Impact of the CCN-proteins CYR61/CCN1 and WISP3/CCN6 on mesenchymal stem cells and endothelial progenitor cells

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
an der Bayerischen Julius-Maximilians Universität Würzburg

vorgelegt von

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Würzburg, 2007

Eingereicht am……

Mitglieder der Promotionskommission
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Publications

Regular papers

Schutze N, Kunzi-Rapp K, **Wagemanns R**, Noth U, Jatzke S, Jakob F.: Expression, purification, and functional testing of recombinant CYR61/CCN1.Protein Expr Purif. 2005 Jul;42(1):219-25.

Schütze N, **Schenk R**, Fiedler J, Mattes T, Jakob F, Brenner RE. CYR61/CCN1 and WISP3/CCN6 are chemoattractive ligands for human multipotent mesenchymal stroma cells. (2007) manuscript submitted to BMC Cell Biol (in revision).

Schenk R., Hofmann A., Eck M., Schmaußer B., Jatzke S., Nöth U., Jakob F. and Schütze N.: Improved *ex vivo* propagation of endothelial progenitor cells by the angiogenic inducer CYR61/CCN1 – implications for bone regeneration (2007) manuscript submitted to Bone

Schenk R., Jatzke J., Nöth U. , Jakob F. and Schütze N.: Angiogenic differentiation of MSC by the extracellular matrix protein CYR61/CCN1 (2007) in preparation

Schenk R, Jatzke S, Klein-Hitpass L, Nöth U, Jakob F, Schütze N. WISP3/CCN6 regulates genes related to anti-angiogenesis, cell survival and interferon response. (2007) manuscript submitted to BBRC

Schütze N., **Wagemanns R.**, Schilling T., Jakob F.; (2006), CCN1. UCSD-Nature Molecule Pages, doi: 10.1038/mp.a003362.01 (online publication).

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Chapter 1

Introduction

1.1 The family of CCN-proteins

The CCN-family is a structural family of secreted proteins that represents extracellular matrix signalling molecules and contains six distinct members. The abbreviation is deviated from the three first members of the family as there are: Cysteine-rich protein 61 (CYR61/CCN1), Connective tissue growth factor (CTGF/CCN2) and Nephroblastoma overexpressed (NOV/CCN3). The other members are wnt1 inducible signalling pathway proteins 1, 2 and 3 (Pennica 1998; Lau and Lam 1999; Perbal 2001; Brigstock 2003; Brigstock 2003).

Characteristic for the CCN-family is the highly conserved structure. Over all they share 30-50 % amino acid sequence identity including 38 highly conserved cysteine residues (less in the case of WISP2), which add to 10 % of the protein mass. N-terminal the proteins contain a secretory sequence, followed by four conserved modules. Domain I shares similarities to the insulin growth factor (IGF) binding proteins and is therefore called IGF-binding protein domain. Domain II is named von Willebrand Type C, domain III thrombospondin-1 domain and the C-terminal domain containing a putative cysteine knot is termed CT domain. Apart from WISP2/CCN5, which lacks the C-terminal domain, all members share the same structure (Lau and Lam 1999; Brigstock 2003). Each domain is encoded by a distinct exon. Therefore, it is suggested that the CCN gene family arose by exon shuffling in evolution (Lau and Lam 1999). The modular structure of the CCN-family is illustrated in fig 1.1.

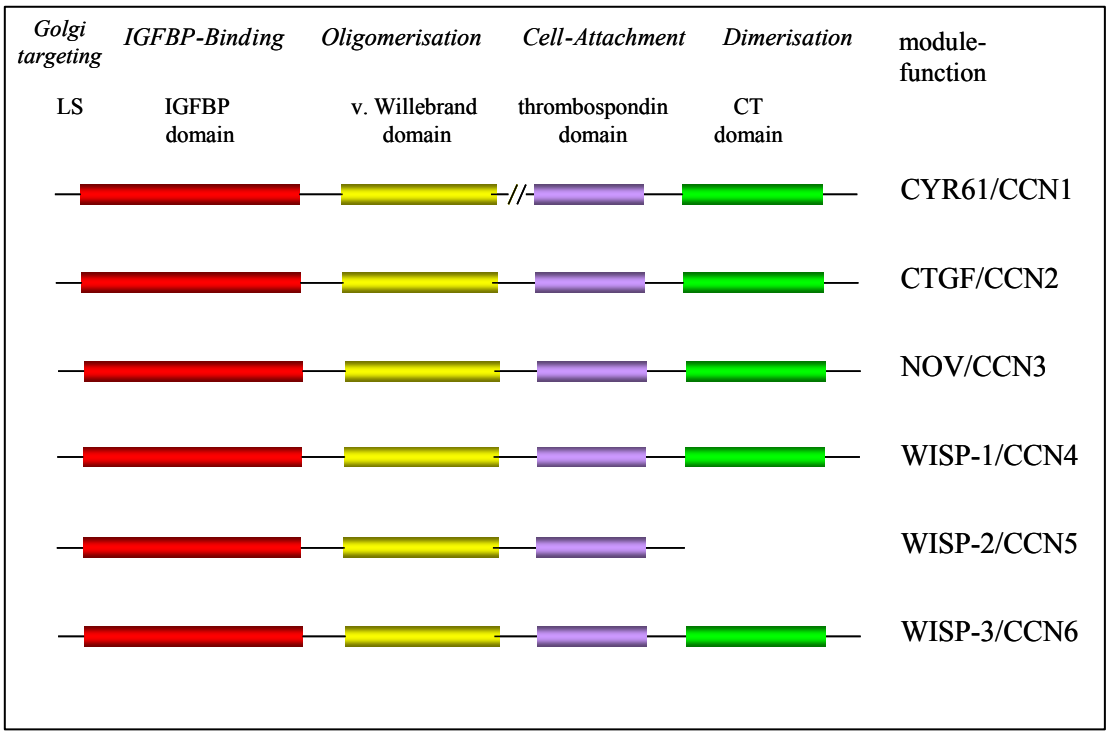


Fig. 1.1: Modular structure of the CCN-family

CCN proteins regulate many cellular processes such as adhesion, migration, mitogenesis, growth arrest, differentiation, survival, apoptosis and angiogenesis in different cell types. The proteins are multifunctional; therefore the property of one CCN protein can be opposed to the property of another. These non-redundant roles of CCN-proteins can be explained by the different domains, each contributing to another function. Additionally, CCN proteins do not bind to specific signal transducing receptors in a classical sense, but rather behave as adaptor proteins that – among others - target integrins, which are also used by different growth factors and cytokines. Beside the integrin binding, CCN proteins exhibit a strong affinity to heparin and can be localized in the extracellular matrix in association to heparin sulphate proteoglycans (Lau and Lam 1999; Brigstock 2003; Kubota and Takigawa 2007).

Due to their multifunctional properties and expression in many different cell types and tissues, CCN proteins are promising molecules in prognostic and diagnostic procedures as well as therapeutical applications (Lau and Lam 1999; Brigstock 2003; Kubota and Takigawa 2007). The CCN-proteins CYR61 and WISP3 are expressed in various cells and tissues of the musculoskeletal system and are therefore of special interest in this field.

1.2 The cysteine-rich protein 61 (CYR61/CCN1)

The cysteine-rich protein (CYR61/CCN1) was first described as an immediately-early induced gene in mouse fibroblasts. The human gene is localised on chromosome 1p31-p22. The protein sequence contains 382 aa, resulting in a 42 kDa product (Lau and Nathans 1987; Brigstock 1999; Lau and Lam 1999). The cysteine-rich protein 61 (CYR61/CCN1) is known as an angiogenic inducer (Brigstock 2002; Kubota and Takigawa 2007). This was proven in activated endothelial cells (Leu 2002), in the corneal implant assay (Babic 1998), in a rabbit ischemic model (Fataccioli 2002) and in the chick chorioallantoic membrane (CAM) assay (Schutze 2005). Interestingly the well-established angiogenic stimulator vascular endothelial growth factor (VEGF) was shown to induce CYR61 in osteoblasts and human umbilical vein endothelial cells (Abe and Sato 2001; Athanasopoulos 2007). Other growth factors such as the fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) (O'Brien 1990), epidermal growth factor (EGF) (Leng 2002) and the transforming growth factor (TGF-β) induce mRNA expression of CYR61, as well as serum which contains many of the previously mentioned factors in different amounts (Lau and Lam 1999). Furthermore CYR61 expression is induced by hormones, vitamin D3, the bone morphogenic protein-2 (BMP-2), cortisol and mechanical stress (Schutze 1998; Brigstock 2002; Grote 2004; Chaqour and Goppelt-Struebe 2006; Parisi 2006).

Besides the angiogenic function of CYR61, the promotion of cell proliferation, apoptosis, migration, differentiation, adhesion and cell survival in different cell types (Kireeva 1996; Lau and Lam 1999; Brigstock 2003; Todorovicc 2005) has been described. Furthermore, CYR61 enhances the cytotoxicity of TNF-α in fibroblasts. The induction is mediated by binding to the integrins $\alpha_v\beta_5$ and $\alpha_6\beta_1$ as well as to syndecan-4 (Chen 2007). The binding of CYR61 to the integrin $\alpha_v\beta_3$ in endothelial cells results in the induction of angiogenic properties as there are adhesion, migration and tubule formation (Leu 2002). Enhanced adhesion of monocytes is mediated by CYR61 binding to $\alpha_M\beta_2$ (Schober 2002). Platelet adhesion is stimulated by CYR61 binding to $\alpha_{IIb}\beta_3$ (Jedsadayanmata 1999). Beside the activation of these five integrin receptors, CYR61 function is also mediated by binding to heparan sulphate proteoglycans (HSP) (Lau and Lam 1999).

As CYR61 is an angiogenic protein it has been revealed to be highly expressed in tumour cells of breast cancer with poor prognosis and high metastatic potential and breast cancer cell

lines (Tsai 2002; Jiang 2004; Xie 2004). In association with the healthy and positive aspects of angiogenesis the protein was found to be expressed at sites of wound healing (Chen 2001). CYR61 is essential for embryonic development as CYR61 null mice suffer from placental vascular insufficiency and compromised embryonic vessel integrity and are therefore lethal in an early phase of development (Mo 2002). Another phenotype of the CYR61 null mice is the development of atrioventricular septal defects (AVSD), which was also discovered in $CYR61^{+/-}$ mutants (Mo and Lau 2006). The finding that CYR61 is highly expressed in cardiomyocytes after myocardial infarction but was almost absent in healthy humans further reveals the relevance of CYR61 for the cardiovascular system (Hilfiker-Kleiner 2004).

The role of CYR61 for the musculoskeletal system is substantiated by the expression of the protein in muscle, bone, chondrocytes, mesenchymal stem cells (MSCs), osteoblasts as well as in osteoblast cell lines such as hFOB (Schutze 1998; Brigstock 1999; Lechner 2000; Parisi 2006) and in the facture callus of mice, rats and sheep (Hadjiargyrou 2000; Lienau 2006; Athanasopoulos 2007). CYR61 gene expression is induced by Wnt3A stimulation (Si 2006), a regulation factor for the osteoblast differentiation of MSCs. Together with the finding that CYR61 inhibits osteoclast differentiation (Crockett 2007), the protein is a potential regulator of bone remodelling. However, the underlying molecular mechanism of CYR61 function in the musculoskeletal system is not fully understood and will be investigated in this study.

1.3 The wnt1 inducible signalling pathway protein 3 (WISP3/CCN6)

WISP3/CCN6 (wnt1 inducible signalling pathway protein 3) is a matrix associated signal molecule with 354 aa resulting in a length of approximately 39 kDa. The gene maps to chromosome 6q21-22 (Kleer 2007).

Among the family of CCN proteins, WISP3 is the only member which is associated to a specific inherited human disease. Mutations of the gene results in two diseases, the progressive pseudorheumatoid dysplasia (PPD) (Hurvitz 1999) and the juvenile idiopathic arthritis (JIA) (Lamb 2005). PPD is an autosomal recessive skeletal disorder which is asymptomatic in the early childhood. Between the age of 3 and 8 patients experience pain and joint swelling resulting in cartilage loss and destructive bone change over the years and often followed by joint replacement surgeries as early as in the third decade of life (Hurvitz 1999). Extraskeletal manifestations of PPD have not been reported. Fourteen mutations responsible for the development of PPD are spread over the whole WISP3 gene in different exons, indicating that every exon is necessary for the protein function. The mutation C52X in the WISP3 protein is found in many patients and might therefore be the ancestral mutation (Delague 2005). A very recent study revealed that heterozygote mutations of the WISP3 gene result in the manifestation of the disease (Zhou 2007). JIA specially the polyarticular-course JIA has similar symptoms as PPD. A potentiality to distinguish both diseases is the inflammatory nature of JIA. The G84A mutation of the WISP3 gene has been described in patients with polyarticular-course JIA (Lamb 2005). Mice models with mutations accordingly to the human WISP3 mutations did not develop the PPD phenotype, nor has overexpression of WISP3 an effect on cartilage size or bone formation. The authors suggest that mice with a much smaller cartilage thickness compared to humans and a higher regeneration potential are not the appropriate model for comparison (Kutz 2005). In a very recent work it was shown that loss of function mutations of the WISP3 gene in zebrafish resulted in affected shape and strength of pharyngeal cartilage. Specifically the overexpression of WISP3 inhibited the BMP and wnt signalling in developing zebrafish. The authors suggest that a dysregulation of BMP and/or wnt signalling contributes to the human disease PPD (Nakamura 2007).

WISP3 mRNA levels are highly upregulated in synovial tissue of rheumatoid arthritis (RA) patients, whereas the protein level remains unchanged compared to normal tissue. Cultured fibroblast-like synoviocytes show the same behaviour (Cheon 2004).

WISP3 expression has been detected in different cell types and tissues as there are mesenchymal stem cells (MSCs) (Schutze 2005), chondrocytes, kidney, testis, placenta, ovary, prostate and small intestine (Sen 2004). In the chondrocyte cell line C28I2 WISP3 has been shown to regulate collagen type II and aggrecan expression via the SOX9 pathway (Sen 2004). Superoxide dismutase expression and activity is promoted both by recombinant WISP3 treatment and WISP3 transduction of C28I2 chondrocytes (Davis 2006). Reactive oxygen species (ROS) levels are low in cells expressing WISP3, whereas mutations of the WISP3 gene lead to an accumulation of ROS and therefore induced oxidative stress (Miller and Sen 2007), which indicates that WISP3 plays a protective role in tissue homeostasis.

WISP3 is known as an inhibitor of angiogenesis (Kleer 2004). In inflammatory breast cancer the expression of WISP3 is lost in 80% of the cases (Zhang 2005). This lead to an induction of VEGF and therefore induced tumour development. Restoration of WISP3 expression in inflammatory breast cancer cells resulted in a decrease of angiogenic factors. Therefore, the protein is discussed to have growth-, invasion-, and angiogenesis-inhibitory functions in inflammatory breast cancer *in vitro* and *in vivo* (Kleer 2004). Frameshift mutations in the WISP3 gene were found in colorectal carcinomas of patients with bad clinical outcome, indicating the protective role of the full-length protein towards cancer related angiogenesis (Thorstensen 2005).

WISP3 is expressed in chondrocytes and MSCs (Schutze 2005) and mutations of the gene results in the bone and joint disease PPD. Although the pathophysiology of PPD is unknown and the role of WISP3 in MSCs is not completely understood, the protein seems to be of high relevance for the musculoskeletal system.

1.4 Angiogenesis

Angiogenesis is defined as the formation of new blood vessels by sprouting from pre-existing blood vessels and is also designated as neovascularisation. By contrast vascularisation is defined as the de novo development of blood vessels (Felmeden 2003). This process mainly occurs during embryonic development. The process of angiogenesis is important for embryonic development but occurs also during adulthood in repair processes as wound and bone fracture healing and female reproductive cycle. Increased angiogenesis is observed in numerous diseases such as psoriasis, diabetic retinopathy, rheumatoid arthritis (RA) and tumour development (Risau 1997; Carmeliet 2003; Felmeden 2003; Costa 2004). Insufficient

angiogenesis is also an undesirable event because it leads to diseases like ischemia, stroke, restenosis, artherosklerosis, osteoporosis and impaired fracture healing (Carmeliet 2003). Angiogenesis is a tightly regulated process by activators and inhibitors. A well established angiogenic inducer is the vascular endothelial growth factor (VEGF). Other essential factors are the fibroblast growth factors (FGFs) and angiopoietins together with their receptors (Felmeden 2003). External stimulators of angiogenesis are hypoxia and shear stress.

Basically, blood vessels consist of endothelial cell monolayers surrounded by mural cells as there are pericytes, smooth muscle cells and fibroblasts in a basement membrane (Griffioen and Molema 2000). First step of the vessel sprouting process is the proteolytic degradation of the basement membrane matrix followed by the migration of endothelial cells towards the angiogenic stimulus. Simultaneously, the endothelial cells start to proliferate, form the lumen and maturate into the functional endothelium (Risau 1997). Many of these developing steps are mediated by VEGF and its receptors Flt-1 (VEGF-receptor 1) and KDR (VEGF-receptor 2). Angiopoietin-2 (Ang-2) destabilizes the basement membrane in the beginning of the process, while Angiopietin-1 (Ang-1) is responsible for the stability and maintenance of the blood vessels after angiogenesis is terminated. Both factors bind to the receptor Tie-2 (Fiedler and Augustin 2006). Other angiogenic inducers like FGF and PDGF (platelet derived growth factor) regulate the migration and proliferation of the endothelial cells (Risau 1997).

Over 30 years ago Judah Folkman postulated that tumour growth is dependent upon angiogenesis (Folkman 1972). Tumour cells express angiogenic activators in order to connect to the oxygen and nutrient supply of the body, afterwards the cells are able to grow to larger tumours. The process is called “angiogenic switch” of tumour cells. New concepts in cancer therapy try to prevent this process. Therefore, angiogenesis activator analogues and antibodies e.g. the anti-VEGF antibody “Avastin” are currently under investigation (Carmeliet 2005). Another approach in cancer therapy uses angiogenesis inhibitors such as endostatin (Folkman 2006). Clinical studies are promising particularly when the therapy is combined with conventional chemotherapies (Carmeliet 2005; Folkman 2006).

Cell based tissue engineered constructs are promising as replacement and treatment of diseased organs. The sizes of the constructs are limited by insufficient nutrient and oxygen supply of the cells within the constructs by diffusion. Therefore angiogenesis is a crucial point in order to supply cells in a tissue engineered construct.

1.5 Bone repair

Bone is a living tissue which is continuously subject to degradation and construction processes. Therefore bone fractures often heal easily in a relatively short time and without external addition of stimulators like growth factors. But if the fracture exceeds a critical dimension (>10 mm gap size), the regeneration potential of bone is low, due to insufficient nutrient and oxygen supply of the regenerative cells (Petite 2000; Logeart-Avramoglou 2005). Fracture repair occurs in four overlapping phases (fig.1.2). Bone fracture is associated with the damage of the containing blood vessels. Their damage leads to a coagulation cascade and the formation of a haematoma, enclosing the fracture side. Angiogenesis is initiated by inflammatory cells, fibroblasts and stem cells in response to released cytokines and growth factors. Soft callus or external callus formation occurs in the meantime by intramembranous ossification of the periosteum. Afterwards the internal callus becomes mineralized and the hard callus of woven bone is formed. In the final phase of bone remodelling the fracture callus is replaced by secondary lamellar bone, the swelling due to the haematoma is degraded and the vascular supply returns to the normal degree (Carano and Filvaroff 2003).

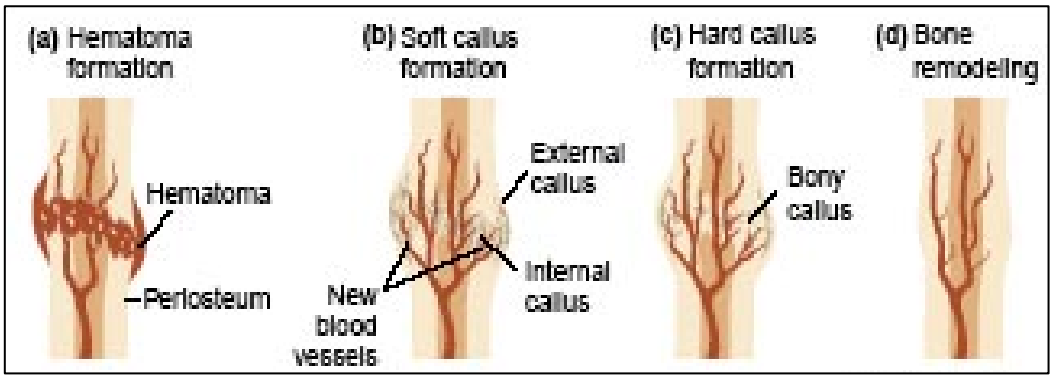


Fig. 1.2: Mechanism of bone repair (Carano and Filvaroff 2003)

Angiogenesis is one of the first steps after bone injury and therefore a crucial step of the repair cascade (Street 2002; Brandi and Collin-Osdoby 2006). Additionally to this early observation it has been shown that many osteogenic factors like BMPs, FGFs and TGF-β also stimulate angiogenesis. Some of these factors do not stimulate angiogenesis directly but via induction of VEGF expression (Carano and Filvaroff 2003). This further strengthens the view of osteogenesis and angiogenesis as closely related processes.

Different bone tissue engineering approaches have been made to improve fracture healing and bone regeneration in critical situations. Many of them use scaffolds composed of organic or

synthetic biomaterials like coral exoskeleton (Petite 2000), poly lactic acid (PLA) (Georgiou 2007), hydroxyapatite-tricalcium phosphate (HA-TCP) or ε-caprolacton (Schantz 2002) and apply growth factors and/or stem cells onto the material *in vitro* (Logeart-Avramoglou 2005). The tissue engineered construct then is implanted into the fracture side in order to replace the damaged bone. Due to the nonexistent vasculature of the construct, nutrient and oxygen supply of the cells occur only by diffusion, hence massive cell death takes place before the cells mineralize the construct and connect to the remaining bone ends (Logeart-Avramoglou 2005). Since these applications so far revealed limited success, new approaches inducing the stimulation of vascularisation of the bone construct using cellular procedures with cells and/or angiogenesis stimulating proteins are needed to improve existing therapeutical procedures.

1.6 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are a subgroup of adult stem cells. The name is derived from the embryonic development. The blastocyst develops three layers of different totipotent cells; ectoderm, endoderm and mesenchyme. Ectodermal cells design the neuronal system, endodermal cells differentiate into inner organs and mesenchymal cells develop the musculoskeletal system with bone, cartilage, muscle, tendon, ligament, dermis, blood vessels and fat (Young and Black 2004). The main characteristics of MSCs are self-renewal and the differentiation capacity (Tuan 2003; Young and Black 2004; Lakshmipathy and Verfaillie 2005; McCulloch and Till 2005; Ulloa-Montoya 2005). Stem cells remain quiescent in the stem cell niche, upon growth factor signalling they start to proliferate. Cell division occurs asymmetrical, producing an identical daughter cell and a second cell with a lineage specific differentiation program (Tuan 2003; Baksh 2004). In opposite to embryonic stem cells, MSCs undergo telomerase shorting and underlie therefore the Hayflick law with the capacity of up to 50 population doublings (Baksh 2004; Grove 2004).

Although other sources of MSCs like fat (Cao 2005), dental pulp (Sloan and Smith 2007) trabecular bone (Noth 2002), placenta (Nishishita 2004) and blood vessels (da Silva Meirelles 2006) are available, bone marrow is the most often used source due to the relative easy accessibility and the enriched population of stem cells (Tuan 2003).

MSCs are usually obtained from bone marrow using extensive washing or gradient centrifugation steps. The whole fraction of isolated cells is cultured in media containing foetal calf serum. Distinction of MSCs from other cells occurs via plastic adherence and fibroblast-

like morphology (Pittenger 1999; Baksh 2004). A single cell marker for MSCs is still missing (Tuan 2003). Some researchers use fluorescence activated cell sorting (FACS) and magnetic bead sorting techniques for certain surface markers to select MSCs (Baksh 2004). The expression of the markers SH2, SH3, CD90 and CD44 together with the absence of expression of the haematopoietic and macrophage markers CD14, CD34 and CD45 are discussed (Pittenger 1999). Another popular marker for MSCs is the stromal cell precursor antibody Stro-1 (Tuan 2003), CD146, previously a marker for mature endothelial cells, received recently attention (Bianco P, personal communication). Many researchers characterise their MSCs retrospectively by differentiation into adipocytes, osteocytes and chondrocytes (Pittenger 1999; Noth 2002; Schutze 2005; Schilling 2007). Differentiation protocols vary slightly. In addition to other substances some research groups prefer the usage of dexamethasone or morphogenic proteins like BMP-2 for the osteogenic differentiation. The most important substances for chondrogenic differentiation are the transformation growth factors TGF-β_1 and TGF-β_3, respectively (Noth 2002; Tuan 2003).

Beside the differentiation to bone, fat and cartilage, MSCs were differentiated to muscle (Dezawa 2005), endothelial cells (Oswald 2004), tendon (Awad 2003), ligament (Hairfield-Stein 2007) and skin. Additionally, the marker expression of non-mesenchymal lineages e.g. of the nervous system and the inner organs of the endodermal linage has been reported (Grove 2004). The differentiation capacity *in vivo* and *in vitro* lead to the interest of researchers for MSCs in cell based applications (Tuan 2003; Baksh 2004; Lakshmipathy and Verfaillie 2005; Ulloa-Montoya 2005). In the field of tissue engineering MSCs are seeded onto scaffolds as there are hydroxyapatite-tricalcium phosphate and poly lactic acids, differentiated into the osteogenic lineage and used as bone replacement (Bianco and Robey 2001; Tuan 2003).

Beneficial for tissue engineering is the fact that MSCs are autologous material, therefore no immunoreactivity after transplantation or ethical constraints occur (Baksh 2004; Young 2005). Another interesting approach to use MSCs are gene therapy applications. Viral transduction occurs typically via adenovirus gene transfer (Bianco 2001; Baksh 2004).

Since angiogenesis is a crucial point in bone repair and cell based tissue engineered approaches for bone reconstruction, the use of cells processing microvessels and neovascularisation within the construct would be an improvement. Therefore cells, such as MSCs, with the potential to obtain angiogenic properties are needed.

1.7 Endothelial progenitor cells (EPC)

Endothelial progenitor cells (EPCs), a new source of stem cells derived from peripheral blood, were described as CD34 positive cells (Asahara 1997). These cells are of high interest since their identification resulted in the formation of a new concept of vasculogenesis in the adult. Furthermore, EPCs could be of therapeutical value in the treatment of ischaemic diseases. Subsequently, it has been shown that EPCs are mobilised from the bone marrow in response to tissue ischemia and vascular injury and differentiate into endothelial cells (Urbich and Dimmeler 2004). Mobilisation of EPCs can also be achieved by patient treatment with GM-CSF (granulocyte macrophage colony stimulating factor), statins, VEGF, erythropoietin and estrogens (Aicher 2005). Early observations showed that EPCs home to sides of injury, e.g. tissue ischemia (Asahara 1999), or wounds, resulting in a more rapid healing process. A growing body of evidence suggests that EPCs have high potential to stimulate neovascularisation in regenerative tissues (Costa 2004; Finkenzeller 2007). Therefore new approaches of tissue engineered bone constructs use EPCs in order to induce neovascularisation in the construct. (Fuchs 2006; Krenning 2007). Also bioartificial small calibre vascular conduits were seeded with EPCs to obtain sufficient functionality (Aper 2007). EPCs are highly discussed in the cardiovascular field as a treatment option for patients suffering acute myocardial infarction. A clinical study (TOPCARE-AMI) showed good regeneration results of the ischemic region one year after application (Schachinger 2004). Another discussed issue in this field is the genetic modification of EPCs (Murasawa 2002; Hristov and Weber 2004) and their application as a treatment tool in cancer therapy due to the homing to ischemic regions and therefore cancer cells in the body.

Despite the progress of the past years the actual characterisation of the EPC cell type is not completely resolved due to differences in reported marker expressions (Zammaretti and Zisch 2005). Thus, terms such as “circulating endothelial cells” (CEC), “early endothelial progenitor cells” (eEPC), “endothelial outgrowth cells” (EOC) and “circulating angiogenic cells” (CAC) have been suggested. This heterogeneity originates from different isolation and culture conditions (Ingram 2005; Khan 2005; Blann and Pretorius 2006). Nevertheless, major conditions have been developed to characterise EPCs. EPCs are defined by the capacity of non-endothelial cells to adapt an endothelial phenotype (Urbich and Dimmeler 2004). There is evidence that EPCs and haematopoietic stem cells are derived from a common precursor, the haemangioblast, which is defined by the surface markers CD34, CD133 and KDR (also

known as vascular endothelial growth factor receptor 2) (Urbich and Dimmeler 2004; Zammaretti and Zisch 2005). Therefore, EPCs are characterised by these markers (Masuda and Asahara 2003; Urbich and Dimmeler 2004) and by the uptake of acLDL as well as positive staining for ulex lectin.

Beneficial for EPC based cellular therapies is the easy access to the circulating cells compared to the isolation of stem cells from bone marrow. A major draw-back, however, is the small number of cells in circulating peripheral blood (between 0.01- 0.0001 % of mononuclear cells) (Blann and Pretorius 2006) and the low EPC cell number that can be isolated. Thus, a stimulating factor, able to induce EPC cell number *in vitro*, is urgently needed to obtain a sufficient number of cells for clinical applications.

1.8 Aim of the study

Mesenchymal stem cells (MSCs) have the potential to differentiate into osteoblasts and other cells associated to the musculoskeletal system. Endothelial progenitor cells (EPC) home to ischemic regions and induce neovascularisation, which is a crucial process for sufficient nutrient and oxygen supply of tissue engineered bone constructs. Therefore, both cell types MSCs and EPCs are of high relevance for bone remodelling approaches.

The cysteine-rich protein 61 (CYR61/CCN1) is known as an angiogenic inducer and is expressed in numerous bone related cells and tissues as for example osteoblasts and bone fracture callus. Thus, this protein could be a candidate factor to improve bone regeneration. This study aims to explore the influence of CYR61 treatment on EPC and the angiogenic differentiation of MSCs in order to create a suitable cell culture system for bone remodelling.

The wnt1 inducible signalling pathway protein 3 (WISP3/CCN6) is expressed in chondrocytes and influences the chondrogenic differentiation of chondrocyte cell lines. Furthermore loss of function mutations of the WISP3 gene lead to the inherited disease PPD, which is characterised with advanced cartilage loss and destructive bone change without inflammatory processes. Since the pathophysiology of this disease is still unknown due to the lack of understanding of WISP3 action at the molecular level, this study aims to analyse the role of WISP3 in MSCs. Therefore MSCs were treated with recombinant WISP3 protein in order to investigate the influence of WISP3 on the global gene expression pattern of MSCs.

Specific aims of this thesis are:

- To establish the expression and purification of CYR61 and WISP3
- To prove that CYR61 and WISP3 recombinant protein are functional
- To elucidate the impact of CYR61 on EPCs
- To prove that the EPC phenotype is not altered by CYR61 treatment
- To elucidate the underlying effect of CYR61 impact on EPCs
- To establish angiogenic differentiation of MSCs
- To investigate the angiogenic potential of CYR61 on MSCs
- To identify MSCs as target cells for WISP3 action
- To analyse the global gene expression pattern dependent on WISP3 in MSCs
- To extract biology from affymetrix data
- To evaluate the potential of WISP3 for application procedure

Chapter 2

Improved *ex vivo* propagation of endothelial progenitor cells by the angiogenic inducer CYR61/CCN1 – implications for bone regeneration

2.1. Abstract

The cysteine-rich protein 61 (CYR61/CCN1) is a matricellular signalling protein of the CCN-family and the expression sites correlate to bone regeneration. A proangiogenic effect of CYR61 on endothelial cells has been revealed. Since osteogenesis and angiogenesis are closely linked, endothelial progenitor cells (EPCs) isolated from human peripheral blood are a promising tool for applications in tissue engineering of bone and beyond. A major limitation until today is the small number of EPCs which can be obtained from one patient. Here we describe the improved *ex vivo* propagation of EPCs obtained from peripheral blood in the presence of CYR61 [0.5 µg/ml]. Cell number achieved was increased up to 7-fold compared to unstimulated control cells. CYR61 treated and control cells were characterised using FACS analysis, immunocytochemistry, uptake of acLDL and concurrent staining for ulex lectin. Both CYR61 treated and untreated cells show the same EPC marker expression of CD34, CD133 and KDR, demonstrating that CYR61 treatment does not affect EPC phenotype. We conclude that the angiogenic inducer CYR61 represents a promising factor for improved *ex vivo* propagation of EPC cell number in short time. This finding could be supportive in cell based applications to stimulate bone regeneration and healing.

2.2 Introduction

Bone reconstruction with cell based therapies of tissue engineering is a growing field in orthopaedic research. Critical size defects do not heal due to the insufficient oxygen and nutrient supply and waste removal of the cells within the biomaterial in tissue engineering applications (Petite 2000). Mass transfer depends on diffusion until the graft is vascularised, therefore many cells die within few days (Logeart-Avramoglou 2005). Hence, to stimulate blood vessel invasion is a key task in bone repair. Many osteogenic factors such as bone morphogenic protein 2 and 4 and fibroblast growth factor 1 and 2 have also angiogenic properties, further tighten the view that angiogenesis and osteogenesis are closely related processes during bone repair (Carano and Filvaroff 2003). A growing body of evidence suggests that endothelial progenitor cells (EPCs) possess high potential to stimulate vascularisation in regenerative tissues. EPCs, a new source of stem cells derived from peripheral blood, were described as CD34 positive cells (Asahara 1997). Subsequently, it has been shown that EPCs are mobilised from the bone marrow in response to tissue ischemia and vascular injury and are able to differentiate into endothelial cells (Urbich and Dimmeler 2004). Their potential has been revealed *in vitro* and *in vivo* e.g. for replacement of segmental bone defects with biomaterials (Logeart-Avramoglou 2005). Also bioartificial small calibre vascular conduits were seeded with EPCs to obtain sufficient functionality (Sreerekha and Krishnan 2006; Aper 2007). Clinical studies using EPCs for treatment of ischemic regions in myocardial infarction are under investigation (Schachinger 2004). The genetic modification of EPCs (Murasawa 2002; Hristov and Weber 2004; Shaw 2004) and their application as a treatment tool in cancer therapy due to the homing to ischemic regions and therefore to cancer cells in the body is recently discussed.

EPCs are defined by the capacity of non-endothelial cells to adapt an endothelial phenotype (Urbich and Dimmeler 2004). CD14 positive monocytes (Fernandez Pujol 2000; Rehman 2003; Romagnani 2005) are discussed to achieve endothelial function. There is evidence that EPCs and haematopoietic stem cells are derived from a common precursor, the haemangioblast, which is defined by the surface markers CD34, CD133 and KDR (also known as vascular endothelial growth factor receptor 2). Therefore, EPCs are characterised by the expression of these markers (Hristov and Weber 2004; Urbich and Dimmeler 2004; Khan 2005; Shantsila 2007) as well as the uptake of acLDL (acetylated low density lipoprotein) and concurrent staining for ulex lectin.

Beneficial for EPC based cellular therapies is the easy access to the circulating cells compared to the isolation of stem cells from bone marrow. A major drawback, however, is the small number of cells in circulating peripheral blood (between 0.01- 0.0001 % of mononuclear cells) (Khan 2005; Blann and Pretorius 2006) and the low EPC number that can be isolated from donors. Thus, a stimulating factor, able to induce EPC number *in vitro*, is urgently needed to obtain a sufficient number of cells for clinical applications in the field of bone regeneration and in ischemic conditions.

The angiogenic inducer cysteine-rich protein 61 (CYR61/CCN1) is a matrix-associated secreted signal molecule. The protein belongs to the structural family of CCN-proteins. Family members have a highly conserved modular structure with 4 domains and 38 conserved cysteine residues (Lau and Lam 1999; Brigstock 2002; Schutze 2005; Chen and Du 2006). These proteins generally function in cell growth and differentiation in an overlapping, yet distinct manner and act cell- and tissue-specific suggesting non-redundant roles (Perbal 2004; Rachfal and Brigstock 2005; Leask and Abraham 2006). CYR61 expression was found in a variety of cell types including mesenchymal stem cells (MSCs), fibroblasts, chondrocytes, osteoblasts and endothelial cells (Lau and Lam 1999; Brigstock 2002; Schutze 2005; Chen and Du 2006). The angiogenic potential of CYR61 has been proven in endothelial cells and in the CAM-assay (Schutze 2005). *In vitro* the CYR61 protein has a proliferative effect on MSCs, osteoblasts and the endothelial cell line EA hy 926 (Schutze 2005). High CYR61 expression was also found in conditions of wound healing, tissue repair, fracture healing and ischemia (Tsai 2002). The protein is induced by a variety of stimuli as VEGF, TNF-α, bFGF, TGF-β and hormones like estrogens and vitamin D (Schutze 1998; Lau and Lam 1999; Brigstock 2002; Hilfiker-Kleiner 2004; Athanasopoulos 2007). Cell adhesion, migration and proliferation of fibroblasts are promoted by CYR61 (Mo and Lau 2006). The functional deletion of CYR61 in mice is embryonically lethal due to insufficient vascular development (Mo 2002), while the heterozygous animals suffer from atrioventricular septal defects (Mo and Lau 2006). Due to the early death of the animals no relevance for bone development was demonstrated in this model, but expression of the CYR61 protein appears to be associated with fracture healing in mice, rats and sheep (Hadjiargyrou 2000; Lienau 2006; Athanasopoulos 2007).

The molecular action of CYR61 relies on binding to several proteins such as integrins and surface heparin sulphate proteoglycans (Lau and Lam 1999; Chen and Du 2006). The binding

of CYR61 to endothelial cells appears to be mediated via the integrin $\alpha_v\beta_3$. Also the integrins $\alpha_v\beta_5$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$ and $\alpha_M\beta_2$ serve as binding partners in a cell specific manner for the influence on fibroblasts and monocytes (Lau and Lam 1999; Schober 2002). The inhibition of osteoclastogenesis is not mediated via the integrins $\alpha_v\beta_3$ and $\alpha_6\beta_1$ (Crockett 2007), indicating that not all CYR61 effects are integrin dependent. CYR61 appears to be an integrator of cellular processes and signal transduction pathways.

In this study we show that treatment with 0.5 µg/ml CYR61 is able to enhance *ex vivo* the EPC number obtained from peripheral blood up to 7-fold without altering EPC phenotype. The CYR61 mediated *ex vivo* propagation is stimulated by improved adhesion of mononuclear cells and further differentiation to EPCs. A sufficient number of EPCs is needed in different applications e.g. the formation of new blood vessels on biomaterials and tissue engineering and the treatment of cardiovascular disorders. Therefore treatment with the CYR61 protein could be a powerful tool for *ex vivo* propagation of EPCs.

2.3 Methods

Volunteers

Written consent was obtained from each volunteer prior to the isolation of EPCs. The local ethical committee of the University of Würzburg approved the isolation of EPCs from peripheral blood. The 24 donors were 8 male and 16 female, aged 14 to 58 and were at healthy conditions.

Isolation and cultivation of EPCs

Isolation of EPCs was initiated from peripheral blood of 24 different donors (50 - 120 ml each) diluted with 0.9 % NaCl buffer and centrifuged via a Ficoll (GE-Healthcare, Freiburg, Germany) gradient (480 g) according to (Fernandez Pujol 2000; Hur 2004; Fuchs 2006). Mononuclear cells were seeded at a cell density of 0.75 $x10^6$ cell/cm² on plastic (for FACS and immunocytochemistry analyses) or on glass dishes (for acLDL uptake and ulex lectin staining as well as for HE staining). EBM-2 medium (Cambrex, Verviers, Belgium) containing 5 % FCS, 50 mM ascorbate-2-phosphate and 1 X penicillin/streptomycin (PAA, Pasching, Austria) was changed three times a week (control cells). Treatment with 0.5 µg/ml of the cysteine-rich protein 61 (CYR61/CCN1) was initiated on the second day of culture or immediately after seeding. Other control cells were treated with 0.19 µg/ml Fc-tag (equimolar amount to 0.5 µg/ml CYR61). Growth and development of the CYR61 treated and untreated cells was monitored and photographed during the time of culture.

Expression and purification of the CYR61 protein and mutant proteins

CYR61 was expressed in baculovirus transduced SF-21 insect cells and purified as an Fc-fusion protein via protein G sepharose according to Schütze and coworkers (Schutze 2005). Prior to the use in EPCs culture, the CYR61 protein function of each protein batch was tested in a proliferation assay (WST-1, Roche, Mannheim, Germany) using the endothelial cell line EA hy 926, resulting in a increased proliferation rate of at least 30 % (Schutze 2005). CYR61 proteins with an inactivating K239E mutation in the integrin $\alpha_6\beta_1$ (T1) binding site and an inactivating D125A mutation in the integrin $\alpha_v\beta_3$ (B3) binding site were described by Lau and coworkers (Chen 2004; Leu 2004). Similar mutations were introduced into a plasmid containing the CYR61 open reading frame via in vitro mutagenesis (Promega, Mannheim, Germany). The mutations were verified by sequencing analyses and the mutated CYR61

proteins T1 and B3 were similarly to CYR61 expressed as Fc-Fusion proteins in insect cells and purified via sepharose G chromatography (Schutze 2005).

HE staining and determination of the cell number

The cell numbers were determined after haematoxylin/eosin (HE) staining. Blue stained cells were counted in three random fields at 100 X magnification with a Zeiss Axiovert microscope.

FACS analysis

The duration of cell culture was between 19 and 21 days to achieve a sufficient number of control cells. Cells were detached with Accutase (PAA, Pasching, Austria) and cell scraper. Cells (1×10^5) were incubated for 30 min with the antibodies for mouse anti human CD31 - FITC, mouse anti human CD34 class II – FITC (both obtained from Serotec, Düsseldorf, Germany), mouse anti human CD133/1 (AC133) – PE (Miltenyi Biotec, Bergisch-Gladbach, Germany) and mouse anti human CD146 – PE (American Diagnostica, Pfungstadt, Germany). As a negative control, cells were incubated with an isotype matched mouse IgG1. FACS analysis was done at the FACS-SCAN and evaluated with the CELL QUEST (both Becton Dickinson, Heidelberg, Germany) software. Each analysis included at least 5×10^4 cells. The surface marker expressions of CYR61 stimulated versus control cells were compared.

Immunocytochemistry

EPCs were cultured for two weeks to achieve a sufficient density of control cells on 8-well chamber slides (Nunc, Wiesbaden, Germany). Preparation of the cells for immunocytochemistry occurred after fixation with acetone/methanol for 8 min. Cells were incubated with the following primary antibodies diluted in 0.1 % BSA/2 % horse serum/TBS at 4°C overnight: mouse anti human CD31 (DAKO Cytomation, Hamburg, Germany), mouse anti human CD34 class II (Dako Cytomation, Hamburg, Germany), mouse anti human CD133/1 (Miltenyi Biotec, Bergisch-Gladbach, Germany), mouse monoclonal anti human KDR (Sigma-Aldrich, Taufkirchen, Germany), mouse anti human Flt-1 (R&D Systems, Wiesbaden, Germany) and mouse anti human VE-cadherin (Chemicon, Hampshire, UK). Mouse serum at the same concentration as the specific antibodies was used as negative control. Secondary antibody rabbit polyclonal anti-mouse (Dako Cytomation, Hamburg,

Germany) was used. Surface marker expression was detected by the APPAP-system combined with fast red staining (Biogenex, Hamburg, Germany). Cell nuclei were counterstained with haematoxylin. CYR61 treated cells were compared to untreated cells and examined using a Zeiss Axiovert microscope and Axiovision software.

RT-PCR

After two weeks of cultivation cells were harvested in lysis buffer. RNA isolation was done according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). cDNA was transcribed from 0.5 µg RNA with random hexamer primers according to manufacturer's instructions (Bioline, Mannheim, Germany). RT-PCR was performed according to the following protocol: 5 min at 94°C, 45 s at 94°C, 45 s annealing temperature, 45 s 72°C and 3 min at 72°C. Steps 2-4 were repeated 23 times for EF1α and 35 times for CYR61. Primer sequences for the housekeeping gene elongation factor 1 alpha (EF1α) and CYR61 are shown in table 2.1. Amplimers were separated in 2% agarose gels containing ethidium bromide. Intron spanning primers were designed using the Primer3 software (Rozen and Skaletsky 2000). Identity of resulting PCR products was checked by sequencing analysis.

gene	**sequence**	**product size**	**annealing temp [°C]**
EF1α for	5´ AGG TGA TTA TCC TGA ACC ATC C 3´	250 bp	54
EF1α rev	5´ AAA GGT GGA TAG TCT GAG AAG C 3´		
CYR61 for	5´CAA CCC TTT ACA AGG CCA GA 3´	206 bp	55
CYR61 rev	5´TGG TCT TGC TGC ATT TCT TG 3´		

Table 2.1: Primer sequences and conditions of RT-PCR

acLDL uptake and ulex lectin staining

Cells were cultured for one or two weeks on glass dishes. While the media were changed, 10 µg/ml acetylated low density lipoprotein (acLDL) (Invitrogen, Karlsruhe, Germany) was added to the cells and incubated for 1h at 37 °C. After multiple washing with PBS, cells were fixed with 4 % paraformaldehyde for 10 minutes. Following multiple washings with NaCl,

cell staining with 10 µg/ml FITC-labelled lectin from *Ulex europaeus* (Sigma-Aldrich, Taufkirchen, Germany) for 1h at 4 °C was done. Cells were washed, stained with DAPI and covered with glass slides. Fluorescence microscopy was performed with a Zeiss Axiovert microscope and the Axiovision software.

Statistical analysis

Statistical evaluation was done according to the Mann-Whitney-U test using data of at least 4 different experiments for each analysis.

2. 4 Results

Since EPCs represent a promising tool to improve blood vessel formation which is important for bone regeneration in tissue engineering applications but are limited in cell number, we aimed to improve EPC number by using an angiogenic factor during cell expansion. Here we show that CYR61 after one week of cultivation enhanced EPC number compared to controls based on results obtained with EPCs from peripheral blood of 24 healthy volunteers.

The stimulation of EPC number by CYR61 was dose dependent in the concentration range between 0.05 and 1.5 µg/ml CYR61 (Fig. 1a) after a week of culture. This timing was chosen since CYR61 treated cells (treated with 0.5 µg/ml and higher from day 2 to day 7) approached confluence. Since the CYR61 protein was expressed as a fusion protein with the IgG-Fc-domain to facilitate purification via protein G sepharose (Schutze 2005) additional control EPCs received the Fc-tag (0.19 µg/ml) in an equimolar amount to 0.5 µg/ml CYR61 (the concentration used in all subsequent experimental procedures). Fig. 2.1a clearly indicates the higher cell number of CYR61 treated cells compared to the control. Treatment with 0.5 µg/ml CYR61 resulted in a 6-fold increase of the cell number compared to control cells (Fig. 2.1b), even a treatment with 0.05 µg/ml CYR61 already increased cell number by 2-fold. Treated cells from different donors showed similar results. Mononuclear cells of 7 different donors were treated from day 2 of cultivation onwards with 0.5 µg/ml CYR61 resulting in a 4.1 fold (SEM: 0.79) increased EPC number compared to control cells (Fig. 2.1c).

To evaluate if the treatment with CYR61 alters the phenotype of EPCs we compared CYR61 treated cells with control cells in regard to EPC marker expressions.

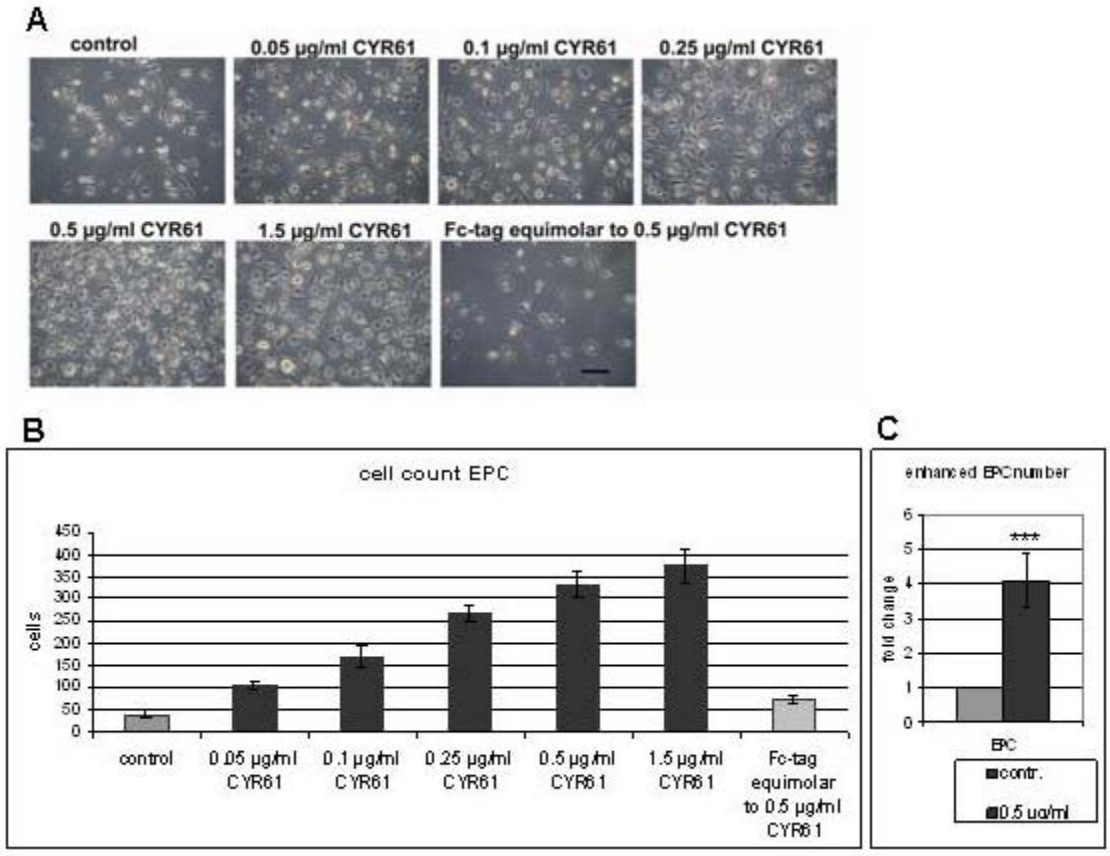


Figure 2.1: CYR61 enhances EPC cell number *in vitro*. *Cells were treated with 0.05, 0.1, 0.25, 0.5, 1.5 µg/ml CYR61, the Fc-tag equimolar to 0.5 µg/ml CYR61 (0.19 µg/ml) for 7 days or remained untreated (control cells). a: inverse microscopy, results shown are characteristic for 6 independent experiments, Bar indicates 100 µm. b: cell count of cells shown in a. c: cell count after treatment with 0.5 µg/ml CYR61 versus control. Average EPC number increase was 4.1 fold (SEM 0.79). Results are obtained from 7 different donors, *** indicate $p<0.001$.*

Results of FACS analysis are shown in Fig. 2.2. Cells were cultivated for 19 to 21 days to obtain a sufficient cell number for all analyses which was particularly important for the control group. Gating for both the control and CYR61 treated cells (0.5 µg/ml) was similar (Fig. 2.2a). Cells were incubated with antibodies against CD133, CD34, CD31 as well as CD146 (Fig. 2.2b) and examined in a FACS unit. Both CYR61 treated and untreated cells were negative for CD133 expression in these analyses. The cells were positive for the haematopoietic stem cell marker CD34 in the control and in 5 of 6 cases of the CYR61 treated cells. Expression of the endothelial cell marker CD31 was positive in all analyses. The marker for mature endothelial cells CD146 was negative for all tests.

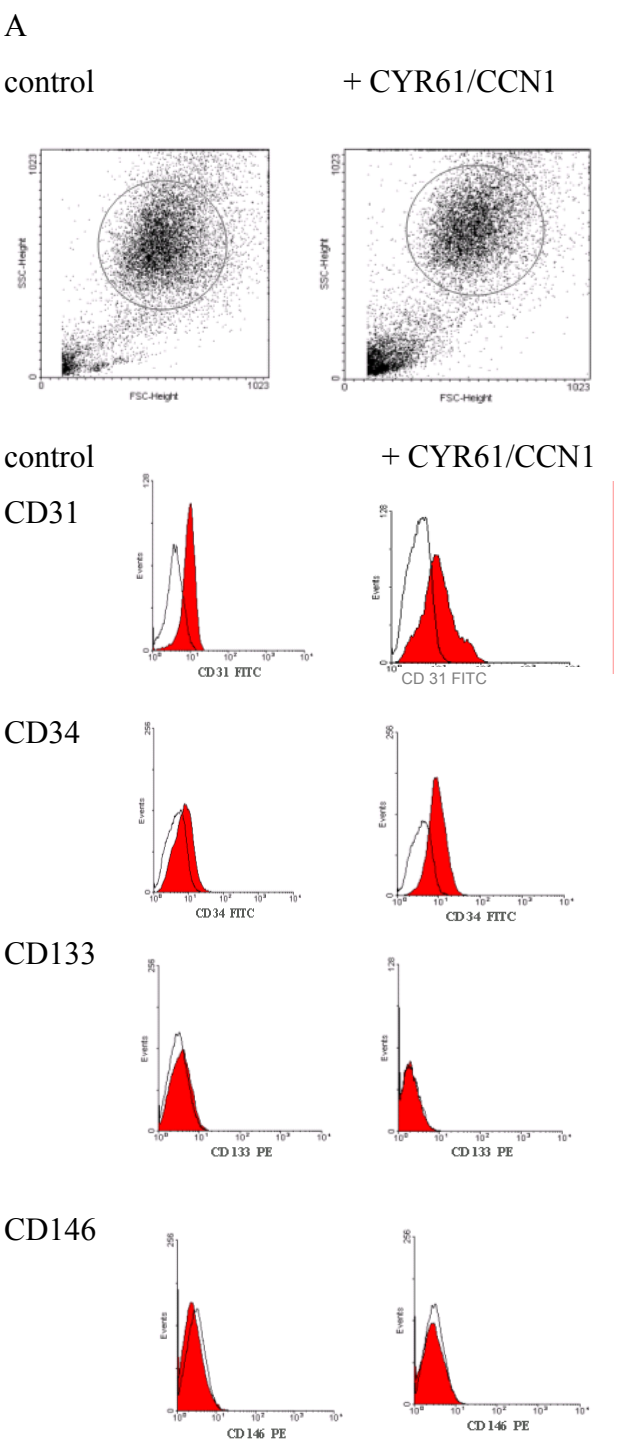


Figure 2.2: CYR61 treated EPCs express the same surface markers compared to the control cells. *FACS analysis of control EPCs (left panel) are compared with 0.5 µg/ml CYR61 treated EPC (right panel). Cell culture duration was between 19 and 21 days. In Fig. 2.2a the gating for both experimental set-ups are shown. Cells were stained with antibodies for CD31, CD34, CD133 and CD146 (Fig. 2.2b). FACS analysis for each antibody was performed at least 3 times.*

Immunocytochemical analyses of the CYR61 treated and control cells are shown in Fig. 2.3. Cells were analysed for the expression of CD34, CD133, KDR, CD31, VE-cadherin and Flt-1. The surface markers were homogenously expressed on the cell monolayer. Both control and CYR61 treated (0.5 µg/ml) cells were weakly positive for CD34. Unlike the FACS data the cells stained positive for the stem cell marker CD133 in immunocytochemistry. In addition the EPC marker KDR was clearly expressed in CYR61 treated and untreated EPCs. Cells were also positive for the endothelial cell surface marker CD31, as indicated by FACS analysis, as well as the marker VE-cadherin and Flt-1.

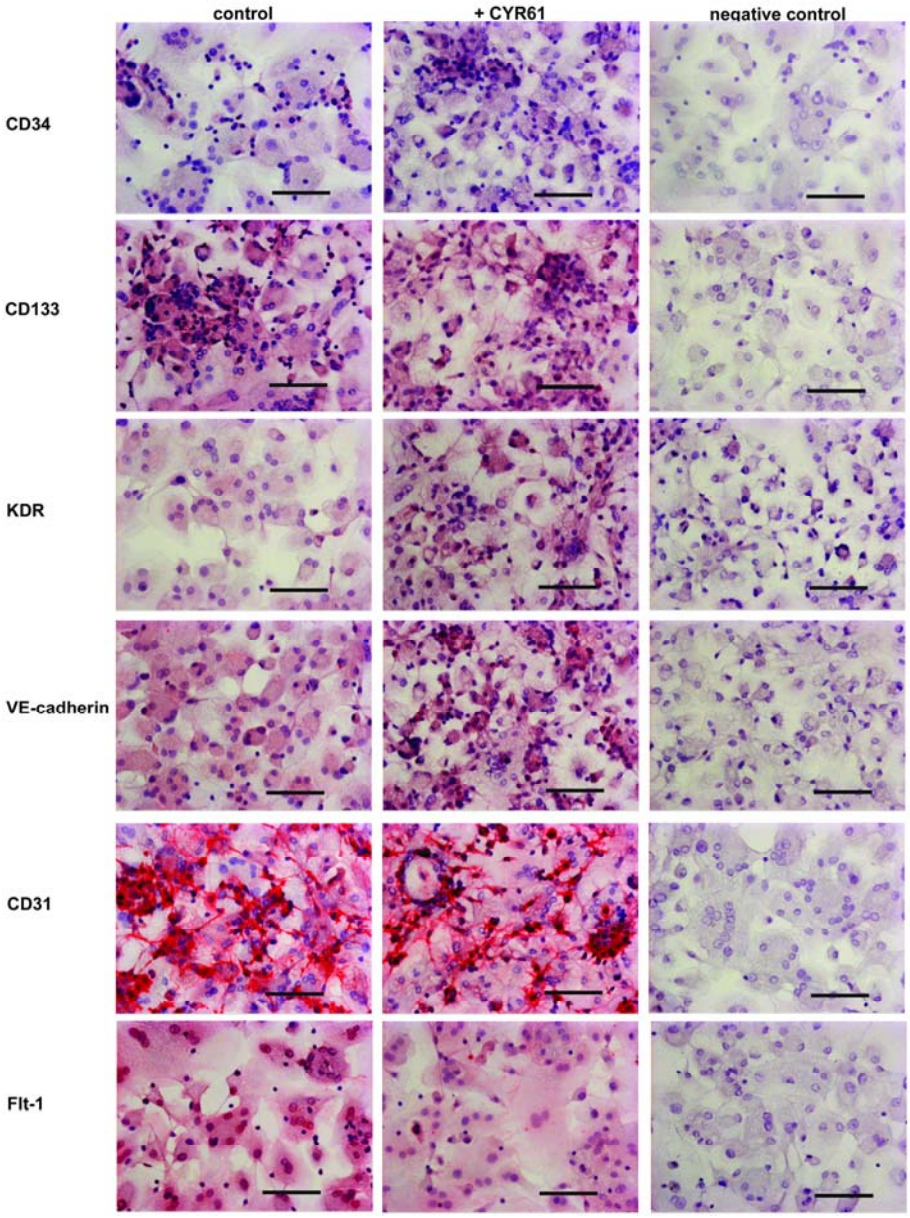


Figure 2.3: Immunocytochemical analyses of CYR61 treated EPC and control cells. *Staining for the antibodies CD34, CD133, KDR, VE-cadherin, CD31 and Flt-1 after 14 days of cultivation are presented. The left panel shows control EPCs. The middle panel relates to CYR61 treated cells (0.5 µg/ml) and the right panel shows the negative control for immunocytochemistry (similar protein concentration of mouse serum as the respective antibody). Bars in the picture indicate 100 µm. Results shown are characteristic for three independent experiments.*

Results of the acLDL uptake and ulex lectin staining of the cells are shown in Fig. 2.4. Both the control (Fig. 2.4a) and the CYR61 treated cells (Fig. 2.4b) displayed the uptake of acLDL and the concurrent binding for ulex lectin in most of the cells.

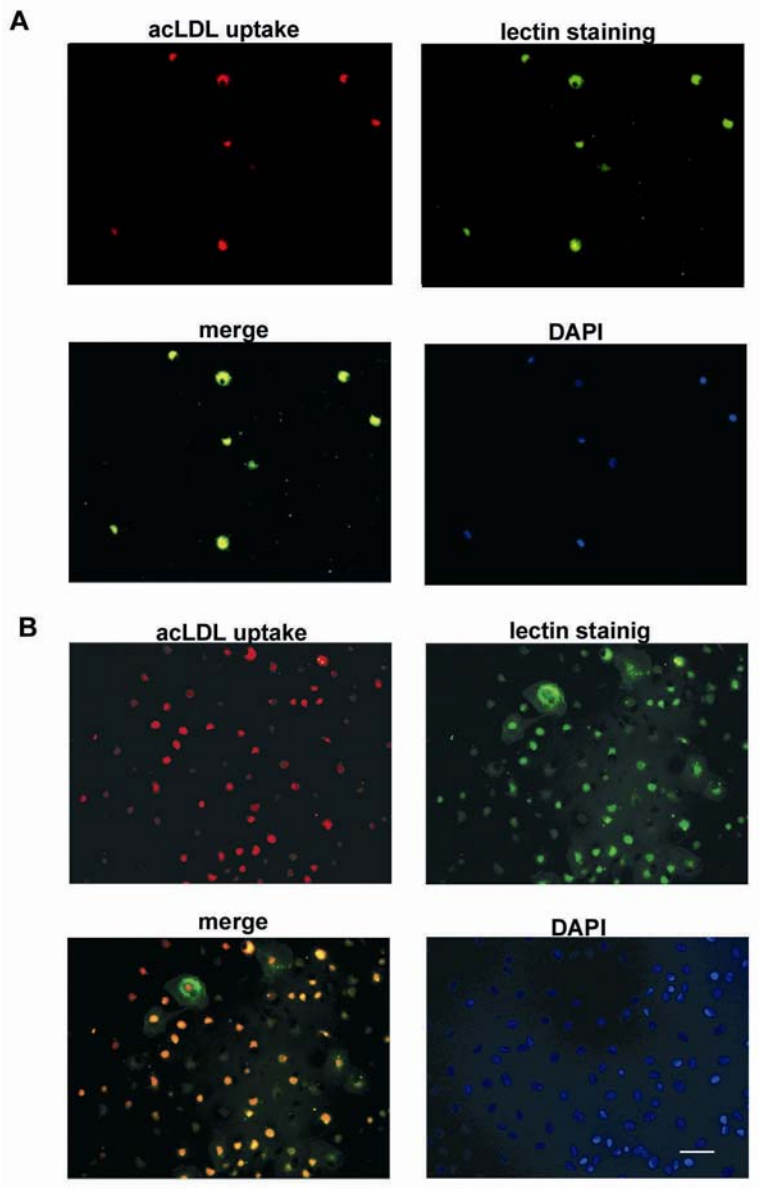


Figure 2.4: acLDL uptake and ulex lectin staining of EPCs treated with CYR61 and control cells. *Fluorescence microscopy shows that adherent cells are positive for uptake of acLDL (red) and binding of ulex lectin (green). Orange cells illustrate cells positive for both, DAPI staining shows nuclei. a) control EPCs, b) EPC stimulated with 0.5 µg/ml CYR61. Cell culture duration was 14 days. Bar indicates 100 µm. Results shown are representative for 5 independent experiments.*

To resolve the question if the observed effect of an induced propagation of EPC *in vitro* is due to an improved proliferation rate or the consequence of increased adhesion of mononuclear

cells to the cell culture surface, the time-points of CYR61 treatment were varied (Fig 2.5). Instead of treatment from day 2 of culture onwards the mononuclear cells were treated with 0.5 µg/ml CYR61 immediately after seeding resulting in a 7-fold higher cell number compared to the untreated control cells. In comparison to the cells treated with CYR61 after plastic adhesion on day 2, the cell number of immediately after cell isolation CYR61 treated cells was 2-fold higher. To evaluate a proliferative effect on EPCs, cells were washed several times with PBS to remove unattached cells on day 2 and were treated with 0.5 µg/ml CYR61 afterwards. The washed and CYR61 treated control cells showed similar cell numbers as the untreated control. HE-staining of the cells is shown in Fig. 2.5a, cell counting of 5 donors in Fig. 2.5b.

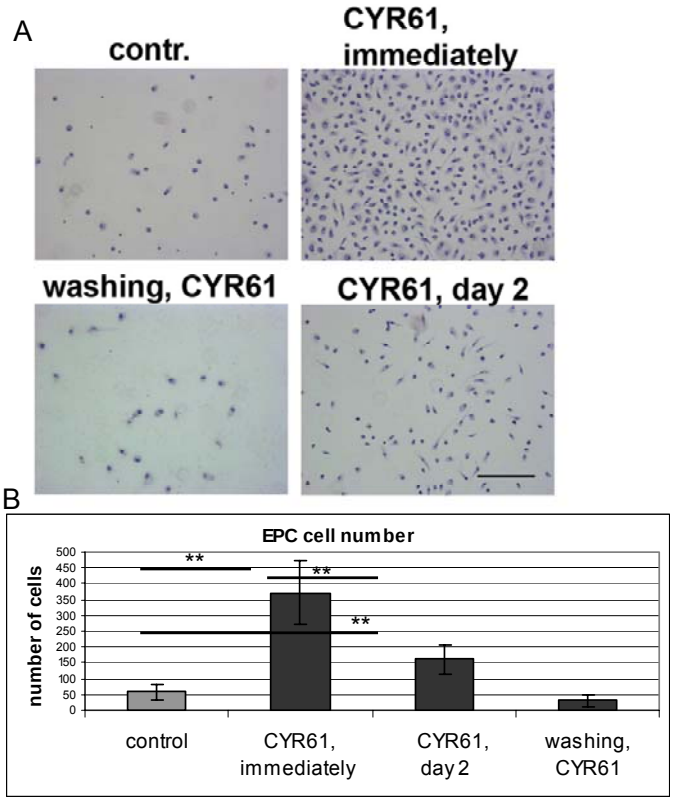


Figure 2.5: Adhesive effect of CYR61 on EPCs. *a): HE-staining of EPC treated immediately after cell isolation with CYR61, treated after cell adhesion on day 2 of culture and treated after removal of unattached cells, versus untreated control cells. b) cell count of cells from 5 donors after the different stimulations in a. EPC were cultured for 7 days. Bar indicates 200 µm, ** indicates $p<0.01$.*

Some effects of CYR61 on different cell types are mediated via integrins, we therefore treated the EPCs with the CYR61 mutants T1 and B3. The protein T1 has a defective binding site for the integrin $\alpha_6\beta_1$, while the B3 mutant is defective in $\alpha_v\beta_3$ binding. Treatment with B3 showed similar results as did the treatment with the same amount (0.5 µg/ml) of CYR61, the cell number after T1 treatment decreased. HE-staining of the cells is shown in Fig. 2.6a, cell counting of 4 donors in Fig. 2.6b.

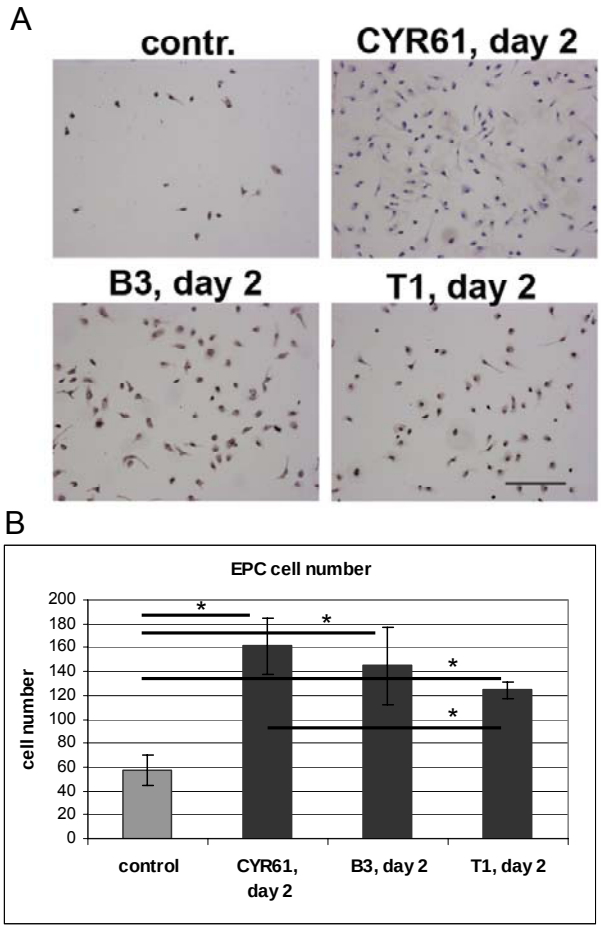


Figure 2.6: Relevance of integrins to the CYR61 effect on EPCs. *a) HE-staining of EPCs treated with CYR61, $\alpha_v\beta_3$ binding mutant B3, $\alpha_6\beta_1$ binding mutant T1 [0,5 µg/ml] versus control cells. b: cell count of cells from 4 different donors. EPC were cultured for 7 days. Bar indicates 200 µm, * indicates $p<0.05$.*

Since the expression of CYR61 was shown in endothelial cells and an endogenous secretion by the EPC could influence the control cells, the expression of CYR61 in EPCs was investigated by RT-PCR. The endothelial cell line Ea hy 926, MSCs of 2 different donors and the human osteoblast cell line hFOB do express CYR61, while no mRNA expression in EPCs of 4 different donors was detectable (Fig. 2.7).

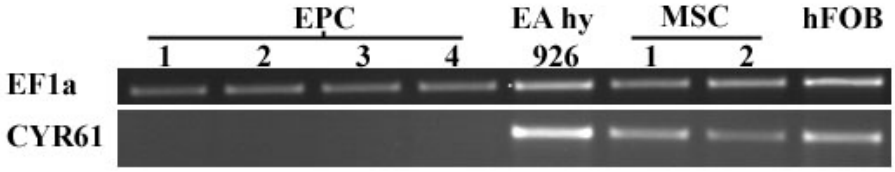


Figure 2.7: CYR61 is not expressed in EPCs. *RT-PCR for CYR61 in EPCs of 4 donors, the endothelial cell line EA hy 926, MSC of 2 donors and the osteoblast cell line hFOB show no expression in EPCs but in all other cell types.*

2.5 Discussion

Endothelial progenitor cells possess high therapeutical potential for the use of these cells in bone regeneration and healing in tissue engineering procedures and the treatment of cardiovascular diseases. The low number of cells which can be obtained from one patient, however, is a major limiting factor. Estimates based on animal experiments suggest that up to 12 l of autologous blood may be necessary to harvest sufficient number of EPCs to induce angiogenesis in patients after intravenous cell infusion (Rehman 2003). Therefore, a stimulating factor for *ex vivo* propagation of EPCs is needed to improve this situation. In this study we show that CYR61 is able to increase EPC number *in vitro* up to 7-fold without altering the endothelial progenitor cell characteristics.

A widely accepted consensus defines cells positive for CD34, CD133 and KDR as EPCs. We proved in FACS analysis, as well as with immunocytochemical staining that both the CYR61 treated cells and the control cells were positive for CD34. The conduct of the surface and stem cell marker CD133 during cultivation of EPC is ambiguously discussed in the literature. Bompais and coworkers (Bompais 2004) showed that the haematopoietic stem cell marker disappears after a few days of cultivation. On the other hand Loges and coworkers (Loges 2004) described that CD133 expression decreased by day 12 of cultivation but increased to up to 56% on day 48. In our experiments CD133 was not detectable after 19 to 21 days cultivation in FACS analysis, neither in the CYR61 treated, nor in the control cells. The more sensitive method of immunocytochemistry showed clearly positive results for CD133 in both the CYR61 treated and control cells. These results indicate that the stimulation with CYR61 protein keeps the cells in an immature stem cell-like stage. Immunocytochemistry of KDR showed positive results for both the CYR61 treated and control cells. Overall the results of the expression pattern of marker proteins suggest that the stimulation with CYR61 protein maintains the EPC phenotype. Immunocytochemical staining and FACS analyses revealed the expression of the endothelial marker CD31. Other endothelial markers like Flt-1, VE-cadherin and vWF were detected in immunocytochemistry. Since the cells have spindle shaped morphology, express the markers KDR and VE-cadherin at a low level, show negative results for CD146 expression in FACS analyses and, failed to form tube-like structures in the matrigel assay (data not shown), they do not represent differentiated endothelial cells, but instead are endothelial progenitor cells (Hur 2004; Khan 2005).

The uptake of acLDL and concurrent staining for ulex lectin are a well accepted proof for the characterisation of EPCs. Both the CYR61 treated and control cells were double stained for acLDL and ulex lectin. This result further strengthened the previous findings that CYR61 enhances EPC number without altering their characteristics.

The differentiation of CD14 positive monocytes obtained from the peripheral blood to endothelial like cells with EPC characteristics is widely discussed in the literature (Fernandez Pujol 2000; Romagnani 2005). These endothelial like cells were cultured on degradable biomaterials in order to obtain a tissue engineered vascular graft (Krenning 2007).

CYR61 is acting on various cell types in a cell-specific manner. Proliferation is induced in mesenchymal stem cells, the osteoblast cell line hFOB and the endothelial cell line EA hy 926 after treatment with CYR61 (Schutze 2005). Differentiation into endothelial cells due to CYR61 was observed in a highly selected $CD34^+$ haematopoietic stem cell population mobilised from bone marrow by GM-CSF (Grote 2007). To elucidate if enhanced proliferation or stimulated cell attachment is responsible for the increase in EPC number we performed proliferation assays using WST-1 reagent and revealed no indication for a proliferative effect of CYR61 on EPCs (data not shown). Rather enhanced cell adhesion is responsible for the effect observed, since immediate treatment with CYR61 leads to a more improved EPC number compared to treatment from day 2 of cultivation onwards when not attached cells were removed.

Previous studies showed an induced cell adhesion initiated by CYR61 via integrins, e.g. the angiogenic effect of CYR61 on activated endothelial is mediated via the integrin $\alpha_v\beta_3$. In this study CYR61 protein with mutations in the binding side for the integrins $\alpha_v\beta_3$ (B3) and $\alpha_6\beta_1$ (T1) were used to investigate the binding mechanism responsible for enhanced adhesion of mononuclear cells after CYR61 treatment. The addition of the $\alpha_v\beta_3$ binding mutant revealed a similar cell number as was obtained by treatment with CYR61. This result indicates that the integrin $\alpha_v\beta_3$ is not responsible for the CYR61 effect. EPC number decreased after treatment with the integrin binding mutant for $\alpha_6\beta_1$. Therefore the integrin $\alpha_6\beta_1$ is partly involved in CYR61 function on EPCs. A study by Schober and coworkers revealed that the integrin $\alpha_M\beta_2$ is the mediator of monocyte adhesion by CYR61 and CTGF (Schober 2002).

An endogenous expression of CYR61 in EPCs was not detectable by RT-PCR. This excludes that an endogenous secretion of CYR61 interfered with the results. In line with this, EPCs do respond to a low concentration of 0.05 µg/ml CYR61 with enhanced cell number, while a

proliferative effect of CYR61 on *in vitro* cultivation of MSCs, hFOB and EA hy 926, cells that express CYR61 endogenously, needed a higher concentration of 0.5 µg/ml CYR61 (Schutze 2005).

Whether CYR61 plays a role in EPC mobilisation *in vivo* is unclear at present. However, it is interesting to note that EPCs home to sites where the CYR61 protein is highly expressed and both EPCs and CYR61 are induced by similar factors. EPCs home to specific regions in the body like wounds, cancer and ischemic regions (Vasa 2001; Hristov and Weber 2004; Aicher 2005). CYR61 expression is upregulated in similar locations (Tsai 2002; Chen and Du 2006). A high expression of CYR61 in fracture callus has also been described (Hadjiargyrou 2000; Lienau 2006; Athanasopoulos 2007) and it would be of high interest to know if EPCs are present at this location which has not been described as yet. Since the fracture callus is a hypoxic region and blood vessel formation is integral part of fracture healing it appears very likely that EPC accumulate in this region since CYR61 is highly expressed.

The mobilisation of EPCs is induced by estrogens and physical exercise (Dimmeler 2001; Strehlow 2003), CYR61 expression is upregulated by estrogens as well as by mechanical stress (Chaqour and Goppelt-Struebe 2006). Stimulation of the human osteoblast cell line hFOB and murine cardiomyocytes with the cytokine TNF-α leads to an upregulation of CYR61 expression (Schutze 1998; Hilfiker-Kleiner 2004). A very recent study revealed an upregulation of CYR61 induced by VEGF in osteoblasts and further promoted fracture healing (Athanasopoulos 2007). Tissue engineered bone reconstruction attempts using mesenchymal stem cells (MSCs) and the crosstalk between MSCs and EPCs has been described (Raida 2006). A further step could be the co-cultivation of MSCs and EPCs in order to obtain sufficient vascularisation of bone grafts. As CYR61 has a strong adhesive effect on EPCs, the coating of biomaterials with the protein could result in the enhanced invasion of EPCs and therefore improved vascularisation of tissue engineered constructs.

In conclusion the results of this study show that the angiogenic inducer CYR61 is a promising tool for the enhancement of EPCs number *in vitro*. This could be beneficial for cell-based therapeutic applications in bone regeneration and fracture healing in tissue engineering procedures.

Chapter 3

Angiogenic differentiation of MSCs by the extracellular matrix protein CYR61/CCN1

3.1 Abstract

Mesenchymal stem cells (MSCs) have the ability to differentiate into various lineages. Therefore the cells are a promising tool for tissue engineering applications. The matrix associated signal molecule cysteine-rich protein 61 (CYR61/CCN1) is known as an angiogenic inducer and has been detected in many angiogenesis related tissues.

In this study we describe an effort to differentiate MSCs into the angiogenic lineage. Varying differentiation protocols using growth factors alone, in combination with 0.5 µg/ml CYR61 and the CYR61 protein alone, respectively were compared to control MSCs. As hypoxia is a discussed influencing factor for angiogenesis, the experiments were repeated under reduced oxygen content. Differentiation approaches were investigated using RT-PCR and immunocytochemistry for different angiogenic factors and in the matrigel angiogenesis assay.

RT-PCR and immunocytochemistry results varied between donors. Matrigel angiogenesis assays revealed similar results for angiogenic induction obtained with growth factors or CYR61 alone, respectively. MSCs formed highly ramified vessel like structures on the whole surface of the matrigel. In contrast, control cells did not displayed connected clusters.

We therefore conclude that CYR61 has angiogenic potential similar to growth factors including VEGF. As CYR61 was detected in a number of angiogenesis associated cells and tissues like tumour cells, placenta, wound healing and bone fracture callus the protein could be relevant for angiogenic processes *in vivo*.

3.2 Introduction

Human mesenchymal stem cells (MSCs) derived from the bone marrow are relatively easy to isolate and expand. In the last years, MSCs received much attention as a tool for tissue engineering and regeneration applications, the cells were for example used in bone reconstruction (Viateau 2007). Characteristics for MSCs are self-renewal and differentiation capacity into various lineages (Noth 2002; Tuan 2003; Baksh 2004; Young and Black 2004; McCulloch and Till 2005). MSCs were differentiated into the osteogenic, adipogenic and chondrogenic lineage by various researchers (Pittenger 1999; Noth 2002; Tuan 2003; Schutze 2005; Schilling 2007). Differentiation to other tissues like muscle, skin, tendon and epithelial cells have been described (Baksh 2004; Dezawa 2005; Shu 2006). However, the differentiation capacity of MSCs is controversially discussed. While some researchers (Jiang 2002) differentiated the cells into lineages of different germlayers and formed the term stem cell plasticity, others (Bianco P, personal communication) could not confirm the results and proclaim that MSCs are elusively progenitors of skeletal tissues. Due to their great therapeutic potential, MSCs are a promising tool for cell based tissue engineering applications.

The cysteine-rich protein (CYR61/CCN1) is an extracellular matrix protein and a member of the structural family of CCN proteins. The family members have a modular structure containing 4 conserved domains and 38 highly conserved cysteine residues (Lau and Lam 1999; Brigstock 2002; Schutze 2005). CYR61 is an angiogenic inducer, which was proven in endothelial cells and in the CAM-assay (Leu 2002; Schutze 2005). Furthermore the protein is expressed in various tissues and conditions associated with angiogenesis like wound healing, placenta, tumour development, fracture healing and the embryonal development of the vascular system (Lau and Lam 1999; Hadjiargyrou 2000; Grzeszkiewicz 2002; Gellhaus 2006; Lienau 2006; Athanasopoulos 2007; Kubota and Takigawa 2007). The functional inactivation of the gene in the mouse is lethal due to insufficient vascularisation (Mo 2002).

In previous studies we demonstrated that the recombinant preparation of CYR61 resulted in a functional protein (Schutze 2005). CYR61 was expressed in baculovirus transfected SF-21 insect cells and purified via affinity chromatography.

Angiogenesis is defined as the development of blood vessels from pre-existing vessels by sprouting (Carano and Filvaroff 2003; Carmeliet 2003). One of the first and crucial steps in bone regeneration is the induction of angiogenesis. Bone fractures are easily repaired without scar formation, but if the defect exceeds a critical size the regeneration capacity is low

(Viateau 2007). Reason is the insufficient vascularisation of the bone reconstruct and therefore deficient oxygen and nutrient supply of the contained cells (Petite 2000; Carano and Filvaroff 2003; Logeart-Avramoglou 2005). Angiogenic differentiated MSCs could be a helpful tool to solve these problems associated with tissue engineering approaches.

In this study we elucidated the effect of the angiogenic factor CYR61 on the angiogenic differentiation potential of MSCs. Angiogenic differentiation was initiated by treatment of confluent MSCs with the growth factors VEGF, bFGF, IGF and EGF in serum reduced medium. Additionally, cells were treated with 0.5 µg/ml CYR61 alone or in combination with the growth factors and compared to untreated control cells. MSCs were cultivated under hypoxic conditions with the angiogenesis inducing factors and compared to cells cultivated under normal oxygen content. Treated cells were investigated for angiogenic induction using RT-PCR, immunocytochemistry and the matrigel angiogenesis assay.

3.3 Materials and Methods

Expression and purification of the CYR61 protein

CYR61 was expressed in baculovirus transduced SF-21 insect cells and purified as an Fc-fusion protein via protein G sepharose as described previously (Schutze 2005).

Cell culture

Mesenchymal stem cells (MSCs) (2 male, 6 female at the age between 34 and 78) were isolated from patients undergoing total hip arthroscopy as described earlier (Schilling 2007). Briefly, trabecular bone was removed and underwent multiple washings. Spindle-shaped MSCs were selected by plastic adherence and propagated in DMEM/Ham´s F12 medium (PAA) containing 10 % FCS, 50 mM ascorbate-2-phosphate and 1 X penicillin/streptomycin (PAA) for approximately two weeks until confluence. Written consent was obtained from each patient prior to isolation of MSCs. Experiments were performed upon approval by the local ethics committee of the University of Würzburg.

Previously MSCs characteristics were proven by differentiation into adipocytes, osteoblasts and chondrocytes and marker expression (Schutze 2005).

Proliferation-assay

Passage 1 MSCs were seeded into 96 multiwell plates (Greiner bio-one) at a cell density of 4000 cells/well. At the second day of culture cells were starved in serum reduced medium (0.5 % FCS) overnight. Afterwards cells were treated with 0.5 µg/ml CYR61 for 24 hours. Control cells received tris/glycin, the eluant for CYR61 and the Fc-tag in an equimolar amount to CYR61 treatments (0.19 µg/ml). Proliferation was analysed using the WST-1 reagent (Roche) according to the manufacturer's instructions.

Angiogenic differentiation

For angiogenic differentiation cells were cultured in serum reduced (2 % FCS) DMEM/Ham´s F12 medium. Confluent MSCs of passage 1 were treated with 0.5 µg/ml CYR61, 50 ng/ml VEGF-165, 1 ng/ml bFGF (both obtained from Promocell), 2 ng/ml IGF and 10 ng/ml EGF (both obtained from Stratmann Biotec) according to table 1.

control	+ CYR61	ang diff	ang diff + CYR61
DMEM/Ham´s F12 10 % FCS	0.5 µg/ml CYR61	50 ng/ml VEGF 1 ng/ml bFGF 2 ng/ml EGF 10 ng/ml EGF	50 ng/ml VEGF 1 ng/ml bFGF 2 ng/ml EGF 10 ng/ml EGF 0.5 µg/ml CYR61

Table 3.1: angiogenic differentiation components

Differentiation of MSCs was performed for one week. Media were changed every 2-3 days.
In addition to the described treatment, angiogenic differentiation of MSCs was performed under hypoxic conditions using 3 % O_2 instead of the atmospheric O_2 content of 21 %. Expression of angiogenic factors under normoxic were compared to hypoxic conditions.

RT-PCR

RNA isolation was done according to the manufacturer's instructions (Macherey-Nagel). cDNA was transcribed from 1 µg RNA with random hexamer primers according to the manufacturer's instructions (Bioline). Intron spanning primers were designed using the Primer3 software (Rozen and Skaletsky 2000). RT-PCR was performed according to the following protocol: 5 min at 94°C, 45 s at 94°C, 45 s annealing temperature, 45 s 72°C and 3 min at 72°C. Steps 2 to 4 were repeated 32 to 41 times. Primer sequences are shown in table 2. Amplimers were separated in 2 % agarose gels containing ethidium bromide. PCR products were normalised to the elongation factor 1 alpha (EF1α) as house keeping gene. Semiquantitative analysis of the agarose gels were done by using LTF Bio 1D software (LTD). Identity of resulting PCR products was verified by sequencing analysis.

gene	Sequence 5´ forward 3´ 5´ reverse 3´	product size	annealing temp [°C]
EF1α	AGG TGA TTA TCC TGA ACC ATC C AAA GGT GGA TAG TCT GAG AAG C	250 bp	54
Ang-1	AGG GAG GAA AAA GAG AGG AAG A CTC CCC CAT TGA CAT CCA TA	150 bp	54
Ang-2	GGG AAG GGA ATG AGG CTT AC CGT TGT CTC CAT CCT TTG TG	159 bp	52
KDR	AGA CCA AAG GGG CAC GAT TC CAG CAA AAC ACC AAA AGA CCA GAC	469 bp	55
Flt-1	GGC ACA GAG ACC CAA AAG AA AGT CCT CAG AGA AGG CAG GA	158 bp	51
Tie-1 r	CAC CGC TGT ACT TTC TGC AC CAC TGT AGA TGC CGC TCG AT	164 bp	51
Tie-2	GCC TTC ACC AGG CTG ATA GT TCT CAC ACG TCC TTC CCA TA	454 bp	48
VE-cad	TGG GCT CAG ACA TCC ACA TA GAC CTC ACC AGC CTT ACC AG	159 bp	48
CD31	TCC GAT GAT AAC CAC TGC AA GTG GTG GAG TCT GGA GAG GA	299 bp	58
CD34	CAC CCT GTG TCT CAA CAT GG GGC TTC AAG GTT GTC TCT GG	191 bp	51
vWF	CAT TGG TGA GGA TGG AGT CC AGC ACT GGT CTG CAT TCT GG	188 bp	55

Integrin α_v	GGG TTG TGG AGT TGC TCA GT AAT GCC CCA GGT GAC ATT AG	264 bp	55
Integrin β_3	TGG CAG CTG TGT CTG TAT CC AAC GGT TGC AGG TAT TTT CG	159 bp	55

Table 2.2: Primer sequences and RT-PCR conditions

Immunocytochemistry

MSCs were cultured in 8-well chamberslides (Nunc). Cells were fixed with acetone/methanol for 8 min and subsequently incubated with the following primary antibodies diluted in 0.1 % BSA/2 % horse serum/TBS at 4°C overnight: mouse anti human CD31 (DAKO Cytomation), mouse anti human CD34 class II (Dako Cytomation), mouse monoclonal anti human KDR (Sigma-Aldrich), mouse anti human Flt-1 (R&D Systems), mouse anti human VE-cadherin (Chemicon) and rabbit anti human vWF (Sigma-Aldrich). Mouse and rabbit sera at the same protein concentrations as the specific antibodies were used as negative control. Secondary antibody was a rabbit polyclonal anti-mouse antiserum (Dako Cytomation) with cross reactivity for both rabbit and mouse. Surface marker expressions were detected by the APPAP-system combined with fast red staining (Biogenex). Cell nuclei were counterstained with haematoxylin. The different treatments were compared and examined using a Zeiss Axiovert microscope and the Axiovision software.

Matrigel-Assay

Matrigel extracellular matrix (BD Bioscience) was prepared according to the manufacturers instructions. An aliquot of 300 µl matrigel was applied to a 24 well multiwell plate and incubated for 30 min at 37°C. MSCs were differentiated, treated with CYR61 and transduced with adenoviral GFP-protein (MOI=100) (kind gift from Dr. Andre Steinert). Cells were detached using 0.25 % trypsin (PAA) and 4 * 10^5 cells were seeded onto the matrigel surface. Alterations of the cell morphology were examined microscopically using a Zeiss Axiovert microscope and recorded.

3.4 Results

Proliferation of MSCs

MSCs were treated for 24 h with CYR61 and compared to control cells. Treatment of MSCs with 0.5 µg/ml CYR61 resulted in an induction of the proliferation rate of 45 %. The results of the experiments are shown in fig. 3.1. Experiments were done with MSCs of three different donors and 12 preparations of the CYR61 protein.

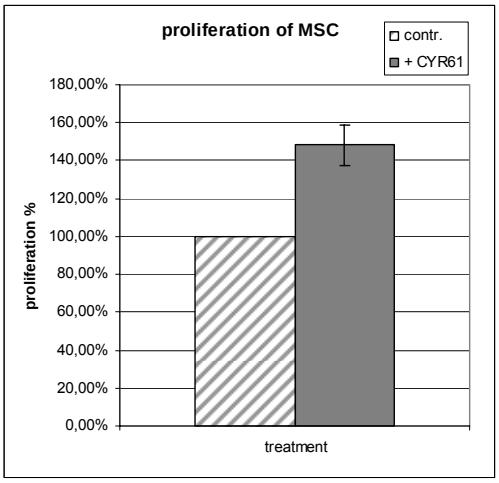


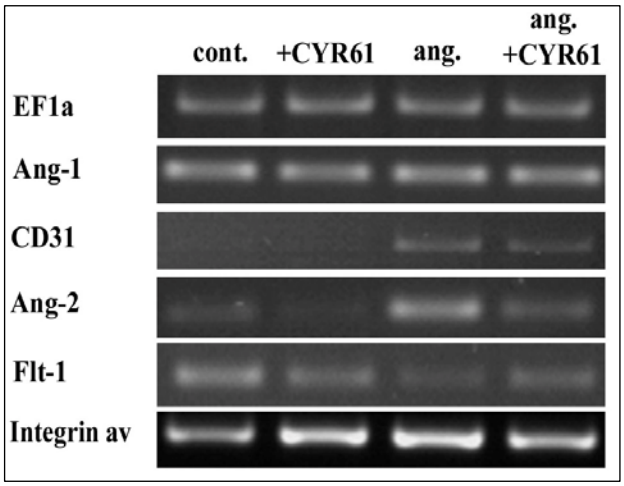
Figure 3.1: MSCs proliferation is induced by CYR61 treatment. *MSCs were treated for 24 h with CYR61 and compared to untreated control cells (set to 100 %) resulting in a 45 % higher proliferation rate. Results are shown for 3 MSCs donors and 12 different CYR61 preparations. Error bar indicates +/- SEM.*

RT-PCR of angiogenic differentiated MSCs

Angiogenic differentiation of MSCs was initiated by treatment of confluent cells with the growth factors VEGF, bFGF, IGF and EGF in serum reduced medium. Recombinant CYR61 (0.5 µg/ml) was added either alone or in combination with the growth factors and compared to untreated control cells. In fig. 3.2A the RT-PCR results obtained with MSCs from one donor are shown, fig. 3.2B shows the semiquantitative evaluation of RT-PCR results from three different donors. Angiopoietin-1 (Ang-1) is differently expressed in the distinct MSCs samples. One donor shows a clear upregulation of RT-PCR product intensity after CYR61 treatment, while the reverse was found on MSCs of another donor. CD31, also known as PECAM, mRNA expression was rarely detectable in the control and CYR61 treated cells Intensities of the RT-PCR products increased after angiogenic differentiation also in combination with CYR61 treatment. The angiopoietin-2 (Ang-2) responds similar. The RT-PCR intensity of the VEGF receptor 1 (Flt-1) expression decreased during CYR61 treatment

and angiogenic differentiation. The integrin α_v was clearly upregulated after CYR61 treatment and angiogenic differentiation.

A



B

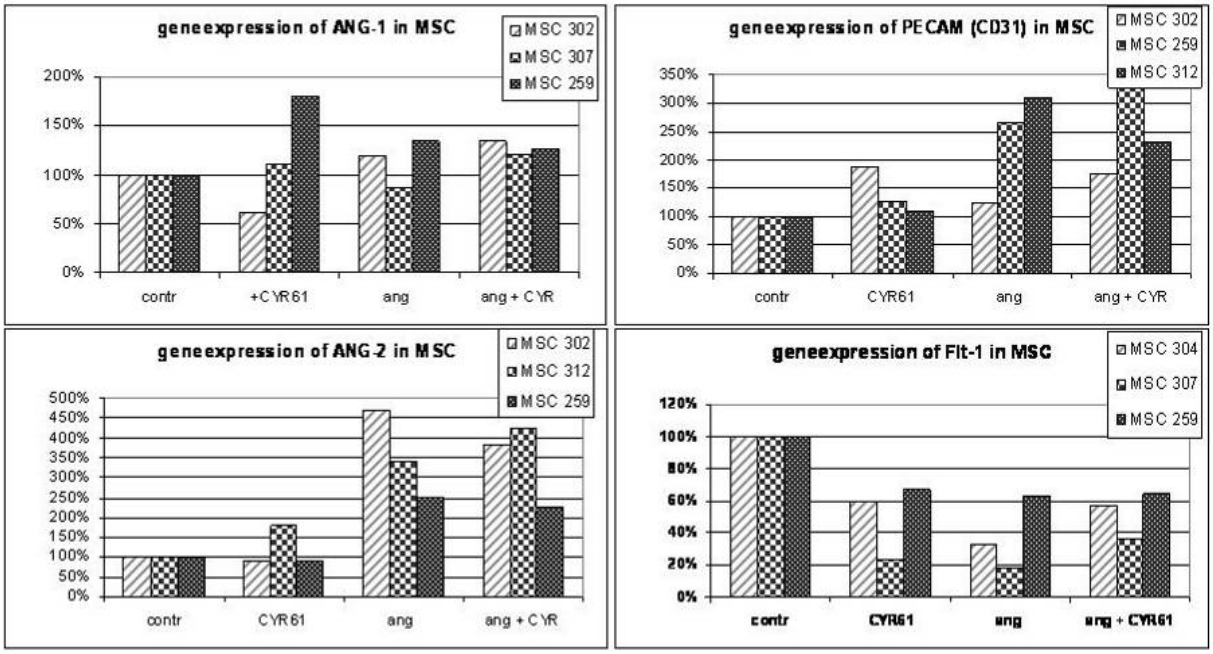
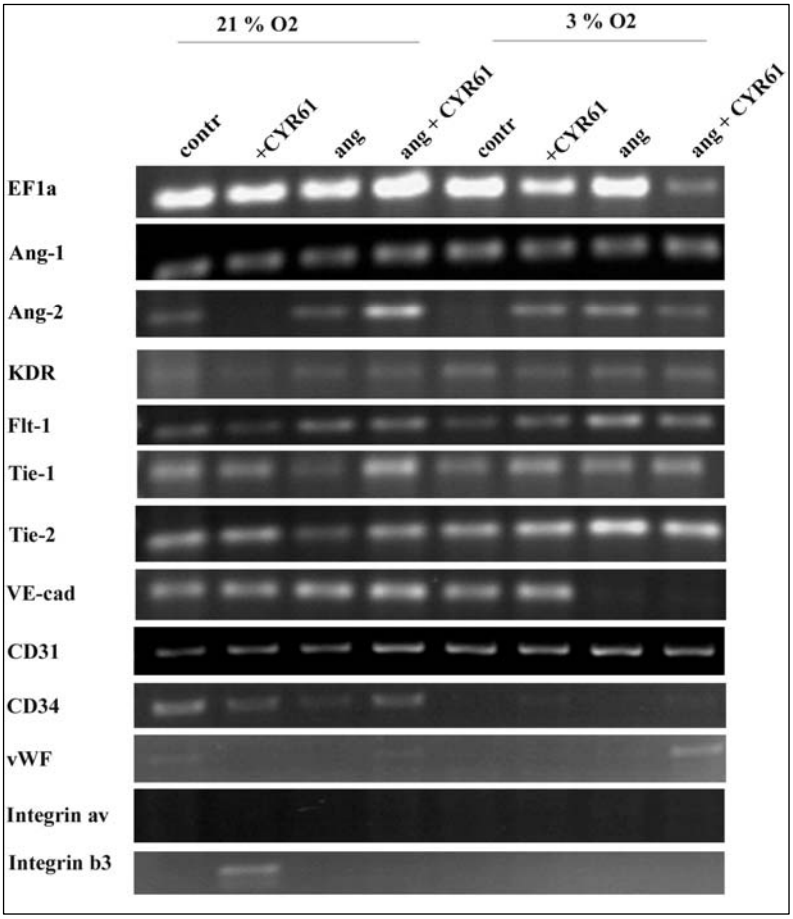


Figure 3.2: MSCs do express angiogenic factors. *A: RT-PCR of angiogenic differentiated MSCs. Results are shown for one donor. B: semiquantitative analysis of angiogenic differentiated MSCs. Results are shown for three independent experiments.*

Additionally, the angiogenic differentiation of MSCs was done applying hypoxic conditions (3% O_2) and compared to the results obtained at normal oxygen concentration. The RT-PCR results of all investigated genes are shown in fig. 3.3A. These results are derived from MSCs of one donor. The experiment was repeated (n=3). The semiquantitative evaluation of some of the investigated genes is shown in fig 3.3B. Expression of Ang-1 and KDR did not change at different O_2 conditions, while Ang-2 and Flt-1 were unregulated at hypoxic conditions. mRNA expression levels of CD31 were downregulated due to hypoxic conditions at two of the three experiments.

A



B

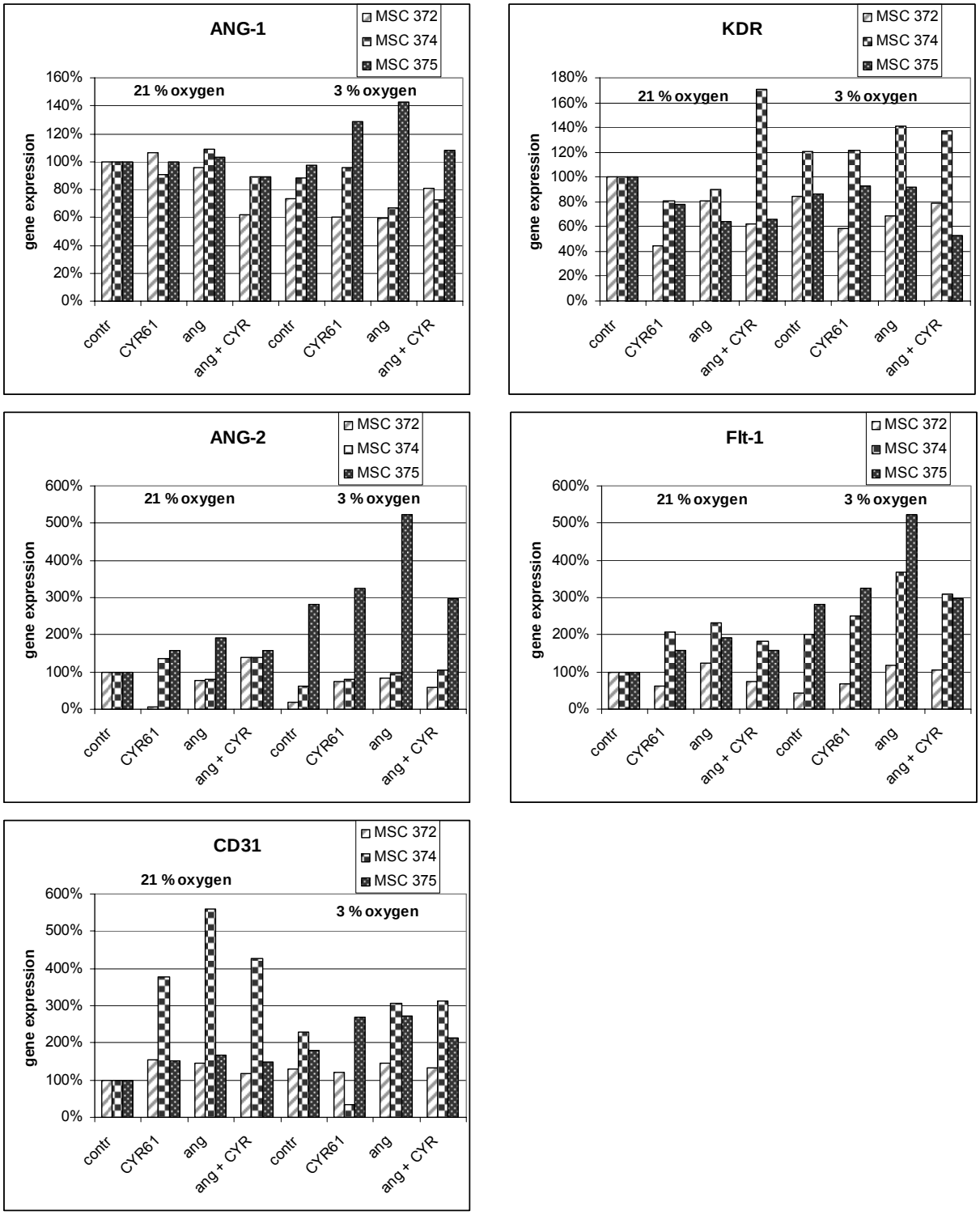


Figure 3.3: Comparison of angiogenic differentiation under hypoxic and normal oxygen conditions. *A: RT-PCR results of angiogenic factors. Results are representative for one donor. B: Semiquantitative RT-PCR evaluation of MSCs from three donors. Cultivation at normal oxygen content is shown on the left side, the right side shows cells cultured at hypoxic conditions.*

Immunocytochemical analysis

The results of the immunocytochemical analyses of one donor are shown in fig 3.4. Apart from the angiogenic differentiated and CYR61 treated sample KDR is hardly detectable. CD31, von Willebrand factor (vWF) and the vascular endothelial cadherin (VE-cad) were detectable in the MSCs, although the expression was not homogenously distributed. The donor variability was not as high as in the RT-PCR results. A reason could be the low signal intensities detected in the immunocytochemical analysis.

Hypoxic culture conditions did not influence the protein expression of the detected angiogenic markers. Negative controls with the equal protein amount of serum as the particular marker revealed negative results in all samples (data not shown).

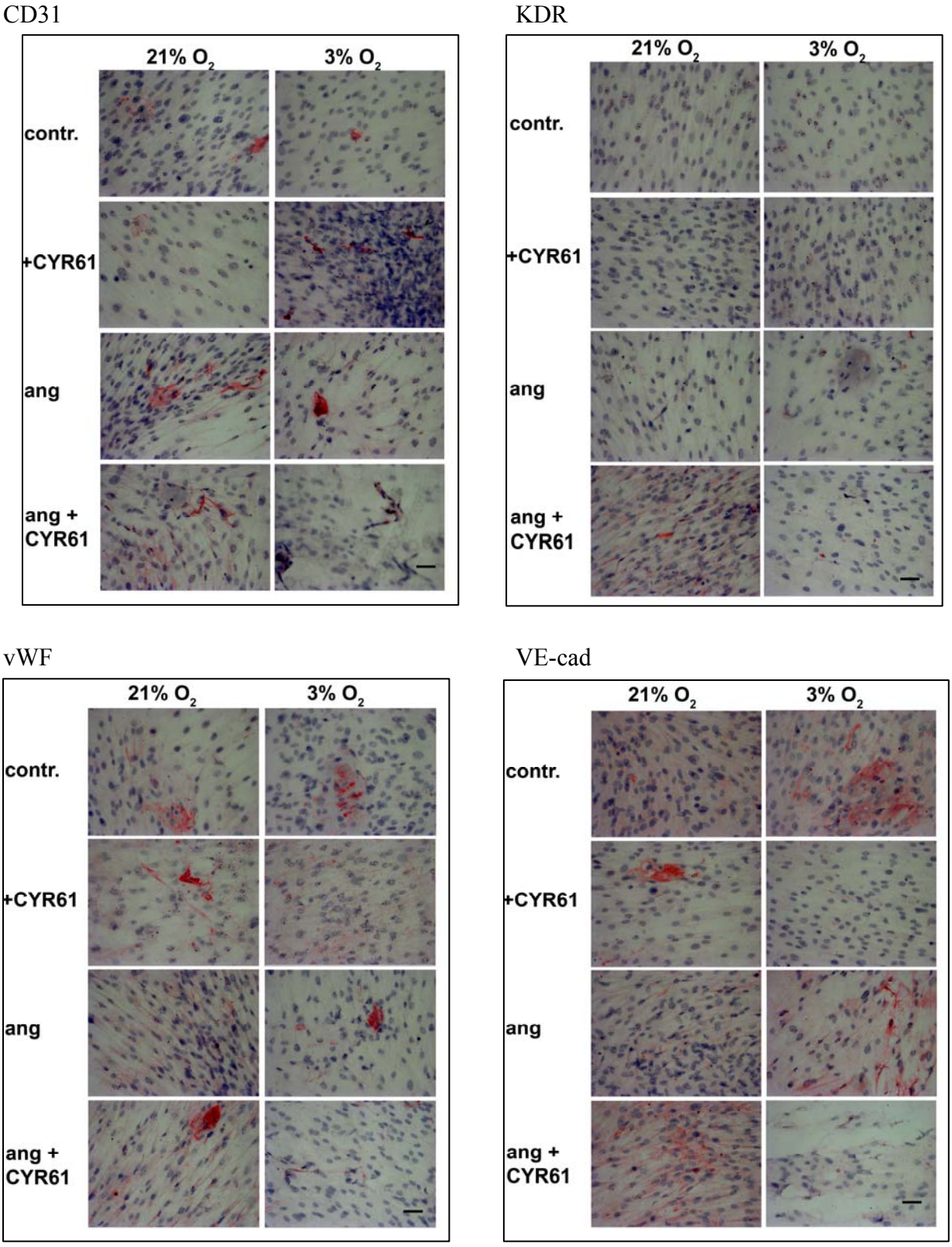


Figure 3.4: MSCs do not respond to the angiogenic differentiation or hypoxic culture conditions. *Immunocytochemistry results of the angiogenic differentiated MSCs are shown for CD31, KDR, vWF and VE-cad expression. Results are representative for MSCs of 3 donors. Bars indicate 100 µm.*

Matrigel angiogenesis assay

MSCs were differentiated for one week, detached, counted and seeded onto the matrigel matrix. The results of the matrigel assay are shown in fig. 3.5. Within 8 to 16 hours the morphology and appearance of the cells changed. The MSCs started to sprout and arranged to vessel like structures. MSCs treated with CYR61, growth factors or both were spreaded over the whole surface of the matrigel. Small clusters of cells displayed vessel like structures with strong ramifications. The untreated control cells were arranged to bigger clusters, not evenly distributed over the surface. Cells started to sprout as well but the much bigger cell clusters were not able to connect. MSCs cultivated under hypoxic conditions showed the same results in the matrigel assay (data not shown).

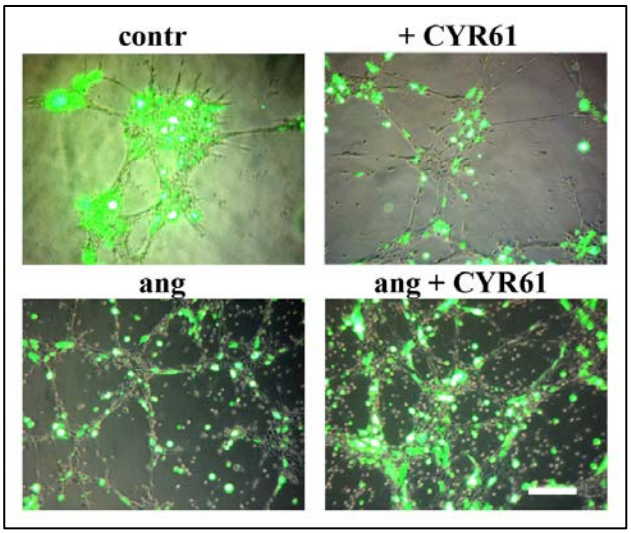


Figure 3.5: MSCs responded to angiogenic differentiation and CYR61 treatment with induced sprouting and ramification of cells in the matrigel assay. *Nuclei are green; cell morphology is visible in phase contrast. MSCs were transduced with GFP protein. Results shown are representative for 5 independent experiments. Bar represents 100 µm.*

3.5 Discussion

Success of cell based reconstruction applications in tissue engineering procedures *in vivo* is limited by oxygen and nutrient supply of the inserted cells. As long as no vascular network is available within the construct the cells are supplied only by diffusion. Therefore the thickness of the construct is limited. Approaches to induce a vascular network by using tubes of different polymers like PTFE and Dracon and coating with endothelial and smooth muscle cells, respectively have been described (Kannan 2005) and maybe useful for the replacement of bigger vessels in cardiovascular treatment. A different approach to restore microvessels and capillaries *in vivo* is therapeutic angiogenesis, which uses cells and/or growth factors to induce neovascularisation of ischemic tissues (Moldovan, 2002). An application of these procedures to tissue engineered constructs aims to create a vascular network in order to supply the cells in the construct with oxygen and nutrients.

Here we analysed the differentiation capacity of mesenchymal stem cells (MSCs) towards an angiogenic phenotype. Differentiation towards the angiogenic phenotype was initiated similar to the method described by Oswald et al. (Oswald 2004). Confluent MSCs were cultured in serum reduced media containing growth factors. Additionally, treatment with 0.5 µg/ml CYR61 alone or in combination with the growth factors was performed. A proliferative effect of CYR61 on different cell systems has been shown before and could be confirmed in this study in MSCs (fig 3.1).

The mRNA level of Ang-1 was not reproducibly influenced by CYR61 or growth factor treatment of MSCs obtained from different donors. Ang-1 is responsible for stability and maintenance of blood vessels. The antagonist Ang-2 destabilises the blood vessel wall in order to induce migration, and hence angiogenesis (Eklund and Olsen 2006). Ang-2 expression was induced in angiogenic differentiated MSCs. Induction of this gene was only detectable in one of three donors after CYR61 treatment alone. Recently a migratory effect of CYR61 on MSCs was described (Schütze, submitted), that theoretically could be dependent on Ang-2. Since Ang-2 expression in MSCs is rarely increased due to CYR61 treatment, the migratory effect of CYR61 on MSCs might not rely on Ang-2.

The endothelial cell marker CD31 was induced in angiogenic differentiated cells as well as in MSCs treated with CYR61 alone. In the case of angiogenic differentiation in combination with CYR61 treatment the mRNA expression level was the largest compared to control, indicating that CYR61 has a fortifying effect on angiogenic differentiation of MSCs.

The VEGF-receptor 1 (Flt-1) was decreased in all differentiation conditions using CYR61, growth factors or the combination of both.

An increase of the endothelial markers KDR, Flt-1, VCAM-1 and VE-cadherin in differentiated MSCs were described in the study of Oswald et al. (Oswald 2004). Additionally, the von Willebrand factor (vWF) and CD34 were increased in the study of Reyes et al. (Reyes 2002). They used multipotent adult progenitor cells, which are more immature compared to MSCs and have a phenotype similar to the angioblast. Several groups (Reyes 2002; Oswald 2004; Cao 2005) used also growth factors such as VEGF and showed that reduced serum conditions resulted in better angiogenic differentiation capacity of MSCs compared to the usually used 10 % FCS. The differentiation system used in this study is similar to the previously described and seems therefore to be promising. The primary cells used in this study were obtained from the femoral head of patients undergoing total hip arthroscopy and might therefore differ from MSCs used in the literature, which are usually derived from iliac crest bone marrow. The MSCs used in this study are possibly differentiated towards the osteogenic or adipogenic lineage and not capable to undergo angiogenic differentiation.

Hypoxia is another discussed condition for angiogenic differentiation (Pugh and Ratcliffe 2003; Potier 2007).We therefore repeated our experiments using a reduced oxygen content of 3 % and compared the results to those which were obtained at normoxic conditions. Semiquantitative evaluation of the RT-PCR results of angiogenic markers revealed an increase of Ang-1, Ang-2, Flt-1 and the VEGF-receptor 2 (KDR) in cells cultivated under terms of hypoxic conditions. CD31 was less expressed in the hypoxic cultivated cells, compared to cells cultivated at normal oxygen content, although this result was not reproducible for the MSCs obtained from the other donors. Immunocytochemical evaluation of the experiments did not indicate an effect of the hypoxic culture conditions towards enhanced expression of angiogenic proteins.

Hypoxia is one of the main factors responsible for angiogenic differentiation (Carmeliet 2003). In this study hypoxic conditions were applied when the angiogenic differentiation of MSCs was initiated. A different approach with the application of hypoxia during expansion of MSCs in addition to angiogenic differentiation could be more promising. D´Ippolito et al. (D'Ippolito 2004) described a related study were bone marrow cell expansion was performed using reduced oxygen conditions. The medium was changed only once a week, which resulted

in the presence of non adherent cells in addition to adherent MSCs. The authors argue that non adherent cells in the medium might provide factors such as cytokines which are needed for maintenance and proliferation of more primitive stem cells. The data indicated good long term culture results and differentiation capabilities of MSCs into the various lineages. Thus, this approach might be also useful for the angiogenic differentiation of MSCs.

One of the main problems associated with differentiation of MSCs is the huge donor variability. Results of the RT-PCR and immunocytochemistry were divergent between donors. In one experiment protein expression of CD31 and CD34 was clearly increased after CYR61 treatment (data not shown). This result was not reproducible with other donors. Therefore we have no clear evidence that the angiogenic differentiation is induced in MSCs by CYR61 treatment. Since CYR61 is endogenously expressed in MSCs a reason for the huge donor variability could be the different amounts of endogenously expressed and secreted CYR61 protein by various donors.

CYR61 treated cells showed the same vessel formation as growth factor treated cells in the matrigel assay. Together with the previously described results of the CAM assay (Schutze 2005) this is a clear indication for the angiogenic potential of CYR61.

We therefore conclude that CYR61 has angiogenic potential but not as strong as the growth factors including VEGF. As CYR61 was detected in a number of angiogenesis associated cells and tissues like tumour cells, placenta, wound healing and bone fracture callus the protein is relevant for angiogenic processes *in vivo*. For *in vitro* applications more investigations concerning culture conditions and applications as solution or adhesion agent have to be done.

Chapter 4

WISP3 regulates genes for anti-angiogenesis, cell survival and interferon response

4.1 Abstract

WISP3/CCN6 (wnt1 inducible signalling pathway protein 3) is a matrix associated, secreted signal molecule. WISP3 is associated with the human disease progressive pseudorheumatoid arthritis, a disease with unknown pathophysiology. Previously we described the expression of WISP3 in mesenchymal stem cells (MSCs). In this study, MSCs were treated for 24 h with 0.5 μg/ml recombinant WISP3 protein and compared to control cells. Global gene expression analysis revealed 111 transcripts regulated by WISP3-treatment. RT-PCR analyses confirmed array results. A cluster of interferon inducible genes including chemokines and members of the TNFSF superfamily were upregulated. RT-PCR analyses of additional time series showed an upregulation of interferon-β due to WISP3-treatment. Detected genes are associated with anti-angiogenic, apoptotic and immune responsive processes which provides important new implications for the role of WISP3 in cartilage protection and tumour response.

4.2 Introduction

WISP3/CCN6 (wnt1 inducible signalling pathway protein 3) is a matrix associated signal molecule and belongs to the structural CCN-family consisting of six members and characterised by 38 (less in the case of WISP2) highly conserved cysteine residues (Lau and Lam 1999). CCN-proteins function in cell growth and differentiation in an overlapping, yet distinct manner, suggesting non-redundant roles (Perbal 2004; Rachfal and Brigstock 2005; Leask and Abraham 2006).

WISP3-expression has been detected in mesenchymal stem cells (MSCs) (Schutze 2005), chondrocytes (Sen 2004), synoviocytes (Hurvitz 1999) and others [6]. In the chondrocyte cell line C28I2 WISP3 upregulates collagen type II and aggrecan expression via the SOX9 pathway (Sen 2004) and superoxide dismutase expression and activity (Davis 2006), thus suggesting a role in cartilage differentiation and homeostasis.

WISP3 is associated with progressive pseudorheumatoid dysplasia (PPD) (Hurvitz 1999), a disease with yet unknown pathophysiology. PPD is an autosomal recessive skeletal disorder initially associated with pain and swellings of the joints at the age between 3 and 8, followed by radiographically detectable cartilage loss, without the inflammatory processes characteristic for rheumatoid arthritis. Thus, patients are at high risk for joint replacement surgery as early as in the third decade of life (Delague 2005).

These findings were not recapitulated in a mouse model with expression of a truncated protein (Kutz 2005). However, gain of function and loss of function studies in the zebrafish revealed that WISP3 appears to modify BMP and Wnt-signalling and influenced pharyngeal cartilage size and shape in development (Nakamura 2007).

Truncated WISP3-proteins and loss of WISP3-expression was observed in inflammatory mammary carcinoma (Kleer 2004; Kleer 2007). Interestingly, a respective gain of function clone overexpressed in WISP3 negative cells displayed a marked reduction in angiogenic and invasion characteristics in a rat aortic ring assay (Kleer 2002), suggesting that WISP3 plays an anti-angiogenic role, acts as a tumour suppressor and could be of use in anti tumour therapy.

Here we investigated the role of WISP3 in MSCs on global gene expression patterns. Array analysis and further investigations by RT-PCR point towards the induction of chemokines, members of the TNFSF superfamily and various interferon inducible genes.

4.3 Materials and methods

Isolation and cultivation of MSCs

MSCs were obtained from patients undergoing total hip arthroscopy according to Schilling et al. (Schilling 2007). Cells were selected by plastic adherence in DMEM/Ham´s F12 medium (PAA) with 10 % FCS, 50 mM ascorbate-2-phosphate and 1 X penicillin/streptomycin (PAA). Written consent was obtained from patients and the procedure was approved by the local ethics committee. MSCs were characterised by differentiation into adipocytes, osteoblasts and chondrocytes as shown previously (Schutze 2005). MSCs from three healthy donors, two males (age 54) and one female (age 61) were analysed.

Purification of WISP3

Cloning, expression and purification of WISP3 as a Fc-fusion protein via affinity chromatography was performed as described (Schütze et. al., submitted).

Preparation of RNA

MSCs at passage 1 were starved in medium containing 0.5 % FCS overnight. Subsequently, recombinant WISP3 was added at 0.5 µg/ml. After 24 h RNA was isolated from treated and untreated MSCs using trizol followed by one-step RNA isolation (Macherey-Nagel). Yield and purity was analysed photometrically and by gel electrophoresis.

Affymetrix analysis

RNA of two patients (both male and age = 54) were evaluated by the Affymetrix Chip HG-U133 Plus 2.0 according to the GeneChip Expression Analysis Technical Manual. Hybridisation signals were analysed by GeneChip Operating software 1.2 and data mining tool 3.1. Expression profiles of WISP3-treated MSCs were compared to control cells. Differential expression was defined by an increase or decrease corresponding to a change P-value <0.001 or >0.999 in at least one of the two compared signals, a signal $\log_2$ ratio (SLR) ≤-1.0 or ≥1.0 (= 2.0-fold change), and a present call in at least one of the two compared samples. Each array experiment was performed twice with MSCs of two donors. The resulting list was analysed using the PathwayAssist software (Stratagene).

Reevaluation of array data

RNA used for array analysis and additional time series RNA plus/minus WISP3 treatment were evaluated by RT-PCR. Intron spanning primers were designed using the Primer3 software (Rozen and Skaletsky 2000). Identity of PCR products was verified by sequencing. MSCs of an additional donor (female, 61 years) was treated for 0, 2, 4, 6, 12, 24 and 48 hours with WISP3 and compared to control cells. 1 µg total RNA was reverse transcribed to cDNA using random hexamers. PCR was performed for: 5 min at 94°C, 45 s at 94°C, 45 s annealing temperature, 45 s 72°C and 3 min at 72°C. Steps 2 to 4 were repeated 32 to 41 times (table 4.1). Amplimers were separated in 2% agarose gels. PCR band intensities were normalised to the house keeping gene elongation factor 1 alpha (EF1α).

gene	Accession No.	Primer 5´- 3´ forward reverse	Size [bp]	Annealing Temp.
EF1α	NM_001402.5	AGGTGATTTCCTGAACCATCC AAAGGTGGATAGTCTGAGAAGC	250	54 °C
CXCL10	NM_001565.1	CCACGTGTTGAGATCATTGC CCTCTGTGTGGTCCATCCTT	180	55 °C
CXCL11	NM_005409.3	CATAGGCCCTGGGGTAAAAG TAAGCCTTGCTTGCTTCGAT	158	55 °C
CCL5	NM_002985.2	CTGCTGCTTTGCCTACATTG TGTACTCCCGAACCCATTTC	216	55 °C
TNFSF10	NM_003810.2	TGAGAACCTCTGAGGAAACCA TGCAAGTTGCTCAGGAATGA	226	55 °C
TNFSF13b	NM_006573.3	AAAGGGGAAGTGCCCTAGAA TTTGCAATGCCAGCTGAATA	238	55 °C
IFIT2	NM_001547.3	AACTGCTCCATCTGCGGTATG GAAAGTCATTTTGGCAAT	160	55 °C

IFIT3	NM_001031683.1	CGGAACAGCAGAGACACAGAG GGGCTGCCTCGTTGTTAC	240	55 °C
IFIH	NM_022168.2	TGCAGTGTGCTAGCCTGTTC TAAGCCTTTGTGCACCATCA	204	55 °C
ISG20	NM_002201.4	GAGCGCCTCCTACACAAGAG AAGCCGAAAGCCTCTAGTCC	188	55 °C
GBP1	NM_002053.1	GGTCCAGTTGCTGAAAGAGC GGTACATGCCTTTCGTCGTC	154	55 °C
GBP2	NM_004120.3	CCTCTCCCCAAGAAACAACA TTCCCGTGAGATGCCTTTAC	213	55 °C
GBP3	NM_018284.1	CAGTTGCTGGAAGAGCAAGA GCCTTGGGTTAGGATGACAG	208	55 °C
OASL	NM_003733.2	CGTGGCAGAAGGGTACAGAT AAGGGTTCACGATGAGGTTG	182	55 °C
GCH1	NM_000161.2	TCTTCACCAAGGGCTACCAG GTAAGGCGCTCCTGAACTTG	300	55 °C
GMPR	NM_006877.2	TTGGAGCTGGAGCAGATTTTG CAGTCCCCCGAGAATATCCAG	241	55 °C
SAMD9	NM_017654.2	ATCACCCGTGACATGGTCTTG CTGTGAATTTCCCCTTTCTGG	150	55 °C
SAMD9L	NM_152703.2	GGCACGGAAAGTGAGTGAGTG GAGCAGAATTTGCCCGTATTG	165	55 °C
ANKRD45	NM_198493.1	GCAGATGCAAGGCTGACTCT ATGGGTTTCCAACCACTCAT	150	55°C

ANGPTL1	NM_004673.3	TATAGACTGCGCCTGGGAAC CACAGGCATTGTACCACCAG	154	55°C
ETV7	NM_016135.2	TCAGCTGCTCCTTGATACCC CAGGGCACGAGACATCTTCT	244	55 °C
PMAIP	NM_021127.1	CAGTGCCAACTCAGCACATTG GTAACGCCCAACAGGAACAC	210	55 °C
BLZF	NM_003666.2	GGGAGAACTGATTGCCCTTT CGAATATCCAGCCCATATCC	206	55 °C
RHEBL	NM_144593.1	CTAGTGGGGAACAAGGCAG GCAGAAGCAAGGCAGTTACC	241	55 °C
Interferon-β	NM_002176.2	AGCACTGGCTGGAATGAGAC TCCTTGGCCTTCAGGTAATG	185	55 °C

Table 4.1: Primer sequences and RT-PCR conditions of investigated genes

4.4 Results

Previously we described the expression of the CCN-protein WISP3 in MSCs (Schutze 2005). Here we analysed the influence of WISP3 on global gene expression profiles in MSCs by array analysis and subsequent RT-PCR analyses. A striking cluster of interferon related genes appears to be upregulated by WISP3 treatment.

Array analyses

Gene expression profiles of WISP3 treated cells using the Affymetrix Chip HG-U133 Plus 2.0 from two donors revealed 107 reproducibly upregulated transcripts due to WISP3-treatment for 24 hours (please see materials and methods for the inclusion criteria). Four transcripts were found to be reproducibly decreased by WISP3-treatment.

Three clusters of related genes and gene families were identified to be upregulated in MSCs. A group of chemokines, namely the members CXCL10, CXCL11 and CCL5 as well as members of the TNFSF gene family and a series of interferon induced genes. Selected results of the array analysis are shown in fig. 4.1. The complete listing of regulated gene products is presented in supplementary table 1.

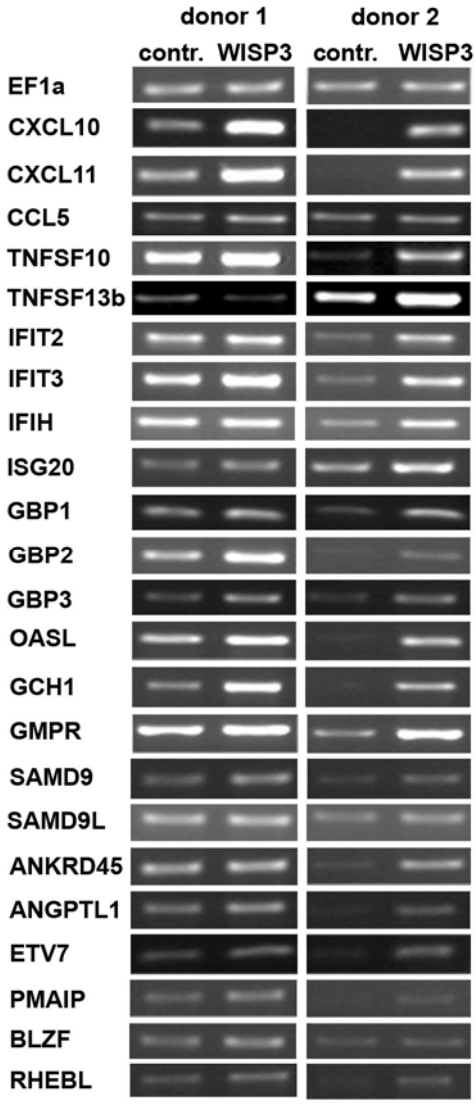


Figure 4.1: WISP3 induces interferon related genes in MSCs. *Identical mRNAs as used in the array analysis were investigated by RT-PCR.*

Reevaluation of array results

RNAs used for the array analyses were investigated by RT-PCR for 23 specific genes. Results are shown in fig. 4.1. Intensities of the majority of the RT-PCR products were higher in WISP3-treated samples and RT-PCR products obtained from mRNA of donor 2 displayed a higher regulation compared to donor 1. These findings are in-line with the array data. The CCL5 RT-PCR product did not change in the WISP3-treated samples. Intensity of the TNFSF13b RT-PCR product slightly decreased in the mRNA of donor 1, not confirming array data. mRNA of donor 2 increased, confirming the array result.

RT-PCR products of SAMD9, BLZF, IFIT2 and RHEBL were upregulated in the WISP3 treated mRNA of one of the donors.

Several of the regulated genes were tested by RT-PCR in an additional time frame experiment. MSCs were treated for 0, 2, 4, 6, 12, 24 and 48 hours with 0.5 µg/ml recombinant WISP3 and compared to untreated control cells as well as cells treated with Fc-tag protein in the equimolar amount (0,19 µg/ml) to the WISP3-protein concentration at the same time points. Additional controls with Fc-tag showed no influence on RT-PCR intensities.

The results of 18 investigated gene products are combined in fig. 4.2. Intensities of the RT-PCR products generally increased after 12 h of WISP3-treatment. Solely the intensities of GBP3 and SAMD9L RT-PCR products were only marginally changed over time and WISP3 treatment.

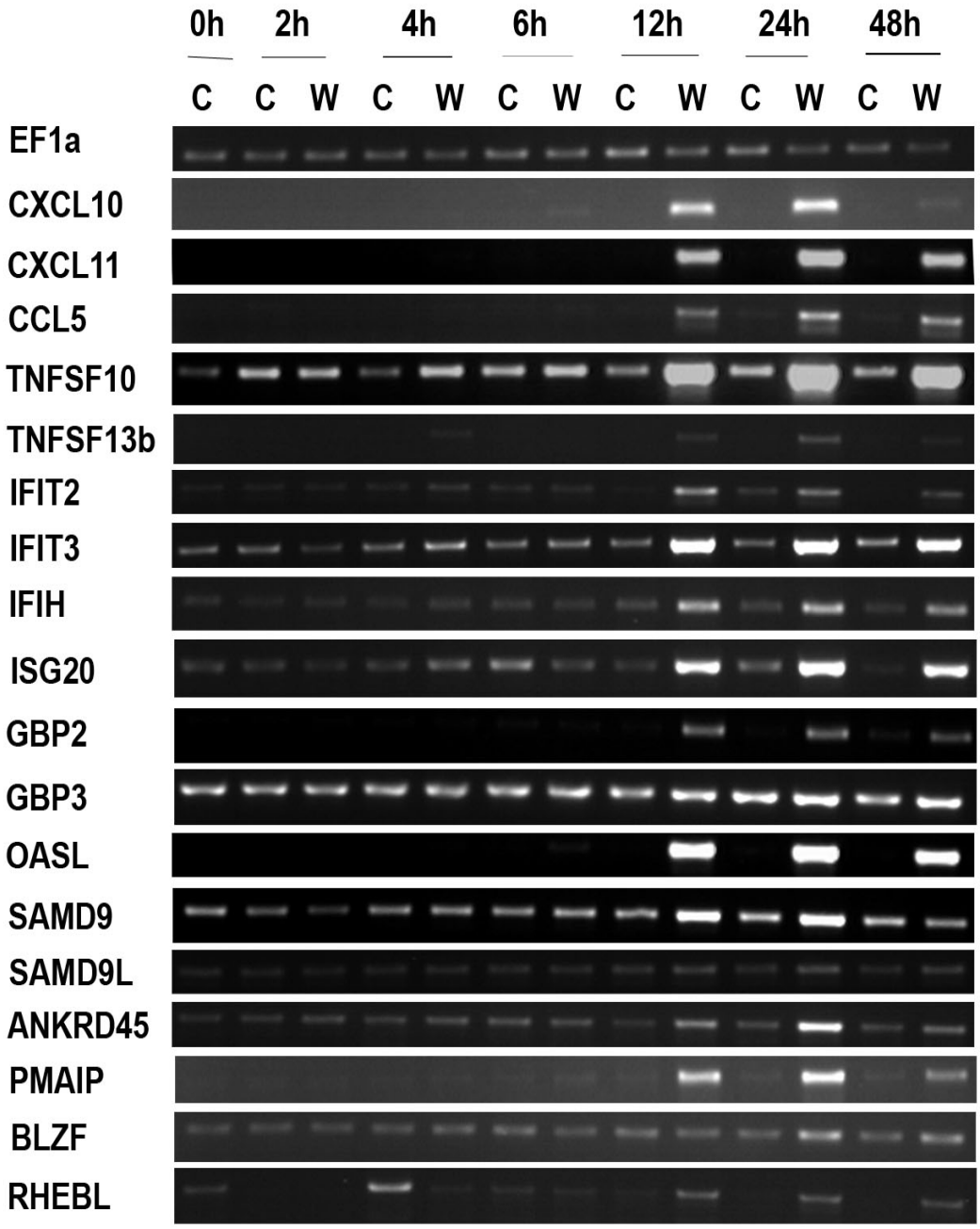


Figure 4.2: WISP3 induces most of the genes after 12 h of treatment. *MSCs were treated for 0, 2, 4, 6, 12, 24 and 48 hours with WISP3 and compared to control cells. C = control MSCs, W = WISP treated MSCs.*

Semi quantitative analyses of RT-PCR products representing the chemokine ligands CXCL10/IP-10, CXCL11/IP-9 (IP = interferon inducible protein) and CCL5/RANTES are shown in fig. 4.3A-C. Analyses of the TNFSF superfamily by RT-PCR are represented in fig. 4.3D and E.

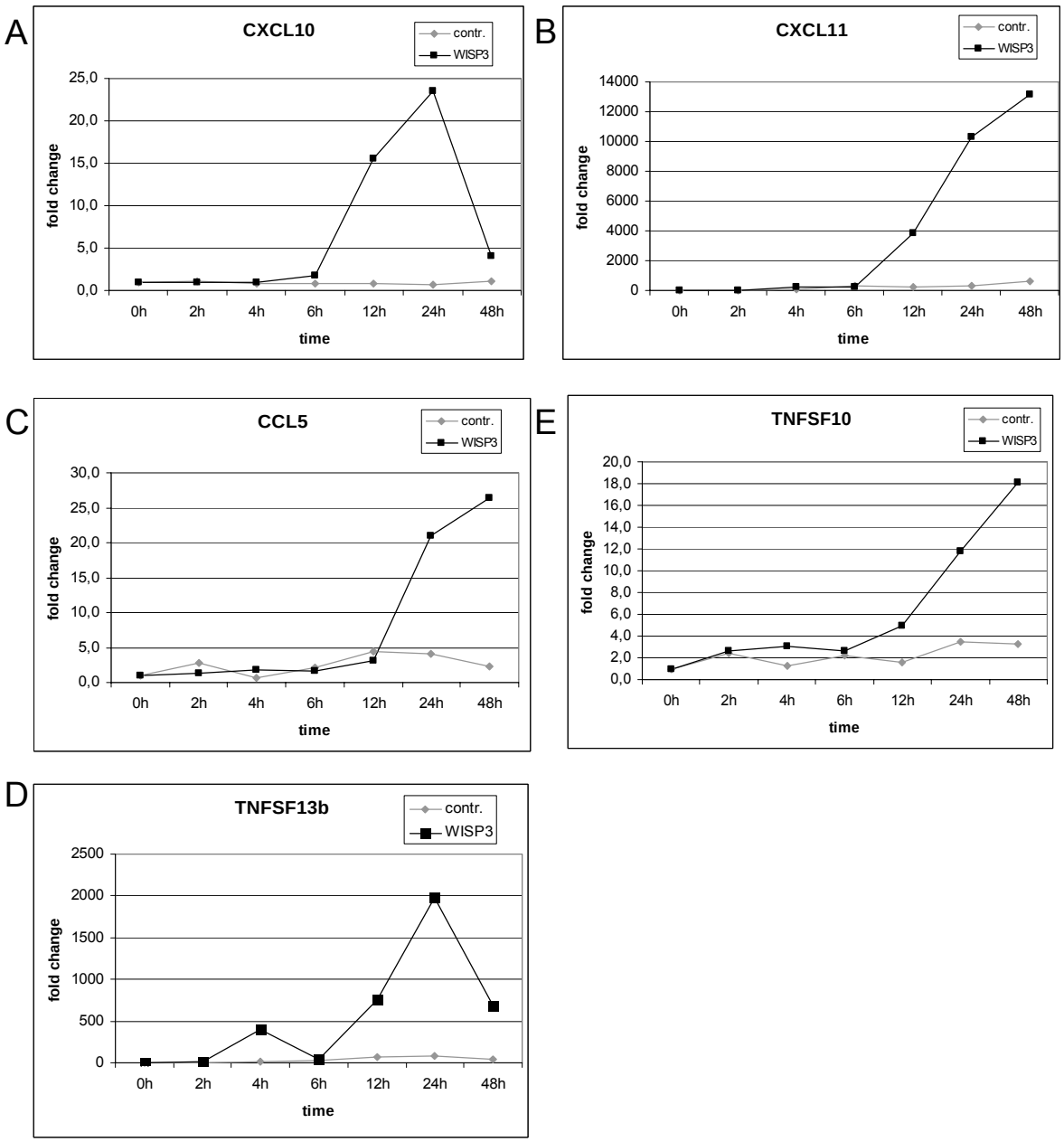


Figure 4.3: *WISP3 induces the chemokines CXCL10, CXCL11, CCL5 and the TNFSF superfamily members TNFSF10 and TNFSF13b in MSCs.*

Semiquantitative RT-PCR evaluation results of the interferon related genes IFIT2, IFIT3, IFIH, ISG20 GBP2, GBP3 and OASL are shown in supplementary fig. 1. Similar to the other results, the gene products were increased after 12 to 24 hours of WISP3 treatment.

Since several interferon inducible genes were found to be upregulated by WISP3 treatment, we investigated whether WISP3 induces endogenous interferon expression. After 4 h of WISP3 treatment interferon-β was found to be increased, with a maximum after 24 hours (fig. 4.4A).

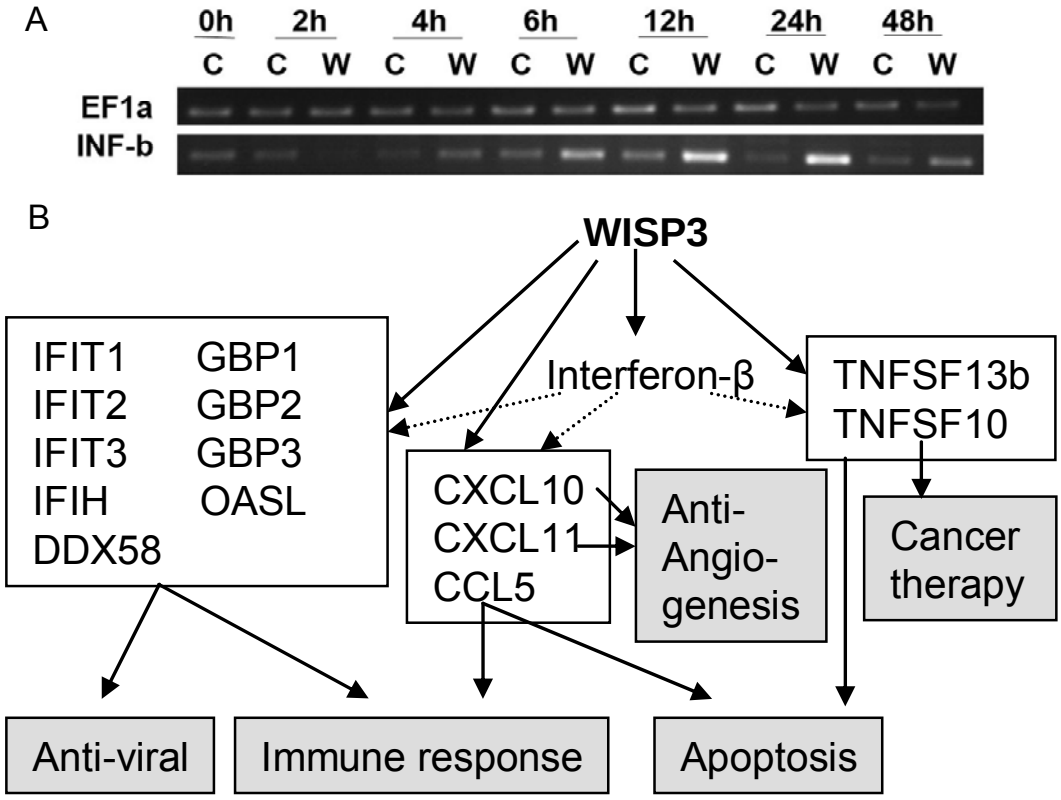


Figure 4.4: Induction of several genes by WISP3 treatment. *A: WISP3 induces interferon-β after 4 h of treatment. B: Several interferon related genes were found to be induced by WISP3 treatment of MSCs.*

Bioinformatic evaluation of the results using PathwayAssist, revealed a relevance of the induced genes to apoptotic and immune responsive processes.

4.5 Discussion

In this study we present for the first time a global gene expression pattern of WISP3 induced genes in MSCs. Many of the detected genes are also induced by interferons (fig. 4.4B).

The chemokines CXCL10 and CXCL11 belong to the subgroup of interferon-inducible non-ELR CXC chemokines (Belperio 2000). Both bind to the receptor CXCR3, a major inhibitor of angiogenesis. Ligands of CXCR3 block proliferation and migration of microvascular endothelial cells (Strieter 2006). Especially CXCL10 has angiostatic function, which was proven in *in vitro* angiogenesis assays (Belperio 2000; Rosenkilde and Schwartz 2004). These findings that WISP3 treatment of MSCs resulted in an upregulation of anti-angiogenic factors further strengthen the view of WISP3 as an inhibitor of angiogenic processes.

Another group of upregulated genes by WISP3 treatment of MSCs was the tumour necrosis factor superfamily (TNFSF). The family member TNFSF10 also known as TRAIL received much attention due to its ability to induce apoptosis selectively in transformed cell lines (Almasan and Ashkenazi 2003). TNFSF10 is therefore considered a potential anti cancer agent (Kumar-Sinha 2002; Clarke 2005). Clinical trials using soluble recombinant TNFSF10 receptor agonists targeting the death receptors TRAIL-R1 and TRAIL-R2 are currently under investigation and revealed good results, particularly, when used in combination with conventional cancer therapies (Schaefer 2007). Besides its pro-apoptotic capacity, TNFSF10 also mediates survival signals via activating MAPKs and PKB/Act signalling cascades (Schaefer 2007). Another WISP3-upregulated member of the TNFSF superfamily is TNFSF13b/BAFF, which represents a key factor controlling B cell survival and maturation and is involved in immune response, cell signalling and cell proliferation (Bossen and Schneider 2006).

A series of interferon induced genes were upregulated in MSCs by WISP3 treatment. The family members IFIT1, IFIT2, IFIT3 and two genes of the 2´-5´-oligoadenylate synthetase group (OAS-1 and OASL) are involved in immune response processes (Justesen 2000). Two genes of the DEAD box proteins (with Asp-Glu-Ala-Asp motif) IFIH and DDX58 were upregulated by WISP3. While IFIH is also a RNA helicase and regulates apoptosis, DDX58 has antiviral properties. The exonuclease ISG20 regulates cell proliferation. GTP binding proteins (GBP2 and GBP3) were detected by array analysis. Little is known about the function of GBP´s, but there is evidence that they have anti-angiogenic and anti-proliferative effects mediated by cytokines (Martens and Howard 2006).

Results of the PathwayAssist analysis revealed that these detected genes are considered to be interferon α and γ-responsive. Expression of these interferons was not found in our MSCs. Therefore, most likely the effect of WISP3 appears to be direct. However, we found interferon-β to be increased due to WISP3-treatment starting at 4 h after initiation of WISP3 treatment which preceded the WISP3-dependent regulation of the cluster of interferon induced genes (12 h and beyond). Thus we cannot exclude that WISP3 acts via interferon-β on the regulation of some of the detected genes. Fig. 4.4B summarises the relationship of the identified gene products.

Interferons mediate anti-tumour effects and are therefore used in cancer therapy (de Veer 2001) and act either indirectly by immunomodulatory or anti-angiogenic processes, or directly by affecting proliferation or differentiation of tumour cells. They have also anti-viral properties and mediate apoptosis (Martens and Howard 2006). Therefore, WISP3 likely represents an upstream regulator of these targets.

WISP3 is discussed as a tumour suppressor. Restoration of WISP3 expression in WISP3 negative human primary inflammatory breast cancer cells has shown a markedly decreased expression of angiogenic mediators like VEGF, interleukin-6 and basic fibroblast growth factor (Kleer 2004). This anti-angiogenic effect is in line with our observation of an induction of anti-angiogenic factors like CXCL10 and CXCL11 after treatment of MSCs with recombinant WISP3.

The results in MSCs could be of relevance for cartilage tissue and the pathophysiology of PPD. Cartilage is a sensitive tissue in terms of inflammation and vascularisation. The induction of immune responsive, cell survival, apoptotic and also anti-angiogenic genes by WISP3 could contribute to a protective role of WISP3 in cartilage homeostasis. Loss of function mutations in the WISP3 gene cause PPD and lead to destruction of cartilage, without symptoms of inflammatory processes. A recent study revealed a decrease in apoptosis rate of chondrocytes obtained from patients suffering PPD (Zhou 2007). This is result is in line with our finding that apoptotic genes are induced by WISP3 treatment. Therefore, it is tempting to speculate that the clinical manifestation of PPD due to the loss of WISP3 at least in part could be explained by the result of a missing protective WISP3-activity via anti-angiogenic, apoptotic and immunomodulatory genes.

4.6 Supplementary

Gene Symbol	Gene Title	Probeset	donor 1: SLR	donor 2: SLR
ANGPTL1	angiopoietin-like 1 /// angiopoietin-like 1	224339_s_at	1,98	2,56
ANGPTL1	angiopoietin-like 1	231773_at	1,67	2,93
ANGPTL1	angiopoietin-like 1	239183_at	2,16	2,81
ANKRD45	ankyrin repeat domain 45	236421_at	3,44	3,01
APOL1	apolipoprotein L, 1	209546_s_at	1,43	1,54
APOL2	apolipoprotein L, 2	221653_x_at	1,25	1,83
APOL6	apolipoprotein L, 6	241869_at	1,85	3
ARL9	ADP-ribosylation factor-like 9	229062_at	1,04	1,76
BLZF1	basic leucine zipper nuclear factor 1 (JEM-1)	1558560_s_at	1,71	1,43
BTC	betacellulin	241412_at	1,95	3,49
C1orf38	chromosome 1 open reading frame 38	207571_x_at	1,3	2,77
C1orf38	chromosome 1 open reading frame 38	210785_s_at	1,21	2,78
CARD15	caspase recruitment domain family, member 15	220066_at	1,57	2,65
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	211366_x_at	1,03	2,83
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	211367_s_at	1,08	3,11
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	211368_s_at	1,11	2,8
CASP1 /// COP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) /// caspase-1 dominant-negative inhibitor pseudo-ICE	1552703_s_at	1,18	2,36
CCL3 /// CCL3L1 /// CCL3L3	chemokine (C-C motif) ligand 3 /// chemokine (C-C motif) ligand 3-like 1 /// chemokine (C-C motif) ligand 3-like 3	205114_s_at	2,71	1,7

CCL5	chemokine (C-C motif) ligand 5	1405_i_at	3,63	1,58
CCL5	chemokine (C-C motif) ligand 5	1555759_a_at	3,34	2,29
CCL5	chemokine (C-C motif) ligand 5 /// chemokine (C-C motif) ligand 5	204655_at	3,28	1,71
COP1	caspase-1 dominant-negative inhibitor pseudo-ICE	1552701_a_at	1,23	1,84
CXCL10	chemokine (C-X-C motif) ligand 10	204533_at	4,08	6,05
CXCL11	chemokine (C-X-C motif) ligand 11	210163_at	4,46	6,52
CXCL11	chemokine (C-X-C motif) ligand 11	211122_s_at	4,57	5,89
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	218943_s_at	1,68	3,95
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	222793_at	1,41	3,51
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	242961_x_at	1,52	3,09
ETV7	ets variant gene 7 (TEL2 oncogene)	224225_s_at	2,19	2,52
FBXO6	F-box protein 6	231769_at	1,16	1,56
FLJ20035	hypothetical protein FLJ20035	218986_s_at	1,11	2,05
FLJ31033	hypothetical protein FLJ31033	228152_s_at	1,84	2,55
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa /// guanylate binding protein 1, interferon-inducible, 67kDa	202269_x_at	1,09	2,43
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	231577_s_at	1,09	2,23
GBP2	guanylate binding protein 2, interferon-inducible /// guanylate binding protein 2, interferon-inducible	202748_at	1,11	1,73
GBP2	guanylate binding protein 2, interferon-inducible	242907_at	1,33	2,09
GBP3	guanylate binding protein 3	223434_at	1,83	1,89

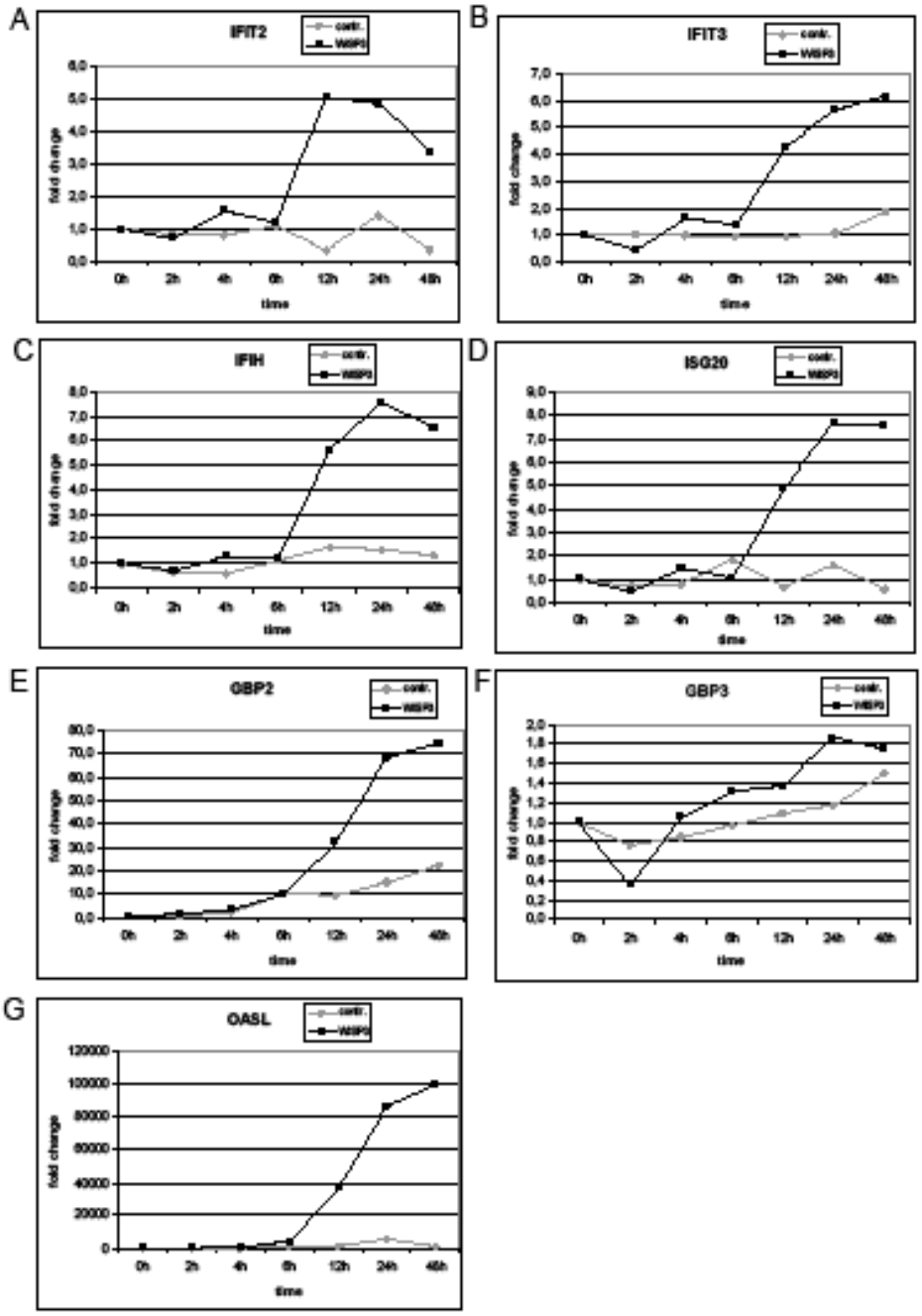
GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	204224_s_at	1,47	3,23
GIMAP2	GTPase, IMAP family member 2	232024_at	1,12	2,95
GMPR	guanosine monophosphate reductase /// guanosine monophosphate reductase	204187_at	1,75	2,82
HERC5	hect domain and RLD 5	219863_at	1,9	4,97
HERC6	hect domain and RLD 6	219352_at	1,01	3,79
HES4	hairy and enhancer of split 4 (Drosophila)	227347_x_at	2,32	1,98
HIST2H2AA	histone 2, H2aa	214290_s_at	1,53	1,5
HIST2H2AA	histone 2, H2aa	218280_x_at	1,6	1,6
HRASLS2	HRAS-like suppressor 2	221122_at	3,18	3,61
IFIH1	interferon induced with helicase C domain 1	219209_at	1,39	3,97
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	217502_at	1,76	5,98
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	226757_at	1,09	5,12
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	204747_at	1,14	5,15
IL15RA	interleukin 15 receptor, alpha	207375_s_at	1,84	1,45
IL18BP	interleukin 18 binding protein	222868_s_at	1,58	2,59
INDO	indoleamine-pyrrole 2,3 dioxygenase	210029_at	5,06	5,86
IRF1	interferon regulatory factor 1	202531_at	1,13	1,38
IRF7	interferon regulatory factor 7	208436_s_at	1,04	2,13
ISG20	interferon stimulated exonuclease gene 20kDa	204698_at	2,06	5,01
ISG20	interferon stimulated exonuclease gene 20kDa	33304_at	1,92	4,06
LOC400759	Similar to calpain (EC 3.4.22.17) light chain - bovine	1570541_s_at	2,12	3,68

LOC441109	hypothetical gene supported by AL713721	230405_at	1,44	2,84
LOC441168	hypothetical protein LOC441168	229391_s_at	1,66	1,34
MGC20410	hypothetical protein BC012330	228439_at	1,8	3,19
MLKL	mixed lineage kinase domain-like	238025_at	1,25	1,73
NT5C3	5'-nucleotidase, cytosolic III	223298_s_at	1,38	1,84
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	202869_at	1,06	3,78
OASL	2'-5'-oligoadenylate synthetase-like	205660_at	1,86	5,47
OASL	2'-5'-oligoadenylate synthetase-like	210797_s_at	1,74	4,93
PARP14	poly (ADP-ribose) polymerase family, member 14	224701_at	1,07	2,97
PDZK3	PDZ domain containing 3	209493_at	2,29	2,29
PIK3AP1	phosphoinositide-3-kinase adaptor protein 1	226459_at	1,44	1,4
PLEKHA4	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4	219011_at	1,21	1,5
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	204285_s_at	1,72	3,06
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	204286_s_at	2,15	2,99
PPM1K	protein phosphatase 1K (PP2C domain containing)	235061_at	1,36	1,7
PRIC285	peroxisomal proliferator-activated receptor A interacting complex 285	228230_at	1,16	1,75
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	204070_at	1,34	1,94
RHEBL1	Ras homolog enriched in brain like 1	1570253_a_at	2,22	1,5
RSAD2	radical S-adenosyl methionine domain containing 2	213797_at	1,5	6,51

RSAD2	radical S-adenosyl methionine domain containing 2	242625_at	1,8	6,7
SAMD9	sterile alpha motif domain containing 9	219691_at	2,67	2,34
SAMD9	sterile alpha motif domain containing 9	228531_at	2,36	2,38
SAMD9L	sterile alpha motif domain containing 9-like	226603_at	1,86	2,46
SAMD9L	sterile alpha motif domain containing 9-like	230036_at	2,33	2,29
SAMD9L	sterile alpha motif domain containing 9-like	235643_at	1,88	2,82
SLC15A3	solute carrier family 15, member 3	219593_at	1,1	2,46
SP100	nuclear antigen Sp100	210218_s_at	1,31	2,12
TDRD7	tudor domain containing 7	213361_at	1,16	1,59
TLR3	toll-like receptor 3	206271_at	1,4	3,22
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10 /// tumor necrosis factor (ligand) superfamily, member 10	202687_s_at	2,64	5,23
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10 /// tumor necrosis factor (ligand) superfamily, member 10	202688_at	2,37	5,61
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10 /// Tumor necrosis factor (ligand) superfamily, member 10	214329_x_at	1,88	5,08
TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	223501_at	1,6	4,75
TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	223502_s_at	1,61	5,46
TREX1	three prime repair exonuclease 1	205875_s_at	1,12	2,34

TREX1	three prime repair exonuclease 1	34689_at	1,08	1,87
TRIM21	tripartite motif-containing 21	204804_at	1,06	1,78
TRIM38	tripartite motif-containing 38	203567_s_at	1,11	1,62
TRIM38	tripartite motif-containing 38	203568_s_at	1,21	1,35
TRIM38	tripartite motif-containing 38	203610_s_at	1,21	1,33
WARS	tryptophanyl-tRNA synthetase	200628_s_at	2,1	1,5
WARS	tryptophanyl-tRNA synthetase	200629_at	1,46	1,45
XRN1	5'-3' exoribonuclease 1	1555785_a_at	1,72	1,38
ZC3HAV1	zinc finger CCCH-type, antiviral 1	220104_at	2,38	2,43
---	Transcribed locus	226725_at	1,04	1,51
---	CDNA FLJ41454 fis, clone BRSTN2011597	230741_at	2,61	1,38
---	CDNA FLJ43172 fis, clone FCBBF3007242	231040_at	2,2	1,63
---	Full length insert cDNA clone ZD88B10	238039_at	1,12	1,73
---	Transcribed locus	239587_at	1,08	2,52
ACO1	aconitase 1, soluble	207071_s_at	-1,85	-1,69
FLJ12442	hypothetical protein FLJ12442	218051_s_at	-1,26	-1,47
TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	227345_at	-1,16	-2,73
---	CDNA FLJ42101 fis, clone TESOP2006704	229384_at	-1,11	-1,34

Supplementary Table 1: *Results of the array analyses*



Supplementary figure 1: *Semiquantitative RT-PCR analyses of the genes for IFIT2, IFIT3, IFIH, ISG20, GBP2, GBP3 and OASL*

Chapter 5

Discussion

5.1 Impact of the CCN-proteins for the musculoskeletal system

CCN-proteins are functional expressed in numerous tissues and cells related to the musculoskeletal system. In murine osteoblasts all CCN-proteins except WISP3 were expressed and regulated by TGF-β, BMP-2 and cortisol (Parisi 2006). Furthermore it was shown that CYR61 regulates the differentiation of a foetal human osteoblast (hFOB) cell line (Schutze 1998).

Several studies revealed that wnt/β-catenin is important for bone formation and osteogenic differentiation of MSCs (Satija 2007). Stimulation of the murine mesenchymal stem cell line C3H10T1/2 with Wnt3A, results in an induced mRNA expression of the CCN-proteins CYR61, CTGF/CCN2, WISP1/CCN4 and WISP2/CCN5. The osteogenic differentiation by Wnt3A, proved through ALP activity assay, was inhibited after siRNA silencing of CYR61 expression. The study further indicates the relevance of CYR61 for osteogenic differentiation of MSCs (Si 2006).

A very recent publication demonstrates that CYR61 inhibits osteoclastogenesis of early osteoclast precursors, while leaving multinucleated osteoclast unaffected. CYR61 expression in osteoclasts was not detected, but was demonstrated in bone associated cells like osteoblasts and MSCs (Crockett 2007). Since CYR61 stimulates the proliferation and differentiation of osteoblasts and inhibits osteoclastogenesis it can be concluded that CYR61 has a protective function in the bone microenvironment.

Evidence is accumulating that osteogenesis and angiogenesis are closely related processes (Carano and Filvaroff 2003; Mayer 2005). CYR61 is a well accepted angiogenic inducer; therefore this is a further hint towards the crucial role of this protein in the bone microenvironment. The crucial role of CYR61 for bone is further strengthened by the finding that the protein is highly expressed in conditions of tissue repair such as in the fracture callus of different animals (Hadjiargyrou 2000; Lienau 2006; Athanasopoulos 2007).

In cartilage associated systems all CCN-proteins were detected. NOV/CCN3, WISP1/CCN4 and WISP2/CCN5 were found slightly increased during chondrogenic differentiation of MSCs (Djouad 2007), while another study revealed a decrease of CYR61, CTGF/CCN2, WISP2/CCN5 and WISP3 expression during chondrogenic differentiation (Schutze 2005).

CYR61 is expressed during chondrogenesis and stimulates the expression of collagen type II and aggrecan in chondrogenic differentiated MSCs. Neutralising anti-CYR61 antibodies block chondrogenesis in MSCs micromass cultures (O'Brien and Lau 1992; Wong 1997). WISP3 stimulates the expression of collagen type II and aggrecan in chondrocyte cell lines (Sen 2004). Lost of function mutations of WISP3 lead to the disease PPD in humans, which is associated with cartilage loss and bone deformation (Hurvitz 1999; Kutz 2005). A very recent study described the morphological changes of the femoral cartilage and the chondrocytes in patients with PPD. Cartilage lost its flexibility and the amount of collagens was decreased. Chondrocytes showed large nuclei and proliferation capacity increased while the rate of apoptosis decreased (Zhou 2007). In the mural system mutations of the WISP3 gene were not associated to a phenotype (Kutz 2005), while loss of function mutations in zebrafish affected the shape and strength of pharyngeal cartilage. Further, the authors could show that overexpression of WISP3 inhibits the BMP and wnt signalling in developing zebrafish (Nakamura 2007). This is the first hint that the human disease PPD could result from a dysregulation in BMP and/or wnt signalling. A connection of the disease juvenile idiopathic arthritis (JIA), with similar symptoms as PPD, to WISP3 mutations has been described (Lamb 2005), further strengthen the relevance of the protein for cartilage development and maintenance.

Therefore, CCN proteins and specially the family members CYR61 and WISP are of high relevance for cells and tissues of the musculoskeletal system.

5.2 Expression and manageability of recombinant CYR61 and WISP3

There are different possibilities to elucidate the impact of proteins on cell function. The intracellular expression of the protein can be reduced by siRNA, the overexpression of the protein within the cell can be initiated or the recombinant protein can be added to the cell culture system. Attempts to eliminate CYR61 and WISP3 by siRNA are under investigation. Since CYR61 as well as WISP3 represent secreted proteins in MSCs, the exact amount of functional protein after cellular overexpression can not be clearly defined and since the exact minimal concentration needed for appropriate signalling is not known, the use of recombinant proteins appears to be the strategy of choice. Therefore the addition of a defined amount of recombinant protein to the cell culture system is the most promising approach to elucidate effects of both proteins in cellular systems.

Recombinant expression of CYR61 has been described before (Kireeva 1996; Kolesnikova and Lau 1998), here we describe the recombinant expression of CYR61 and WISP3 as a Fc-fusion protein produced in baculovirus transfected Sf-21 insect cells and purified via SepharoseG affinity chromatography. Various attempts using bacterial and yeast expression systems for CYR61 were fruitless. Therefore researchers used eukaryotic expression systems like insect cells. Human and murine CYR61 was expressed in Sf-9 insect cells and purified via cation-exchange chromatography (Kireeva 1996; Kolesnikova and Lau 1998). The advantage of the Fc-tagged system used in our study is the induced solubility of the obtained protein and the ease of purification based on Fc-tag and protein-G interaction. Due to the 10 % high amount of cysteine residues within the protein sequence, the protein is very adhesive to different surfaces. Long term storage studies revealed that the concentration of recombinant protein decreased due to adhesion to the storage vials and by precipitation. The remaining protein in solution, however still remained functional, which was proven in proliferation assays with the endothelial cell line EA hy 926 (data not shown). Functionality of the recombinant CYR61 protein was also proven in the CAM-assay (Schutze 2005) and in the matrigel assay (chapter 3).

A commercially available full length recombinant CYR61 protein is produced by the Taiwanese manufacture “abnova” and has been used in several studies (Athanasopoulos 2007; Grote 2007). CYR61 is expressed as a glutathione transferase (GST) fusion protein; therefore an additional enzymatic step using prescission protease to cleave the GST from CYR61 and the proof of the cleavage by gelelectrophoresis and silver staining is needed. The Fc-CYR61 used in this study is ready to use without further preparation.

The stability of the commercial CYR61 is defined with 3 months; the Fc-CYR61 used in this study is stable for at least 6 weeks. Several tests using the Fc-tag, in the same molecular amount as the functional protein, as additional control revealed that the tag does not influence proliferation rate, adhesion characteristics, migration or gene expression profile of the different cell systems used in this study.

Recombinant WISP3 protein was expressed in SF-21 insect cells according to the CYR61 protocol as a Fc-tagged protein. MSCs did respond to 0.5 µg/ml of WISP3 with an induced proliferation rate of 10 to 30 % (data not shown). This was the first functional proof of the recombinant protein. In contrast to the other recombinant produced CCN-protein CYR61, WISP3 appears to be more stable during long term storage. WISP3 concentration decreased

only marginal, which indicates a different adhesion profile. Although both proteins belong to the same structural family they share only 35 % sequence homology, explaining the differences in behaviour.

5.3 The cysteine rich protein 61 (CYR61)

5.3.1 Impact of CYR61 on EPCs

Endothelial progenitor cells received much attention due to their ability to induce neovascularisation in ischemic tissues (Werner and Nickenig 2006; Tse and Lau 2007), as for example in tissue engineered bone regeneration applications or the treatment of cardiovascular diseases. Cells can be easily obtained from the peripheral blood but are limited in number. Enhancement of cell number can be achieved *in vivo* by infusion of stimulating factors like granulocyte macrophage colony stimulating factor (GM-CSF), statins, VEGF or estrogens (Aicher 2005). *In vivo* induction of EPC number needs preparation, is time consuming and is therefore not always applicable. A protocol to enhance *in vitro* propagation of EPC number is therefore of high interest for the development and improvement of therapeutical procedures.

In chapter 2 of this study the enhanced *ex vivo* propagation of EPC number by CYR61 treatment is described. The study revealed an up to 7-fold induced cell number within one week of cultivation without altering the EPC phenotype.

EPCs were obtained from peripheral blood and treated with different concentrations (0.05 – 1.5 µg/ml) CYR61, resulting in an enhanced cell number already at the lowest concentration. Saturation was achieved at 1.5 µg/ml CYR61 treatment, therefore the concentration of 0.5 µg/ml was used in further studies. EPCs were characterised by the expression of the markers CD34, CD133 and KDR as well as by the uptake of acLDL and the concurrent staining for ulex lectin. Both the CYR61 treated and the untreated control cells displayed similar properties, which indicates that the EPCs characteristics are not altered by CYR61 treatment.

A proliferative effect of CYR61 on EPCs was not detectable. In further studies the time point of initiation of CYR treatment was varied. CYR61 was additionally added to the crude mononuclear cell preparation prior to adhesion. The coating of the cell culture surface with CYR61 revealed that the observed effect of enhanced cell number is caused by improved cell adhesion. The adhesion of $CD14^+$ monocytes and further differentiation to endothelial like cells with similar properties as EPCs is widely discussed in the literature (Fernandez Pujol

2000; Rehman 2003; Romagnani 2005). It is therefore possible that CYR61 treatment leads to an enhanced adhesion of these monocytes and further differentiation to EPCs.

In order to elucidate the mechanism responsible for the observed effect of CYR61 on EPCs, cells were treated with mutated CYR61 proteins with defect binding sides for the integrins $\alpha_v\beta_3$ (B3) and $\alpha_6\beta_1$ (T1). Treatment with B3 did not influence the EPCs behaviour whereas the cell number decreased due to treatment with T1, indicating that the integrin $\alpha_6\beta_1$ at least partly mediates the binding of EPCs to the surface. Monocyte adhesion is mediated by the integrin $\alpha_M\beta_2$ (Schober 2002), it has to be elucidated in the future if this integrin affects the adhesion of EPCs.

This study revealed that EPCs do not express CYR61 endogenously. Therefore results obtained in control cells can not be influenced by endogenous secretion of the protein. It is interesting to note that osteoclasts as well do not express CYR61. Both EPCs and osteoclasts responded to very low concentrations (0.05.µg/ml) of the protein (Crockett 2007), whereas CYR61 expressing cells like MSCs, the endothelial (Ea hy 926) and the osteoblast cell line (hFOB) needed higher concentrations of 0.5 µg/ml to observe effects (Schutze 2005).

A connection between EPCs and the angiogenic inducer CYR61 was revealed for the first time by Grote et al (Grote 2007). They described a promoted adhesion and migration of $CD34^+$ cells after treatment with recombinant commercial available CYR61. Although the authors did not call their cells EPCs, the isolation method by gradient centrifugation as well as the $CD34^+$ magnetic bead separation and further cultivation is characteristic for this cell type. Some of the presented results are in line with the observations described in chapter 2 of this study. Thus, the authors (Grote 2007) describe an effect of CYR61 at low concentrations, found that CYR61 stimulates adhesion via integrins and could not detect endogenous expression of CYR61 in their $CD34^+$ cells.

Whether CYR61 plays a role in EPC mobilisation *in vivo* is unclear at present. However, it is interesting to note that EPCs home to sites where the CYR61 protein is highly expressed and both EPCs and CYR61 are induced by similar factors. The mobilisation of EPCs is induced by estrogens and physical exercise (Dimmeler 2001; Strehlow 2003), CYR61 expression is upregulated by estrogens as well as by mechanical stress (Chaqour and Goppelt-Struebe 2006). EPCs home to specific regions in the body like wounds, cancer and ischemic regions (Vasa 2001; Hristov and Weber 2004; Aicher 2005). CYR61 expression is upregulated in similar locations (Tsai 2002; Chen and Du 2006). A high expression of CYR61 in fracture

callus has also been described (Hadjiargyrou 2000; Lienau 2006; Athanasopoulos 2007) and it would be of high interest to know if EPCs are present at this location which has not been described as yet. Since the fracture callus is a hypoxic region and blood vessel formation is integral part of fracture healing it appears very likely that EPCs accumulate in this region since CYR61 is highly expressed.

In conclusion the results of this study show that the angiogenic inducer CYR61 is a promising tool for the enhancement of EPCs number *in vitro*. This could be beneficial for cell-based therapeutic applications in bone regeneration and fracture healing in tissue engineering procedures.

5.2.2 Impact of CYR61 on MSCs

The CCN-protein CYR61 is expressed in numerous tissues and cell systems including MSCs. Different functions of the protein as angiogenic, proliferative and migrative have been described. A migratory effect of CYR61 on endothelial cells and fibroblasts has been proven before (Grzeszkiewicz 2001; Leu 2002). In this study the migratory effect of CYR61 on MSCs and osteogenic differentiated MSCs has been elucidated. The MSCs and differentiated MSCs responded to a treatment with 1 to 2 µg/ml CYR61 by induced migration. The effect was further specified as chemotaxis, which means the targeted migration in contrast to chemokinesis as a random migration towards an effector (Schütze et. al., 2007 submitted). Chemotactic effects of CYR61 are known to be mediated by integrin $\alpha_v\beta_3$ in endothelial cells and by integrin $\alpha_v\beta_5$ in fibroblasts, therefore the effect of both integrins was tested in MSCs. Preincubation of MSCs with antibodies for both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were performed and further analyzed in the migration assay. The migration potential of both MSCs and osteogenic differentiated MSCs were not influenced by either of the integrins, indicating that they do not mediate the migration. Further studies to elucidate if other integrins, which are known to be influenced by CYR61, like $\alpha_6\beta_1$, $\alpha_M\beta_2$, $\alpha_{IIb}\beta_3$ and heparan proteoglycans have to be done.

Chapter 3 of this study describes an approach to differentiate MSCs to the angiogenic lineage. Cells were either treated with 0.5 µg/ml CYR61, with growth factors including VEGF or with both together and compared to untreated control cells. Additionally the influence of reduced oxygen content (3 %) on the differentiation capacity was elucidated and compared to normal oxygen conditions. Differentiated MSCs were analyzed using RT-PCR, immunocytochemistry and a matrigel angiogenesis assay. Gene expression analysis of

angiogenic factors like CD31 (PECAM), Angiopoietin-1 (Ang-1) and -2 (Ang-2) revealed an upregulation after angiogenic differentiation with growth factors, whereas the VEGF-receptor 1 (Flt-1) decreased in all samples. Hypoxic culture conditions resulted in induced mRNA expression of the VEGF-receptor 2 (KDR), Flt-1 and Ang-2 compared to normoxic cultivated control cells. mRNA levels of Ang-1 was basically unchanged, whereas CD31 level decreased after cultivation of MSCs in hypoxic conditions. Divergent data were obtained from MSCs of three donors. While cells of one donor responded very strong to the treatment, MSCs of another donor were only marginal affected and the third MSC population showed no reaction. This could be due to various levels of the intracellular expression and secretion of CYR61 in MSCs of different donors. The endogenously expressed amount of CYR61 may vary between donors, resulting in different results. Analyses of angiogenic factor protein expression by immunocytochemistry did not clarify the picture. The angiogenic factors CD31, KDR, VE-cadherin (VE-cad) and von Willebrand factor (vWF) were similarly expressed in all samples.

The culture conditions used in this study are similar to these previously described (Reyes 2002; Oswald 2004; Cao 2005). However other researchers detected a clear induction of angiogenic factors due to treatment with growth factors. A reason for this difference could be that the MSCs used in this study were obtained from bone marrow of the femoral head from patients undergoing hip replacement surgery. It is therefore likely that the MSCs have osteogenic or adipogenic properties. Many other researchers use MSCs obtained from the iliac crest bone marrow and select cells with special surface markers before propagation. This might result in a more immature stem cell phenotype and therefore better differentiation capacity into various lineages.

The differentiation capacity of MSCs is controversially discussed in the literature. Some researchers (Jiang 2002) differentiated MSCs even into other lineages of the neuroectodermal and endodermal germlayer and suggest that this finding relates to the term stem cell plasticity. Other researchers (Bianco P, personal communication) could not confirm these results and proclaim therefore that MSCs have limited differentiation capacity only into cells of the musculoskeletal system. It therefore not fully clarified if MSCs have the potential to differentiate into the endothelial lineage with angiogenic properties.

The matrigel angiogenesis assay revealed a clear indication for the angiogenic potential of CYR61. MSCs treated with CYR61 started to sprout with strong ramification of the vessel like structures on the matrigel surface. This result is similar to the picture obtained from

MSCs treated with growth factors, whereas the untreated control cells responded with sprouting but without ramification and connection of the structures. The angiogenic potential of CYR61 is therefore proven in the established matrigel assay and CYR61 treated MSCs responded similar to cells treated with growth factors. Hypoxic culture conditions did not influence the results.

The angiogenic potential of CYR61 on MSCs was proven by the matrigel assay. Furthermore a proliferative (chapter 3 and (Schutze 2005)) and migrative effect of CYR61 (Schütze et al., submitted) on MSCs has been demonstrated, identifying MSCs as CYR61 target cells. Both processes are essential for angiogenesis (Risau 1997). These findings strengthen the view of CYR61 as an angiogenesis stimulator. Several studies revealed that osteogenic and angiogenic processes are closely related processes (Carano and Filvaroff 2003; Peng 2005; Athanasopoulos 2007). CYR61 is induced by the potent angiogenic factor VEGF and promotes fracture healing (Athanasopoulos 2007). The relationship of the different bone associated cell types to CYR61 and the relevance of the protein for bone remodelling is depicted in figure 5.1.

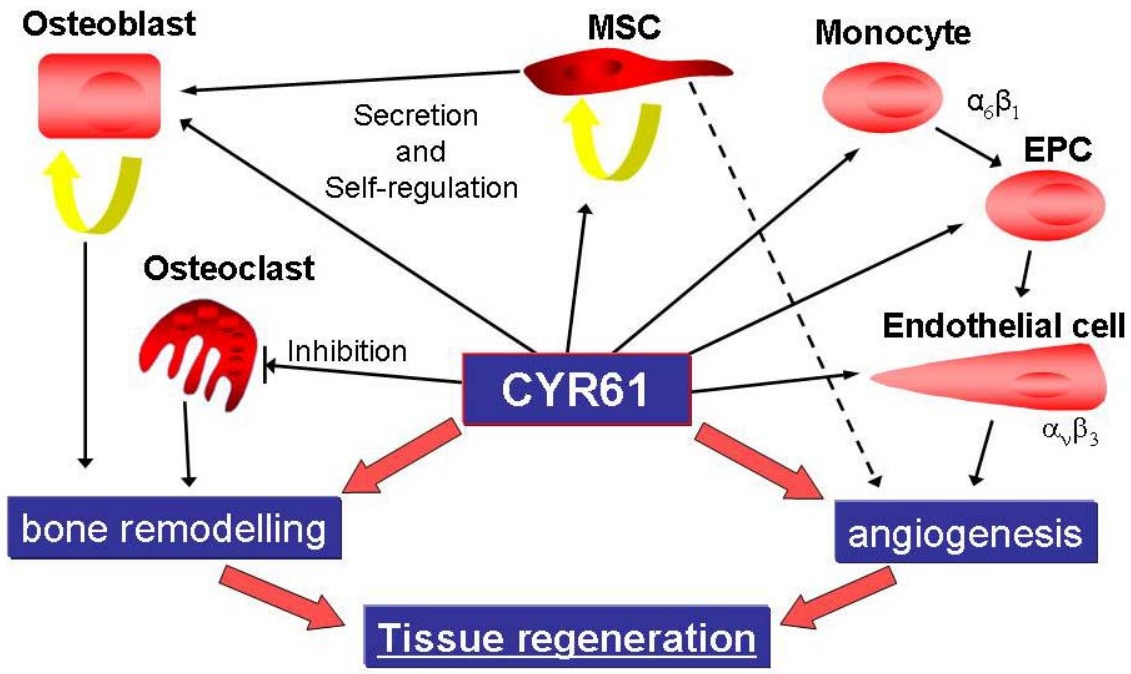


Figure 5.1: Impact of CYR61 on different cell types of the musculoskeletal system. *The CYR61 protein is expressed in osteoblasts and MSCs. Both respond to the protein with an enhanced proliferation rate. The differentiation capacity towards bone is improved by CYR61 treatment of osteoblasts. On the other hand, osteoclasts are inhibited by CYR61. Therefore CYR61 has a crucial role in bone remodelling processes. Angiogenic differentiation of MSCs is induced by CYR61 treatment. The adhesion of monocytes is enhanced by CYR61 treatment via the integrin $\alpha_6\beta_1$. These monocytes obtain EPC characteristics and differentiate subsequently to endothelial cells, which are crucial for angiogenesis. CYR61 is involved in Bone remodelling and angiogenesis. Both processes are closely related and of intrinsic value for bone tissue regeneration.*

5.3 Impact of WISP3 on MSCs

In a previous study it was shown that MSCs do express the CCN-protein WISP3 (Schutze 2005). In another study (Schütze et al., 2007, submitted) a migratory assay by using modified Boyden-chamber revealed a chemotactic effect of WISP3 on MSCs as well as on osteogenic differentiated MSCs, thereby identifying MSCs as WISP3 target cells. The most effective concentration was 2 µg/ml WISP3. The impact of CCN-proteins is not mediated by a specific receptor, but rather by integrins and proteoglycans and perhaps additional binding partners. In order to elucidate the mechanism responsible for the migratory effect of WISP3 on MSCs, cells were preincubated with antibodies against the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. While the $\alpha_v\beta_3$ antibody had only marginal inhibitory effect on the migratory potential of the WISP3 treated MSCs, the antibody $\alpha_v\beta_5$ entirely prevented the migration of the MSCs as well as the osteogenic differentiated MSCs. Therefore the integrin $\alpha_v\beta_5$ seems to be the mediator for the migratory effect of WISP3 on MSCs. This finding is the first hint towards the mechanism involved in WISP3 impact on MSCs and may be relevant for MSC recruitment and migration in the context of skeletal development and repair.

MSCs are target cells of WISP3 function, but the molecular mechanism is not understood as yet. Therefore this study elucidated the gene expression pattern of WISP3 treated MSCs. In chapter 4 the affymetrix gene expression study of MSCs treated with 0.5 µg/ml WISP3 was described. MSCs were treated for 24 h and compared to untreated control MSCs. In the gene expression study a number of interferon related genes including chemokines and the TNFSF superfamily were detected as WISP3-dependent gene products and verified by RT-PCR. The detected transcripts are associated to processes like anti-angiogenesis, cell survival, immune response and apoptosis. An additional time series whereas the MSCs were treated for 2, 4, 6, 12, 24 and 48 hours confirmed the array results and revealed an upregulation of most of the detected gene products after 12 h of treatment with a decrease until 48 h. Bioinformatic analyses of the array results identified a cluster of transcripts inducible by the interferons α and γ. Both interferons were not detected by RT-PCR in MSCs, therefore most likely the regulated transcripts are directly dependent on WISP3. However, interferon β was upregulated in the MSCs by WISP3 beginning after 4 h of treatment until 24 h. Therefore we can not exclude the possibility that (some of) the detected transcripts are not induced by WISP3 treatment directly but rather by interferon β which is induced by WISP3.

The gene TNFSF10 was detected in array analysis. This finding is consistent with the literature since the corresponding protein is discussed as a potent anti cancer agent and WISP3 is known to be downregulated during inflammatory breast cancer and therefore described as a tumour suppressor and anti-angiogenic factor (Kleer 2004). In line with the anti-angiogenic potential is the finding that the anti-angiogenic chemokines CXCL10 and CXCL11 (Rosenkilde and Schwartz 2004) were increased in MSCs after WISP3 treatment. Established markers associated to cartilage or chondrogenic differentiation were not detected in the array analysis, although WISP3 has been described to induce collagen type II and aggrecan expression in the chondrocyte cell line C28I2 (Sen 2004). This could be due to the selected time point of 24 h, which is possibly too early to detect genes of the slow metabolic cartilage. Though, immune responsive and the already mentioned anti-angiogenic genes were detected. They could participate in the protection of the cartilage tissue from inflammatory and invasive processes. In line with this the human disease PPD is characterised by cartilage destruction and is caused by lost of function mutations of the WISP3 gene. Since PPD is an inherited disease and shows no symptoms until early childhood, it is likely that WISP3 has no strong cartilage differentiation supporting constitutive function but rather plays a protective role in cartilage homeostasis. A recent study revealed that chondrocytes obtained from patients suffering PPD show decreased apoptosis rates (Zhou 2007). Interestingly numerous apoptosis inducing genes were found to be increase in MSCs after WISP3 treatment. Therefore the findings of the study are congruent with the literature and further strengthen the relevance of WISP3 for cartilage protection and maintenance. Continuative studies with chondrocytes and chondrocyte cell lines based on the results obtained from WISP3 treated MSCs may give hints towards the pathophysiology of PPD.

5.4 Future perspectives

This study revealed that EPC number is enhanced by CYR61 treatment, but the responsible mechanism is widely unknown. The CYR61 impact in different cells is mediated by the integrins $\alpha_v\beta_3$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, $\alpha_M\beta_2$ and heparan sulphate proteoglycans. Several questions arise from this knowledge: Which integrins are expressed on EPCs? Do specific antibodies against the integrins and proteoglycans block the enhanced propagation of EPCs by CYR61?

Furthermore it would be interesting to know which fraction of the isolated mononuclear cells from peripheral blood does respond to CYR61. This question could be answered by the use of a tagged antibody against CYR61, separation of the CYR61 binding and non-binding fractions and separated cultivation and analysis of the cells.

As various factors induce EPCs mobilisation *in vivo,* it would be interesting to investigate which effect has intravenous application of CYR61 to the organism.

MSCs and EPCs seem to be involved in bone repair mechanisms. A first attempt to elucidate the crosstalk between both cell types has been made (Raida 2006). A co-culture model using MSCs and EPCs in context with CYR61 in order to understand cell communication in osteogenic and angiogenic differentiation could be an interesting approach.

Hypoxic conditions in the field of MSCs propagation and further differentiation should be investigated. As angiogenesis and osteogenesis are closely related processes, hypoxia could be beneficial for both differentiation systems.

Tissue engineered bone constructs are under investigation. As soon as the construct exceeds a distinct size cell based applications are difficult to supply with oxygen and nutrients. The immediate induction of angiogenesis within the construct is therefore a fundamental step to improve tissue engineering approaches. Different strategies to induce angiogenesis by using CYR61 are possible. First of all pretreatment of cells with CYR61 (MSCs or EPCs) before application to the construct is a possibility. Furthermore the construct could be coated with CYR61 in order to attract cells like EPCs, which induce neovascularisation within the construct. The third approach could be the use of slow release constructs supplemented with CYR61 in order to release the protein constantly to the surrounding area.

WISP3 is of relevance for cartilage protection and maintenance and the association to the disease PPD is widely accepted. Therefore further investigations to clarify the underlying mechanisms have to be done. An approach for such an investigation could be a gene expression study of WISP3 treated chondrocytes or chondrocyte cell lines. Furthermore

functional studies, as for example an apoptosis test with WISP3 treated MSCs and chondrocytes, should be done.

Another unresolved question deals with the function of the single domains of CYR61 and WISP3. Single domains and segments with two or three domains of CYR61 were expressed as purified Fc-tagged proteins. This work has to be done for the WISP3 protein. The established functional tests, such as proliferation and apoptosis, should be repeated with the single domains of the proteins and compared to the full length protein.

Apart from the musculoskeletal system CYR61 and WISP3 are of interest for anti-cancer therapies. WISP3 as an anti-cancer drug, since a number of anti-cancer and anti-angiogenic factors are induced by WISP treatment and anti-CYR61 antibodies in order do suppress angiogenesis in tumour cells.

Chapter 6

6.1 Summary

CYR61 and WISP3 belong to the family of CCN-proteins. These proteins are characterised by 10% cysteine residues whose positions are strictly conserved. The proteins are extracellular signalling molecules that can be associated with the extracellular matrix. CCN-proteins function in a cell- and tissue specific overlapping yet distinct manner. CCN-proteins are expressed and function in several cells and tissues of the musculoskeletal system.

In this study the impact of the angiogenic inducer cysteine-rich protein 61 (CYR61/CCN1) on endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) as well as the wnt1 inducible signalling pathway protein 3 (WISP3/CCN6) on MSCs were elucidated.

EPCs are promising cells to induce neovascularisation in ischemic regions as tissue engineered constructs. A major drawback is the small amount of cells that can be obtained from patients; therefore a stimulating factor to induce *in vitro* propagation of EPCs is urgently needed. In this study, mononuclear cells obtained from peripheral blood were treated with 0.5 µg/ml CYR61, resulting in an up to 7-fold increased cell number within one week compared to untreated control cells. To characterise if EPCs treated with CYR61 display altered or maintained EPC phenotype, the expression of the established markers CD34, CD133 and KDR as well as the uptake of acLDL and concurrent staining for ulex lectin was analysed. Both CYR61 treated and untreated control cells displayed EPCs characteristics, indicating that CYR61 treatment induces EPC number without altering their phenotype. Further studies revealed that the stimulating effect of CYR61 on EPCs is due to enhanced adhesion, rather than improved proliferation. Usage of mutated CYR61-proteins showed that the adhesive effect is mediated, at least partly, by the integrin $\alpha_6\beta_1$, while the integrin $\alpha_v\beta_3$ has no influence. Endogenous expression of CYR61 was not detectable in EPCs, which indicated that control cells are not influenced by endogenous secretion of CYR61 and also could explain the dose-dependent effect of CYR61 that is measured at a low concentration of 0.05 µg/ml.

MSCs were treated with 0.5 µg/ml CYR61, a combination of growth factors including VEGF, both together and compared to untreated control cells. Matrigel angiogenesis assay revealed an induction of angiogenesis, detected by induced sprouting of the cells, after CYR61 treatment of the MSC. Induced sprouting and vessel like structure formation after CYR61

treatment was similar to the results obtained after treatment with growth factors including the established angiogenesis inducer VEGF. This result clearly demonstrates the angiogenic potential of CYR61 on MSCs. Further studies revealed a migrative and proliferative effect of CYR61 on MSCs. Both properties are crucial for the induction of angiogenesis thus further strengthening the view of CYR61 as an angiogenic inducer.

MSCs and EPCs are promising cells for tissue engineering applications in bone remodelling and reconstruction. MSCs due to their potential to differentiate into other lineages; EPCs induce neovascularisation within the construct. Both cell types respond to CYR61 treatment. Furthermore EPCs home to sides were CYR61 expression is detectable and both are induced by similar stimulators. Therefore CYR61 is a promising factor for tissue engineered bone reconstruction applications.

WISP3 is expressed in cartilage in vivo and in chondrocytes in vitro. Loss of function mutations in the WISP3 gene are associated to the inherited human disease progressive pseudorheumatoid dysplasia (PPD), that is characterised by cartilage loss and bone and joint destruction. Since MSCs also express the protein, the aim of this study was to elucidate if recombinant protein targets MSCs. A migratory effect of WISP3 treatment on MSCs and osteogenic differentiated MSCs has been proven in this study. To elucidate if global gene expression patterns are influenced by WISP3, cells were treated with 0.5 µg/ml WISP3 and compared to untreated control MSCs. Gene expression study by using affymetrix technology revealed an induction of interferon inducible genes including CXCL chemokines and members of the TNFSF family. Reevaluation by RT-PCR on identical RNA and an additional time series confirmed the results. Although no established cartilage associated genes were detected as regulated genes within this 24h treatment, anti-angiogenic and immunosuppressive genes indicate a protective role of WISP3 for the cartilage, which is sensitive to inflammatory processes.

Both CCN-proteins CYR61 and WISP3 are valuable for the musculoskeletal system. This and previous studies revealed the role of CYR61 for osteogenesis and angiogenesis of tissue engineered applications. WISP3 is responsible for development, protection and maintenance of cartilage. Therefore further studies with the proteins in the musculoskeletal system are of high relevance.

6.2 Zusammenfassung

CYR61 und WISP3 gehören zur Familie der CCN-Protein. Diese Proteine werden durch ihre Cysteinreste charakterisiert die10 % der Proteine ausmachen und hoch konserviert sind. Die Proteine sind extrazelluläre Signalmoleküle und können an die extrazelluläre Matrix gebunden sein. CCN-Proteine wirken Zell- und Gewebeabhängig in einer spezifischen und doch überlappenden Weise. CCN-Proteine werden exprimiert und wirken gleichzeitig in einigen Zellen und Geweben des muskoloskeletalen Systems.

In dieser Arbeit wurde der Einfluss des angiogen wirkenden Cystein-reichen Proteins 61 (CYR61/CCN1) auf endotheliale Progenitorzellen (EPCs) und mesenchymale Stammzellen (MSCs), sowie die Wirkung vom wnt indizierbaren Signalweg Protein 3 (WISP3/CCN6) auf MSCs untersucht.

EPCs sind viel versprechende Zellen für die Behandlung und Neovaskularisierung von Ischämien wie zum Beispiel in Konstrukten aus dem Tissue Engineering. Von Nachteil ist die geringe Zellzahl, die von einem Patienten gewonnen werden kann. Aus diesem Grund ist ein Stimulator notwendig, der die *in vitro* Vermehrung der Zellen induziert. In dieser Studie wurden mononukleäre Zellen aus dem peripheren Blut von Spendern mit 0,5 µg/ml CYR61 behandelt. Die Zellzahl der CYR61 behandelten Zellen nahm innerhalb von einer Woche um das 7-fache im Vergleich zu den unbehandelten Zellen zu. Um die CYR61 behandelten EPCs zu charakterisieren wurde die Expression der etablierten Oberflächenmarker CD34, CD133 und KDR sowie die Aufnahme von acLDL mit der gleichzeitigen Anfärbbarkeit für Ulex lektin untersucht. Sowohl die CYR61 behandelten als auch die unbehandelten Zellen zeigten die charakteristischen Merkmale für EPCs. Somit ist der Nachweis erbracht, dass die EPC Zellzahl durch die CYR61 Behandlung erhöht wird ohne den Phänotyp der Zellen zu ändern. Weitere Studien ergaben dass der beobachtete Effekt eher auf verstärkter Adhäsion an die Zellkulturoberfläche als auf eine Induktion der Proliferationsrate beruht. Die Verwendung von mutierten CYR61 Proteinen zeigte, dass der adhäsive Effekt zumindest zum Teil über das Integrin $\alpha_6\beta_1$ vermittelt wird, während das Integrin $\alpha_v\beta_3$ keinen Effekt zu haben scheint. Eine endogene Expression von CYR61 in EPCs konnte nicht nachgewiesen werden, was die Ansprechbarkeit der EPCs schon bei niedrigen dosis-abhängigen Konzentrationen von 0,05 µg/ml erklären könnte.

MSCs wurden mit 0,5 µg/ml CYR61, einer Kombination von Wachstumsfaktoren inklusive VEGF und beiden zusammen behandelt und mit unbehandelten Kontrollzellen verglichen. Im

Matrigel Angiogenese Assay konnte die Induktion von Angiogenese, ermittelt durch die Induktion der Zellsprossung, durch die Behandlung der MSCs mit CYR61 nachgewiesen werden. Die beobachtete Sprossung und Bildung von Gefäß-ähnlichen Strukturen nach der CYR61 Behandlung war dem Effekt nach der Behandlung mit Wachstumsfaktoren inklusive dem etablierten angiogenen Stimulator VEGF ähnlich. Dieses Ergebnis ist der Beweis für das angiogene Potential von CYR61 auf MSCs. Weitere Studien bewiesen einen migrativen und proliferativen Effekt von CYR61 auf MSCs. Beide Eigenschaften sind entscheidend für die Induktion von Angiogenese, wodurch das Bild von CYR61 als angiogener Induktor verstärkt wird.

MSCs und EPCs sind viel versprechende Zellen für die Rekonstruktion und den Umbau von Knochen mittels Tissue Engineering. MSCs durch ihr Potential in verschiedene Richtungen zu differenzieren und EPCs durch die Möglichkeit der Neovaskularisierung der besiedelten Konstrukte. Beide Zellarten reagieren auf CYR61 Behandlung. Weiterhin akkumulieren EPCs an ähnlichen Stellen im Körper an denen CYR61 exprimiert wird. Außerdem werden beide durch die gleichen Faktoren stimuliert. Deshalb stellt CYR61 einen viel versprechenden Faktor für Knochenrekonstruktions-Anwendungen mittels Tissue Engineering dar.

WISP3 wird *in vivo* im Knorpel und *in vitro* in Chondrozyten exprimiert. Außerdem sind Funktionsverlust-Mutationen im WISP3-Gen mit der vererbten Krankheit Progressive Pseudorheumatoide Dysplasie (PPD) assoziiert. Die Krankheit ist durch den Verlust von Knorpel und dem Abbau von Knochen gekennzeichnet. MSCs exprimieren WISP3, aus diesem Grund sollte in der Studie geklärt werden welche Wirkung das rekombinate Protein auf MSCs hat. Ein migratorischer Effekt von WISP3 auf MSCs und osteogen differenzierte MSCs wurde in dieser Studie nachgewiesen. Um den Einfuß der WISP3 Behandlung auf das globale Genexpressionsmuster der MSCs zu ermitteln, wurden diese mit 0,5 µg/ml WISP3 behandelt und mit unbehandelten Zellen verglichen. Genexpressionsstudien mittels Affymetrix Technologie zeigte eine Induktion von interferon stimulierten Genen, unter anderem CXC Chemokine und Mitglieder der TNFSF Familie. Die Ergebnisse wurden durch RT-PCR an identischer RNA und einer zusätzlichen Zeitreihe bestätigt. Obwohl keine eindeutig knorpelrelevanten Gene detektiert wurden, stellen die gefundenen anti-angiogen und immunsupressiv wirkende Gene eine schützende Funktion für den im Zusammenhang mit immuninflamtorischen Prozessen empfindlichen Knorpel dar.

Sowohl CYR61 als auch WISP3 sind wichtig für das muskoloskeletale System. Diese und vorherige Studien haben gezeigt das CYR61 einen Einfluss auf die Osteogenese und Angiogenese vom MSCs hat. WISP3 ist verantwortlich für die Entwicklung, den Schutz und Erhalt von Knorpel. Deshalb sollten weitere Studien zur Funktionsaufklärung der Proteine im muskoloskeltalen System durchgeführt werden.

Chapter 7

Appendix

7.1 References

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7.2 Abbreviations

Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
CAC	circulating angiogenic cells
CAM-assay	chick chorioallantoic membrane assay
CCL5	chemokine (CCL-motif) ligand 5
CD marker	cluster of differentiation marker
CEC	circulating endothelial cells
CTGF	connective tissue growth factor
CXCL10	chemokine (CXC-motif) ligand 10
CXCL11	chemokine (CXC-motif) ligand 11
CXCR3	chemokine (CXC-motif) receptor 3
CYR61	cysteine-rich protein 61
eEPC	early endothelial progenitor cells
EF1α	elongation factor 1 alpha
EGF	epidermal growth factor
EPCs	endothelial progenitor cells
Flt-1	VEGF receptor-1
GBP	guanylate binding protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione transferase
HA-TCP	hydroxyapatite-tricalcium phosphate
HSP	heparin sulphate proteoglycan
IFIT	interferon-induced protein with tetratricopeptide repeats
IGF	insulin-like growth factor
ISG20	interferon stimulated exonuclease gene 20kDa
JIA	juvenile idiopathic arthritis
KDR	VEGF receptor-2
MSCs	mesenchymal stem cells

NOV	nephroblastoma overexpressed
OASL	2'-5'-oligoadenylate synthetase-like
OEC	endothelial outgrowth cells
PDGF	platelet-drived growth factor
PLA	poly lactic acid
PPD	progressive pseudorheumatoid dysplasia
RA	rheumatoid arthritis
SAMD9	sterile alpha motif domain containing 9
SLR	signal $\log_2$ ratio
Stro-1	stromal cell precursor antibody
TGF	transforming growth factor
TNFSF10	tumour necrosis factor family member 10
TNFSF13b	tumour necrosis factor family member 13B
TNF-α	tumour necrosis factor α
VEGF	vascular endothelial growth factor
WISP3	wnt1 inducible signalling pathway protein 3

7. 3 Curriculum vitae

Personal details

Name	Rita Schenk
Birth name	Wagemanns
Address	Waldalgesheimerstr. 7
	55545 Bad Kreuznach
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08/81-06/84	elementary school, Born
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08/91-06/94	grammar school “St. Wolfhelm”, Waldniel
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Academical career

10/96-04/04	studies of biotechnology at the Technical University Berlin
10/01-02/02	student research project at the Max Vollmer Institut für biophysikalische Chemie und Biochemie, Fachgebiet Biochemie und molekulare Biologie. Titel: „Detektion unbekannter nichtribosomaler Peptidsynthetase Gene in filamentösen Pilzen“
02/03-04/04	diploma thesis at Medizinische Klinik mit Schwerpunkt Rheumatologie und Klinische Immunologie, Labor für Tissue Engineering, Charite, Berlin. Titel: „Molekulare Charakterisierung humaner 3D-Knorpelzell und Pannus-Kulturen als *in vitro*-Modell für die rheumatoide Arthritis“
university degree	Dipl.-Ing. Biotechnology
09/04	start of PhD thesis at the centre for musculoskeletal research, University of Würzburg
08/94-08/96	Two stays abroad, among others in Thailand, Australia, New Zealand, USA, Ecuador and Canada. In between side jobs for financing.

Award

2007	conference scholarship of the DArdoW Young investigator Symposium 2007 (YIOSS) to the Osteologie in Wien.

7.4 Acknowledgements

I would like to thank….

PD Dr. Norbert Schütze, for giving me the opportunity to make this scientific thesis at the centre for musculoskeletal research. Furthermore I would like to thank for the support and helpful discussions in the last three years and the opportunity to present the work at national and international symposia.

Prof. Roland Benz, for accepting the position as an assessor, thereby enabling this scientific doctorate at the faculty of medicine.

PD Dr. Ullrich Nöth, for supervising and making this thesis possible.

Prof. Franz Jakob, for the revision of this work and many helpful advices and scientific discussions.

Prof. Jochen Eulert, for the support of the basic scientific research at the orthopaedic clinic Würzburg.

The interdisciplinary centre for clinical research (IZKF) Würzburg**,** for the funding of this project.

Susanne Jatzke**,** for excellent technical support and many helpful ideas and discussions.

Anke Hoffmann**,** for her excellent and zealous work in this project.

Nadja Karl for the introduction in the field of adenoviral gene transfer.

Viola Monz**,** Christa Amrehn and Manu Kunz**,** for excellent technical support.

Martina Regensburger**,** for isolation and providing of the primary MSCs and many helpful technical advice.

Dr. Regina Ebert, Andrea Heymer, Dr. Catarina Limbert, Dr. Lothar Seefried, Dr. Tatjana Schilling and Meike Weber**,** for sharing the office with me and many scientific and non-scientific discussions.

Heike Ruoff, Bettina Hainke and Jens Jäger**,** for many interesting questions to the research.

Doris Schneider, Jutta Meißner-Weigl, Sabine Zeck and Sigrid Müller-Deubert**,** for technical advice.

Monika Hofmann**,** for the kind helps in organisational tasks.