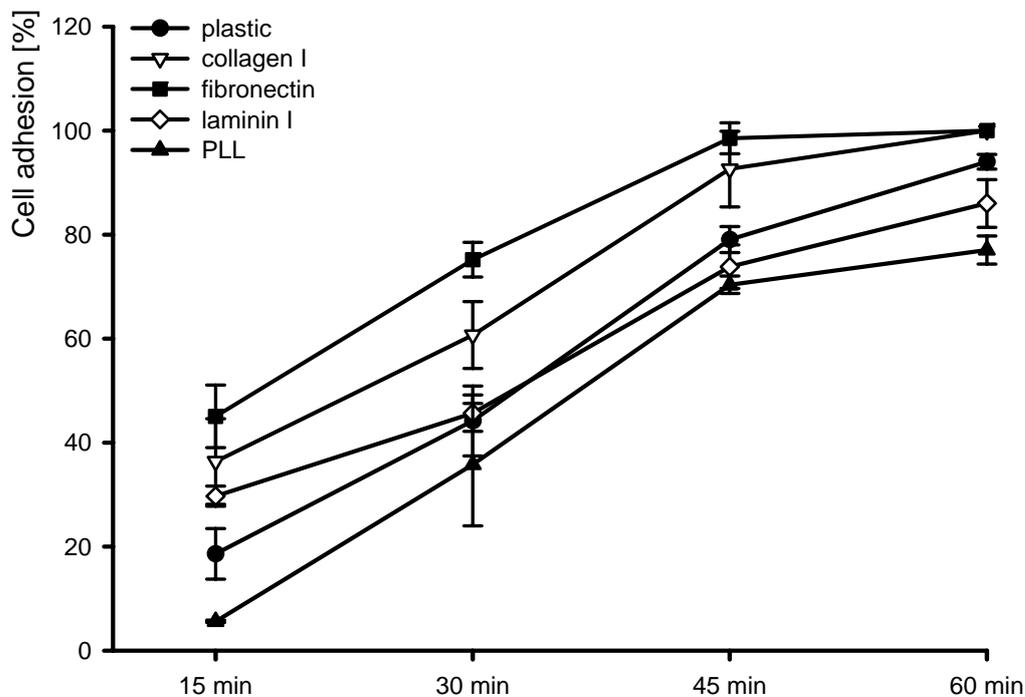


Fig. 8. HMSC adhesions to different ECM proteins. A representative experiment consisting of triplicates.

#### 4.1.3. Influence of different ECM proteins of hMSC proliferation capability

Next, we analyzed the influence of the different ECM proteins on hMSC proliferation capability by BrdU assay (fig. 9). The highest cell proliferation rate was observed when hMSC were propagated on CollI protein, followed then by FN and laminin I while the lowest cell proliferation was detected on PLL surface. Importantly, here we found that CollI protein pro-

vides the best proliferation stimuli for hMSC, even though the cells propagation was limited only to 24 hour because of the BrdU toxicity effect.

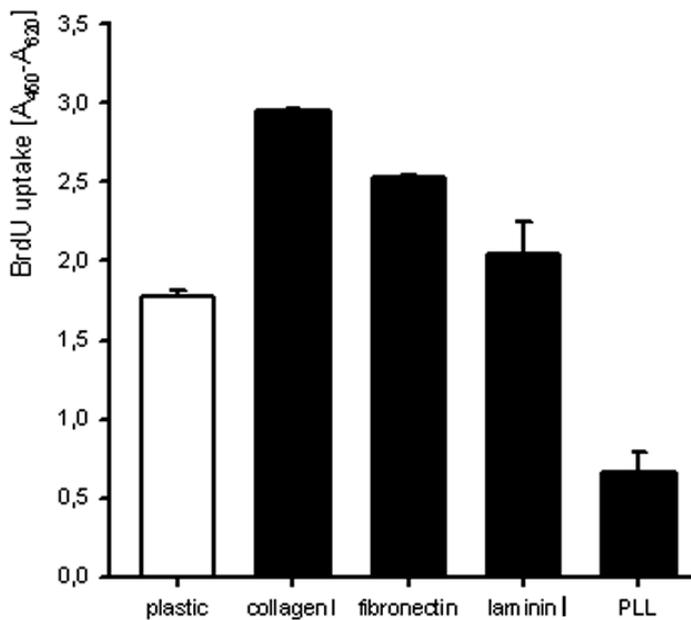


Fig. 9. hMSC proliferation on different ECM proteins measured by BrdU uptake for 24 hours. The chart consist of 3 independent experiments.

#### 4.1.4. Integrin expression in hMSC

Considering the ability of hMSC to attach to various ECM proteins, we next investigated the corresponding to this protein integrins expression. hMSC were screened for the expression of eight alpha (α1-α6, α11 and αV) and three beta (β1, β3 and β5) integrin subunits by RT-PCR (fig. 10). In addition, we compared the integrin expression of hMSC to human osteoblasts (hOB). All tested integrins subunits were expressed by hMSC; however their expression levels were different. The only exception was the integrin α4 subunit which was found expressed only by hOB. This was not surprising taking in account the importance of this integrin receptor for the OB and HSC interactions. Interestingly, among the all tested integrin subunits, α1 and α11 had the highest expression levels in hMSC. Moreover, we found also an increased expression of integrin α2, α4, β3 and β5 in hOB in comparison to hMSC.

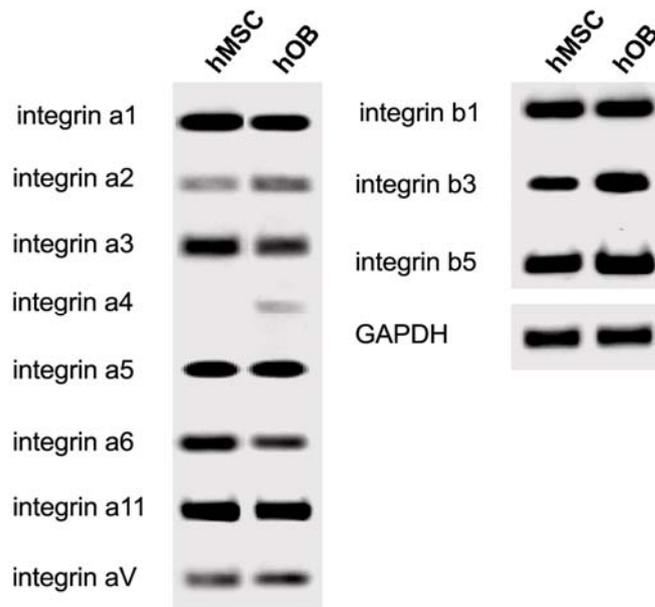


Fig. 10. Integrin expression by hMSC and hOB. A representative experiment shows the mRNA expression level of 8 a- and 3 b-subunits.

#### 4.1.4.1. Expression of ColI-binding integrin in hMSC

Taken together, our analysis of ECM affinity and integrin expression profile suggested the ColI importance for hMSC behavior. Therefore, we performed a more detailed investigation of ColI-binding integrin expression (integrin a1, a2 and a11) in hMSC. The initial RT-PCR screening analysis showed that these integrins were differently expressed (fig. 11A). The following quantitative analysis performed by LC-PCR in the three different hMSC donors (fig. 11B) confirmed the data observed by RT-PCR. This allowed us to grade the expression levels of ColI-binding integrin in hMSC as integrin a11 had the most abundantly expression, then followed by integrin a1 and the weakest was integrin a2.

Next, we investigated the protein expression levels of integrin a2 and a11 in hMSC XI by WB analysis. The total protein input was normalized according to the b-actin WB. As expected, we detected a strong a11 integrin expression which was approx. 1.8 – fold higher than the expression of integrin a2 in hMSC (fig. 11C).

To investigate the formation and localization of integrin a2 and a11 in the cells, we performed an immunofluorescence staining (fig. 11D). In hMSC stained for integrin a11, we detected strong focal adhesion formation, localized within the all cell body. However, when hMSC were stained for integrin a2, despite of the positive cell staining no visible clustering was detected.

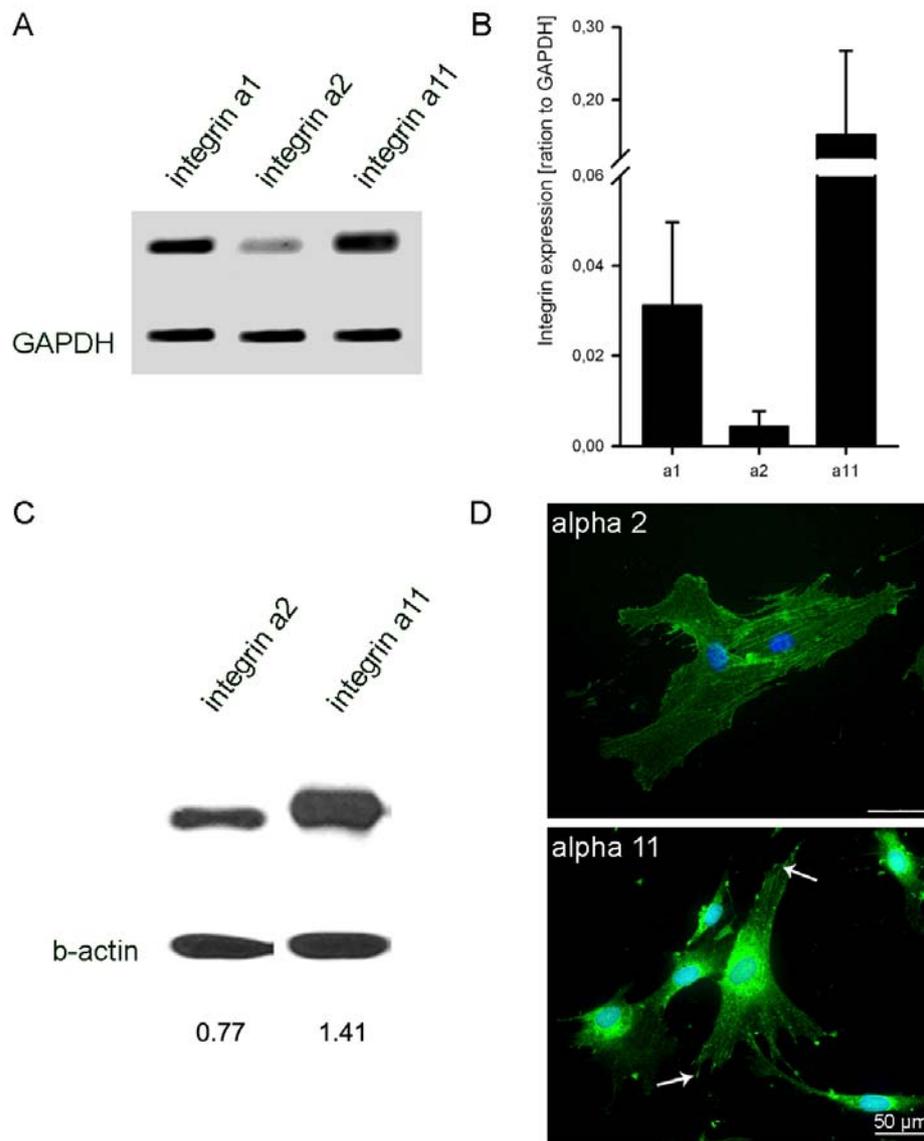


Fig. 11. ColI-binding integrin expressions in hMSC. A) RT-PCR, a representative experiment with hMSC XI; B) LC-PCR with three different donors; C) a representative WB experiment and D) immunofluorescence staining of a2 and a11 integrins (green) and nuclear counter-staining (blue) with hMSC XI. Bar 50 $\mu$ m.

#### 4.1.5. Integrin expression changes upon osteogenic stimulation

To investigate whether upon osteogenic differentiation the expression of ColI-binding integrins changes, the three different hMSC donors were stimulated for 21 days with osteoinductive media according to Böcker *et al.*, 2007 [213]. Then, the RT-PCR analysis and Alizarin Red (AR) staining were performed. The AR staining of two independent stimulations of hMSC XI and single stimulation of hMSC XIII and XV showed a consistently strong matrix calcification in all donors (fig. 12A). In order to compare the differentiation potential of the different hMSC donors we performed a quantification of the AR staining using ELISA colorimetric as-

say (fig. 12B). The results showed that all hMSC donors were able to osteogenically differentiate but the extent of matrix mineralization was donor dependent as donor hMSC XIII differentiated the most.

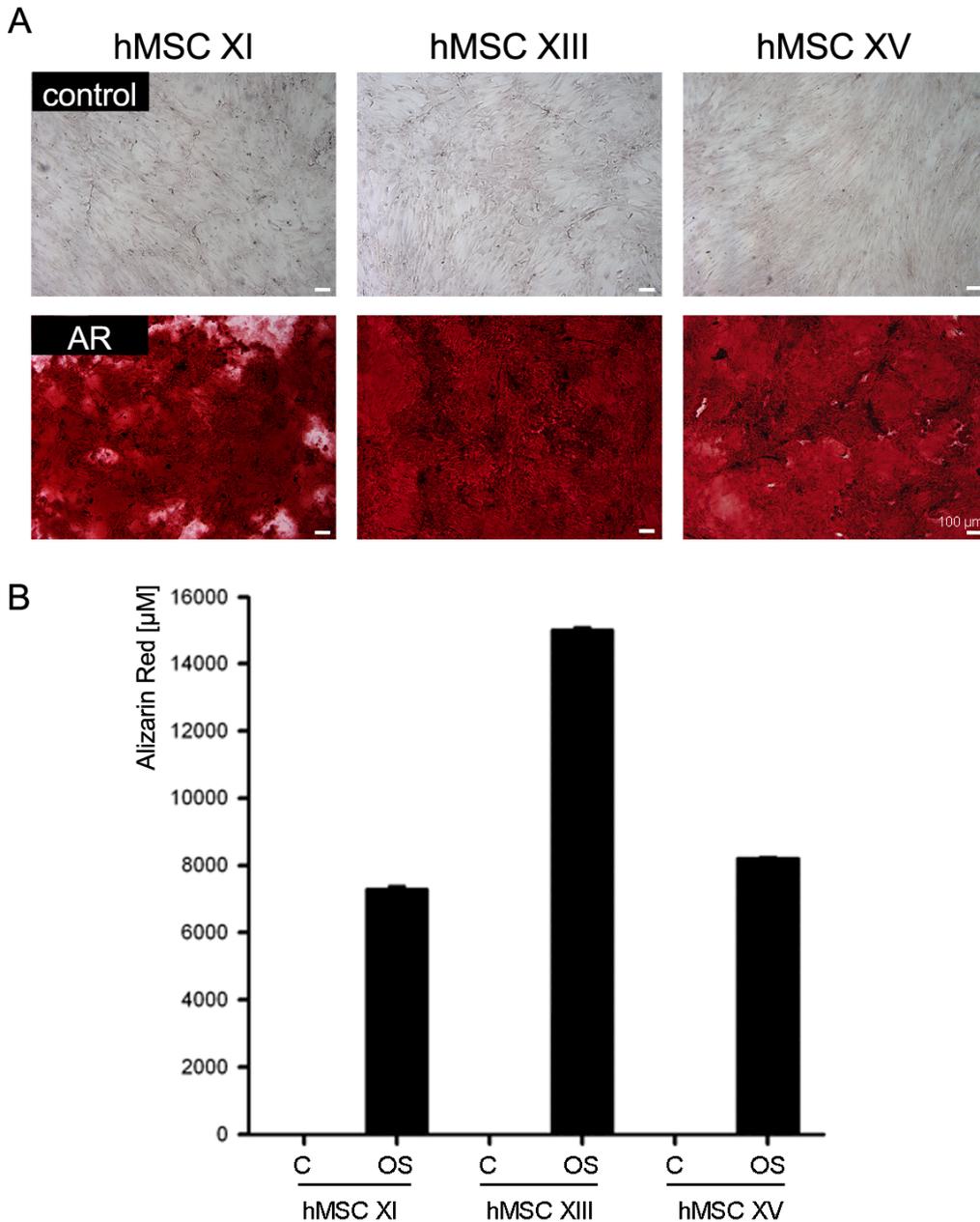


Fig. 12. Osteogenic differentiation of hMSC XI, XIII and XV. A) A representative pictures of AR staining and B) a representative quantification. Bar 100µm.

#### 4.1.6. Changes in integrin a1, a2 and a11 expression upon osteogenic stimulation

The integrin expression of hMSC prior and post osteogenic differentiation was assessed by LC-PCR (fig. 13A-C) and WB (fig. 13D). The LC-PCR analysis showed upregulation of the

mRNA level of integrin  $\alpha 2$  (fig. 13B) and  $\alpha 11$  (fig. 13C) but no changes in integrin  $\alpha 1$  (fig. 13A). This data was confirmed also on protein level by WB analysis (fig. 13D). Both, integrin  $\alpha 2$  and  $\alpha 11$  were clearly upregulated upon OS stimulation with 2-fold for integrin  $\alpha 2$  and 5-folds for integrin  $\alpha 11$  in the three hMSC donors in comparison to non-stimulated sample.

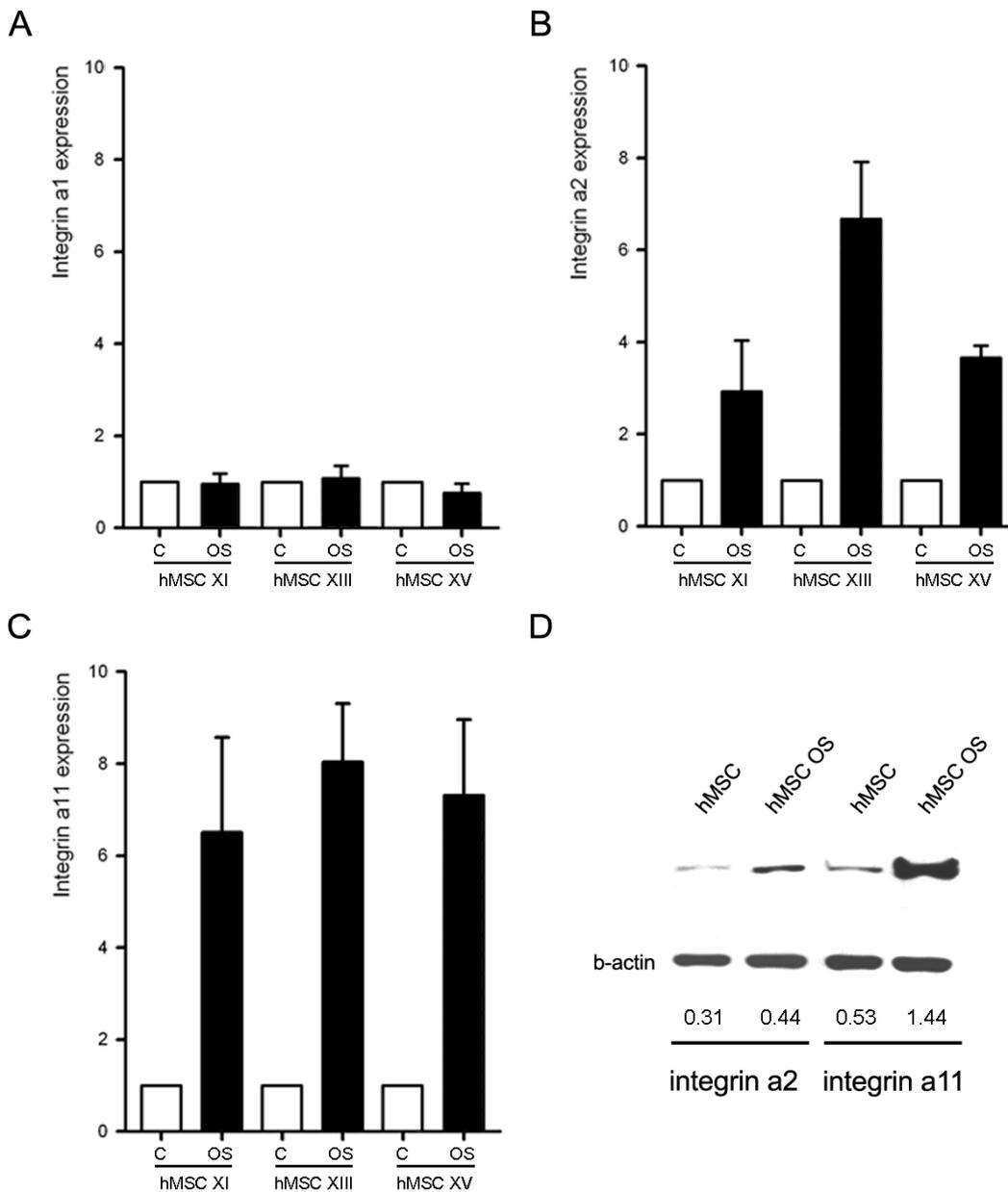


Fig. 13. Colli-binding integrin expression change upon OS stimulation. Integrin  $\alpha 1$  (A),  $\alpha 2$  (B) and  $\alpha 11$  (C) mRNA expression levels were analysed by LC-PCR in two independent differentiations of hMSC. (D) WB was reproduced twice with hMSC XI.

In addition, we estimated the effect of integrin expression on the OS capacity. We found an interesting correlation between the matrix mineralization and the upregulation of integrin  $\alpha 2$

(fig. 14) as more matrix mineralization was detected in the donor expressing higher levels of integrin a2.

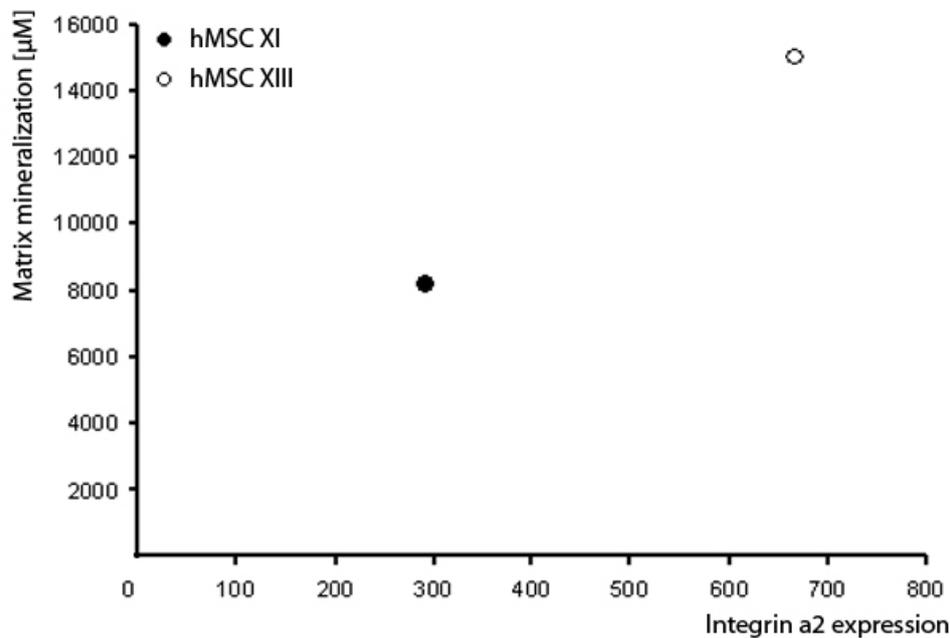


Fig. 14. Integrin a2 dependent matrix mineralization upon OS stimulation in two different hMSC donors.

## 4.2. Establishment of a stable knockdown system for Coll-binding integrins

### 4.2.1. Cloning of lentiviral constructs for expression of a1, a2 and a11 shRNA

For the establishment of the stable integrin knockdown we used a lentiviral delivery system based on the shRNA technology. Target-specific shRNA pre-design sequences were cloned in pENTR/U6 plasmid by ligation reaction (fig. 15A). Next we verify the cloned shRNA by a digestion analysis as we cut out the inserted shRNA sequence from the plasmid backbone (fig. 15B). The clones with correct digestion profile were then sequenced for verification of the shRNA quality (fig. 15C). Finally, the plasmids which had the proper digestion pattern and correct DNA sequence were used for the next step of cloning – the transfer of the shRNA sequence into pLenti4-BlockIT plasmid.

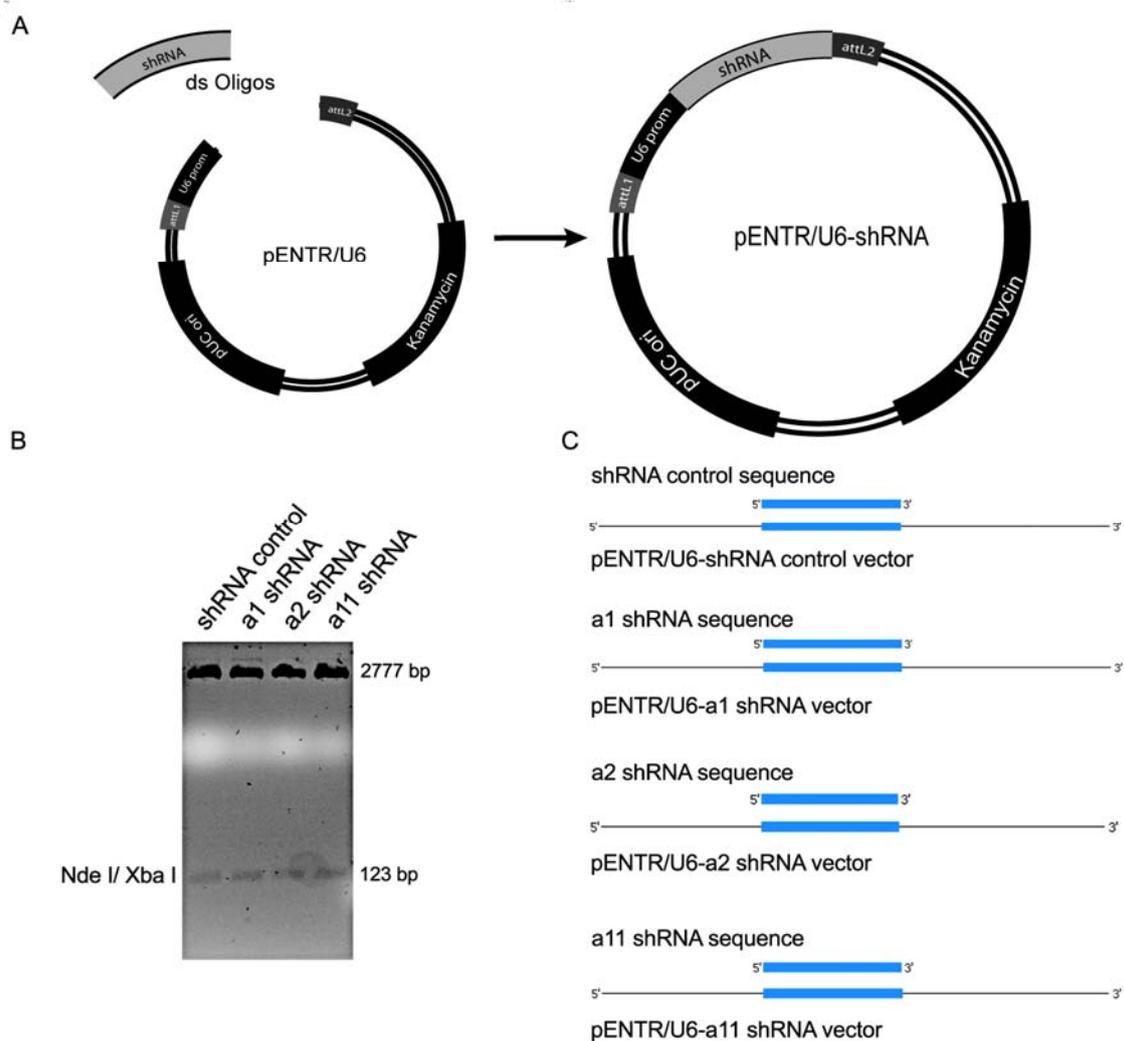


Fig. 15. Cloning of shRNA in pENTR/U6 and its verification. A) Schematic presentation of shRNA and pENTR/U6 ligation procedure. B) Digestion analysis of clone plasmids by cutting out of shRNA inserts (123bp) with NdeI/XbaI restriction enzymes. C) Comparison of the reference sequence and the sequencing results of the obtained pENTR/U6-shRNA plasmids.

For the transfer of the shRNA sequence into pLenti4-BlockIT plasmid we performed a cloning reaction catalyzed by the LR Clonase enzyme. The reaction is based on the recombination of attL1 and 2 sites from pENTR/U6 plasmid with attR1 and 2 from pLenti4-BlockIT final destination vector. This results in the formation of pLenti4/U6-shRNA plasmid (fig. 16A). The desired plasmids were again selected based on their digestion pattern. For this purposes we performed three types of plasmid digestion: 1) we cut the insert with NdeI/XhoI restriction enzymes, 2) we cut part of the insert and the backbone with KpnI restriction enzyme and 3) we performed a plasmid linearization by a single cutting NdeI restriction enzyme (fig. 16B). Again, the properly digested plasmids were sequenced for validation of the not changed shRNA region (fig. 16C) and then used for a lentivirus production.

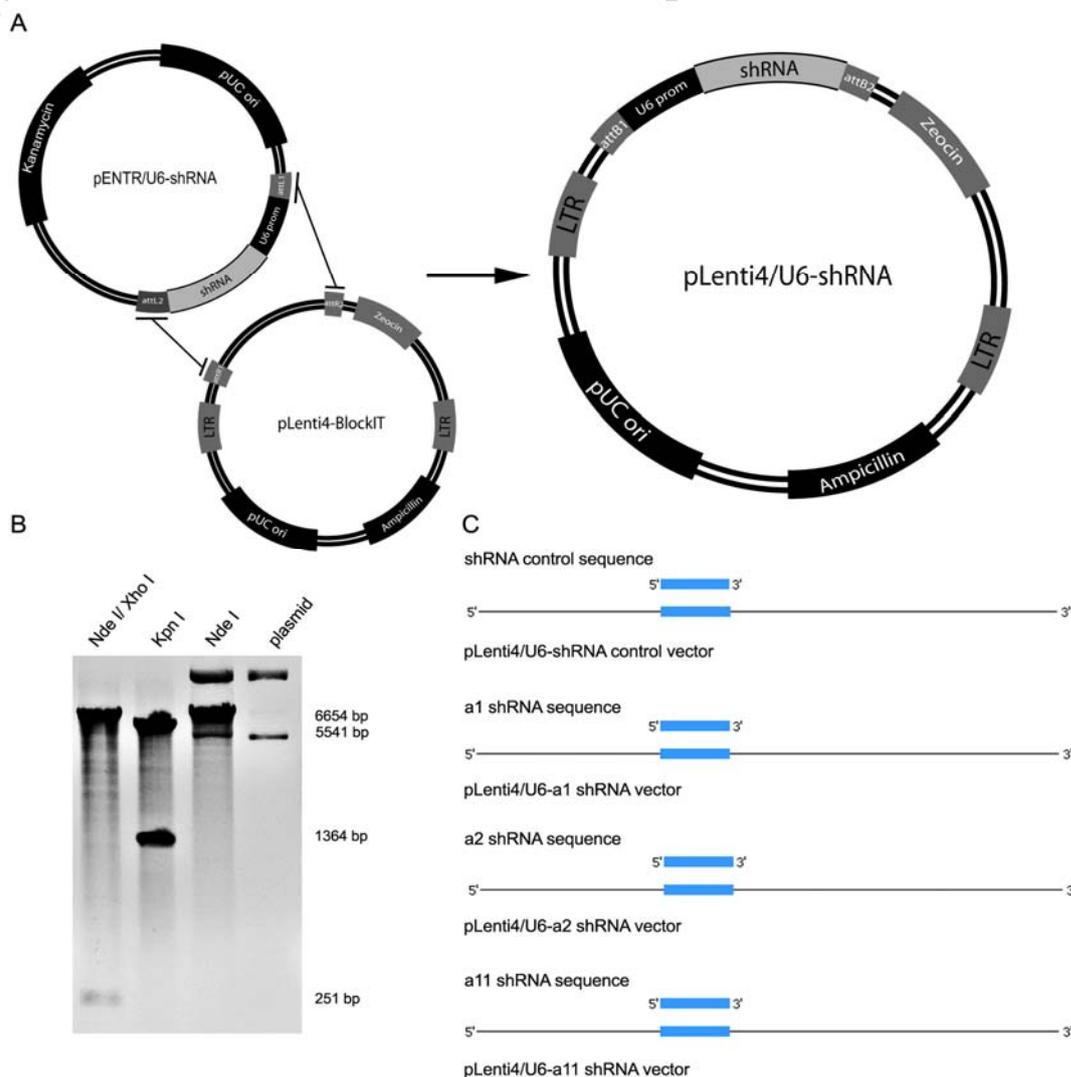


Fig. 16. Cloning of the shRNA in pLenti4-BlockIT final destination vector. A) Schematic presentation of clonase reaction between pENTR/U6-shRNA and pLenti4-BlockIT vector. B) pLenti4/U6-shRNA control digests for insert out (251bp) with NdeI/XhoI, cutting inside the insert and the backbone (1364bp) with KpnI and plasmid linearization (6654bp) with NdeI restriction enzymes and C) Sequencing verification of the shRNA region.

#### 4.2.2. Knockdown of integrins a1, a2 and a11 in hMSC

hMSC were infected with integrin a1, a2 and a11 shRNA containing lentiviruses. Then RT-PCR analysis was performed. We detected a clear downregulation of the targeted integrin mRNA as shown in a representative experiment performed with hMSC XI donor (fig. 17A). Next we assessed the overall knockdown efficiency by LC-PCR (fig. 17B). The observed integrin downregulation in the three hMSC donors was  $96\pm 4\%$  for a1,  $85\pm 14\%$  for a2 and  $95\pm 4\%$  for a11.

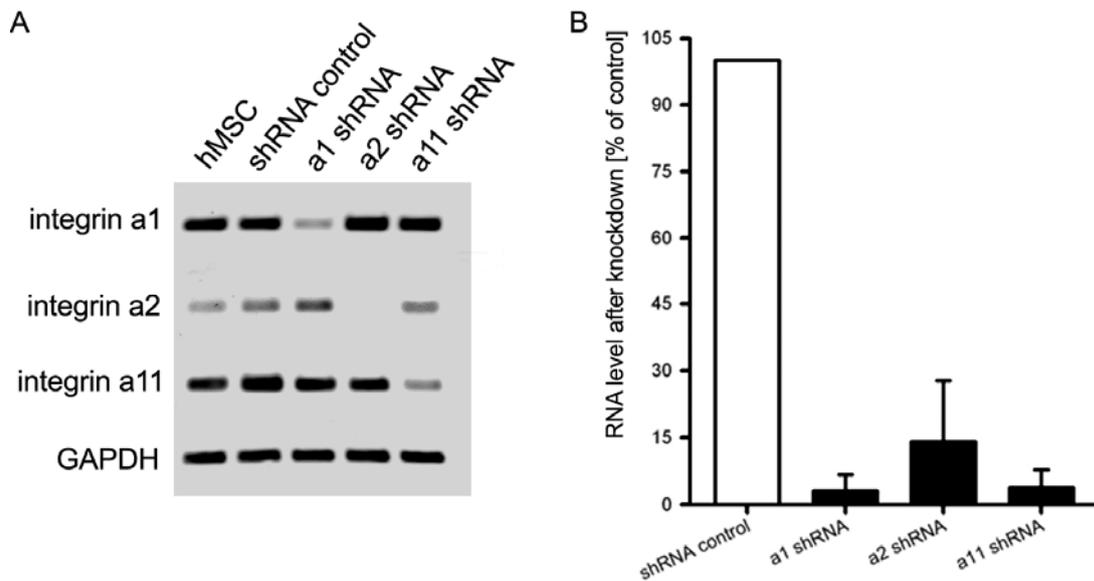


Fig. 17. Knockdown of integrin a1, a2 and a11 in hMSC. A) RT-PCR analysis of hMSC XI donor and B) LC-PCR analysis of the three donors consist of all performed infection as the level of integrin a1, a2 and a11 mRNA left after knockdown was calculated as a percent of the control cells.

Moreover, we confirmed the knockdown of integrin a2 and a11 also on protein level by WB as shown for hMSC XI (fig. 18). In all three hMSC donors transduced with a2 or a11 shRNA we observed a clear downregulation of in the production of corresponding integrins. In particular, integrin a2 protein was downregulation with 75% and for a11 with the amazing 96%. Interestingly, we observed an upregulation of the integrin production of the other two members of the  $\text{Col1}$ -binding integrin family. When integrin a1 was knockdown, the levels of expression in a2 and a11 protein increased with 0.42- and 1.1-folds, respectfully. Additionally, when integrin a2 was knockdown we observed an upregulation of integrin a11 with 1.1-folds. However, no upregulation of integrin a2 was observed in response to a11 knockdown.

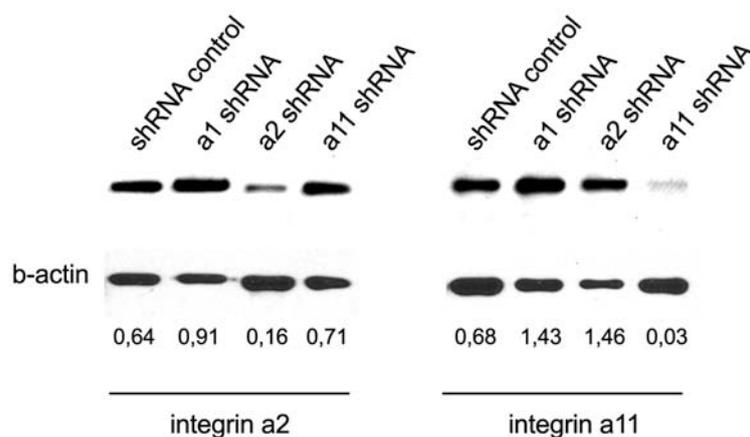


Fig. 18. WB analysis for integrin a2 and a11 after knockdown of integrin a1, a2 or a11 in hMSC XI. A representative experiment from two independent repeats.

#### 4.2.3. Morphological and growth characterization of shRNA-transduced hMSC

The hMSC transduced with four different shRNA were characterized by morphological appearance and growth capacity for a period of 30 days. No obvious differences concerning cell morphology was detected in the shRNA transduced hMSC, however during the cell culture after infection and selection we observed a constant cells lost within the a2 and a11 shRNA-transduced cells populations. In contrast, the cell number of shRNA control and a1 shRNA hMSC continuously increased (fig. 19).

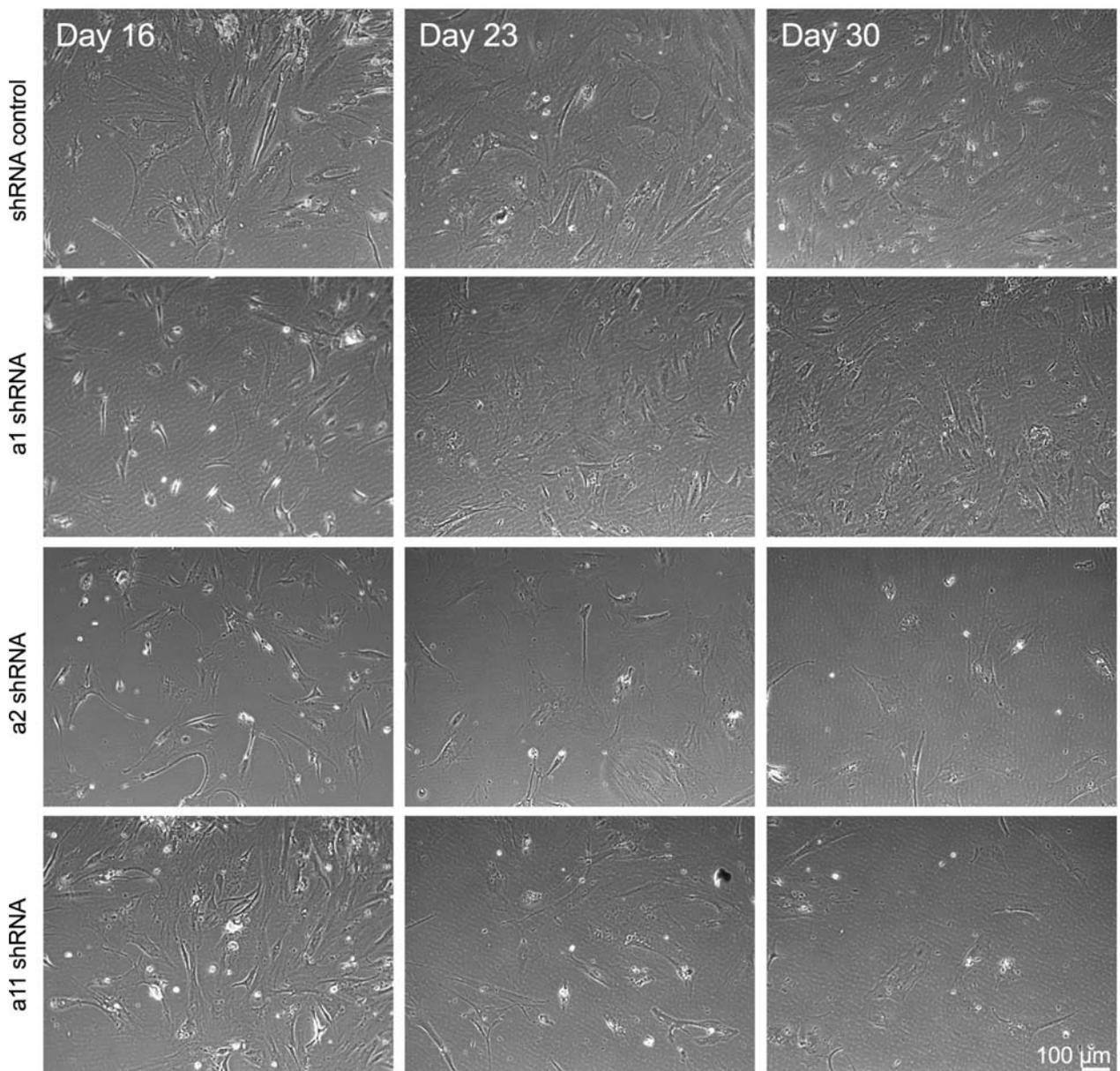


Fig. 19. Morphological appearance and cell density of shRNA-transduced hMSC XI cells. Bar 100 $\mu$ m.

In order to estimate the rate of the observed cell loss we performed a “death curve” analysis of hMSC XI (fig. 20) by counting the number of cells in 12-well plates during 14 days of period (fig. 20A). Two different areas of 1.1cm<sup>2</sup> were used for the analysis of each shRNA-

transduced hMSC. Next, we calculated in percentage the cumulative lost of cell between day 0 and 14 day (fig. 20B). Thus, at day 14 shRNA control and a1 shRNA cell populations increased with  $55.8\pm 16\%$  or  $76.3\pm 42\%$  correspondingly, whereas a2 and a11 shRNA cells reduced with  $83.3\pm 0.5\%$  and  $73.1\pm 12.4\%$ , respectively.

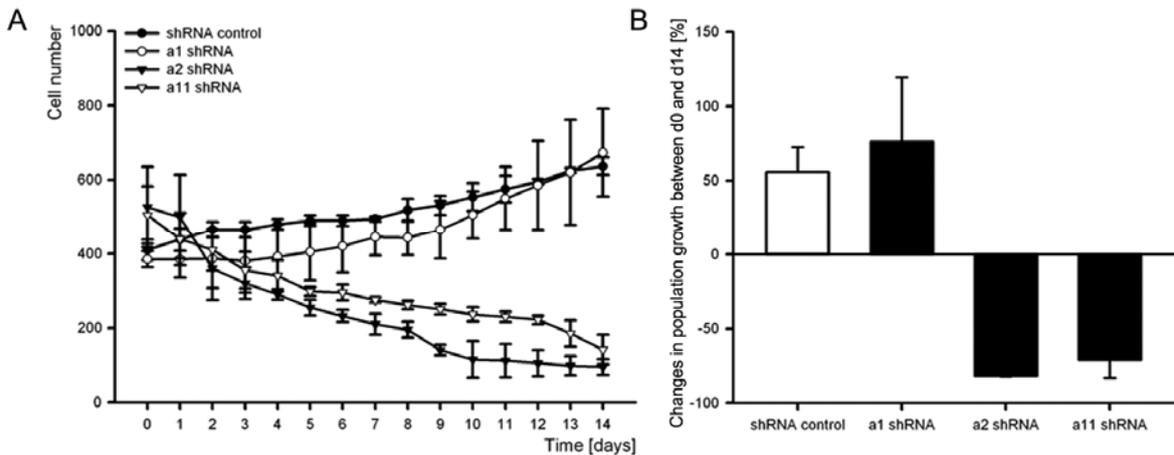


Fig. 20. Growth analysis of shRNA-transduced hMSC XI. A) Cell counting experiment over 14 days culture period. B) Estimation of the total amount of gained and lost cells at the end point of the growth analysis.

#### 4.2.4. RT-PCR analysis of genes associated with IFN pathway

Since two of the integrin knockdown hMSC were dying out over time, we first investigated whether IFN pathway was activated in response to the virus infection or presence of dsRNA. We analyzed by RT-PCR the gene expression of OAS1 and 2, RIG-I and ISG56 in all shRNA-transduced hMSC as for a positive control was used IFN-beta induced hMSC (fig. 21). In all three independent infections we detected a basal expression of the IFN-related genes in hMSC which varied between the genes and the infections. For example, OAS1 had the weakest and OAS2 the highest basal. However, we observed no upregulation of the IFN-related genes in comparison to the positive control.

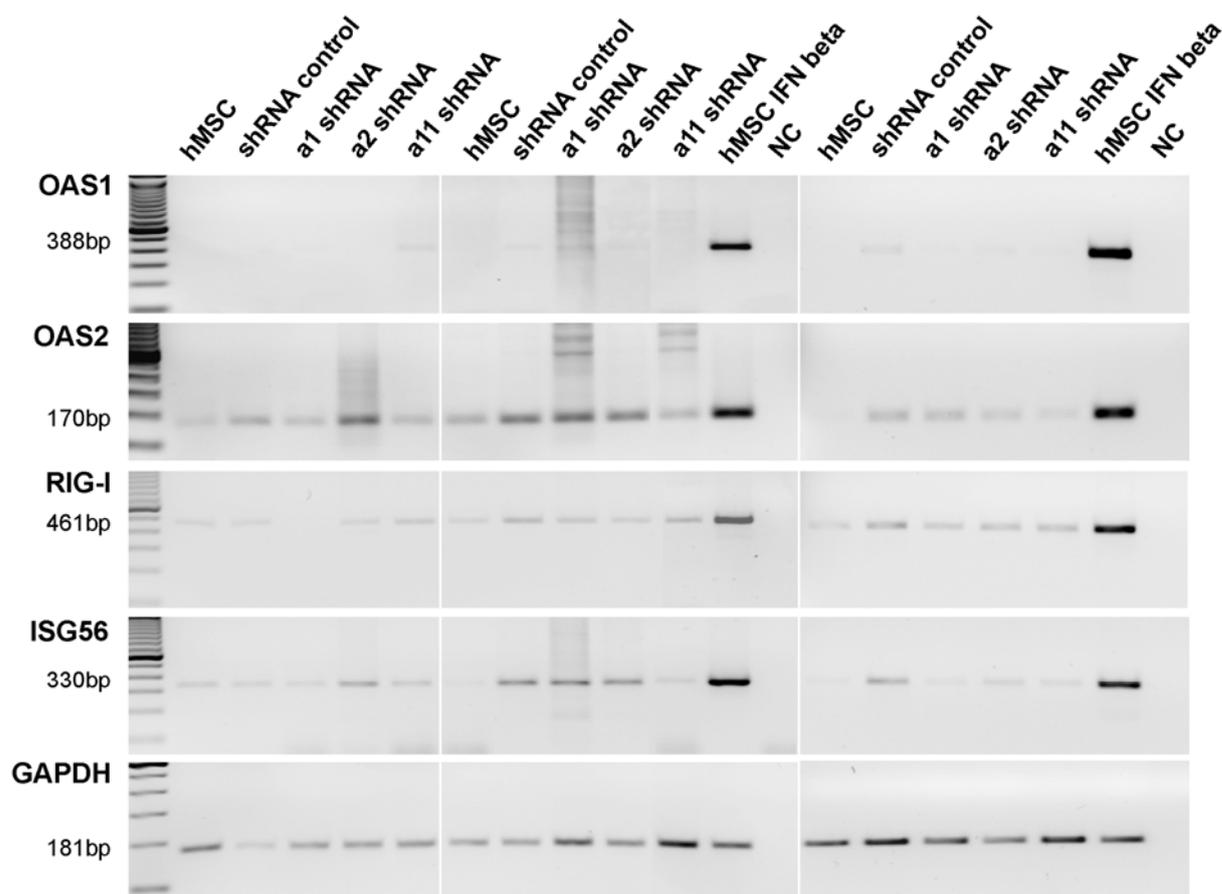


Fig. 21. Analysis of genes related to the IFN pathway. Gene expression of OAS1 and 2, RIG-I, ISG56 was assessed by RT-PCR in control and shRNA-transduced hMSC XI cells.

#### 4.2.5. Adhesion, spreading and migration analysis of shRNA-transduced hMSC

In order to investigate the reason for the observed cell death, which was not caused by IFN upregulation, the adhesion, spreading and migration capacity of the shRNA-transduced hMSC were next analyzed.

First, we analyzed the shRNA-transduced hMSC adhesion on ColII (fig. 22). We observed a high initial adhesion of shRNA control and a1 shRNA cells with  $64.6 \pm 5.5\%$  and  $51.7 \pm 6.4\%$ , respectively. Furthermore, after 90 min all hMSC transduced with shRNA control were attached ( $98.8 \pm 1.8\%$ ) whereas for a1 shRNA cells this process required 120 min ( $91.6 \pm 6.5\%$ ). In comparison, the a2 knockdown cells showed much slower adhesion on ColII as we detect an initial adhesion of  $33.1 \pm 2.3\%$  which was approx. half of the shRNA control or a1 shRNA cells attached. However, after 120 min  $79.1 \pm 3.5\%$  of the a2 shRNA hMSC were attached. In contrast to all other cell types, a11 shRNA cells had an extremely low initial adhesion of  $16.2 \pm 1.2\%$  and throughout the whole experimental period not more than  $30.1 \pm 3\%$  of the knockdown hMSC attach to ColII.

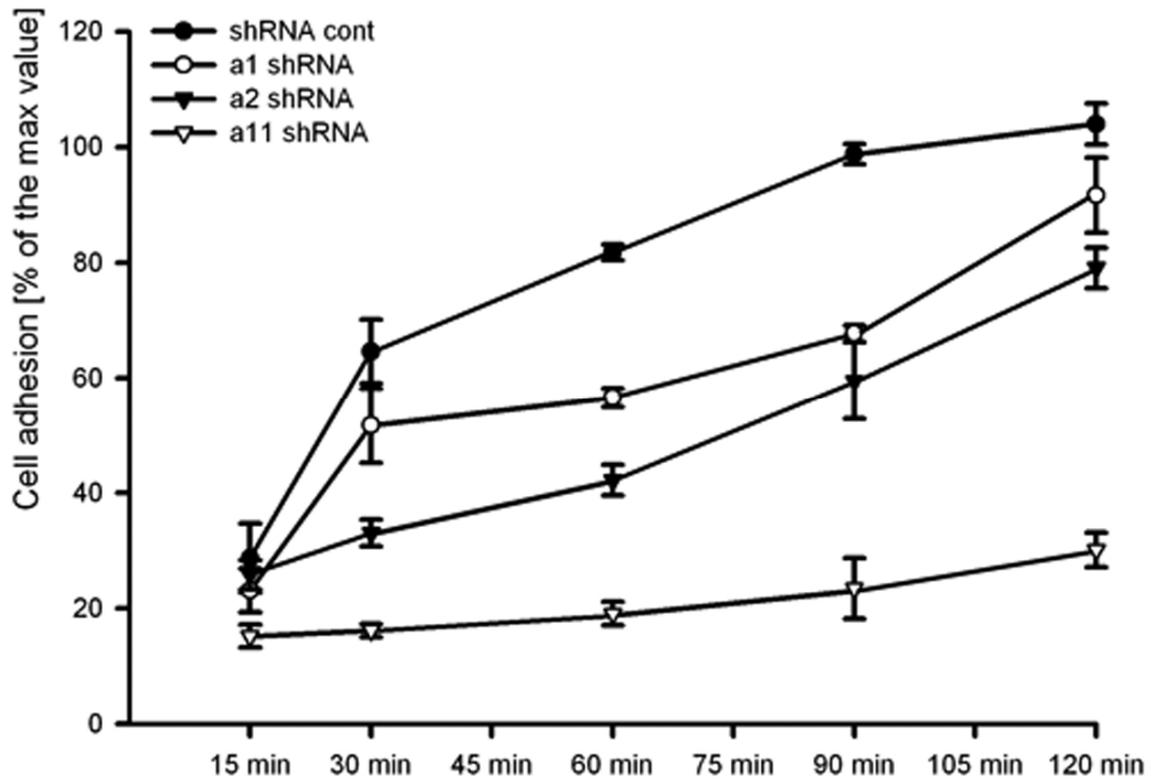


Fig. 22. Analysis of shRNA-transduced hMSC cell adhesion to ColII matrix for a period of 120 min. This experiment represents data from three different donors - hMSC XI, XIII and XV.

Second, we analyzed the cell spreading time of the four different shRNA hMSC by time lapse experiments. Cell spreading was analyzed for a period of 80 min (fig. 23). The spreading time of shRNA control and a1 shRNA cells was similar and took  $34.2 \pm 1.4$  and  $33.3 \pm 1.4$  hours, respectively. In comparison them, integrin a2 and a11 shRNA cells showed a delay in spreading which took twice longer as the spreading time of integrin a2 knockdown was  $80.8 \pm 11.5$  and for a11-  $61.7 \pm 6.3$  hours.

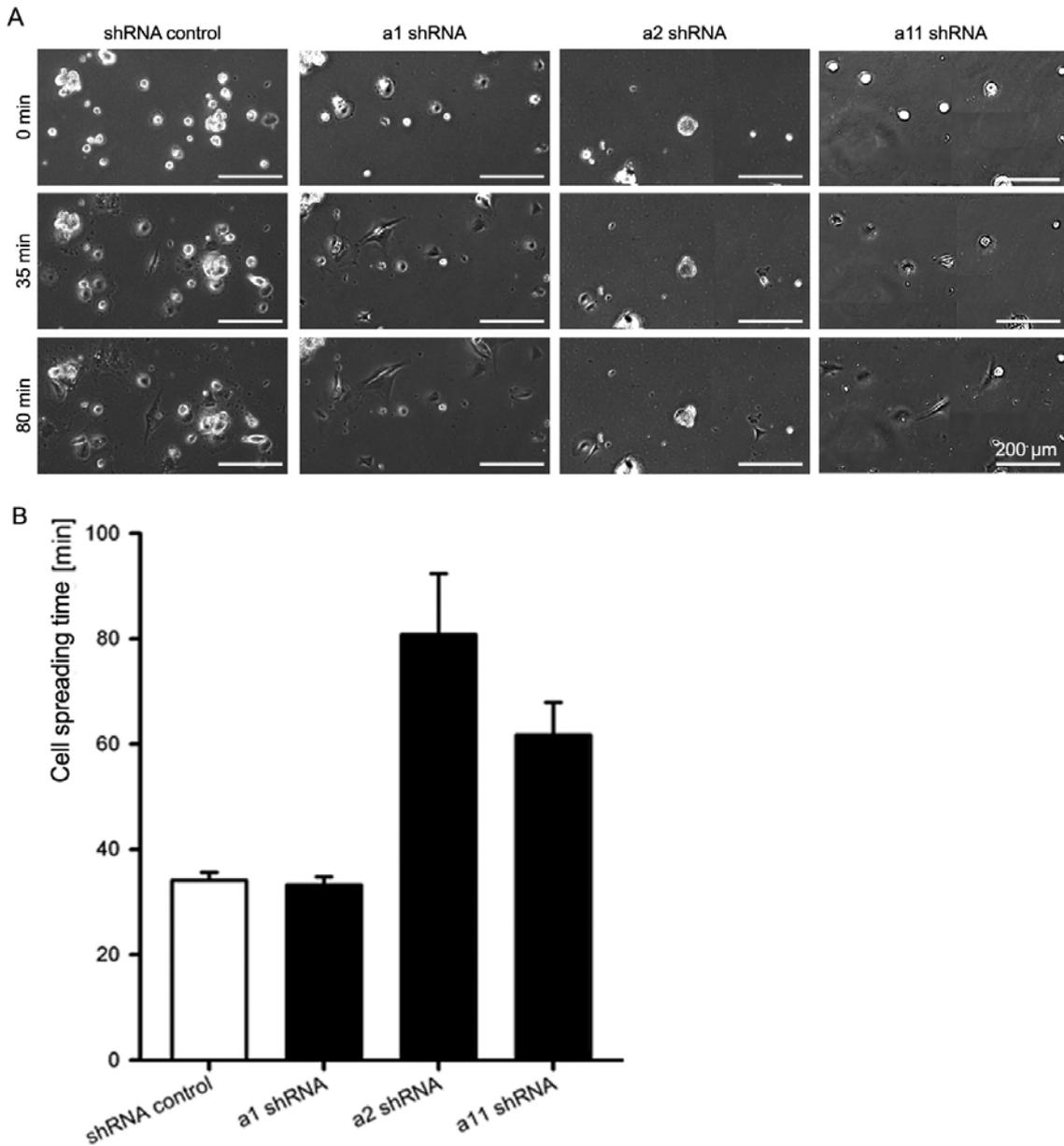


Fig. 23. Cell spreading of shRNA-transduced hMSC XI on CollI matrix. A) Representative images taken at 3 different time points. B) Quantification of the time for cell spreading hMSC XI and XIII. Bar 200 $\mu$ m.

In order to observe the effect of decreased adhesion and longer time for cell spreading, we next performed a cell migration analysis. Two independent infections of donor hMSC XI and one of hMSC XV were used to determine migration distance and cell velocity (fig. 24). The average pathway which the shRNA control cells passed was  $171.3 \pm 31.3 \mu\text{m}$  with mean velocity of  $11.4 \pm 2.1 \mu\text{m/h}$ . Integrin a1 knockdown cells migrated to a bit longer distance of  $208.8 \pm 6.6 \mu\text{m}$  with a mean velocity of  $13.9 \pm 0.4 \mu\text{m/h}$ . Integrin a2 and a11 knockdown cells showed similar migration pattern on CollI, as for a2 shRNA cells the migrated cell distance was  $81.6 \pm 4.1 \mu\text{m}$  with velocity of  $5.4 \pm 0.2 \mu\text{m/h}$  and for a11 shRNA cells the distance was  $73.5 \pm 11.7 \mu\text{m}$  with the velocity of  $4.9 \pm 0.8 \mu\text{m/h}$ .

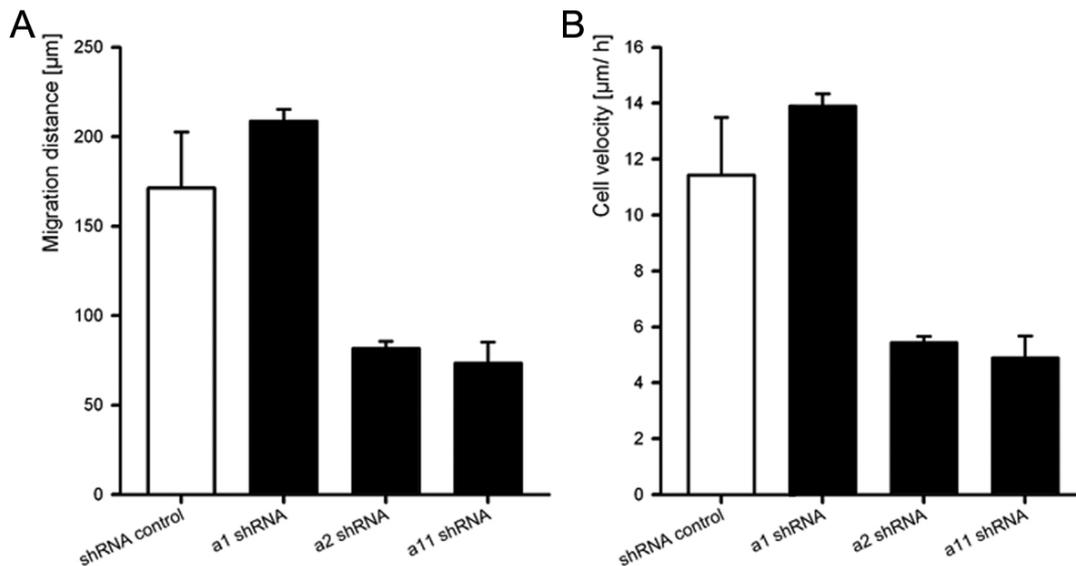


Fig. 24. Migration analysis of shRNA-transduced hMSC on ColI. Cell migration was evaluated by measurement of A) distance and B) velocity of two hMSC XI and one hMSC XV viral infections.

#### 4.2.6. Investigation of apoptosis in integrin a2 and a11 shRNA-transduced hMSC

Based on the observation that a2 and a11 shRNA hMSC cells are gradually lost during cultivation and that this is not caused by any IFN gene upregulation, we next investigated if these cells might be lost because of the cell apoptosis. For this purpose we performed an apoptosis assay which detects the changes in the mitochondrial membrane. JC-1 staining (fig. 25) in non-apoptotic cells aggregates at the mitochondria wall in the intact mitochondria as stains the mitochondria red. In apoptotic cells which exhibit a mitochondria leakage, JC-1 monomer resides in the cytoplasm and stains the cells green. For prove of the principle, we included a positive control in which we induce apoptosis in hMSC by FAS antibody stimulation. The results from the performed JC-1 staining with to different hMSC donors demonstrated that shRNA control and a1 shRNA cells were not apoptotic as in these cells the intact mitochondria were stained in red. In contrast, hMSC with integrin a2 or a11 knockdown exhibited a leakage in their mitochondria since the majority of the cells were stained only green. Thus it suggests that these cells undergo apoptosis. Similar to staining of integrin a2 and a11-deficient cells, the apoptotic induced hMSC control demonstrated only green cytoplasm staining.

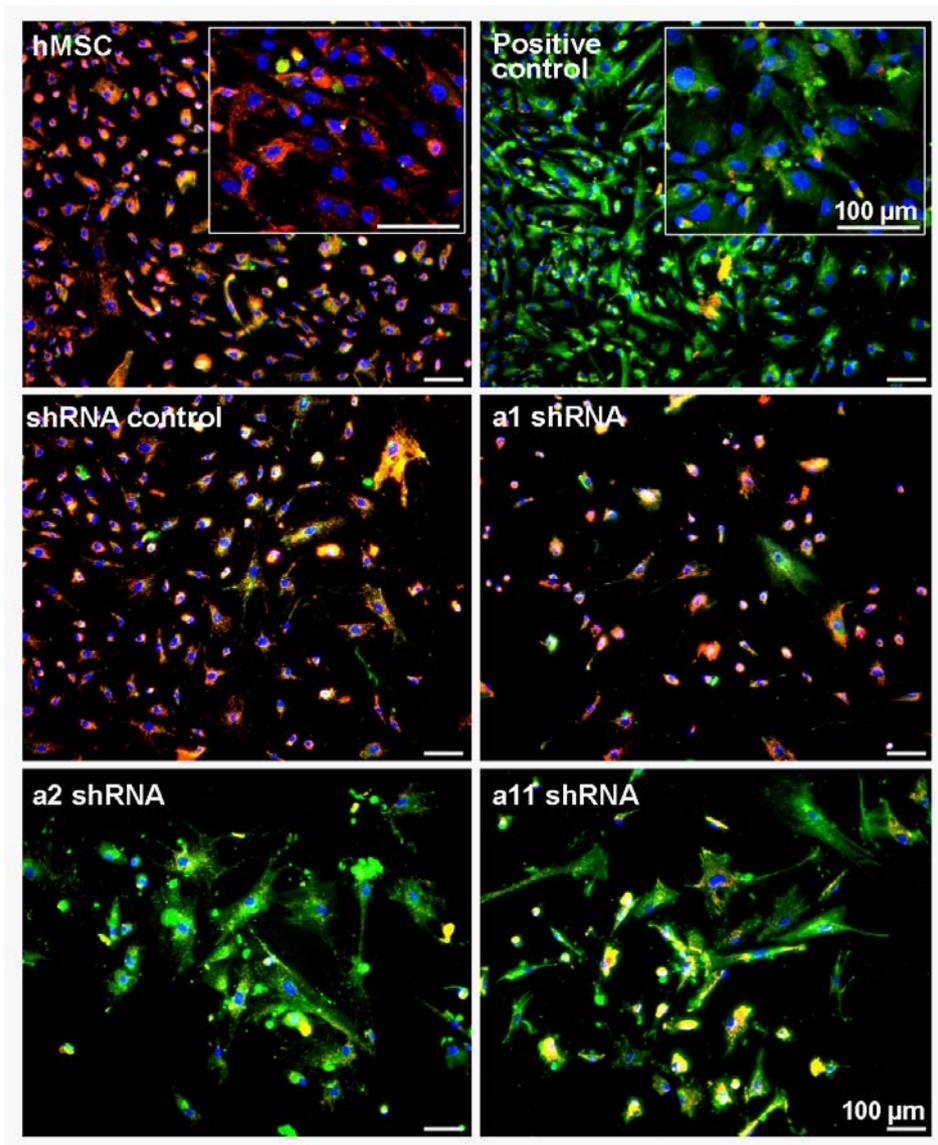


Fig. 25. A representative apoptosis analysis of cells from hMSC XI donor after knockdown of a1, a2 and a11 integrin. Positive control - hMSC treated with FAS antibody. Bar 100 $\mu$ m.

#### 4.2.7. Osteogenic differentiation of shRNA-transduced hMSC

To analyze whether the knockdown of ColI-binding integrins can influence also the good osteogenic potential of hMSC, the four different shRNA cells were osteogenically stimulated for 21 days (fig. 26). The results manifested that both, shRNA control and a1 shRNA cells, were able to differentiate and mineralize the matrix. In contrast, integrin a2 and a11 knockdown cells did not manage to mineralize the matrix and the AR staining was negative. Moreover, during the differentiation process of three hMSC donors, we again detected a tremendous loss of cells with a2 and a11 knockdown and after 21 days in the osteogenic media, only very few cells were left.

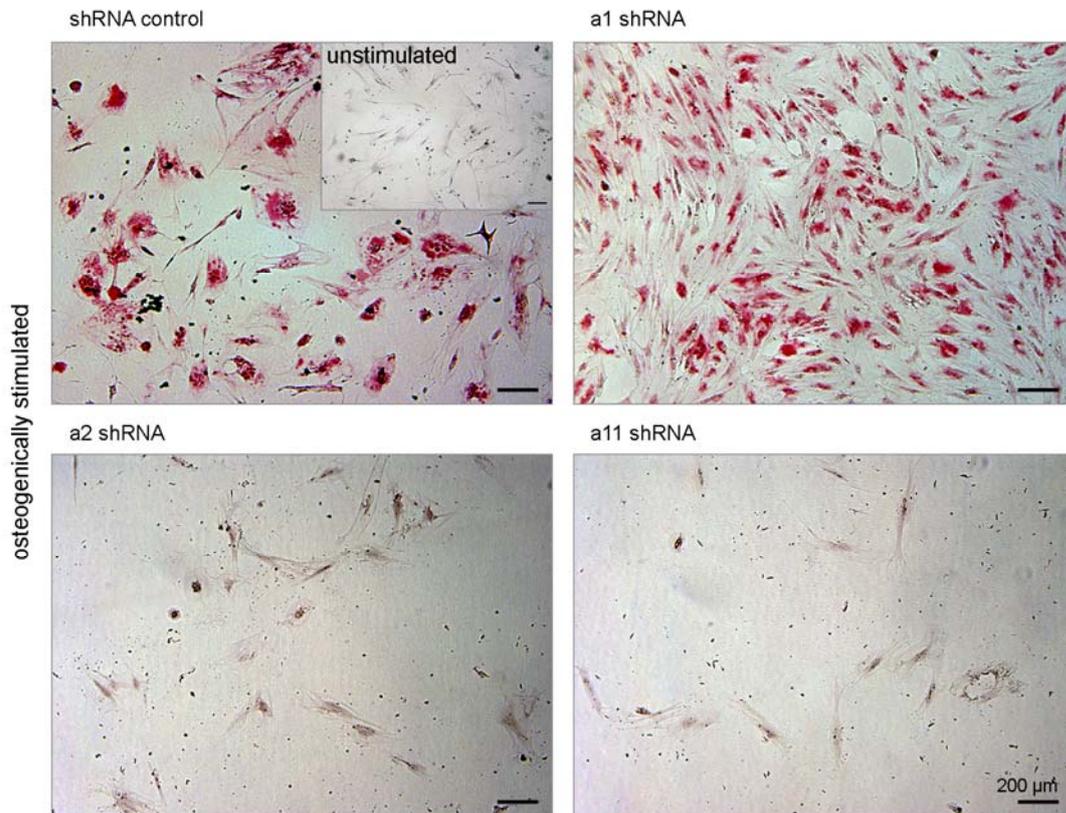


Fig. 26. Osteogenic differentiation of shRNA-transduced hMSC. A representative experiment with hMSC XI. Bar 200 $\mu$ m.

#### 4.2.8. Investigation of compensatory mechanism between the Coll-binding integrins

The cell behavior of a1, a2 and a11 shRNA knockdown cells provoked us to investigate a possible compensatory mechanism in-between the Coll-binding integrins. Therefore, we analyzed in details the integrin mRNA changes of a1, a2 and a11 shRNA hMSC by LC-PCR (fig. 27) and by WB (fig. 18). We compared the integrin expression levels in the three individual integrin knockdowns to the shRNA control cells. We found that when integrin a1 was lost, the expression levels of a2 and a11 mRNA increased with  $1.7\pm 0.1$ - and  $3.7\pm 0.5$ -folds respectively. Similar changes occurred also when integrin a2 was diminished. This resulted in upregulation with  $2.4\pm 0.6$ - of integrin a1 and  $2.1\pm 0.5$ -folds of a11 integrins. Interestingly, lack of integrin a11 led only to increase of integrin a1 mRNA expression with  $1.7\pm 0.1$ -folds. The data obtained on protein level by WB analysis also confirmed the described above integrin changes in a1, a2 and a11 shRNA-transduced hMSC. We detected an upregulation of a2 and a11 protein in a1 shRNA cells and a1 and a11 proteins in a2 shRNA cell, but no elevation of a2 protein was detected in a11 shRNA cells (fig. 18).

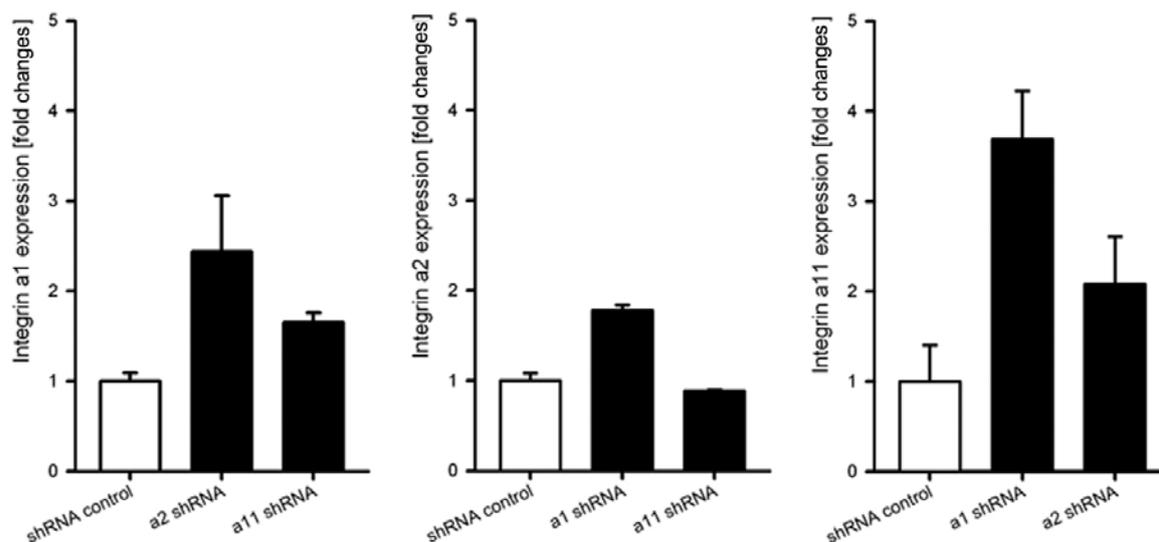


Fig. 27. Cross-talk between Coll-binding integrins in shRNA-transduced hMSC assessed by LC-PCR.

#### 4.2.9. Analysis of the expression levels of Coll-binding integrins in normal and osteoporotic hMSC

Finally, we investigated the integrin a1, a2 and a11 expression in primary hMSC isolated from three healthy and three osteoporotic patients (the cells were kindly provided by Dr. Christian Prall). We want to test whether there might be changes in the expression levels of Coll-binding integrins due to this disease. The donors used for the experiments are summarized in tab. 4. For osteoporotic were claimed to be cells isolated from patients having bone mineral density factor or T-value lower than -2.5 and as healthy - cells isolated from donors with T-value bigger than -1.

	Donor	Age	T-value
Healthy	3	86	-1
	27	93	-0,5
	29	68	-0,9
Osteoporotic	32	81	-2,7
	35	85	-3,5
	38	83	-3,2

Tab. 4. HMSC donors used to compare a1, a2 and a11 integrin expression in healthy and osteoporotic conditions.

Interestingly, we detected a striking 21.8-folds downregulation of integrin a2 expression in the osteoporotic hMSC (fig. 28). In addition, we observed also an elevated expression of in-

tegrin a1 and a11 with 1.49- and 1.33-folds, respectively. These results correlated with our observation that a1 and a11 integrins upregulate in response to knockdown of a2 (fig. 27).

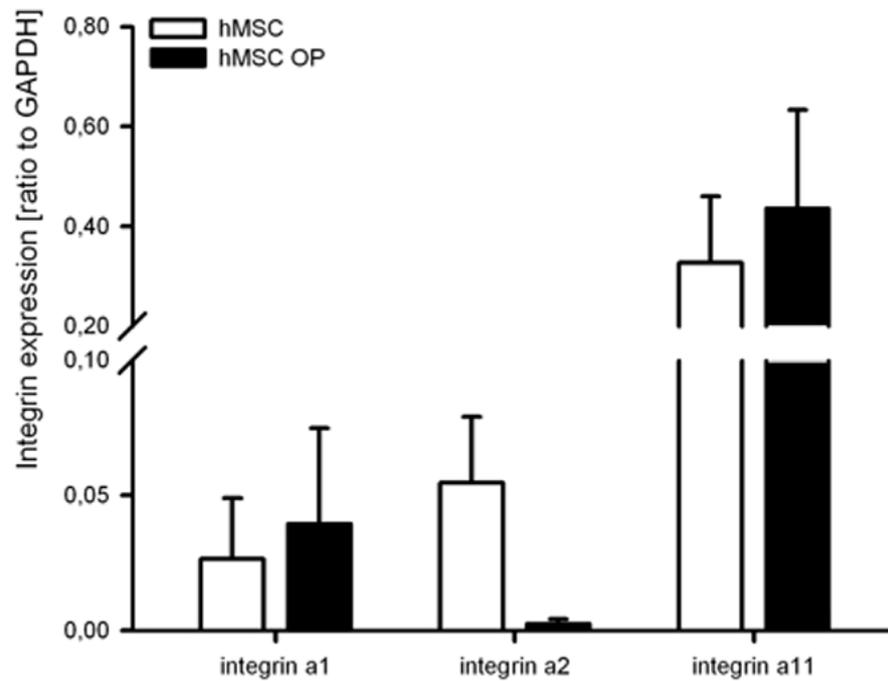


Fig. 28. Integrin a1, a2 and a11 expression in primary hMSC. Three healthy and osteoporotic patients were tested.

## 5. Discussion

hMSC, with their regenerative potential, have been one of the major studying subjects in the last decades. Despite the numerous data collected, there are still a lot of open questions left. In this doctoral thesis we aimed to investigate the integrin expression of hMSC and to functionally analyze the effect of single collagen I-binding integrin knockdown on hMSC behavior *in vitro*. For these purposes were used three hMSC donors having a similar morphology and population doublings.

### **hMSC adhesion and proliferation on different extracellular matrix proteins**

First, we investigated hMSC matrix preference by analyses of their cell attachment and proliferation on different proteins. As a member of the BM stem niche, we speculated that hMSC can bind to all major proteins in BM - collagens, FN and laminins. Earlier, *Salaszniyk et al.* [39] investigated the hMSC adhesion toward different ECM proteins. The author reported a high binding affinity of hMSC towards ColI and IV, FN and Vitronectin. In our cell adhesion experiments, we observed an increased hMSC attachment to ColI, FN and laminin I proteins as the cell affinity was the greatest towards ColI and FN. Apparently, those two ECM proteins were reported to be strongly expressed in BM [216].

The observed laminin I attachment of hMSC was delayed in comparison to ColI and FN. Similarly, *Salaszniyk et al.* [39] also reported a minimal hMSC attachment to laminin I after the first 30 min, while after 120 min all cells attached. The hMSC adhesion toward laminins was also investigated by *Klees et al.* [217], who showed distinct adhesion properties of different laminins after 30 min. The authors reported that hMSC attachment towards laminin I was 4-fold less effective than to laminin V. Additionally, the cell adhesion towards laminin V was similar as to the ColI, ColVI and FN proteins.

In addition, in our adhesion experiments we used PLL as a control for an integrin-independent adhesion. It is known that PLL mediated the cell adhesion by the establishment of ionic bindings between the cell membrane proteins and PLL surface. Our data showed that the hMSC attachment to PLL was always weaker in comparison to the natural substrates or polystyrene. Such lower effect on cell adhesion towards PLL was also reported earlier by *Salaszniyk et al.* [39]. In addition, the authors observed no changes in the phosphorylated form of focal adhesion kinase (FAK). FAK is a key player in the formation of focal adhesion complexes and is a directly affected by cell integrins. Thus, this proved the PLL as a surface, promoting the integrin-independent adhesion and can be used as a good control of the unspecific cell adhesion.

In conclusion, hMSC affinity toward the two natural substrates - ColI and FN, widely expressed into the BM niche, was the strongest and a clear cell adhesion induction was observed.

Second, we analyzed the hMSC proliferation on ColI, FN, Laminin I, PLL and polystyrene. We observed that hMSC favored again the natural substrates - ColI and FN. However, some differences between our results and those published earlier are noteworthy. Our BrdU data showed an increased cell proliferation on ColI, FN and laminin I when compared to the plastic control. The highest cell proliferation was detected on ColI, followed by FN and laminin I. In comparison, *Gronthos et al.* [137] showed also an enhancement of the cell proliferation by the natural proteins. With clonogenic analysis, performed in serum-depleted media authors showed that hMSC plated on laminin I protein had the higher amount of colony. Cells proliferated then better on FN, Vitronectin and ColIV. At ColI and ColIII, hMSC produced approximately 80% of the colonies obtained in normal culture media. The reason for the differences observed by *Gronthos et al.* [137] and our results might be method used and the time period for analysis. We assessed the hMSC proliferation by a colorimetric assay, detecting the BrdU incorporation for 24 hour time period whereas *Gronthos et al.* applied a colony forming assay and maintained the cells in culture for 14 days.

Alike to the cell adhesion, hMSC cultured on PLL showed a weaker proliferation in comparison to the polystyrene control. Similar adhesion was reported also by *Lu et al.* [218] and *Semenov et al.* [219] which observed an inhibition of hMSC proliferation on PLL surface. In fact, *Lu et al.* [218] showed that the proliferation inhibition is highly dependent on the PLL concentration and the presence of the serum in the media.

In conclusion, we detected a positive effect of the natural substrates - ColI and FN for cell adhesion and proliferation. The effect of the laminin I was also positive, but only for cell proliferation whereas the cell adhesion was delayed.

### **HMSC integrin expression**

Next, we investigated the expression of 8 alpha and 3 beta integrin subunits in hMSC and compared them to human OB. Both cell types showed a similar integrin expression pattern. The hOB expressed all of the tested integrins whereas in hMSC integrin a4 was not detected. Similar results was observed also by *Kumar et al.* [220] and *Majumdar et al.* [55] which showed by immunofluorescence and FACS analysis that integrin a4 was not presented on the surface of hMSC. The expression of integrin a4 in OB was linked to the interactions between OB, leukocytes and HSC in BM cell niche [221]. Moreover, it is known that integrin a4 binds

to VCAM receptor which was shown to be an essential for the initial capturing, rolling and firm attachment of the leukocytes to the OB [220].

All of the other tested integrin subunits were expressed in both cell types but there were clear differences in their expression levels. We detected an abundant expression of integrin  $\alpha 1$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 11$  and all tested beta-subunits in both cell types. Similar results were reported also by other groups [55, 137, 217, 222]. *Majumdar et al.* [55] and *Gronthos et al.* [137] indentified the expression of the integrins  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\alpha V\beta 3$  and  $\alpha V\beta 5$  in hMSC by FACS analysis; *Klees et al.* [217] reported integrin  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$ ,  $\alpha V$ ,  $\beta 1$  and  $\beta 4$  by studying the effect of blocking antibodies and *Chastain et al.* [222] detected integrin  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ ,  $\beta 5$  and  $\beta 6$  by RT-PCR. Although there are several contradictions regarding the integrin expression in hMSC, within the above articles, the expression of integrin  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$  and  $\beta 5$  was proved.

In conclusions, we found that hMSC expressed integrins  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 11$ ,  $\alpha V$ ,  $\beta 1$ ,  $\beta 3$  and  $\beta 5$ . Interestingly, hMSC did not expressed integrin  $\alpha 4$ , detected in OB cells. Also we observed differences in the expression level between the tested integrins in hMSC.

### **Expression of Coll-binding integrins in hMSC**

Among all tested alpha subunits, we found that integrin  $\alpha 1$  and  $\alpha 11$  had one of the highest expressions. This data correlate with the earlier observed highest hMSC affinity towards Coll. Therefore, we further analyzed the expression of integrins belonging to the group of Coll-binding integrins. We found differences between the  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 11$  integrin basal expressions on mRNA and protein level. On mRNA, the expression of integrin  $\alpha 11$  was the strongest, followed by  $\alpha 1$  and  $\alpha 2$ . The analysis on protein level confirmed the expression differences between integrin  $\alpha 2$  and  $\alpha 11$  observed on mRNA level. Integrin  $\alpha 11$  was expressed twice more in comparison to the integrin  $\alpha 2$ . Moreover, this integrin formed distinguishable focal adhesion complexes when the cells were cultured on Coll protein. Similar observation were reported by *Zhang et al.* [223] who analyzed the Coll-binding integrins expression in mouse dermal fibroblast. The integrin  $\alpha 11$  shown the higher levels of protein production, followed by  $\alpha 1$  and  $\alpha 2$ .

In conclusion, we observed a higher levels of expression of Coll-binding integrin in hMSC as integrin  $\alpha 11$  was the strongest, followed by  $\alpha 1$  and the weakest was  $\alpha 2$ . The highest expression of integrin  $\alpha 11$  suggests that this integrin have a great importance for hMSC adhesion.

**Integrin  $\alpha 2$  and  $\alpha 11$  expression change upon osteogenic (OS) differentiation**

The reported elevated expression of CollI upon OS differentiation [224,225] suggested that there might be a corresponding increase in the expression levels of the CollI-binding integrins in hMSC. Indeed, all three analyzed hMSC donors, which were proven to strongly differentiate into osteoblasts lineage, were found to increase the integrin  $\alpha 2$  and  $\alpha 11$  mRNA and protein levels upon OS stimulation. An increased integrin  $\alpha 2$  expression after OS differentiation was also reported by Meyers *et al.* [226]. By the use of Western blot analysis, the authors found an increase of the integrin  $\alpha 2$  expression with  $136 \pm 21\%$  in stimulated compare to unstimulated hMSC after 7 days of differentiation. Moreover, the authors even proposed a model of integrin  $\alpha 2$ -dependent increase in Runx2 expression. Runx2, a key osteogenic transcription factor, was stimulated by the activation of MAPK which is a known downstream target of the integrins. Integrin  $\alpha 2$  and  $\alpha 11$  upregulation was reported also by Foster *et al.* [227] who assessed the integrin changes in hMSC undergoing OS differentiation by quantitative proteomic analysis. The increase of integrin  $\alpha 2$  and  $\alpha 11$  protein levels was with  $2.0 \pm 1.0$  and  $3.0 \pm 2.3$ -folds, respectively in OS stimulated hMSC.

In contrast to integrin  $\alpha 2$  and  $\alpha 11$ , no expression change was observed for integrin  $\alpha 1$  in hMSC upon OS differentiation. Interestingly, Rider *et al.* [228] reported that in hMSC subpopulation FACS sorted for increased integrin  $\alpha 1$ , had enhanced plasticity as these cells readily underwent adipo-, osteo- and chondrocytes differentiation *in vitro*. Thus, we suggest that integrin  $\alpha 1$  is important for hMSC stemness rather than their OS differentiation.

Finally, when we correlated the integrin expression to the extent of matrix mineralization, we observed that the high integrin  $\alpha 2$  and  $\alpha 11$  levels, but not  $\alpha 1$ , correspond to the high mineralization capability. Interestingly, we reported here a strong linear correlation between the increased integrin  $\alpha 2$  expression and the amount of mineralize by hMSC matrix. This effect might be due to the increased CollI expression during the OS differentiation as reported earlier [224,225]. Similar effect was observed also for integrin  $\alpha V\beta 3$  by Gordon *et al.* [229] who analyzed the influence of bone sialo protein (BSP) expression on MC3T3 differentiation. By overexpressing of BSP, the OS differentiation in MC3T3 increased. Moreover, this increase was strongly dependent on the integrin  $\alpha V\beta 3$  level and on the activation of its downstream effectors belonging to the MAPK pathway.

In conclusion, we found an increased expression of both integrins  $\alpha 2$  and  $\alpha 11$  in osteogenic differentiated hMSC, suggesting the higher importance of these two integrins for osteogenesis.

### **Establishment of integrin knockdown in hMSC**

The suggested, from our research and published literature, importance of ColI-binding integrins for hMSC behavior, urged to develop a stable and reliable method for integrin down-regulation and to perform loss-of-function cell studies. For this purpose, we selected a knock-down technology mediated by short hairpin RNA (shRNA). Till now, the study of ColI-binding integrins was based majorly on a data obtained by blocking antibodies or siRNA sequences. By using these approaches, the effect of integrin silencing was analyzed only for a short period of time - from few hours [230,231] up to 7 days [232]. Another disadvantage of both methods is the heterogeneity of the cell population since no selection was possible. Those two major disadvantages we overcome by the applying of lentiviral delivered shRNA into the cells.

Knockout mouse models of  $\alpha 1$  [140],  $\alpha 2$  [144] and  $\alpha 11$  [147] integrins did not showed a severe phenotype changes in the animals. This suggested that the loss of single integrin was eventually compensated by the remaining ColI-binding integrins. The data obtained from knockout mice is informative, but the results can not be directly transmitted to human because of the genetical and phenotypical differences between both species. Therefore, in order to study the integrin importance for hMSC, we selected the virally delivered shRNA method. It allows us to establish a stable knockdown and to select a homogenous cell population based on the introduced antibiotic resistance. Thus, this method provides a suitable model system for studying of a gene loss in hMSC since the existing until now methods did not allow a stable and efficient gene downregulation.

Commonly, the gene silencing achieved by shRNA is greater than 50% and highly depends on the type of transduced cells. Cell lines are known to be more easily transducible than primary cells and therefore the downregulation efficiency is higher. Using our integrin knock-down system, we obtained more than 80% silencing of the targeted genes. This result was first assessed on mRNA level by LC-PCR. Additionally, Western blotting analyses of integrin  $\alpha 2$  and  $\alpha 11$  shRNA-transduced hMSC showed nearly a complete loss of the targeted protein.

Since hMSC were infected with viruses containing dsRNA, we introduced a control-hMSC population transduced with shRNA against non-human gene. This bacterial gene is responsible for the synthesis of  $\beta$ -galactosidase enzyme which catalyzes the hydrolysis of  $\beta$ -galactosides to its monosaccharides. Such shRNA control cells are commonly used and they are essential for the evaluation of the effects of the virus infection and the presence of the dsRNA into the cells. Importantly, our shRNA control-transduced hMSC showed similar cell

behavior and growth characteristics as the non-transduced hMSC and therefore, we used them as a control group throughout the whole study.

In conclusion, we established a knockdown system for each ColI-binding integrin which showed approximately 80% knockdown efficiency in all tested hMSC donors.

### **Lack of integrin a2 and a11 led to cell loss**

After the establishment and validation of a1, a2 and a11 knockdown in hMSC, we analyzed in details the hMSC behavior. Our first impression was that a2 and a11 shRNA cells were decreasing in number during the cultivation. Moreover, by counting the cell number during 14 days, we observed a tremendous effect of a2 and a11 shRNA on hMSC survival - more than 80% of the cell population was lost, whereas the number of transduced with shRNA control and a1 shRNA hMSC constantly increased. Similar impact of integrin a2 on the cell survival was observed also by *Kozlova et al.* [233] and *Rezgui et al.* [234] who used blocking antibodies and detected clear induction of cell death in different human carcinoma cells due to the inhibition of integrin a2.

Interestingly, the phenotypes of single knockout mouse models for integrin a2 and a11 were described as vital and fertile. Nevertheless, these animals exhibited few minor defects. For example, the integrin a2 knockout mouse model showed a deficient adhesion of platelets to ColI [144]. The integrin a11 knockout mouse had reduced body size, exhibited higher mortality and showed pronounced periodontal ligament defects [147]. Interestingly, the a11 mutant mice remained smaller even when they were fed with a soft food, indicating that the growth defect exists independently to the reported tooth phenotype. Therefore, more detailed cellular studies, engaging MSC and OB, are required to completely evaluate the phenotype of the single integrin knockouts. Furthermore, a generation of double or triple knockouts mouse strains can provide additional information about the role and the importance of the ColI-binding integrins *in vivo*.

In conclusion, knockdown of integrin a2 and a11 in hMSC led to a cell loss whereas the integrin a1 and shRNA control hMSC remained vital and proliferative.

### **Cell death in a2 and a11 shRNA-transduced hMSC is not caused by IFN pathway induction.**

Regarding the application of siRNA technology, several drawbacks have been recognized such as off-target effects [202,235] and possible activation of a subset of genes in response to the viral infection or dsRNA, leading to cell death [236]. The problem with si/shRNA off-

targeting is that it can lead to misinterpretation of the observed results due to non-specific gene silencing. In addition, viral infection as well as cell transduction with dsRNA can activate the cellular self-defense mechanism as viral RNA and dsRNA molecules can be recognized and bind to RIG-I receptor. RIG-I is localized in the cytoplasm and can trigger the IFN pathway (e.g. ISG56) and thus, cell death can be induced. The induction of the IFN pathway can be assessed by investigating the expression of downstream factors such as OAS1 and OAS2 which activate RNaseL and inhibit cell proliferation. In order to investigate whether our cells were dying because of IFN pathway activation, we analyzed the expression levels of OAS1 [237,238], OAS2 [239], RIG-I [240] and ISG56 [238,241] genes. Our results, based on a three independent infections, clearly demonstrated that the presence of shRNA in the cells did not upregulate any of the tested genes, even though slight fluctuations were detected. As a positive control in this experiment, we introduced hMSC treated with IFN-beta [242]. The applied 2000U/ml IFN-beta concentration was shown to be in the physiological range in the study of human amniotic membrane cells [243].

Our data conclusively showed that the observed cell loss in hMSC transduced with integrin  $\alpha 2$  or  $\alpha 11$  shRNA was not caused by induction of the any IFN pathway.

### **Integrin $\alpha 2$ and $\alpha 11$ influence hMSC adhesion, spreading and migration**

Next, we analyzed the adhesion, spreading and migration of the control and integrin knock-down hMSC on ColI. Studies using mouse fibroblast lacking integrin  $\alpha 2$  [223] or  $\alpha 11$  [147] reported an interesting change of the cell attachment to ColI. *Zhang et al.* [223] showed that  $\alpha 2$ -deficient dermal fibroblasts had 33% reduction of ColI attachment, whereas there was no difference when same cells attached to other proteins, such as ColIV, laminin 1 and 5. Moreover, these cells showed markedly reduced cell attachment to different ColI concentrations in short term experiments in comparison to the wild-type cells. The critical effect of the  $\alpha 2$  integrin for adhesion to ColI was also observed in  $\alpha 2$ -defective keratinocytes [244] and osteoblasts [245]. The keratinocytes showed that absence of integrin  $\alpha 2$  led to an inefficient cell adhesion on ColI even after 1 hour. In  $\alpha 11$ -deficient skin fibroblasts, the integrin loss caused even stronger reduction of the cell attachment which was only 20% of the cell input. Additionally, *Popova et al.* [147] observed a significant impact of integrin  $\alpha 11$  on cell proliferation since approximately 60% of the mouse embryonic fibroblasts were arrested in G0/G1 phase when compared to the wild-type cells. Our data obtained from the cell adhesion experiments was in line with the knockout studies. We observed a clear delay of the hMSC attachment to ColI upon the knockdown of  $\alpha 2$  and  $\alpha 11$ . In particular for  $\alpha 2$ -deficient cells this delay was de-

tected only for a short term and then the cells were able to attach to 80%. In case of integrin  $\alpha 11$  knockdown, we observed a severe reduction of the hMSC attachment as not more than 30% of the cells was able to adhere to ColII. In contrast,  $\alpha 1$ -deficient and shRNA control hMSC showed similar tendency in the attachment to ColII. Nevertheless, a slight reduction of the adhesion of  $\alpha 1$  shRNA cells in comparison to the control cells was observed. Similarly, *Zhang et al.* [223] also detected a slight influence of integrin  $\alpha 1$  on mouse fibroblast adhesion when cells were blocked with antibody.

Our spreading analysis similar to the adhesion assay also showed distinguishable differences between shRNA control, integrin  $\alpha 2$  and  $\alpha 11$  knockdown hMSC as the knockdown of integrin  $\alpha 1$  seems to not influence hMSC behavior on ColII. In comparison to shRNA control and integrin  $\alpha 1$  knockdown,  $\alpha 2$ - and  $\alpha 11$ -deficient hMSC required double time for spreading. Interestingly, the integrin  $\alpha 2$  knockdown showed the higher spreading delay despite of the fact that those cells were able to attach to ColII. Moreover, integrin  $\alpha 11$ -deficient hMSC displayed a better spreading capacity than integrin  $\alpha 2$  knockdown, even that those cells demonstrated a very low attachment on ColII. The opposing results observed in integrin  $\alpha 2$ - and  $\alpha 11$ -knockdown cells adhesion and spreading suggested the different importance of those integrins for the hMSC. We can propose that integrin  $\alpha 2$  and  $\alpha 11$  participate differently in the adhesion and spreading mechanisms as integrin  $\alpha 11$  is important for the initial cell adhesion and formation of the focal contacts, while integrin  $\alpha 2$  is more important for the cell spreading and formation of later focal complexes in hMSC on ColII. Moreover, because of the deficient cells adhesion and spreading, we can suggest that knockdown of integrin  $\alpha 11$  leads to incompetence of hMSC to bind to ColII which in resulted in reduction of hMSC number. Similar result were observed by *Disatnik et al.* [246] who showed the inability of integrin  $\alpha 5$ -deficient myoblasts to attach and spread on FN and to activate FAK. In this study, upregulation of integrin  $\alpha 4$  was shown to compensate the missing integrin  $\alpha 5$  and to recover the cell spreading and survival.

Fibroblasts obtained from integrin  $\alpha 2$  knockout mice, showed no difference in skin wound closure when compared to the wild-type *in vivo* [144, 223]. In contrast, *Grenache et al.* [244] using a scratch assay, reported that the migration of  $\alpha 2$ -deficient keratinocytes on ColII was significantly impaired, as 40% of the scratch remained open. Regarding the  $\alpha 11$ -deficient cell migration, *Popova et al.* [247] demonstrate that these cells were able to migrate faster than the wild-type. Moreover, analysis of embryonic fibroblast which lack integrin  $\alpha 11$  revealed not only increased migration capability on ColII, but also on collagen II, III and V [247]. The observed differences between *in vivo* and *in vitro* studies can be explained by the difference in the used cell types. *Chen et al.* and *Zhang et al.* [144,223] investigate the influence of integrin

a11 on the cell behavior in dermal a2-deficient fibroblast, whereas *Grenache et al.* [244] used a2-knockout keratinocytes and *Popova et al.* [247] used a11-knockout embryonic fibroblasts. Moreover, the techniques used in these studies to analyze cell migration were different. *Chen et al.* [144] used animal wound healing, whereas *Grenache et al.* [244] and *Popova et al.* [247] assessed the cell migration by scratch assay or transwell migration. Our migration analysis revealed comparable migration pattern of shRNA control and a1 shRNA cells as both cell lines migrated to a distance of 200 $\mu$ m. Similar to the cell spreading data, the groups of integrin a2- and a11-deficient hMSC exhibited reduced migration compare to the control cells. Nevertheless, both cell lines exhibited similar migration as cells migrated to only 75 $\mu$ m for 15 hours.

In conclusion, the delay of cell attachment, spreading and reduced migration on Coll upon integrin a2- and a11-knockdown demonstrated the importance of these two integrins for hMSC behavior. Moreover, integrin a2 and a11 seem to participate differently in the cell adhesion and spreading, but equally into the cell migration mechanism. Therefore, the abnormal adhesion, spreading and migration might be an additional factor to the observed cells loss.

### **Integrin a2 and a11 knockdowns lead to mitochondrial leakage**

The knockdown of integrin b1 in blastocyst cells was shown to trigger apoptosis due to inadequate or inappropriate cell–matrix interactions [248-250]. The classical pathway of apoptosis in cells is through the activation of the “death” receptors such as Fas or TNFR which lead to initiation of an apoptosis-induced signaling, processed via caspase pathway and finishes with cells death. Another type of apoptosis occurs when integrin receptors were missing. This process is known as anoikis and was described by *Fisher and Screaton* [251]. It involves activation of the pro-apoptotic Bcl-2 proteins localized on the surface of the mitochondria. When activated, those proteins participate in the formation of pores on the mitochondrial membrane resulting in release of the cytochrom C in cytoplasm. Subsequently, cytochrom C participates in formation of caspase-activation complex which activates number of caspases and results in cell death.

In order to investigate whether the observed cell loss is due to initiation of apoptosis, we next performed apoptosis-related analyses of shRNA-transduced hMSC based on JC-1 staining. This dye is used to evaluate the integrity of mitochondrial membrane. When we compared the four different shRNA-transduced hMSC, we observed mitochondrial leakages only in integrin a2- and a11-deficient hMSC. To prove that the observed effects were caused by initiation of apoptosis, we introduced a positive control by activation of caspase pathway with FAS anti-

body [252,253]. Upon activation with FAS antibody, mitochondrial leakage, similar to the observed in  $\alpha 2$  and  $\alpha 11$  knockdowns was detected.

In summary, the loss of  $\alpha 2$  and  $\alpha 11$  integrin in hMSC led not only to disturbance in the cell adhesion, spreading and migration, but also resulted in the formation of mitochondrial membrane pores which triggered apoptosis.

### **Integrin knockdown effect on osteogenic differentiation**

*Schneider et al.* [245] reported a dose dependent inhibition of matrix mineralization in mouse osteoblasts treated with different concentrations of integrin  $\alpha 2$  blocking antibody. Similar effect was observed also by *Xiao et al.* [254] who blocked integrin  $\alpha 2$  in mouse MSC. In comparison to these studies, which were based on blocking antibodies, we analyzed the matrix mineralization capability of stably transduced with shRNA hMSC. As expected, the shRNA control and  $\alpha 1$ -deficient cells were able to differentiate. The osteogenic capability was assessed by an Alizarin red staining which visualized the mineralized matrix. In the case of integrin  $\alpha 2$  and  $\alpha 11$  knockdown, we observed again the constant cell loss during the stimulation process. Furthermore, no differentiation was observed in the remaining cells. However, it remains unclear if of integrin  $\alpha 2$  and  $\alpha 11$  have a direct effect on OS or the lack of OS is due to the lower cell number in result to the apoptosis induction.

In conclusion, lack of integrin  $\alpha 2$  and  $\alpha 11$  led to impair of osteogenic differentiation which can be a direct effect of integrin downregulation or a consequence of the ongoing apoptosis.

### **HMSC ColI-binding integrin compensation**

The absence of severe abnormality within integrin  $\alpha 2$  and  $\alpha 11$  knockout mice suggested eventual compensatory mechanism between these integrins. As mentioned earlier, *Tiger et al.* [148] and *Zhang et al.* [223] studied the effect of integrin  $\alpha 2$  and  $\alpha 11$  on ColI attachment in mouse myoblasts and fibroblasts. *Tiger et al.* [148] investigated the integrin cross-talk in mouse myoblastic lineage C2C12 lacking the expression of the integrins  $\alpha 1$  and  $\alpha 11$ . These cells are known to have a reduced adhesion and migration capacity on ColI. By overexpression of integrin  $\alpha 2$  or  $\alpha 11$ , the cell attachment towards ColI improved as integrin  $\alpha 11$  had a stronger effect than  $\alpha 2$ . Moreover, transduction with either integrin  $\alpha 2$  or  $\alpha 11$  strongly enhanced the ability of the myoblasts to contract ColI gels. *Zhang et al.* [223] investigated also the compensatory effects of integrins  $\alpha 1$  and  $\alpha 11$  for the ColI cell adhesion in  $\alpha 2$ -deficient mouse skin fibroblasts. The authors found that when blocked with integrin  $\alpha 1$  antibody, the cell adhesion of mouse fibroblasts to ColIV was completely inhibited as the presence of in-

tegrin a2 and a11 was not sufficient to substitute the missing a1 integrin. Differently to integrin a1 blocking, knockdown of a2 integrin was successfully substituted by integrin a1 and a11.

We investigated the compensatory cross-talk between the ColII-binding integrins in absence of a single a1, a2 or a11 integrin in hMSC. The loss of integrin a1 led to an increased expression of integrin a2 and a11, and loss of integrin a2 stimulated the expression of integrin a1 and a11. Interestingly, knockdown of integrin a11 led to a slight increase only in integrin a1 expression. These findings are summarized in tab. 5.

Cell type	Integrin a1	Integrin a2	Integrin a11
a1 shRNA	<b>na</b>	++	++
a2 shRNA	++	<b>na</b>	++
a11 shRNA	+	-	<b>na</b>

Tab. 5. Changes of integrin expression upon single ColII-binding integrin knockdown. Abbreviations: (++) strong, and (+) slight gene upregulation; (-) no changes in gene expression.

Here, we demonstrated that the missing of a1 or a2 ColII-binding integrin led to an increased expression of the other two members of the family. However, this finding was not true for integrin a11. An enhanced expression of integrins a2 and a11 was also reported in a1-deficient mouse mioblasts or skin fibroblasts by *Tiger et al.* [148] and *Zhang et al.* [223]. Interestingly, they showed that integrin a1 had a higher binding affinity towards ColIV rather than ColII. The cells lacking integrin a1 were unable to bind ColIV while the upregulation of integrin a2 and a11 expression increased the cell attachment towards ColII.

In our study, the integrin a2 knockdown upregulated integrin a1 and a11 and the cells were still able to attach to ColII, despite of the observed delay. Similarly, *Zhang et al.* [223] also demonstrate that blocking of integrin a2 in mouse skin fibroblasts did not strongly influence the cell adhesion towards ColII. Additionally, this observation was confirmed also in the a2 knockout cells. The loss of the most abundantly expressed in hMSC integrin a11 led to increase only in the expression of integrin a1, while the level of integrin a2 remained unchanged. This fact can explained why the a11-deficient cells had a minimal cell attachment towards ColII and the adhesion never exceeded more then 30%. Thus, the upregulation of integrin a1 and the basal expression of integrin a2 did not substitute the missing integrin a11 and therefore, the hMSC adhesion towards ColII was impaired. Knockdown of integrin a1 led to an upregulation of a2 and a11 expression which ensured the cell attachment to ColII. These

cells showed similar spreading, migration and differentiation capacity as the control hMSC. It remains still to be tested the cell behavior on ColIV.

Loss of integrin  $\alpha 2$  resulted in reduction of cell adhesion, spreading and migration of hMSC and furthermore, initiated apoptosis despite of the compensatory upregulation of both integrin  $\alpha 1$  and  $\alpha 11$ . This effect can be explained with the difference in the signaling pathways mastered by integrin  $\alpha 2$  and  $\alpha 11$  [148]. Integrin  $\alpha 2$  signaling pathway is partially known. It had been shown a direct influence of integrin  $\alpha 2$  on the activation of MAPK. Also integrin  $\alpha 2$  dependent activation of ERK1/2 had been shown to be important for the cell survival [255]. In comparison to integrin  $\alpha 2$ , nothing is known about the integrin  $\alpha 11$  signaling pathway. Nevertheless, it is known that TGF- $\beta$  has a positive effect on integrin  $\alpha 11$  expression [256]. The loss of integrin  $\alpha 11$  in hMSC led to impaired cell adhesion, spreading and migration of hMSC on ColI. Additionally, the absence of integrin  $\alpha 11$  was not efficiently substituted by the integrin  $\alpha 1$  and  $\alpha 2$  and we observed an induction of apoptosis.

#### **HMSC derived from osteoporotic patients have downregulated integrin $\alpha 2$ expression**

The fact that integrin  $\alpha 2$  inhibit osteogenesis in mouse MSC *in vitro* [254] and the recent discovery of integrins are involved in some bone-related diseases, such as osteoarthritis [257] and osteoporosis [258], provoked an investigation of the ColI-binding integrin expression in hMSC, isolated from healthy and osteoporosis suffering patients. As reported by *Kanis et al.* [259], osteoporosis is a disease characterized by reduction of the bone mineral density, altering of the bone microarchitecture and expression changes of non-collagenous proteins. We speculated that there might be differences in the ColI-binding integrin expression due to the osteoporosis. We found, that in comparison to healthy hMSC, in osteoporotic cells the expression of integrin  $\alpha 2$  was inhibited. Interestingly, there was also a slight upregulation of integrin  $\alpha 11$  expression which was in line with our *in vitro* results. These conclusions were drawn from analysis of three patients per group. Nevertheless, more healthy and osteoporosis patient donors need to be further analyzed.

In conclusion, we observed an inhibition of integrin  $\alpha 2$  and increase integrin  $\alpha 11$  mRNA expression in osteoporotic hMSC. Interestingly, this observation was in line with the results obtained by our integrin  $\alpha 2$  knockdown model in hMSC. However, further experiments are required for understanding of the importance of the integrin  $\alpha 2$  in osteoporosis.

## 6. Conclusions

1. HMSC showed a pronounced affinity towards collagen I and fibronectin, since these two proteins enhanced hMSC adhesion and proliferation.
2. HMSC expressed integrins  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 11$ ,  $\alpha V$ ,  $\beta 1$ ,  $\beta 3$  and  $\beta 5$  subunits but not integrin  $\alpha 4$  subunit.
3. Among the collagen I-binding integrins,  $\alpha 11$  was the most expressed in hMSC, followed by integrin  $\alpha 1$  and then  $\alpha 2$ .
4. Upon osteogenic differentiation, integrin  $\alpha 2$  and  $\alpha 11$  were significantly upregulated.
5. A stable and very efficient knockdown of integrin  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 11$  by lentiviral delivery of shRNA was successfully established in hMSC.
6. The genetically modified hMSC showed no IFN-related gene upregulation in response to the viral infection or presence of dsRNA.
7. Integrin  $\alpha 2$  and  $\alpha 11$ -deficient hMSC showed reduction in cell adhesion, spreading and migration on collagen I, whereas integrin  $\alpha 1$ -deficient cells were similar to control hMSC.
8. Integrin  $\alpha 2$ - and  $\alpha 11$ -deficient hMSC, but not  $\alpha 1$ -knockdown cells, reduced in number during cultivation and showed mitochondrial leakage suggesting activation of apoptosis.
9. Upon osteogenic stimulation, integrin  $\alpha 2$  and  $\alpha 11$ -deficient hMSC further reduced in number and failed to mineralize the matrix.
10. Loss of integrin  $\alpha 1$  and  $\alpha 2$  led to an upregulation of the other two remaining ColI-binding integrins, whereas upon knockdown of integrin  $\alpha 11$  hMSC upregulated only integrin  $\alpha 1$ .
11. Preliminary investigation showed a tremendous downregulation of integrin  $\alpha 2$  in hMSC derived from patients suffering of osteoporosis.

In conclusion, our results strongly suggested that both integrins  $\alpha 2\beta 1$  and  $\alpha 11\beta 1$  mediate an indispensable signaling for hMSC survival. Once these receptors were ablated from cell surface, hMSC reduced their cell spreading, adhesion, migration and survival rates. Our integrin knockdown models can be used for a further investigations and understanding of the integrins  $\alpha 2\beta 1$  and  $\alpha 11\beta 1$  importance and signaling in hMSC and hOB since we observed a strong downregulation of integrin  $\alpha 2$  expression in osteoporosis.

## 7. Summary

**Introduction:** Human mesenchymal stem cells (hMSC) are easily obtainable from bone marrow and possess the ability to differentiate into osteoblasts. Therefore, they have been suggested as a suitable source for bone regeneration. HMSC are equipped with a variety of integrins that mediate essential cell-matrix interactions. Collagen I represent approximately 90% of the bone protein content. Cell attachment to collagen I is mediated by three members of the integrin receptor family named  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  and  $\alpha 11\beta 1$  integrins. The main aim of this doctoral thesis was to investigate the basal expression of those integrins in hMSC and to functionally analyze the knockdown effect of a single collagen I-binding integrin on hMSC behavior *in vitro*.

**Materials and methods:** HMSC were cultured on collagen I-coated surface. A lentiviral transfer of  $\alpha 1$ -,  $\alpha 2$ - and  $\alpha 11$ -specific shRNA was applied for downregulation of the corresponding integrin mRNA. Quantitative PCR and western blot analysis were used to assess the basal expression, knockdown efficiency and integrin compensation. Colorimetric adhesion assay was used for estimation of the extent of cells attachment. HMSC spreading and migration was observed by time lapse experiments. JC-1 staining was used for investigation of the initiation of apoptosis.

**Results:** Quantitative PCR were used to assess the basal expression of collagen I-binding integrins in three hMSC donors. We found that these integrins are differently expressed as integrin  $\alpha 11$  had the highest and integrin  $\alpha 2$  the lowest expression. Next, we applied lentiviral delivery of target-specific short hairpin RNA (shRNA) in order to knockdown each of the collagen I-binding integrins and compared them to the hMSC transduced with a sequence against a non-human gene abbreviated as shRNA control. We achieved significant downregulation (> 80%) of the collagen I-binding integrin mRNA and protein. Subsequently to the transduction, we did not noticed pronounce morphological cell changes, however, a clear decrease of  $\alpha 2$ - and  $\alpha 11$ -knockdown hMSC numbers was observed during cultivation. Using a quantitative adhesion assay, we estimated that 120 min after plating only 30% of integrin  $\alpha 11$ -deficient cells were able to attach to collagen I. In contrast, at the same time point, 70% of integrin  $\alpha 2$ -knockdown hMSC were attached while integrin  $\alpha 1$ - and shRNA control hMSC have already reached 100% cell adhesion. Furthermore, a time lapse-based investigation showed that integrin  $\alpha 1$ - and shRNA control hMSC need approximately 35 min to fully spread on collagen I. In contrast, integrin  $\alpha 2$ - and  $\alpha 11$ -knockdown hMSC took approximately double more time for spreading in comparison to shRNA control hMSC. Additionally, we analyzed the migration

capability of the four different hMSC lines. The average path which integrin  $\alpha 1$ - and shRNA control hMSC passed was approximately 170  $\mu\text{m}$  with mean speed of 11.5  $\mu\text{m}/\text{h}$ . In parallel integrin  $\alpha 2$  and  $\alpha 11$ -deficient hMSC migrated to a distance of approximately 70  $\mu\text{m}$  with a velocity of 5  $\mu\text{m}/\text{h}$ . Since it was observed a lost of  $\alpha 2$ - and  $\alpha 11$ -deficient hMSC, next we performed JC-1 staining that visualizes mitochondrial leakage, a hallmark of apoptosis. The majority of integrin  $\alpha 2$ - and  $\alpha 11$ -knockdown hMSC exhibited mitochondrial leakage whereas integrin  $\alpha 1$ - and shRNA control hMSC showed intact mitochondria. Finally, we used quantitative PCR to investigate whether there were compensatory effects between the three integrin receptors. We detect that knockdown of integrin  $\alpha 1$  led to upregulation of  $\alpha 2$  and  $\alpha 11$ . Similarly, when integrin  $\alpha 2$  was downregulated, integrin  $\alpha 1$  and  $\alpha 11$  expression increased. Interestingly, knockdown of integrin  $\alpha 11$  caused only a slight increase in integrin  $\alpha 1$  but not in  $\alpha 2$  expression. We also observed that upon osteogenic stimulation, integrin  $\alpha 2$  and  $\alpha 11$ -deficient hMSC further reduced in number and did not mineralize the matrix even on a single cell level. Moreover, our preliminary investigation in hMSC-derived from osteoporosis suffering patients showed a tremendous downregulation of integrin  $\alpha 2$ .

**Conclusions:** Our results strongly suggested that integrins  $\alpha 2\beta 1$  and  $\alpha 11\beta 1$  mediate an indispensable signaling for hMSC. Once these receptors were ablated from cell surface, hMSC reduced their spreading, adhesion, migration and survival rates. Our integrin knockdown models can be used for further investigations and understanding of the integrins  $\alpha 2\beta 1$  and  $\alpha 11\beta 1$  importance and signaling in hMSC and hOB since we observed a strong downregulation of integrin  $\alpha 2$  expression in osteoporosis.

## Zusammenfassung

Einleitung: Human mesenchymale Stammzellen (hMSC) können auf einfache Weise aus dem Knochenmark gewonnen werden und haben die Fähigkeit, sich in Osteoblasten zu differenzieren. Daher scheinen sie eine geeignete Quelle für die Regeneration von Knochen zu sein. HMSC enthalten eine Vielzahl an Integrinen, die essenzielle Zell-Matrix Wechselwirkungen vermitteln. Kollagen I macht ungefähr 90% des Proteingehalts im Knochen aus. Die Anhaftung der Zelle an Kollagen I wird durch drei Mitglieder der Integrin Rezeptor Familie, den Integrinen  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  und  $\alpha 11\beta 1$ , vermittelt. Das Ziel dieser Doktorarbeit war, die basale Expression dieser Integrine in hMSC zu untersuchen sowie eine funktionelle Analyse des Knockdown Effektes eines einzelnen Kollagen I-bindenden Integrins auf das hMSC Verhalten *in vitro* durchzuführen.

Material und Methoden: HMSC wurden auf Kollagen I beschichteten Oberflächen kultiviert. Mittels lentiviralen Gentransfers mit  $\alpha 1$ -,  $\alpha 2$ - und  $\alpha 11$ - spezifischer shRNA (short hairpin RNA) wurde für die entsprechende Integrin mRNA herunterreguliert. Quantitative PCR und Westernblot dienten dazu, die basale Expression, die Knockdown Effizienz und kompensatorische Effekte zu erfassen. Durch den Einsatz eines kolorimetrischen Adhäsionsassays wurde das Ausmaß der Zelladhäsion ermittelt. Die Ausbreitung und Migration der hMSC wurde durch Zeitraffermikroskopie beobachtet. Eine JC-1 Färbung wurde zur Untersuchung des Apoptoseverhaltens verwendet.

Ergebnisse: Mittels quantitativer PCR wurde die basale Expression von Kollagen I-bindenden Integrinen von drei hMSC Spendern ermittelt. Wir haben herausgefunden, dass diese Integrine in unterschiedlichem Maß exprimiert werden. Integrin  $\alpha 11$  zeigte die stärkste und Integrin  $\alpha 2$  die schwächste Expression. Um einen Knockdown in den jeweiligen Kollagen I bindenden Integrinen zu erreichen, wurde ein lentiviraler Transfer mit shRNA durchgeführt. Als Kontrolle dienten hMSC, welche mit einer Sequenz gegen ein nicht humanes Gen transduziert wurden, welche als shRNA Kontrolle abgekürzt wurde. Wir erzielten eine signifikante Herunterregulierung (> 80%) der Integrin-mRNA und -Proteine. In Folge der Transduktion konnten wir keine ausgeprägten morphologischen Veränderungen feststellen, jedoch stellten wir während der Kultivierung einen eindeutigen Rückgang der Zellzahl von  $\alpha 2$ - und  $\alpha 11$ -Knockdown hMSC fest. Im quantitativen Adhäsionsassay zeigte sich, dass 120 Minuten nach dem Ausplattieren nur 30% der  $\alpha 11$ -defizienten Zellen fähig waren, an eine mit Kollagen I beschichteten Oberfläche zu adhären. Dagegen waren nach der gleichen Zeitspanne 70% der  $\alpha 2$ -Knockdown hMSC an Kollagen I und bereits 100% der  $\alpha 1$ -

Knockdown und shRNA Kontroll hMSC adhären. Des Weiteren stellte sich bei einer Zeitraffermikroskopie heraus, dass  $\alpha 1$ -Knockdown und shRNA Kontroll hMSC ungefähr 35 Minuten benötigten, um sich auf Kollagen I völlig auszubreiten. Dagegen brauchten  $\alpha 2$ - und  $\alpha 11$ -Knockdown hMSC ungefähr die doppelte Zeit zum Ausbreiten. Zusätzlich untersuchten wir die Migrationsfähigkeit der Zellen. Die durchschnittliche Strecke, die  $\alpha 1$ - und shRNA Kontroll hMSC zurücklegten, betrug ca. 170  $\mu\text{m}$  mit einer mittleren Geschwindigkeit von 11.5  $\mu\text{m}/\text{h}$ , während  $\alpha 2$  and  $\alpha 11$ -defiziente hMSC ca. 70  $\mu\text{m}$  weit wanderten mit einer Geschwindigkeit von 5  $\mu\text{m}/\text{h}$ . Da ein Verlust an  $\alpha 2$ - and  $\alpha 11$ -defizienten hMSC zu verzeichnen war, führten wir zunächst eine JC-1 Färbung durch, mittels derer mitochondrische Schäden als Kennzeichen von Apoptose sichtbar gemacht werden können. Die Mehrzahl der  $\alpha 2$ - and  $\alpha 11$ -Knockdown hMSC wiesen mitochondrische Schäden auf, während  $\alpha 1$ - und shRNA Kontroll hMSC intakte Mitochondrien hatten. Zuletzt untersuchten wir mögliche kompensatorische Effekte zwischen den drei Rezeptoren mittels quantitativer PCR. Wir konnten feststellen, dass der Knockdown des  $\alpha 1$  Integrins zu einer Hochregulierung von  $\alpha 2$  and  $\alpha 11$  führte. Ein ähnliches Ergebnis zeigte sich bei der Herunterregulierung von Integrin  $\alpha 2$ , die eine erhöhte Expression der  $\alpha 1$  and  $\alpha 11$  Integrine hervorrief. Interessanterweise bewirkte der Knockdown des  $\alpha 11$  Integrins einen leichten Anstieg der  $\alpha 1$  aber nicht der  $\alpha 2$  Integrin Expression. Wir haben zudem festgestellt, dass sich die Zellzahl der Integrin  $\alpha 2$  und  $\alpha 11$ -defiziente hMSC nach osteogener Stimulierung weiter reduzierte und die Matrix auch auf Einzelzellebene nicht mineralisiert war. Außerdem zeigte unsere vorausgegangene Untersuchung mit hMSC von Osteoporosepatienten eine enorme Herunterregulierung von Integrin  $\alpha 2$ .

Zusammenfassung: Unsere Ergebnisse deuten daraufhin, dass  $\alpha 2\beta 1$  und  $\alpha 11\beta 1$  Integrine unentbehrliche Signale in hMSC vermitteln. Sobald den Zellen diese Rezeptoren fehlten, wurden die Ausbreitung, die Adhäsion, die Migration und die Überlebensraten reduziert. Insbesondere durch unsere Feststellung der Herunterregulierung der Integrin  $\alpha 2$  Expression in Osteoporosepatienten, können unsere Integrin Knockdown Modelle in weiterführenden Untersuchungen eingesetzt werden, um das Verständnis über die  $\alpha 2\beta 1$  und  $\alpha 11\beta 1$  Integrin Signalwege in hMSC und hOB auszudehnen.

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**Appendix**

Supplementary table 1. Nucleotide sequence of the used in the study PCR primer pairs.

Genes	Primer pairs	Product size, bp	Annealing temp, °C	Reference	
<b>Integrins</b>					
a1	F	ACATCAGCCAAGTCAATGTTTCG	241	51	[260]
	R	AGCATTAACAGCAACAATCCGG			
a2	F	GCTGCTGTGCATTAGATATTAG	217	48	[260]
	R	CTGTAACTTCTGGTGAAATCCT			
a3	F	ATCTTGAGAGCCACAGTCA	201	52	[260]
	R	CTGGGTCCTTCTTTCTAGTTC			
a4	F	AATGGATGAGACTTCAGCACT	278	48	[260]
	R	CTCTTCTGTTTTCTTCTTGTAGG			
a5	F	ACTAGGAAATCCATTACAGTTC	201	52	[260]
	R	GCATAGTTAGTGTCTTTGTTGG			
a6	F	CTTGGAGAAGATGGGTTTATT	213	48	[260]
	R	GAATACAGATAGGGGAGGAAA			
a11	F	TGGGCGCACCCATGTA CTTC	223	55	Self de- signed
	R	ATGGCTCCTGCGTGGTTGTC			
aV	F	GGAGCACATTTAGTTGAGGTAT	274	46	[260]
	R	ACTGTTGCTAGGTGGTAAACT			
b1	F	ATGAATGAAATGAGGAGGATTACTTCG	322	52	[260]
	R	AAAACACCAGCAGCCGTGTAAC			
b3	F	CTGCTGTAGACATTTGCTATGA	211	52	[260]
	R	GCCAAGAGGTAGAAGGTAAATA			
b5	F	CTGTGGACTGATGTTTCCTT	407	54	[260]
	R	GTATGCTGGTTTTACAGACTCC			
<b>Interferon-related genes</b>					
OAS1	F	GCTGGAAGCCTGTCAAAGAG	388	55	[261]
	R	GAGCTCCAGGGCATACTGAG			
OAS2	F	CAGGAAGTTGTGGAGCTAGG	170	49	[262]
	R	AAGGGAAGAATGGATGTGAG			
RIG-I	F	TCCTTTATGAGTATGTGGGCA	461	50	[263]
	R	TCGGGCACAGAATATCTTTG			
ISG56	F	GCCATTTTCTTTGCTTCCCCTA	330	53	[264]
	R	TGCCCTTTGTAGCCTCCTTG			
<b>House-keeping gene</b>					
GAPDH	F	CAACTACATGGTTTACATGTTC	181	50	[265]
	R	GCCGTGGCTCCACGAC			

\* F- forward, R- reverse

Supplementary table 2. Antibodies used in the study.

<b>Name</b>	<b>Manufacturer</b>	<b>Host</b>	<b>Application</b>	<b>Dilutions</b>
<b>Primary antibodies</b>				
Anti-human alpha 2	BD Bioscience	Mouse	WB and ICH	1: 200
Anti-human alpha 11	R&D systems	Rat	WB	1: 200
Anti-human alpha 11	Prof. D.Gullberg	Rabbit	ICH	1: 200
<b>Secondary antibodies</b>				
Anti-mouse HRP	Rockland	Rabbit	WB	1: 4000
Anti- mouse AF488	Invitrogen	Donkey	ICH	1: 1000
Anti-rat HRP	Santa Cruz	Goat	WB	1: 10000
Anti-rabbit AF488	Invitrogen	Goat	ICH	1: 500

\* WB – Western blotting, ICH - immunohistochemistry

**List of abbreviations**

293FT cells	Human embryonal kidney cell line (HEK)
a1 shRNA cell	HMSC transduced with shRNA sequence against integrin a1
a2 shRNA cell	HMSC transduced with shRNA sequence against integrin a2
a11 shRNA cell	HMSC transduced with shRNA sequence against integrin a11
AAV	Adeno-associated virus
Abl	Abelson murine leukemia viral oncogene
Ago2	Argonaute 2 enzyme
AR	Alizarin red solution
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2 family	Pro- or anti-apoptotic genes
bFGFR	Basic fibroblast growth factor receptor 1
Bim/ Bmf	Bcl2 modifying factor
BM	Bone marrow
BMPRIA	Bone morphogenic protein receptor type IA
BrdU	Bromodeoxyuridine
Cbl gene	Casitas B-lineage lymphoma gene
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD105	Endoglin, homodimeric transmembrane protein
CD11b	Integrin alpha M (ITGAM)
CD14	Monocyte differentiation antigen
CD146	Melanoma cell adhesion molecule (MCAM)
CD166	Activated leucocyte cell adhesion molecule (ALCAM)
CD19	B-lymphocyte antigen
CD271	Low affinity nerve growth factor receptor (LNGFR)
CD31	Platelet/endothelial cell adhesion molecule (PECAM-1)
CD34	Primitive hematopoietic progenitor monomeric cell surface antigen
CD44	Hyaluronan receptor
CD45	Protein tyrosine phosphatase, receptor type, C (PTPRC)
CD73	5'-nucleotidase (endonuclease)
CD90	Thy-1 cell surface antigen
ColI	Collagen type I
Cdks	Cyclin-dependent kinases
Crk, Nck and Grb-2	Adapter protein binds to several tyrosine-phosphorylated proteins
CXC, CC, C or CX3C	Chemokines

CXCR	CXC chemokine receptors
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbeco's minimal essential media
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA dsOligos	Double-stranded RNA oligonucleotides
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
Erk	Extracellular signal-regulated kinases
Etk	Tyrosine kinase
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
Fas	Death receptor (CD95)
FBS	Fetal bovine serum
FN	Fibronectin
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFOGER / GASGER	Collagen binding motifs
HeLa	Human cervix carcinoma cell
HGFR	Hepatocyte growth factor receptor
HIV	Human immunodeficiency virus
HLA-DR	Cell surface receptor, MHC class II
HMSC	Human mesenchymal stem cells
Hoechst 33342	Nuclear staining dye
HOB	Human osteoblasts
HSC	Hematopoietic stem cells
ICAM-1 or -2	Inter-Cellular Adhesion Molecule 1 or 2
IGFR	Insulin growth factors receptor
JC-1	5,5,6,6-tetrachloro-1,1,3,3 tetraethyl benzimidazolyl carbocyanine iodide
Jnk	c-Jun N-terminal kinases
LB media	Luria-Bertani bacterial cultivation media
LC-PCR	Light cycler polymerase chain reaction
MAPK	Mitogen-activated protein kinase
Mek	Mitogen-activated protein kinase kinase
mRNA	Messenger RNA
MSC	Mesenchymal stromal cells or mesenchymal stem cells
NEAA	Non essential amino acids

NPAG	P-nitrophenyl N-acetyl- $\beta$ -d-glucosaminide
Oligos	Oligonucleotides
Opti-MEM	Modified eagle's minimum essential media
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
pDNA	Plasmid DNA
Pen/Strep	Penicillin / Streptomycin mix
PI(3)K	Phosphoinositide 3-kinases
PKA	Membrane-associated protein kinase A
PKC	Protein kinase C
PLC $\gamma$	Phosphoinositide phospholipase C gama
PLL	Poly-L-lysine
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone -related protein
PUC	Plasmid cloning vector
PVDF	Polyvinylidene Fluoride
Pyk2	Protein tyrosine kinase 2
Ras/ Raf	GTPases, function in signal transduction as GTP/GDP-regulated switches
RGD motif	Arginine -Glycine –Asparagine motif
Rho, Rac and Cdc42	GTPases of the Rho-subfamily
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonucleic acid nuclease
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
S.O.C. media	Bacterial culture media
SDF-1	Stromal cell-derived factor-1
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
siRNA	Short-interfering RNA
Src family kinases	Proto-oncogenic tyrosine kinases
SSEA-4	Thiosulfate sulfurtransferase
Stbl3	Competent <i>E. coli</i> strain
STRO-1	Stem cell surface marker
Syk	Spleen tyrosine kinase
TGF $\beta$ RI / RII	TGF beta receptors
TOP10	Competent <i>E. coli</i> strain
U6 promoter	Constitutively expressed Polymerase III promoter

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VCAM-1	Vascular cell adhesion molecule 1 (CD106)
VEGFR	Vascular endothelial growth factor receptor
aMEM	Eagle's minimum essential medium

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**Publications**

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