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VLIV O-GLYKOSYLACE V ONKOFETÁLNÍM FIBRONEKTINU NA KOSTNÍ FORMACI

Diplomová práce

Vypracováno na
Institutu imunologie
Ruprecht-Karls-University
Heidelberg

Školitelé:

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Heidelberg, Hradec Králové 2011 Alžběta Lejsalová

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THE INFLUENCE OF THE O-GLYCOSYLATION IN ONCOFETAL FIBRONECTIN ON THE BONE FORMATION

Diploma thesis

Performed at

Institute of Immunology

The Ruprecht-Karls-University

Heidelberg

Supervisors:

Prof. Dr. Inaam Nakchbandi
Doc. PharmDr. Tomáš Šimůnek, Ph.D.

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I declare that this work is my original author work, which I had developed by myself. All literature and other sources, which I had used, all of them are given in the list of used literature and they are quoted in text regularly. The work was not used to get same or another title.

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2011

Alžběta Lejsalová

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Abstrakt

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Katedra biochemických věd

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Název diplomové práce: Vliv O-glykosylace v onkofetálním fibronektinu na kostní formaci

Hepatická osteodystrofie je jednou z nejčastějších komplikací pacientů s chronickým cholestatickým onemocněním jater, které se významně podílí na snížení kvality života. Bylo zjištěno, že zvýšené hladiny onkofetálního fibronektinu u pacientů s primární biliární cirhózou korelují se ztrátou kostní hmoty a sníženou kostní formací. Onkofetální fibronektin je charakterizován O-glykosylací v místě variabilní domény. Kromě tohoto místa ke O-glykosylace dochází ještě na NH_2-konci molekuly fibronektinu. Cílem této práce bylo připravit O-deglykosylované formy fibronektinu a provést genový silencing fibronektinu v savčích buňkách pro další výzkum, který má ověřit efekt O-glykosylace na kostní formaci.

Abstract

Charles University in Prague
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Performed at
Institute of Immunology
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Candidate: Alžběta Lejsalová
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Title of diploma thesis: The Influence of the O-glycosylation in oncofetal fibronectin on the bone formation

Hepatic osteodystrophy is a major complication in patients with chronic cholestatic liver diseases which significantly contributes to decreased quality of life. It was established that increased levels of the oncofetal domain of fibronectin correlated with bone loss and decrease in bone formation in patients with primary biliary cirrhosis. The oncofetal fibronectin is characterized by the O-glycosylation in the variable domain. Another O-glycosylation is found on the NH_2-terminus. The aim of this project was to prepared O-deglycosylated fibronectin isoforms and fibronectin knockdown mammalian cells for further research on the effect of O-glycosylation on the bone formation.

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1 Introduction

1.1 Fibronectin

Fibronectin is a glycoprotein present in most body tissues and fluids. Fibronectin can bind to different molecules and themselves and thus it has an important role in various physiological and pathological processes. Fibronectin plays a major role in cell adhesion, growth, migration and differentiation (Vartio et al. 1987; Hynes 1990). Fibronectin is distinguished into two types according to solubility and presence of alternative spliced domains: soluble plasma fibronectin (pFN) and less soluble cellular fibronectin (cFN). Plasma fibronectin is produced mostly in liver by hepatocytes. It circulates in blood and other body fluids, where it is thought to enhance blood clotting, wound healing and phagocytosis. Cellular fibronectin is synthesized by fibroblast and other cell types. It regulates the shape of cells, the organization of the cytoskeleton and it is important for migration and cellular differentiation of many cell types (Hynes 1990; Tajiri et al. 2005).

1.1.1 Structure

Fibronectin is a high molecular weight glycoprotein having two subunits with a total molecular weight about 500 kDa. The two polypeptide chains are held together by a pair of interchain disulfides bonds at the C-terminal end. Moreover each subunit has about 30 interchain disulfide bonds (Hynes, 1990). The fibronectin monomer consists of three different types of repeats. There are 12 types I repeats, two type II repeats and 15-17 type III repeats. Although the different isoforms of fibronectin exist, they are always a product of a single gene. The resulting multiple forms of fibronectin arise from alternative splicing and posttranslational modifications (Hynes 1990; Murray et al. 2002; Pankov and Yamada 2002).

1.1.2 Posttranslation glycosylation

Glycosylation is a posttranslation modification that occurs in fibronectin, where seven potential N-glycosylation sites and two O-glycosylation sites are found. O-glycosylation is located at amino acid threonine. It is located either at Thr 278 or at Thr 279 in the connecting segment between the NH_2-terminal and a collagen-binding domain. The second one is in variable region either at Thr 2064 or Thr 2065 (Tajiri et al. 2005).

The carbohydrates probably stabilize fibronectin against hydrolysis and influence its affinity to some substrates (Pankov and Yamada 2002; Sano et al. 2008).

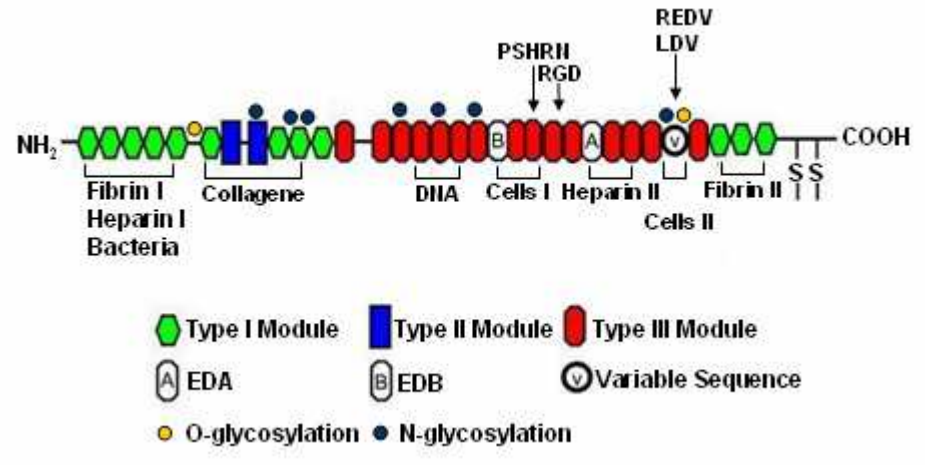


Figure 1: The structure of the fibronectin domain. The main functional domains with interaction regions, variably spliced module and glycosylation sites are shown (Wang et al. 2008).

1.1.3 Fibronectin isoforms

The variations of amino acid sequence at three different sites are consequences of the alternative splicing of the mRNA. This regions are extra domain B (also called EDB, EIII-B or EDII), extra domain A (EDA, EIII-A or EDI) and variable region (V or IIICS). EDA and EDB domains are either present or not. The variable region can be either partially or completely included or excluded (Pankov and Yamada 2002). The subdivision of variable domain is species dependent. 5 variants can be produced in humans (Schwarzbauer et al. 1989). As a result there are 20 potentially different forms of fibronectin in humans (Ffrench-Constant 1995).

1.1.3.1 The EDA domain

Fibronectin containing the EDA domain is characteristic in embryogenesis and play an important role in promoting cell proliferation and migration. The EDA positive fibronectin could be found also in adult tissues, namely in liver tumors or during wound healing, where cell migration is also a prominent feature (Vartio et al. 1987; Ffrench-Constant et al. 1989; Oyama et al. 1989; Brown et al. 1993; Manabe et al. 1999).

Inclusion of EDA and EDB domain in fibronectin is promoted by transforming growth factor beta (TGF-β) (Borsi et al. 1990). This plays a role in connection with fibrosis. TGF-β is present at sites of fibrosis and EDA is necessary for the differentiation of fibroblasts into fibrogenic myofibroblasts (Muro et al. 2008). Increased levels of EDA was also observed in patients with acute vascular tissue injury associated with major trauma or sepsis syndrome (Peters et al. 1989).

1.1.3.2 The EDB domain

The expression of fibronectin containing the EDB domain is similar to expression of the EDA positive fibronectin. EDB domain is also typical for proliferating tissue. It is a marker of angiogenesis (Castellani et al. 1994). As well as EDA also EDB positive fibronectin can be found in tumour tissue. The elevated amount of EDB was noted in lung tumor tissues (Oyama et al. 1990).

1.1.3.3 The variable domain

The variable domain is always present in one monomer of plasma fibronectin and almost in both chains of cellular fibronectin. These two forms have temporally and structurally specific roles in the tissue repairing. Soluble plasma fibronectin is important in the coagulation pathway, whereas cellular fibronectin forms are specialized for assembly into insoluble fibrils in the extracellular matrix. The variable region is required for fibronectin dimers formation and for the stability of this molecule (Schwarzbauer et al. 1989; Wilson and Schwarzbauer 1992) .

1.1.3.4 The oncofetal fibronectin

There is one specific isoform of fibronectin called oncofetal fibronectin (oFN). This isoform is defined by an O-linked glycosylation in the variable domain. The oncofetal fibronectin occurs in fetus and amniotic tissue, but also in carcinomas or by patients with chronic liver disease (Matsuura and Hakomori 1985; Matsuura et al. 1989; Kawelke et al. 2008). A raised level of this isoform in the cervix or vaginal secretion may serve as a predictor of preterm delivery (Nageotte et al. 1994).

1.1.4 Interactions

Fibronectin can bind to different molecules, such as glycosaminoglycans, collagen, fibrin and can be a ligand for a dozen members of the integrin receptor family (Plow et al. 2000).

Several integrin-recognition sequences are along the fibronectin molecule (Pankov and Yamada 2002). The cell adhesion RGD sequence (Arg-Gly-Asp amino acid triplet) is located in III10 and it occurs not only in fibronectin but also in other cell adhesion proteins. The RGD sequence is the dominating recognition motif for integrin $\alpha_V\beta_3$. This sequence acts in synergy with PHSRN (Pro-His-Ser-Arg-Asp), which is located in fibronectin repeat III9 and promotes specific $\alpha_5\beta_1$ integrin binding to fibronectin (Johansson et al. 1997).

There are two other cell-recognition sequences LDV (Leu-Asp-Val) and REDV (Arg-Glu-Asp-Val) identified in the alternatively spliced variable region. Both of them are recognized by $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (Pankov and Yamada 2002). The EDA type III repeat has also an adhesion activity for $\alpha_4\beta_1$ and $\alpha_9\beta_1$ and enhances the cell-adhesive activity of fibronectin by interaction of fibronectin with integrin $\alpha_5\beta_1$ (Xia and Culp 1994; Manabe et al. 1997; Liao et al. 2002).

Fibronectin contains two major heparin-binding domains that interact with heparan sulfate proteoglycans. The stronger heparin-binding site is located in the C-terminal part, the weaker binding domain is situated at the NH_2-terminal end of the protein. Syndecans, the transmembrane heparan sulfate proteoglycans, cooperate with integrins. Syndecan-4 mediates cell response to matrix fibronectin (Midwood et al. 2004) and initiates signals that enhance the fibronectin fibril assembly (Mao and Schwarzbauer 2005). Syndecan-1 also regulates fibronectin fibrillogenesis and the loss of it alters the activity of integrins that bind fibronectin (Stepp et al.) Fibronectin also contains two major fibrin-binding sites (Fibrin I and Fibrin II). The major site is in the N-terminal domain and is formed by type I repeats 4 and 5. The interaction of fibronectin with fibrin is mediated by factor XIII transglutaminase and is important for cell adhesion or cell migration into fibrin clots.

Repeats I and II of fibronectin include collagen-binding site (Pankov and Yamada 2002).

1.1.5 Functions

Not only because of interactions with a number of molecules fibronectin performs many vital functions. Fibronectin has important roles in wound healing, embryogenesis, fibrosis and in bone formation.

1.1.5.1 Wound healing

Fibronectin plays a significant role in wound healing. Together with von Willebrand factor and fibrinogen it belongs to the group of major ligands thought to mediate platelet adhesion and aggregation (Matuskova et al. 2006). Fibronectin incorporated in the clots serves as a chemoattractant for a various cells involved in wound healing, such as leucocytes, macrophages, fibroblasts, endothelial cells and epithelial cells and (Jo et al. 1991). It provides a provisional matrix for movement of fibroblasts into the clot and epithelial cells over the new basement membrane. It can also act as an arrestment for myofibroblasts. Fibronectin stabilizes collagens fibers by cross-linking with them. Mice with complete deficiency of plasma fibronectin had delayed thrombus formation and growth (Ni et al. 2003). Fibronectin can work also as an opsonin for dead cells and tissue debris (Jo et al. 1991).

1.1.5.2 Embryogenesis

The presence of fibronectin and other components of extracellular matrix is required for correct embryogenesis (Krolo et al. 1998). In developing mouse embryos, fibronectin is first expressed in blastocysts (George et al. 1993). It has been suggested that fibronectin may promote migration of parietal endoderm and trophoblast outgrowth during development (Sutherland et al. 1988; George et al. 1993; Krolo et al. 1998). The mutation of fibronectin gene involves multiple mesodermal defects, an arrest in axis elongation and the absence of somites, organized notochord and a defect in vasculogenesis in the yolk sac (Georges-Labouesse et al. 1996). Mice lacking fibronectin exhibit early embryonic lethality (George et al. 1993).

1.1.5.3 Fibrosis

The process of fibrosis is characterized by matrix remodelling and increased formation of fibrous connective tissue. Because fibronectin has an ability to bind connective

tissue and cells and mediates many cell-matrix interactions, it is important in situations where cellular and matrix components are remodelled (Rennard and Crystal 1982). It is one of the first matrix proteins increased in fibrosis (Rodés et al. 2007). Fibronectin and also TGF-β produced by activated macrophages can act as a chemoattractant for fibroblasts. Collagen produced by fibroblasts accumulates that can lead to the development of fibrosis (Rennard et al. 1981).

1.1.5.4 Bone formation

1.1.5.4.1 Fibronectin and osteoblasts

The presence of integrins on bone cells suggest a functional role of fibronectin for cell-matrix interactions in the cellular bone metabolism (Hughes et al. 1993). After addition of an anti-fibronectin antibody to the culture of osteoblasts the formation of mineralized nodules is inhibited. Fibronectin plays a role in regulating osteoblast differentiation (Moursi et al. 1996) and may support the survival of mature osteoblasts (Globus et al. 1998).

Osteoblasts themselves produce fibronectin during proliferation and differentiation, although the main source of continuous fibronectin provision would be the liver (Kawelke et al. 2008). The osteoblast-derived fibronectin seems to be a control of the number and function of the osteoblasts whereas the circulating FN affects the bone mineralization and contribute to bone health by maintenance of matrix integrity (Bentmann et al. 2010)

1.1.5.4.2 Fibronectin and collagen network

The bone matrix is composed mostly of collagen. The most abundant type in the extracellular matrix is collagen type I. Collagen type I is secreted as a triple helix, which then assembles to form fibrils (Murray et al. 2002) . Fibronectin plays a key role in collagen network formation, in the polymerization of fibrillar type I and III collagens (Velling et al. 2002). Fibroblasts unable to produce fibronectin and cultured in the absence of this glycoprotein failed to assemble collagen (Shi et al. 2010). Continues presence of fibronectin is crucial for regulation of composition and stability of extracellular matrix (Sottile and Hocking 2002).

1.2 Bones

Bones are a rigid and hard organ that form together with cartilages and joints the endoskeleton. The importance of bone is principally mechanical rigidity, support and protection of internal organs. Its surface forms the attachment site for muscles, tendons and ligaments to allow locomotion. Skeleton has also an essential metabolic function, serves as a reserve of calcium and phosphate. Red and white blood cells are formed in bone marrow. The human body has approximately 206 bones. The proportions vary according to type and region. Bones make up about 9% of body mass and 17% of body weight (Cooke 1955).

1.2.1 Anatomy

Skeleton consist of different bones forms. The four general categories of bones are long bones, short bones, flat bones, and irregular bones.

Outer surface of bone is covered with membrane called periosteum. The endosteal membranous structure covers the inner surface of cortical and trabecular bone and it is in contact with bone marrow space.

The bone mass of the adult skeleton consist of two tissue types, cortical or compact, and cancellous, spongy or trabecular bone. Cortical bone, which comprises 80% of the skeleton, is dense and solid. It constitutes the outer part of all skeletal structures and surrounds the marrow space. The functional unit of cortical bones is osteon called Haversian system. Osteons are formed of concentric layers or lamellae and central Haversian canal that contains nerves and blood vessels. Volkmann's canals running within osteons interconnect osteons with periosteum. Cancellous bone represents 20% of the skeletal mass. It is less dense and more elastic. It is composed of network of plates and rods interspersed in bone marrow compartment. The turnover rate is higher than in cortical bone and thus it has major metabolic function. The cancellous bone is placed towards the end of the bones and near the internal surface of the cortex. (Steele D.G. and Bramblett C.A. 1994; Felsenberg 2001; Hadjidakis and Androulakis 2006)

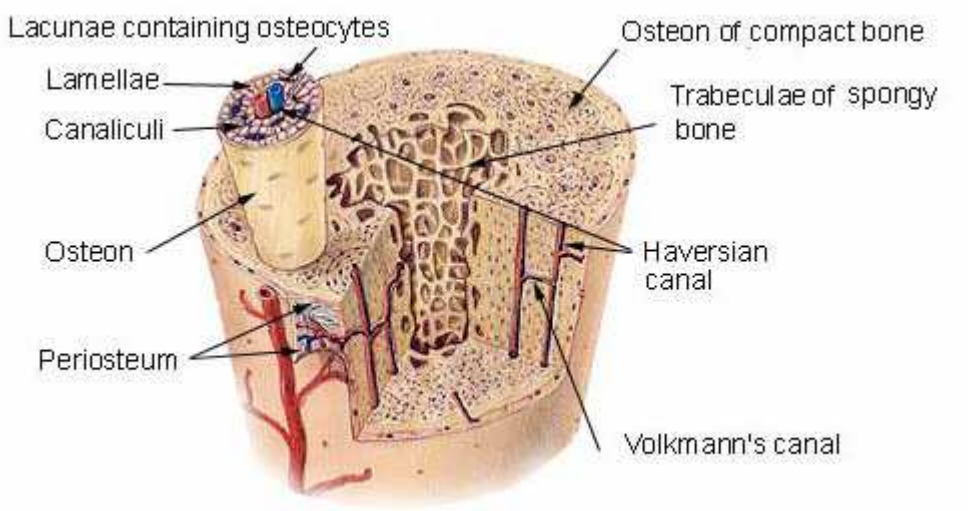


Figure 2: The structure of cortical and cancellous bone. Compact bone consists of osteons with central Haversian canal, which is surrounded by lamellae of matrix and osteocytes located between matrix in lacunae. Volkmann's canals interconnect osteons with periosteum. In the middle part of the bone the cancellous bone is situated. (http://training.seer.cancer.gov/anatomy/skeletal/tissue.html, date 3.5.2011)

1.2.2 Bone matrix

Bone is composed of 50 to 70% mineral, 20 to 40% organic matrix, 5 to 10% water, and less than 3% lipids. Bone mineral provides mechanical rigidity and bearing strength, whereas the organic matrix provides elasticity and flexibility. The mineral content of bone is mostly hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], with small amounts of carbonate, magnesium, and acid phosphate (Clarke 2008). The primary mineralization process is characterized by a rapid increase of minerals during few days up to 70% of the final value, followed by a slow and gradual maturation of the mineral component, called secondary mineralization (Ruffoni et al. 2007).

Approximately 90% of the unmineralized organic portion (osteoid) of bone matrix consists of bone-specific collagen type I. This protein is constituted by three polypeptide chains forming a triple-helix structure. Collagen type I is characterized by repeating $(GLY\text{-}X\text{-}Y)_n$ triplets. In the X and Y position are often proline and hydroxyproline. Collagen is synthesized by osteoblasts as procollagen, which is subsequently cleaved by proteinases to form mature collagen. This is influenced by fibronectin that is essential for collagen polymerization (Velling et al. 2002; Holt et al. 2007). The rest organic part of the bone matrix consists of non-collagenous proteins such as osteocalcin, osteonectin and sialoprotein.

1.2.3 Bone cells

Three types of bone cells are recognized, differentiated on the basis of form, function and location. These are osteoblasts, osteocytes and osteoclasts.

Osteoblasts are bone forming cells originated from mesenchymal stem cells. Their development is divided into three basic phases: preosteoblast, mature osteoblast and osteocyte, which is regulated mainly by two transcription factors CBFA1/Runx2 and Osterix and Wnt/β-Catenin signalling pathway (Yu et al.; Hill et al. 2005). Osteoblasts are small, mononucleated cells found near to periosteum and endosteum. Osteoblasts are responsible for production of osteoid containing bone matrix proteins and also for mineralization of the osteoid matrix. During matrix maturation the expression of alkaline phoshatase is higher. It increases the local concentration of inorganic phosphate, a mineralization promoter and decreases the concentration of pyrophosphate, an inhibitor of formation (Evans et al. 2000; Orimo 2010). Upon termination of bone matrix synthesis, osteoblasts either undergo cell death by apoptosis or differentiate into osteocytes or flat lining cells that overlap 80-95% inner surface of bone. Osteoblasts produce a range of growth factors and contain receptors for different hormones (Steele D.G. and Bramblett C.A. 1994).

Osteocytes originate from osteoblasts and are the most abundant cells in bone making up 95% of all bone cells (Franz-Odendaal et al. 2006). Osteocytes are housed within lacunae and canaliculae in lamellar bone. A network of canaliculi connects them with osteoblasts and flat lining cells and provides the contact with adjacent cells, internal and external surfaces of bone and with the blood vessels. Osteocytes are active in the process of final or secondary mineralization (Steele D.G. and Bramblett C.A. 1994).

Osteoclasts are large multinucleated cells. They are derived from mononuclear precursor cells of the monocyte-macrophage lineage. Cytokines, RANKL and CSF are essential for osteoclastogenesis. Osteoclasts are associated with bone resorbing. They secrete large quantities of hydrogen ions and proteolytic enzymes such as collagenases and cathepsins. Hydrogen ions are crucial for the removal of the mineral component and enzymes digest bone organic matrix (Steele D.G. and Bramblett C.A. 1994; Clarke 2008). For binding of osteoclast to bone matrix the integrins are required (Horton and Davies 1989).

1.2.4 Embryogenesis

Skeleton formation starts in ninth week of prenatal development. It is either intramembranous (direct) or chondral (indirect). Intramembranous ossification begins with hardening and capillarisation of mesenchyme. Mesenchymal cells differentiate into osteoblast precursors and with extension and multiplication of organelles into osteoblasts. By this process parietal bones are formed. Chondral ossification is typical for long and short bones that differentiate from mesenchyme into cartilage that is gradually replaced by bone (Steele D.G. and Bramblett C.A. 1994; Schiebler and Korf 2007).

1.2.5 Bone remodelling

Bone remodelling serves such as mechanism of adaptation on mechanical changes and helps to repair microdamages in bone. It also plays an important role in maintenance of plasma calcium homeostasis. Bone remodelling results from the action of osteoblasts and osteoclasts and involves the removal of mineralized bone and formation of bone matrix that subsequently become mineralized.

The remodelling process consists of three successive phases: resorption, reversal and formation. Resorption begins with activation of osteoclasts which resorb a mineralized bone matrix. Subsequently the osteoprogenitor cells migrate into the resorption lacuna and inhibit osteoclasts and stimulate osteoblasts. The formation phase follows. Osteoblasts converge at the bottom of resorption cavity and form osteoid until the resorbed bone is completely replaced by new. Then the surface is covered with lining cells and a prolonged resting period begins (Hill 1998; Hadjidakis and Androulakis 2006).

1.2.5.1 Regulation of bone remodeling

The process of bone remodelling is controlled by hormones and many other proteins. The regulation is systematic and local. The major regulators are parathyroid hormone, calcitriol, growth hormone, glucocorticoids, thyroid hormones and sex hormones (Hadjidakis and Androulakis 2006).

1.2.5.1.1 Systematic regulation

The most important regulator of calcium homeostasis is parathyroid hormone (PTH). It maintains serum calcium concentration by stimulating bone resorption, increasing renal tubular reabsorption and calcitriol production by conversion of 25-hydroxycholecalciferol to 1, 25-dihydroxycholecalciferol, the active form of vitamin D_3. PTH can stimulates bone formation when given intermittently (Liu et al. 2009).

Calcitriol plays an indirect role in mineralization. It enhances intestinal calcium and phosphorus absorption and thus promotes bone mineralization.

Calcitonin produced by thyroid reduces levels of serum calcium. It inhibits the activity of osteoclasts and decreases the reabsorption of calcium in kidney.

The growth hormone/insulin like growth factor (GH/IGF-1) system determinates bone mass acquisition during growth period. In adults they have a critical role in bone mass maintenance. IGF-1 is mostly synthesized in liver in response to GH. It functions as an anabolic regulator of bone cell activity, decreases collagen degradation and increases bone matrix deposition (Yakar et al. 2002; Adami et al. 2009).

Glucocorticoids have both stimulatory and prevailing inhibitory effects. They stimulate osteoblast maturation by promoting their differentiation from mesenchymal progenitors but at the same time decrease their activity. Furthermore they increase osteoclast recruitment and affect the secretion of bone regulators (Hadjidakis and Androulakis 2006).

Sex steroids are also essential for skeletal growth and bone turnover. They are needed for the completion of epiphyseal maturation during puberty. Patients with hypogonadism have reduced bone mass density (BMD) and increased fracture risk.

1.2.5.1.2 Locally regulation

Bone cells produce several types of cytokines, growth factors and other mediators that are implicated in bone metabolism. These local factors are also produced by cells of immune or haematological system.

RANKL/RANK/OPG system is critical for skeletal health. Its disruption leads to numerous bone diseases. Receptor activator of nuclear factor kappa B (RANK) is a transmembrane molecule expressed on osteoclast precursor cells and mature osteoclasts. Receptor activator of nuclear factor kappa B ligand (RANKL) is expressed by osteoblasts. The RANK ligation with RANKL is required for osteoclast progenitor

cells to differentiate into osteoclasts and for activation of mature osteoclasts. Osteoprotegerin (OPG) produced by osteoblasts has an osteoprotective role, because it blocks the RANK/RANKL interaction and thus inhibits the osteoclast maturation (Simonet et al. 1997; Boyce and Xing 2007).

Macrophage colony-stimulating factor (M-CSF) is also necessary for osteoclast development. Mice which do not express functional M-CSF have osteopetrosis (Karsenty and Wagner 2002). M-CSF expressioned by osteoblast binds to its receptor c-fms at the surface of pre-osteoclast cells and activates an intracellular cascade that leads to proliferation and survival of osteoclasts. M-CSF production is stimulated by a number of cytokines such as TNF-α or IL-10 (Hadjidakis and Androulakis 2006).

IL-1 is a stimulator of bone resorption. It increases proliferation and differentiation of osteoclast precursors and osteoclastic activity (Chiang et al. 1999). TNF is also potent stimulators of bone resorption. It induces the resorption either indirectly by production of osteoclast differentiation factor RANKL or directly by enhancing proliferation and activity in the osteoclast lineage (Pfeilschifter et al. 1989; Boyce et al. 2005). IL-6 also stimulates bone resorption and it acts synergistically with PTH (de la Mata et al. 1995). The bone formation is stimulated by TGF-β. TGF-β enhances osteoblastic differentation and synthesis of bone matrix proteins (Erlebacher et al. 1998). Further stimulators of bone formation are IGF-1, fibroblast growth factor (FGF) and platelet-derived growth factor PDGF. They enhace the differentation of mesenchymal stem cells (Ng et al. 2008).

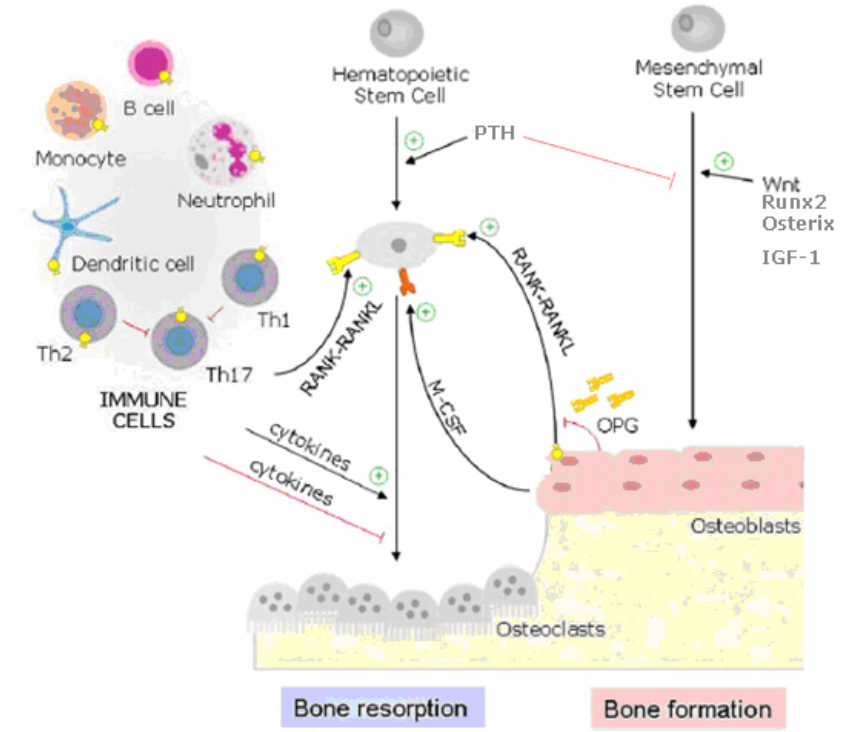


Figure 3: Bone remodelling regulation. Bone remodelling is infuenced by various molecules. Osteoclastogenesis is induced by RANK-RANKL, M-CSF and PTH. RANKL is express also by immune cells. Bone formation is induced by Wnt, Runx2, osterix and IGF-1. Osteoprotective role have OPG and some cytokines produced by the immune system. OPG-osteoprotegerin; RANK-receptor activator NF-κB; RANKL-receptor activator NF-κB ligand ;
M-CSF-macrophage colony-stimulating factor; IGF-1-insulin like growth factor; PTH-parathormone (Caetano-Lopes et al. 2009).

1.3 Hepatic osteodystrophy

The term hepatic osteodystrophy encompasses secondary osteoporosis associated with chronic liver disease as a result of decreased bone formation. Osteomalacia, with typical defective mineralization of bone matrix, is reported less frequently. Hepatic osteodystrophy is a complication of parenchymal liver disease and cholestatic liver disease. It has been shown that patients with cholestatic liver disease have increased prevalence of osteoporosis compared to other chronic liver diseases (van der Merwe et al. 2000; Collier 2007; Mounach et al. 2008; Goral et al. 2010).

1.3.1 Cholestatic liver disease

Cholestasis is defined as a reduction of bile flow through the hepatic biliary tree. There are various causes of cholestasis, including extrahepatic and intrahepatic disorders. The chronic cholestasis results in the retention of bile acids and other substances those excretions are depended on bile flow. The accumulation of these substrates leads to liver damage and effects on other organs (Sylvester 2001). Primary biliary cirrhosis is the most frequently cholestatic liver disease. Second most common is primary sclerosing cholangitis (Hauser S. C. et al. 2008).

Primary biliary cirrhosis (PBC) is an autoimmune liver disease which affects women in over 90% of cases and is associated with varying extrahepatic autoimmune syndromes. PBC is characterized by progressive destruction of intrahepatic bile ducts together with cholestasis, portal inflammation and fibrosis which may lead to cirrhosis and to its complications (Nguyen et al.; Crosignani et al. 2008; Mauss S. et al. 2009).

Primary sclerosing cholangitis (PSC) is characterized by the progressive destruction of large intra and extrahepatic bile ducts and affects predominantly male patiens with an age around 40. PSC is mostly associated with ulcerative colitis (Mauss S. et al. 2009)
The most common symptoms of both diseases are fatigue and pruritus. Osteopenic bone disease occurs as a serious complication of long standing cholestasis (Beuers U. et al. 2009).

1.3.2 Osteoporosis

Osteoporosis is a common complication with advanced forms of liver diseases. It appears more in patients with PBC because they are usually elderly women, who are naturally prone to osteoporosis (Mounach et al. 2008). Osteoporosis is characterized by low bone mass and microarchitectural impairment that results in increase of bone fragility and susceptibility to fracture. Typical fractures are hip or vertebral body fractures. Causes of osteoporosis may be various. Osteoporosis is classified as a primary and secondary type. The primary type is seen by older persons and by postmenopausal women. Secondary osteoporosis results from variety conditions, such as endocrine disease, drugs or liver disease. The best predictor of fracture of fracture risk is a diagnosis of mineral bone density (BMD). The measurement is done using the dual-energy x-ray absorptiometry (Sambrook and Cooper 2006; Goral et al. 2010).

1.3.3 Pathogenic mechanism of hepatic osteodystrophy

The process of the continuous bone remodelling is regulated by two systems: the immune and the endocrine system. Altering concentration of many hormones, growth factors and cytokines is found by liver diseases as a result of reduced liver metabolic activity and incident inflammatory. This may affect the bone remodelling, increase of the bone resorption or decrease of the bone formation.

Inflammatory cells such as monocytes, neutrophiles, lymphocytes B and lymphocytes T have ability to produce RANKL and thus induce osteoclast differentiation and, consequently, bone resorption. Also, these cells produce a variety of pro-inflammatory cytokines. The cytokines TNF-α and interleukin IL-1, IL-3, IL-6, IL-7, IL-11, IL-15 and IL-17 potentiate bone loss either by increasing osteoclast generation and activation or by inducing RANKL expression by the osteoblasts. Macrophage-colony stimulating factor (M-CSF) activates proliferation and survival of osteoclasts. On the other hand, interleukins IL-4, IL-5, IL-10, IL-12, IL-13, IL-18 and interferons IFN-α, IFN-β and IFN-γ are inhibitors of osteoclastogenesis by blocking RANKL signalling (Herman et al. 2008; Caetano-Lopes et al. 2009; Nakchbandi and van der Merwe 2009).

A loss of synthetic function of liver is common in patients with advanced liver disease. This leads to fall of production of substance such as IGF-1. Serum levels of IGF-1 are low in patients with chronic liver disease (Gallego-Rojo et al. 1998; Ormarsdottir et al.

2001) that can correlate with low bone mass, because IGF-1 stimulates bone formation.

Bone mineralization is also affected by vitamin D_3. It is synthesized in the skin and hydroxylated in the liver by the enzyme 25-hydroxylase. It was suggested that a decrease activity of this enzyme might be responsible for the bone loss associated with chronic liver disease. But most studies in patients with PBC showed that vitamin D_3 levels are normal (van der Merwe et al. 2000) and its supplementation does not correct the osteodystrophy (Floreani et al. 1997).

Bilirubin and bile acids accumulate in patients with cholestasis. *In vitro* studies showed, that unconjugated bilirubin decreased osteoblast proliferation, but in vivo data from animal and clinical studies did not prove this inhibitory role (Guanabens and Pares; Nakchbandi and van der Merwe 2009).

1.3.4 Association between fibronectin and hepatic osteodystrophy

Fibronectin is necessary for osteoblast differentiation *in vitro* (Moursi et al. 1996). It was found, that patients with liver disease produce altered fibronectin isoforms that are not formed under physiological conditions (Xu et al. 1997). This can result in impaired function of osteoblasts. Oncofetal fibronectin is one of the isoforms, produced by diseased activated stellate cell in liver. A relationship between fibronectin isoforms and hepatic osteodystrophy was examined in cross-sectional study in humans, *in vitro* experiments on osteoblasts and in vivo experiments in mice (Kawelke et al. 2008).

Table 1: Circulating levels of fibronectin isoforms in patients with PBC compared to control (Kawelke et al. 2008).

Substance	*Normal range*	*Patients*	*Controls*	*P-values*
Total fibronectin (mg/liter)	200-400	181 ± 26	209 ± 23	0.43
Oncofetal fibronectin (mg/liter)	Unknown	4.3 ± 0.6	3.0 ± 0.2	<0.05
EDA fibronectin (mg/liter)	Unknown	1.6 ± 0.2	1.2 ± 0.1	<0.05
EDB fibronectin (mg/liter)	Unknown	49.4 ± 10.6	27.2 ± 4.6	0.08

This study showed that fibronectin containing the oncofetal domain and fibronectin containing the EDA domain were significantly higher in the patients (tab. 1). Furthermore, circulating levels of oFN in patients with PBC correlated significantly negative with osteocalcin, a marker of bone formation (Kawelke et al. 2008)

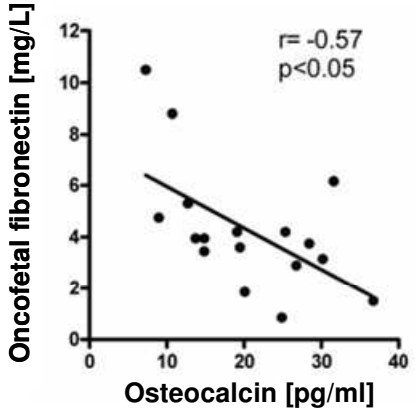


Figure 4: Oncofetal fibronectin correlates negatively with osteocalcin in the patients (Kawelke et al. 2008).

The effects of both glycosylation (N & O) that are located in oncofetal fibronectin molecules were further examined. It was found that the deglycosylation of fibronectin has an impact on function of murine osteoblasts *in vitro*. The O-deglycosylation of oncofetal fibronectin led to loss of inhibition and nodule formation of osteoblasts. However, it was not determinated which of the O-glycosylation is responsible for the effect (data from the laboratory).

2 Aims of the project

This thesis is a part of a project which focuses on a possible role of oncofetal fibronectin in the development of osteoporosis. Previous study in patients showed that increased levels of oncofetal fibronectin correlates negatively with osteocalcin, a marker of bone formation. *In vitro* results from the laboratory demonstrated that the reason may be the O-glycosylations present in oncofetal fibronectin. This is a posttranslation modification of fibronectin and the O-glycosylation located in the variable domain is characteristic for oncofetal fibronectin.

Thus one of the aims of this work was to prepare mutated fibronectin isoforms in which the O-glycosylation sites are not present anymore: namely both separately and also together in one molecule. The second part of the project was to knockdown the fibronectin gene in mammalian cells to obtain a possibility to express the mutated fibronectin isoforms and to purify them.

3 Materials

3.1 Materials

- Cryo tubes (Greiner bio-one GmbH; Frickenhausen, GE)
- Culture flask (Thermo Fisher Scientific Inc; Waltham MA, USA)
- Cuvettes (Brand Gmbh + CO KG, Wertheim, GE)
- Falcon tubes (Greiner bio-one GmbH; Frickenhausen, GE)
- Flat bottom culture plates (Thermo Fisher Scientific Inc; Waltham MA, USA)
- Microcentrifuge tubes (Sarstedt AG & Co.; Nümbrecht, GE)
- Pasteur capillary pipettes (WU Mainz, Bamberg, GE)
- Pipettes (Greiner bio-one GmbH; Frickenhausen, GE)
- Pipettes tips (Greiner bio-one GmbH; Frickenhausen, GE)

3.2 Instruments

- Autoclave (Systec GmbH; Wettenberg, GE)
- Camera EOS 350 Digital (Canon Inc.; Tokyo, Japan)
- CASY cell counter (Roche; Mannheim, GE)
- Centrifuge (Thermo Fisher Scientific Inc; Waltham MA, USA)
- ELISA Reader (Labinstuments Deutschland GmbH; Crailsheim, GE)
- Freezer -20 °C (Privileg; Stuttgart, GE)
- Freezer -80 °C (New Brunswick Scientific; Edison NJ, USA)
- Gel system (Peqlab Biotechnologie GmbH; Erlangen, GE)
- Gel documentation (Bio-Rad Laboratories GmbH; Düsseldorf, GE)
- Heating block (Eppendorf; Hamburg, GE)
- Thermomixer compact (Eppendorf; Hamburg, GE)
- Incubator (New Brunswick Scientific; Edison NJ, USA)
- Laminar flow cabinet (Thermo Fisher Scientific Inc; Waltham MA, USA)
- Liquid nitrogen tank (Messer Group GmbH; Sulzbach, GE)
- Microplate Washer (ASYS Hitech GmbH; Eugendorf, Austria)
- Microscope (Leica Microsystems; Wetzlar, GE)
- Microwelle (LG Electronics; Seoul, Korea)
- pH meter (Knick GmbH + Co. KG; Balingen, GE)
- Pipetboy Comfort Classic (IBS Integra Biosiences; Fernwald GE)
- Shaking incubator (New Brunswick Scientific; Edison NJ, USA)
- Thermo cycler (Biometra; Göttingen, GE)

- UV spectrophotometer (Eppendorf; Hamburg, GE)
- Vortex (Bender and Hobein AG; Zürich, Switzerland)
- Weight (Kern and Sohn; Balingen, GE)

3.3 Chemicals, reagents

- Acetic acid anhydrous (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- Agar (Becton Dickinson GmbH; Heidelberg, GE)
- Agarose (Gibco, Invitrogen; Karlsruhe, GE)
- Ampicillin (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Blasticidin (Gibco, Invitrogen; Karlsruhe, GE)
- Boric acid (AppliChem GmbH; Darmstadt, GE)
- Bromophenol blue (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- BSA (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Crystal violet (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- DMEM (Gibco, Invitrogen; Karlsruhe, GE)
- DMSO (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- DNA Ladder Mix (Fermentas GmbH; St. Leon-Rot, GE)
- DPBS (Gibco, Invitrogen; Karlsruhe, GE)
- EDTA (AppliChem GmbH; Darmstadt, GE)
- Fast Digest Green Buffer (Fermentas GmbH; St. Leon-Rot, GE)
- FCS (Pan Biotech GmbH; Aidenbach, GE)
- Fugene Transfection reagent (Roche; Mannheim, GE)
- Geneticin (Gibco, Invitrogen; Karlsruhe, GE)
- Glucose (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Glycerol (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- H_2SO_4 (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Iodoacetic acid (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- IPTG (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Kanamycin (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- KCl (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- KH_2PO_4 (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- L-glutamine (Gibco, Invitrogen; Karlsruhe, GE)
- Lipofectamin 2000 (Gibco, Invitrogen; Karlsruhe, GE)
- Lysozym (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- MEM Non Essential Amino Acids (Gibco, Invitrogen; Karlsruhe, GE)
- N-ethylmaleimide (AppliChem GmbH; Darmstadt, GE)

- NaCl (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- $NaHCO_3$ (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- Na_2HPO_4 (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- Na_2O_3Se (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Not I digest enzyme (Fermentas GmbH; St. Leon-Rot, GE)
- Opti-MEM Medium (Gibco, Invitrogen; Karlsruhe, GE)
- Penicillin/streptomycin (Gibco, Invitrogen; Karlsruhe, GE)
- Potassium acetate (Grüssing GmbH; Filsum, GE)
- Red Save (Intron Biotechnology, Korea)
- RNAse (Fermentas GmbH; St. Leon-Rot, GE)
- SDS (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Sodium deoxycholate (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Sodium pyruvate (Gibco, Invitrogen; Karlsruhe, GE)
- TMB (Merck Chemicals Ltd.; Darmstadt, GE)
- Trypsin/EDTA 0.05 % (Gibco, Invitrogen; Karlsruhe, GE)
- Tryptone (Becton Dickinson GmbH; Heidelberg, GE)
- Tris (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Tris HCl (Carl Roth GmbH + Co. KG; Karlsruhe, GE)
- Tween 20 (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- X-gal (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- Yeast Extract (Carl Roth GmbH + Co. KG; Karlsruhe, GE)

3.4 Antibodies

- anti-human-Fibronektin-IgG from Rabbit (Sigma-Aldrich Chemie GmbH; Taufkirchen, GE)
- anti-human-Fibronektin-HRP-konj.-IgG from Rabbit (Dako Deutschland GmbH; Hamburg, GE)

3.5 Media, solutions, buffers

Blocking buffer (ELISA)	3 % BSA / Wash Buffer
Cell culture medium for HuH-7 cells	DMEM / 10% FCS / Penicillin/Streptomycin 50 U/50 µg/ml for KD cells 2 µg/ml of Blasticidin

○ Cell culture medium for 293 FT cells	DMEM / 10% FCS Penicillin/Streptomycin 50 U/50 µg/ml 0.1 mM MEM Non Essential Amino Acids / 1 mM Sodium Pyruvate 2 mM L-glutamine 500 µg/ml Geneticin
○ Cell freezing medium for HuH-7	DMEM / 40% FCS / 10% DMSO
○ Cell freezing medium for 293 FT	DMEM / 10% DMSO
○ Coating buffer (ELISA)	14,71 mM Natriumcarbonat 35,71mM Natriumhydrogencarbonat Add up 500 ml millipore ddH_2O
○ DNA loading buffer	50% Glycerin in 4x TBE Add Bromphenol blue
○ Dilution buffer (ELISA)	1% BSA / Wash Buffer
○ DOC lysis buffer	2% Sodium deoxycholate 20 Mm Tris-HCl, pH 8.8 Proteinase inhibitor 1:100 2 mM EDTA / 2 mM iodoacetic acid 2mM N-ethylmaleimide
○ LB Agar	10 g NaCl / 10 g Tryptone 5 g Yest extract / 20 g Agar Adjust to pH 7.0 with NaOH Add up 1000 ml dH_2O Autoclave Add antibiotic (100 µg/ml Ampicilin or 30 µg/ml Kanamycin) or 80 µg/ml X-gal and 20 mM IPTG Pour into petri dishes

- LB Medium

  10 g NaCl / 10 g Tryptone
  5 g Yest extract
  Adjust to pH 7.0 with NaOH
  Add up 1000 ml dH_2O
  Autoclave
  Add antibiotic (100 µg/ml Ampicilin or 30 µg/ml Kanamycin)

- Maxi prep solutions

  Solution I

  50 mM Glucose / 10 mM EDTA
  25 mM Tris-HCl, pH 8
  Just before add 100 µl/ml RNAse
  2 mg/ml Lysosym

  Solution II

  0.2 M NaOH / 1% SDS

  Solution III

  3 M Kaliumacetate
  11.5 % Acetic acid anhydrous

  TE buffer

  10 mM Tris-HCl, pH 8 / 1mM EDTA

- PBS

  100 ml PBS (10x)
  Add up 1 l dH2O,

- PBS (10x)

  400 g NaCl / 58 g Na_2HPO_4/
  10 g KH_2PO_4 / 10 g KCl pH
  Addjust to pH 7.0
  Add up 5 l mit dH2O

- Lyses buffer

  20 mM Tris pH 7.4
  150 mM NaCl / 10% Glycerin
  0.5 % Triton x 100 / 2 mM EDTA
  10 mM Proteinaseinhibitor
  376.75 ddH_2O

o TBE	100 ml TBE (10x) Add up 1 l dH_2O
o TBE (10x)	108 g Tris / 55.6 g Boric Acid 4.65 g EDTA Add up 1 l dH_2O
o Wash buffer (ELISA)	100 ml PBS (10x) 0.5 ml Tween 20 900 ml dH_2O

3.6 Cells

- HuH-7 (Health Science Research Resources Bank; Japan)
- 293 FT HEK Cells (Invitrogen; Karlsruhe, GE)
- One Shot TOP10 *E.coli* (Invitrogen; Karlsruhe, GE)
- One Shot Stbl3 *E.coli* Invitrogen; Karlsruhe, GE)
- XL10-Gold *E.coli* (Agilent Technologies; Santa Clara, USA)

3.7 Kits

- BLOCK-iT ™ Lentiviral RNA Expression System (Invitrogen; Karlsruhe, GE)
- BLOCK-iT ™ U6 RNAi Entry Vector Kit (Invitrogen; Karlsruhe, GE)
- MicroBCA ™ Protein Assay Kit (Thermo Fisher Scientific Inc.; Waltham MA, USA
- peqGOLD Plasmide MiniPrep Kit (Peqlab Biotechnologie GmbH; Erlangen, GE)
- peqGOLD Taq DNA Polymerase Kit ((Peqlab Biotechnologie GmbH; Erlangen, GE)
- Qiagen Plasmide Maxi Kit (Qiagen GmbH; Hilden, GE)
- QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies; Santa Clara, USA)

3.8 DNA

3.8.1 shRNA oligonucleotides (Biomers.net GmbH; Ulm, GE)

- 207 (for 5' UTR)

5'-CACCGAAGCAAACTTGGTGGCAACTCGAAAGTTGCCACCAAGTTTGCTTC-3'

5'-AAAAGAAGCAAACTTGGTGGCAACTTTCGAGTTGCCACCAAGTTTGCTTC-3'

- 8215 (for 3' UTR)

5'-CACCGGAGATAGCTTTACACTTTCTCGAAAGAAAGTGTAAAGCTATCTCC-3'

5'-AAAAGGAGATAGCTTTACACTTTCTTTCGAGAAAGTGTAAAGCTATCTCC-3'

- 8393 (for 3' UTR)

5'-CACCGAGATTGCCTGCAAGGGAAATCGAAATTTCCCTTGCAGGCAATCTC-3'

5'-AAAAGAGATTGCCTGCAAGGGAAATTTCGATTTCCCTTGCAGGCAATCTC-3'

3.8.2 Primers

3.8.2.1 Site directed mutagenesis:

- Control primer (Invitrogen; Karlsruhe, GE)

fwd 5'-CCATGATTACGCCAAGCGCGCAATTAACCCTCAC-3'

rev 5'-GTGAGGGTTAATTGCGCGCTTGGCGTAATCATGG-3

- 1. O-gly (Biomers.net GmbH; Ulm, GE)

fwd 5' -CACACCTCTGTGCAGTCCTCATCGAGCGGATCTG-3'

rev 5'-CAGATCCGCTCGATGAGGACTGCACAGAGGTGTG -3'

- 2 O-gly (Biomers.net GmbH; Ulm, GE)

fwd 5'- CCACACCGCCCTCATCGGCCACCCCC-3'

rev 5'-CAGATCCGCTCGATGAGGACTGCACAGAGGTGTG-3'

- NH_2-term (Biomers.net GmbH; Ulm, GE)

5'-GTAGATTGTACTCGCCTGGG -3'

- V 120 (Biomers.net GmbH; Ulm, GE)

5'-CTGGAACCGGGAACCGAAT-3'

3.8.2.2 RNAi

- U6 (Biomers.net GmbH; Ulm, GE)

fwd 5'- GGACTATCATATGCTTACCG -3'

rev 5'- TAATACGACTCACTATAGGGG -3'

4 Methods

4.1 Cell culture

4.1.1 HuH-7 cells culture

The HuH-7 cells were cultured in DMEM supplemented with 10 % FCS and 1 % penicillin-streptomycin, which was replaced every 3 days. Cells were incubated at 37 °C in a 5% CO_2 setting. The passaging was done according to cells density.
For determination of fibronectin production the cells were cultured in DMEM containing 10% FCS without fibronectin or complete without FCS. In this case the FCS was substituted with 0.03 µM Na_2O_3Se (sodium selenit). Otherwise the cell can not live (Nakabayashi et al. 1982).

4.1.2 293 FT (HEK) cells culture

The 293 FT cells were used for the production of lentviral stock. The 293 FT cells were cultured in DMEM medium containing 10 % FCS supplemented with 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin, 500 µg/ml geneticin. The culture medium was changed every 3 days. Cells were grown at 37 °C in a 5% CO_2. The passaging was done according to cells density.

4.1.3 Subculturing cell

At about 80-90 %confluence, the cells were subcultured. After washing with DPBS, cells were detached with 0.05 % trypsin/EDTA and mixed with DMEM containing FCS. The suspension was centrifuged at 1500 rpm for 3 min. The supernatant was removed and cells were resuspended in fresh complete culture medium. The viability and cell density was determinated using CASY apparature.

4.1.4 Freezing cells

The HuH-7 cells were resuspended in DMEM containing 40% FCS and 10% DMSO. Cells were stored in -80 °C in freezing container overnight and then transfered to liquid nitrogen.

4.1.5 Blasticidin killing curve

In order to generate stable cell lines expressing the shRNA, it was necessary to determine the minimum amount of blasticidin required to kill non-transduced cells. This was done by generating a blasticidin kill curve.
The day before testing cells were plated in approximately 25% confluence. Next day the culture medium was substituted with a medium containing various concentrations of blasticidin: 0, 1, 2, 3, 4, 5, 7, 9 µg/ml. Every 3 days it was replaced with freshly prepared selective medium. The cells were daily monitored. After 14 days the appropriate concentration of blasticidin was determinated like the minimum concentration of antibiotic that killed all the cells.

4.2 Bacterial culture

Bacteries were cultured after transformation with plasmid DNA. The *E.coli* were spread onto LB agar selective plate (containing 100 µl/ml ampicillin or 20 µl/ml kanamycin) and incubated overnight at 37 °C. A single colony was picked and added to 5 ml of selective LB medium (containing 100 µl/ml ampicillin or 20 µl/ml kanamycin). The *E. coli* culture was incubated overnight at 37 °C shaking at 250 rpm.

4.2.1 Freezing E.coli

An aliquot from each bacterial culture was freezed in cryotubes. 900 µl of bacterial culture was mixed with 100 µl glycerol. The mixture was freezed in liquid nitrogen and stored in -80°C.

4.3 DNA Plasmid isolation

The Mini-Plasmid preparation was done using 5ml overnight *E.coli* culture. For Maxi-Prep isolation 100 µl of this culture was added to 100 ml of selective medium and incubated at 37 °C with constant shaking overnight. For low copy of plasmid DNA the peqGOLD Plasmide MiniPrep Kit (peqLab) was used. For Maxi-plasmid preparation the solutions noted in materials part were used.

4.3.1 Mini-plasmid isolation

Plasmid DNA was purified according to the kit manufacturer´s protocol. The centrifuged *E.coli* cells were resuspended in 250 µl of buffer 1. Then 250 µl of lysis buffer 2 was

added and the lysate was neutralised adding 350 µl of buffer 3. The lysate was cleared by centrifugation and the supernatant was transfered to a column, where the DNA plasmid was adsorbed during centrifugation. Protein contamination was removed by washing step with 500 µl of PW-buffer and 750 µl of wash buffer. The column matrix was dried and the plasmid DNA was eluted using 100 µl elution buffer. The DNA content was quantified by photometric measurement at 260 nm.

4.3.2 Maxi-prep isolation

The pelleted cells were resuspended in 5 ml solution I supplemented with RNase A and lysozyme just before use. After addition 10 ml of solution II, 5 min incubation at RT followed. Finally 7.5 ml of solution III was added. After 20 min incubation on ice, the precipitate was filtred. 6-fold volume of 70% isopropanol was added to the filtrate to purify the plasmid DNA. After 15 min incubation at RT the DNA was centrifuged at 4000 rpm, 4 °C for 30 min. 5 ml 70 % ethanol was added to the pellet and centrifuged at 4000 rpm, 4 °C, 10 min. After drying on air the pellet was resuspended in TE buffer. The plasmid DNA concentration was measured by photometric method at 260 nm.

4.4 Restriction of plasmid DNA

Since the plasmid DNA is circular, a good size of the DNA can not be verified, because of movement with different speeds in an agarose gel. Therefore the digestion of DNA with a restriction enzyme was done to obtain a linear piece that was compared to a standard of DNA using the gel electrophoresis method.

The procedure was done in the following sequence: 1 µg of plasmid DNA was added with 2 µl of fast digest green buffer and 1 µl of fast digest NotI enzyme into water. The total volume was 20 µl. The reaction was mixed and incubated for 30 min at 37 °C and afterwards inactivated at 80 °C for 5 min. Then the gel electrophoresis was performed.

4.5 Agarose Gel Electrophoresis

Gel electrophoresis was done to verify the presence of an insert in the vector.

1% agarose gel was prepared suspending dry agarose in 1 x TBE buffer. Agarose was boiled in a microwave to completely dissolve. Then the red save, a green fluorescent dye was added (1µl per 50ml gel). The gel was poured into a suitable former and a comb was inserted. It was allowed to cool to form a rigid gel. Afterwards the gel was

poured over with 1 x TBE buffer and the comb was removed. To 10 µl of sample 5 µl of DNA loading buffer was added and this 15 µl of was pipetted into wells. The first well was loaded with a marker (DNA Ladder mix). The running parameters were 150 V and 30 min. After electrophoresis the gel was exposed to UV light to detect the DNA and the bands were compared to the marker.

4.6 ELISA (Enzyme-Linked ImmunoSorbent Assay)

To investigate the levels of fibronectin produced by knockdown the HuH-7 cell line, a total fibronectin ELISA was performed. The medium containing fibronectin free FCS was collected from the cells after 3 days in culture. The ELISA plate was coated with coating buffer (50 µl pro well) containing the anti-human fibronectin antibody in concentration of 0.12 µg/ml and incubated overnight at 4 °C. After 3 times washing with wash buffer, blocking buffer was added (50 µl pro well) and incubated at RT for 1 hour. During this time, the standard series was prepared by diluting human plasma fibronectin in dilution buffer (concentrations: 2000, 1000, 500, 250, 100, 50, 25, 12.5 ng/ml). Samples were diluted 1:10. After blocking, the plates were again washed 3 times and the standard series and samples were applied (50 µl per well). The plate was incubated for 2 hours at RT. Then the plate was 5 times washed and 50 µl pro well of secondary antibody solution (polyclonal rabbit anti-human antibody in Dilution Buffer 1:2000) was added. After 45 min incubation at RT and 5 times washing 50 µl of TMB pro well was pipetted. It was followed by 25 min incubation at RT in dark.
Just before the photometric measurements the reaction was stopped by the addition of 50 µl 0.5 M H_2SO_4. The plates were measured at 450 nm with an ELISA reader.

4.7 Determining the protein concentration

The amount of total protein was determined using a BCA protein assay kit. This was carried out according to the manufacturer.

4.8 Site Directed Mutagenesis

Site directed mutagenes is a technique used to creat a point mutation in a defined sequence of DNA molecule that results in corresponding changes in protein structure and function (Chalker and Davis 2010). This method was used for replacing the amino acid threonine where the both O-glycosylations are found.

The procedure requires an oligonucleotide encoding the desired mutation that serves as a primer for initiation of DNA synthesis. The primers were synthesized using the program The QuikChange® Primer Design Program by Agilent Technologies. The procedure was done with QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and the reactions were prepared according to manufacturer's recommendations.

Three different fibronectin isoforms of cellular fibronectin (cFN) were prepared. First, the O-glycosylation sites in NH_2-terminus (called first O-glycosylation, or 1) and variable domain (called second O-glycosylation, or 2) were mutated separately. Then the vector containing the fibronectin insert with mutated variable domain served as a template and the O-glycosylation place in the NH_2-terminus was mutated to obtain fibronectin isoform lacking O-glycosylation (indicated 1+2 O-glycosylation).

The fibronectin cDNA was placed in pmax cloning vector.

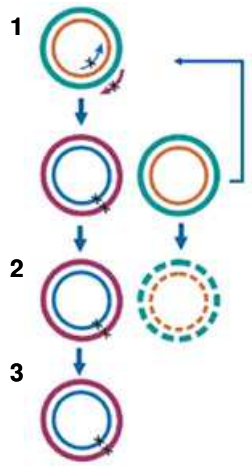


Figure 5: Overview of the Site directed mutagenesis. Mutant strand synthesis (1) is followed by *Dpn*I digestion of the parenteral DNA template (2) and trasformation of mutated molecule into compotent cells (3) (QuikChange II XL Site-Directed Mutagenesis Kit).

Mutant Strand Synthesis Reaction

The mutant strand synthesis reaction was prepared as indicated in tab. 2.

Table 2: Reagents for mutant strand synthesis reaction.

Control reagents	Sample reagents	Amount
10x reaction buffer		5 µl
pWhitescript 4.5-kb control plasmid	dsDNA template	10 ng
nucleotide control primer fwd.	oligonucleotide primer fwd.	125 ng
nucleotide control primer rev.	oligonucleotide primer rev.	125 ng
dNTP mix		1 µl
QuikSolution reagent		3 µl
ddH_2O		final volume of 50 µl

Then 1 µl *PfuUltra* High Fidelity DNA polymerase was added and the reactions were cycled using the following cycling parameters indicated in tab. 3.

Table 3: PCR parameters for mutant strand synthesis reaction.

Segment	Cycles	Temperature	Time
1	1	95°C	1 min
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	10 min
3	1	68°C	7 min

The reaction tubes were placed on ice for 2 min to cool to < 37°C.

Dpn I Digestion of the Amplification Products

To each reaction 1 µl of *Dpn*I restriction enzyme was added, the tubes were centrifuged for 1 min and the reactions were incubated at 37 °C for 1 hour to digest the parenteral supercoiled dsDNA.

Transformation into competent cells

The XL10-Gold ultracompetent cells were thawed on ice. For each reaction 45 µl was used. After addition of 2 µl of β-mercaptoethanol, cells were incubated on ice for 10 min, swirling gently every 2 min. 2 µl of the *Dpn*I treated DNA was added. After 30 min

incubation on ice the reaction tubes were put for 30 s in 42 ℃ warm water bath, 2 min incubated on ice and then 0,5 ml of 42 ℃ preheat SOC medium was added. The tubes were incubated at 37 ℃ for 60 min shaking at 250 rpm. Then 250 µl of each reaction was plated onto seclective agar plates containing 100 µg/ml ampicillin for control, 20 µg/ml kanamycin for sample. The plates were incubated overnight at 37 ℃. The next day clones of samples were picked and the overnight culture in kanamycin culture LB medium was prepared for plasmid preparation.

4.9 DNA sequencing

DNA sequencing was done to determinate the precise sequence of nucleotides in the mutated part of DNA. DNA sequencing was carried out by GATC Biotech Company. Sequencing was performed using primers: NH_2-term and V 120 (see materials). Plasmid concentration was 100 ng/µl and primer concentration was 10 µM.

4.10 RNA interference

RNA interference (RNAi) is a mechanism that uses a short antisense RNA to inhibition of gene expression via the messenger RNA (mRNA) degradation. The short RNAs (siRNA) are generated by cleavage of double stranded RNA (dsRNA) precursors by RNase III type enzyme Dicer. After binding to a specific protein complex RISC (RNA-induced silencing complex) with endonuclease activity they are able to cleavage the target mRNA. Depending on degree of complementarity to the target mRNA the RNAi can induce either translation repression or transcriptional inhibition (Matzke and Birchler 2005).

Short hairpin RNA (shRNA) is an artificially designed class of RNA molecule that can trigger gene silencing through interaction with mRNA (Paddison et al. 2002). shRNA molecule contains sense and antisense sequences connected by a short section of nucleotides that enables the formation of a loop structure. shRNA uses a vector introduced into cells and utilizes the U6 promotor. It is transcribed by RNA polymerase III. The hairpin structure is cleaved by Dicer and then bound to the RISC which cleaves mRNA.

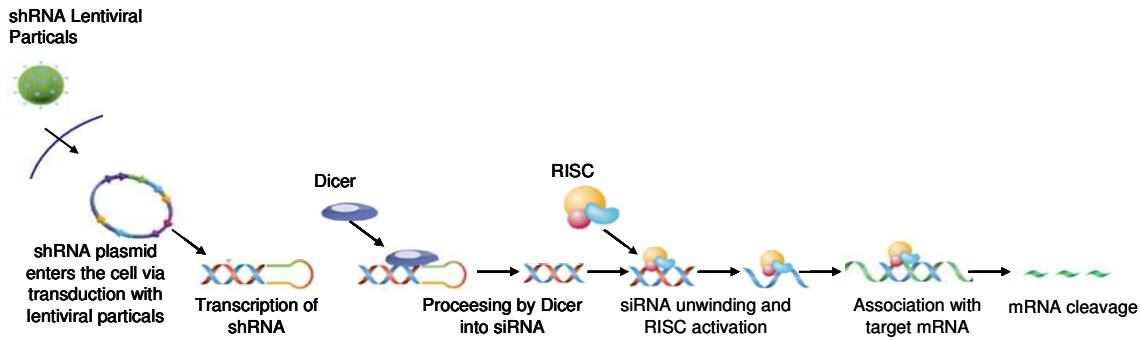


Figure 6: RNA interference scheme (http://www.scbt.com/gene_silencers.html, date 3.5.2011)

For design of double-stranded DNA for subsequent expression of shRNA the BLOCK-iT™ RNAi Designer (Invitrogen) programme was used. The sequence for fibronectin gene was given, as region for target sequence the untranslated region was selected and the oligonucleotides were chosen according to properties recommended in the protocol to BLOCK-iT ™ U6 RNAi Entry Vector Kit (Invitrogen) that was used for RNA interference together with BLOCK-iT ™ Lentiviral RNA Expression System (Invitrogen). The untranslated region as a shRNA target sequence enables

the expression of mutated fibronectin isoforms from the plasmid, which contains only the coding sequence after performing the transduction of the knockdown cells.

Generating the Double-Stranded Oligo (ds oligo)

The DNA single-stranded oligos were annealed to generate double-stranded oligos. The reactions (tab. 4) were set up at RT, incubated at 95 °C for 4 min, cooled to RT and microcentrifuged briefly for 5 s. The annealing mixtures were diluted to final concentration of 5 nM.

Table 4: Annealing reaction reagents.

Reagent	Volume
"Top strand" DNA oligo (200M)	5 µl
"Bottom strand" DNA oligo (200M)	5 µl
10X Oligo Annealing Buffer	2 µl
DNase/Rnase-Free Water	8 µl

The ds oligos were cloned into the pENTR^{TM}/U6 vector. The ligation reaction was performed using reagents in tab. 5.

Table 5: Ligation reaction reagents.

Reagent	Volume
5X Ligation Buffer	4 µl
pENTR/U6 (0.5 ng/µl)	2 µl
ds oligo (5 nM)	1 µl
T4 DNA Ligase (1 U/µl)	1 µl
Dnase/Rnase-free water	12 µl

Transforming into competent cells

The ligation mixture was transformed into competent *E.coli*. One Shot TOP10 Competent *E. coli* supplied by the kit was used. 2 µl of the ligation reactions was added into the vials with *E.coli* and mixed gently. Reactions were incubated on ice for 15 min, then heat-shocked for 30 s at 42 °C and the tubes were transferred to ice. 250 µl of

room temperature SOC medium was added and the tubes were shaked horizontally at 37 ℃ for 1 hour at 200 rpm.
50 µl and 100 µl was plated on ampicillin (100 µg/ml) LB agar plates. The next day clones were picked and the overnight culture in selective culture LB medium was prepared for plasmid preparation.

Analyzing transformants

To confirm the presence of the ds oligo inserts the PCR was performed. 2.5 µl of 10X Buffer, 2 µl of enhancer, 1 µl of dNTP mix, 1 µl of U6 primer pair, 0.25 µl of *Taq* DNA Polymerase, 1.5 µl of $MgCl_2$ and 100 ng of template DNA and ddH_2O up to 25 µl was set up and placed into the thermocycler to start PCR. The products were run on a gel to see inserts and compared to a positive control.

Table 6: PCR programme.

Segment	Cycles	Temperature	Time
1	1	94℃	5 min
2	35	94℃	1 min
		43℃	30 s
		72℃	30 s
3	1	72℃	7 min

LR Recombination Reaction

To generate an expression clone the recombination reaction using pENTR™/U6 vector and pLenti6/BLOCK-iT™-DEST vector was performed. 150 ng of pENTR™/U6 vector containing the ds oligo insert was mixed at RT with 1 µl of pLenti6/BLOCK-iT™-DEST vector which contains the elements required to packaging of the expression construct into virions. The TE Buffer, pH 8.0 was added up to 8 µl.
2 µl of LR Clonase II was added to each sample, incubated at 25 ℃ for 1 hour. 1 µl of the Proteinase K was added and the reactions were incubated for 10 min at 37 ℃.

Transforming into competent E.coli

The One Shot Stbl3 cells were used in this step of experiment. 3 µl of the LR recombination reaction was added to one vial of thawed *E.coli.* The reactions were incubated on ice for 30 minutes. The cells were heat-shocked for 45 s at 42 ℃ water

bath and placed on ice for 2 min. 250 µl of pre-warmed SOC Medium was added and the tubes were incubated at 37 °C for 60 min shaking at 250 rpm. 50 and 100 µl of transformation mix were spread on a pre-warmed plates containing 100 µg/ml ampicillin and incubate overnight at 37 °C. The next day the clones were picked and the preparation for maxi prep was done.

Producing Lentivirus in 293FT cells

Before creating a stably transduced cell line expressing shRNA, it was necessary to produce a lentiviral stock by co-transfecting the pLenti6/BLOCK-iT^{TM}-DEST vector with ViraPower^{TM} Packaging Mix into 293FT cell line. This was performed using lipofectamine method.

DNA – lipofectamine complex was prepared: 9 µg of the ViraPower^{TM} Packaging Mix and 3 µg of plasmid DNA were diluted in 1.5 ml of Opti-MEM Medium without serum. In a separate tube 36 µl of lipofectamine was diluted in 1.5 ml of Opti-MEM Medium without serum and incubated for 5 min at RT. Then the diluted DNA with diluted lipofectamine was combined and incubated for 20 min at RT.

293 FT cells were resuspended at a density of $1.2x10^6$ in the growth medium. The DNA-lipofectamine complex was added into a 10 cm^2 tissue culture plate containing 5 ml of growth medium without antibiotic and 5 ml of the 293 FT cells suspension ($6x10^6$ total cells). The cells were incubated overnight at 37 °C.
The next day the media containing the DNA-lipofectamine complexes were removed and 10 ml of the complete culture medium was added. 72 hours posttransfection the virus-containing supernatant was harvested, centrifuged at 3000 rpm for 5 min at 4°C and the supernatans was stored in 1 ml aliquots at -80°C.

Transduction and Titering Procedure

The day before transduction the HuH-7 cells were plated into 6-wells plate in about 50 % confluence. The second day serial dilutions of lentiviral stock with HuH-7 complete culture medium (virus concentration was 10^{-2},10^{-3},10^{-4},10^{-5},10^{-6}) were prepared to a final volume of 1 ml. This medium was added to the cells and one well was used as a negative control (without virus). The next day the medium was replaced with 2 ml of complete culture medium and the day after with complete culture medium containing the appropriate amount of blasticidin (2 µg/ml) to select the transduced

cells. The medium containing blasticidin was every 3 days replenished. After 13 days dead cells in the mock well were observed.

The titer of lentiviral stock was performed to determinate the multiplicity of infection (MOI). MOI is the number of virus particles per cell and correlates with the number of integration events.

13 days after transduction the medium from one 6-well plate was removed and the cells were washed twice with 1 ml of DPBS. 1 ml pro well of 1 % crystal violet in 10 % ethanol solution was added and after 10 min incubation at RT removed. The cells were washed twice with DPBS. The blue-stained colonies were counted. The individua blue-stained colonies were appeared in these concentrations of virus 10^{-4}, 10^{-5}, 10^{-6}. The titer was calculated as an average of number of colonies multiplied by concentration of the virus.

The HuH-7 cells were transduced with appropriate amount of virus (at a suitable MOI).

Transduced HuH-7 cells

13 days after transduction the blasticidin-resistent colonies were picked and transfered into 96-wells with 200 µl of medium containing blasticidin. The clones were left to grow and expand to assay for knockdown of the target fibronectin gene. The prescreening of fibronectin production was performed using ELISA assay. The best clones were chosen and left to rised. The cells were cultured for three days in medium without FCS to precise verification of the fibronectin production.

4.11 Transient Transfection

To test the functionality of modificated fibronectin gene the transient transfection was performed. For transfection the Fugene transfection reagent was used and the procedure was done according to manufacturer's recommendations. The knockdown HuH-cells were transfected with the plasmids DNA containing the mutations in O-glycosylation sites at the NH_2-terminus, in variable domain and both together. As a positive control cFN was used. Results were compared to knockdown and original HuH-7 cells.

One day before transfection the cells were plated in 6 wells dishes in density of 60% confluency. The cell culture medium used for the experiment was free of fibronectin and antibiotics. The next day the Fugene reagent was diluted 3:1 with DMEM and after 5 min incubation at RT 1 µg of plasmid DNA was added. After 15 min incubation at RT the tranfection reagent-DNA complex was slowly added to the cells.

Three days after starting the experiment the medium was collected from the cells. Half of the transfected cells was used for DOC solubility assay and the second half was lysated in Lysis Buffer.

Cell lysates

At first the cells were washed with PBS, detached with 0.05% trypsin/EDTA and mixed with PBS. After centrifugation the pellet was resuspended in 200 µl of lysis buffer. After 10 min shaking the samples were centrifuged again and the supernatants were collected. The concentration of fibronectin was examinated using ELISA.

Fibronectin Matrix Assembly – a DOC-Solubility Assay

The principle of biochemical analysis of fibronectin matrix is a differential solubility in the deoxycholate detergent (DOC). The stable fibrillar fibronectin matrix is in 2% DOC insoluble, whereas the cells are lysed (Wierzbicka-Patynowski et al. 2004).
The DOC-solubility assay was performed 3 days after Fugene transfection.
Cells were washed with PBS and 0.5 ml of DOC lysis buffer was added to each well. Cells were scraped off and the cell lysate was centrifuged. Supernatant was removed and insoluble pellet was resuspended in 171 µl of Lysis Buffer. To determinate the amount of fibronectin in samples the ELISA was performed.

5 Results

5.1 Knockdown of the fibronectin gene in mammalian cells

5.1.1 The confirmation of presence of ds oligo insert in pENTR/U6 vector

At first the DNA single-stranded oligos were annealed and cloned into the pENTR/U6 vector. In order to know whether the pENTR/U6 vector contains the ds oligo inserts the PCR was performed. Vector containing 50 bp insert should be 275 bp long.

After running an agarose gel the all of sequences was between 300 and 200 bp and the size can not be exactly determined (figure 7 a). Therefore the PCR was performed again with chosen samples and compared to better control. Figure 7 b confirms the plasmid present with desired size 275 bp.

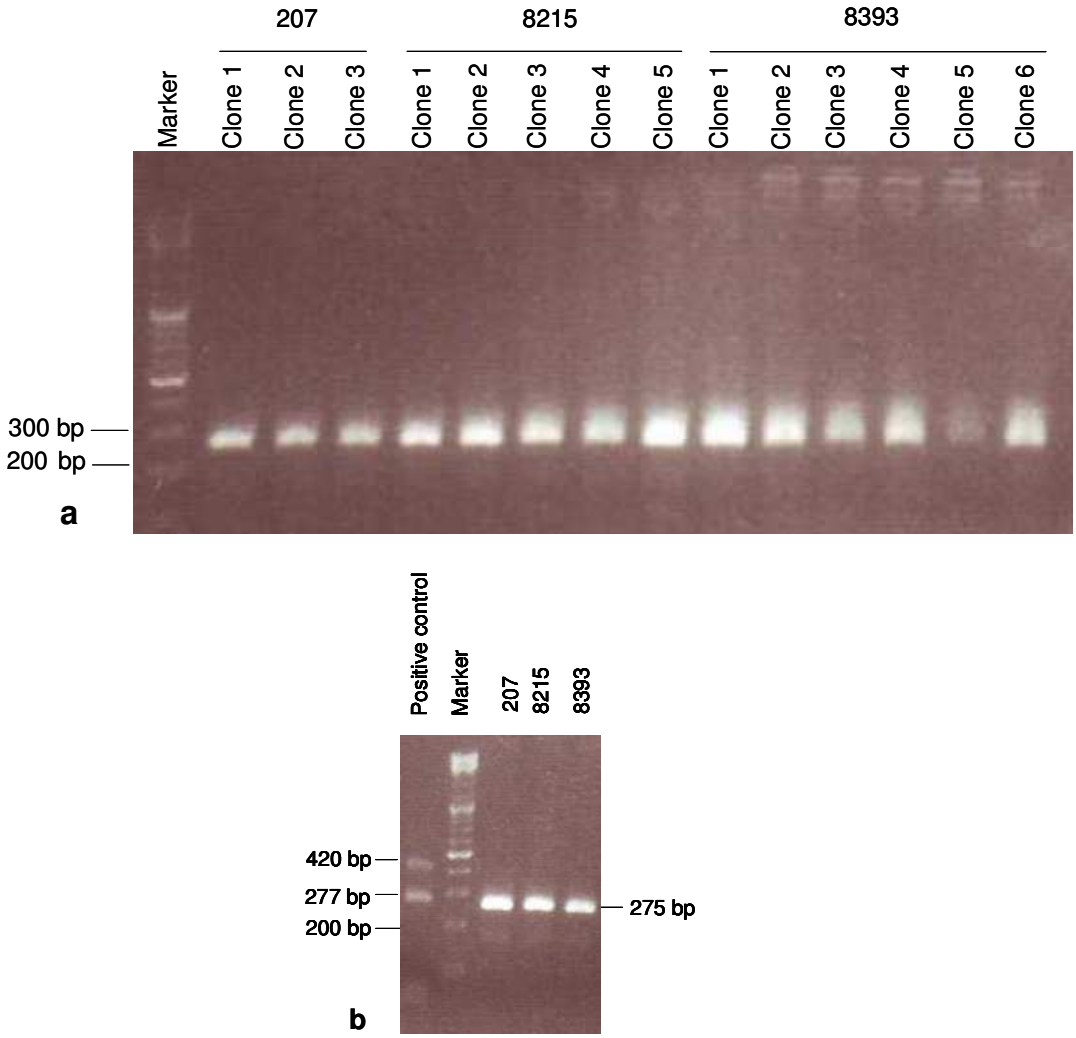


Figure 7: The confirmation of insert present in pENTR/U6 vector.

The figure a shows all of clones after performing PCR. The picture b shows the 275 bp sequence which corresponds to the length of the desired sequence containing the insert.

5.1.2 Blasticidin kill curve

In order to recognize cells expressing the shRNA we determinated the lowest number of blasticidin that killed 100% of the HuH-7 cells. 13 days after addition of antibiotic it was observed that 2 µg/ml of antibiotic is the minimum necessary toxic concentration.

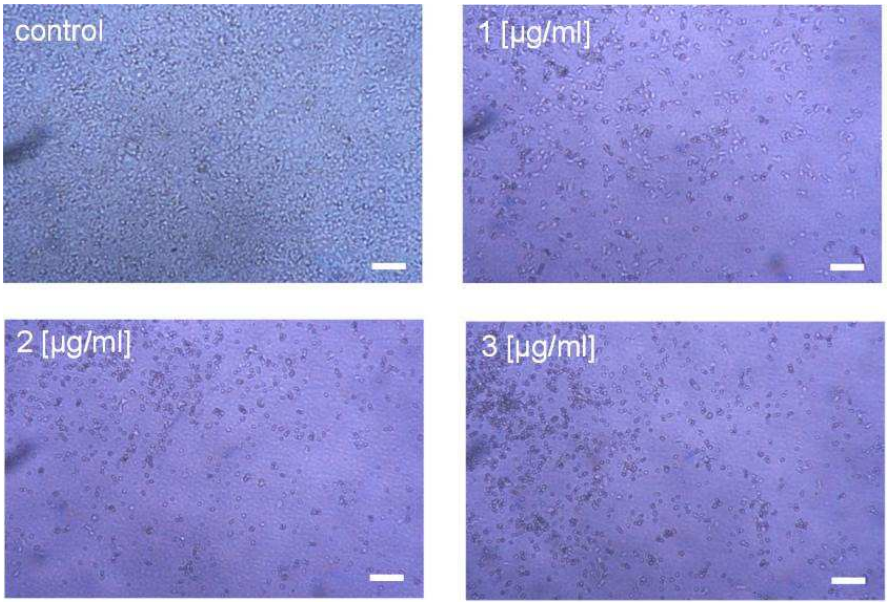


Figure 8: Blasticidin kill curve. Death cells are seen in medium containing 2 and 3 µg/ml blasticidin compared to cells cultured without antibiotic. Bars represent 200 µm.

5.1.3 Titer determination, transduction of mammalian cells

To determinate the MOI the HuH-7 cells were transduced using different virus concentration. 13 days after transduction the evident difference between the amounts of virus was observed. The not transduced cells were dead.

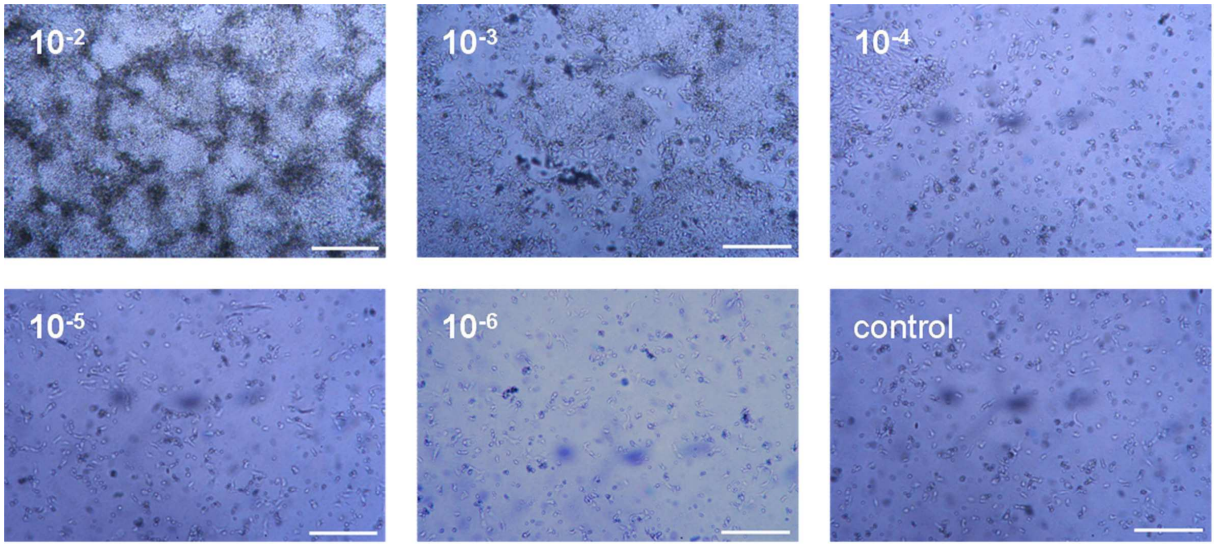


Figure 9: Transduction procedure. Obvious difference between different concentrations of the virus was observed. Cells cultured in medium containing higher virus concentration were mostly alive, while cells cultured in medium containing the lowest concentrations were dead. Bars represent 500 µm.

Titer determination

13 days after transduction the titer was determined. The cells were stained using crystal violet and colonies were counted in wells with 10^{-4}, 10^{-5} and 10^{-6} of virus concentration. The titer (transduction units pro ml) was calculated as an average of number of colonies multiplied by concentration of the virus (tab. 7).

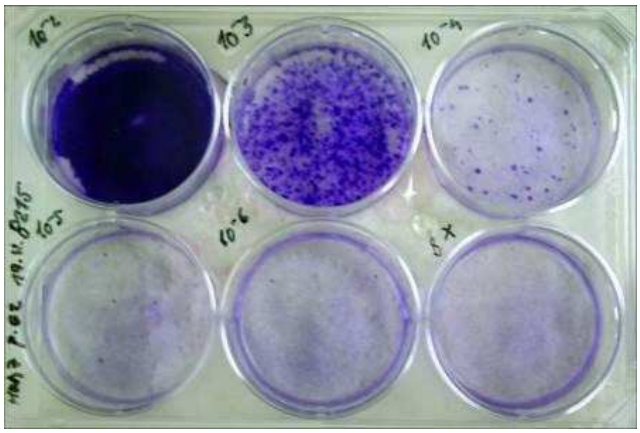


Figure 10: Titering procedure. The plate after cristal violet staining. The countable clones are in wells of 10^{-4}, 10^{-5} and 10^{-6} virus concentration.

Table 7: Titer of virus stock. The titer was counted for each of shRNA.

shRNA	TU/ml
207	1.517×10^{-6}
8215	0.723×10^{-6}
8393	2.350×10^{-6}

5.1.4 Confirmation of knockdown

The transduced blasticidin-resistent colonies were picked and left to grow. To evaluate the level of fibronectin gene silencing the ELISA assay was performed. First the prescreening of fibronectin production was examined in samples of culture medium (fig. 11). The best knockdown clones were left to grow.

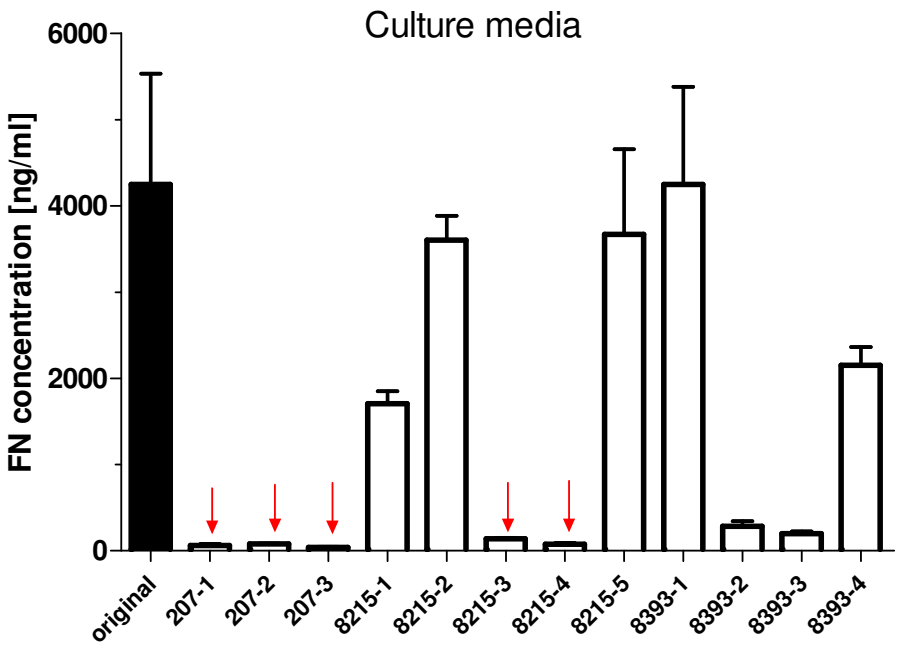


Figure 11: Prescreening of FN production: FN ELISA was performed with 1:10 diluted medium that was collected after three days in culture in FN free conditions. HuH-7 without shRNA insert were used as a control. Red arrows indicate the best knockdown clones used for further work.

After obtainment a sufficient number of knocdown clones, the cells were cultured for three days in FCS free medium containing 0.03 µmol/ml Na_2O_3Se. The presence of fibronectin was determinated in medium and cell lysates and it was corrected to the protein content (fig. 12). The best reduction of fibronectin production: 94.7 % in medium, 93.9 % in cell lysate compared to original HuH-7 was observed by clone 207/1 that was used for further experiment.

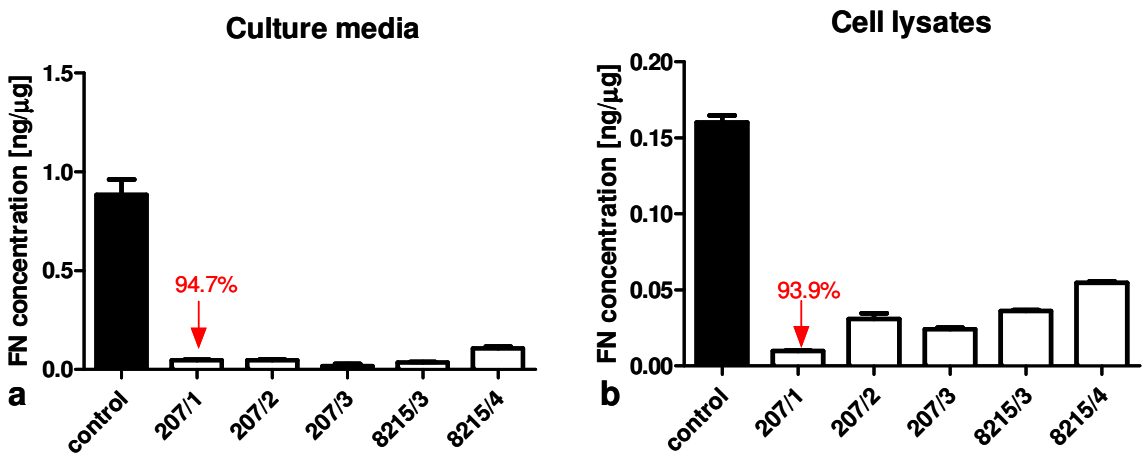


Figure 12: Efficient knockdown of FN gene in HuH-7 cells. The fibronectin concentration was measured using ELISA in FCS free media containing Na_2O_3Se (a) and cell lysates (b) and corrected to the total protein content in the cells. The best reduction of fibronectin production was determinated by clone 207/1. This reduction was by 94.7% and 93.9 % compared to the non-silenced control. Clone 207/1 was used for further work.

5.2 Production of mutated fibronectin isoforms

Mutant strand synthesis reaction

To demonstrate the effectiveness of site directed mutagenesis method, the control plasmid and primers were used. The control plasmid contains a stop codon TAA at the position where codon CAA would normally appear in the β-galactosidase gene. *E.coli* transformed with this control plasmid appear white on LB ampicillin plates containing IPTG and X-gal, because the β-galactosidase gene is damaged. The control primers create a point mutation that reverts the thymidine in stop codon to cytosine and the function of β-galactosidase gene is renewed. Following transformation, blue colonies can be seen.

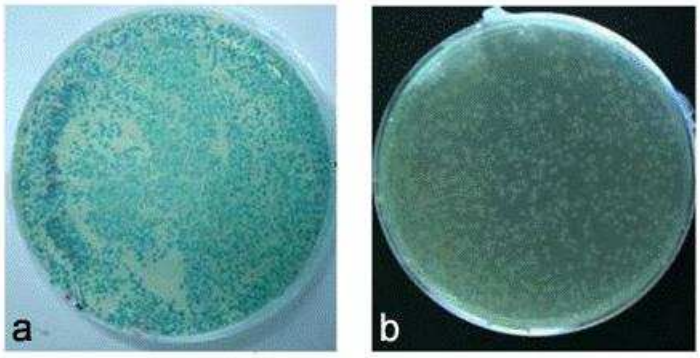


Figure 13: Mutagenesis control transformation.

Blue colonies of mutagenesis control are observed on the agar plate containing IPTG and X-gal (a). The use of control primers restored the origin of β-galactosidase gene and hence its function. Picture b shows white colonies containing fibronectin insert in pmax cloning vector.

Confirmation of plasmid DNA present

Before sequencing we verified the present and length of plasmid DNA in samples from maxi prep. It was perfomed using Not I restriction enzyme to linearized the plasmid and visualized using gel electrophoresis. The observable bands correspond to the size of 10 328 bp (10 328 bp of fibronectin, 2875 bp of pmax vector).

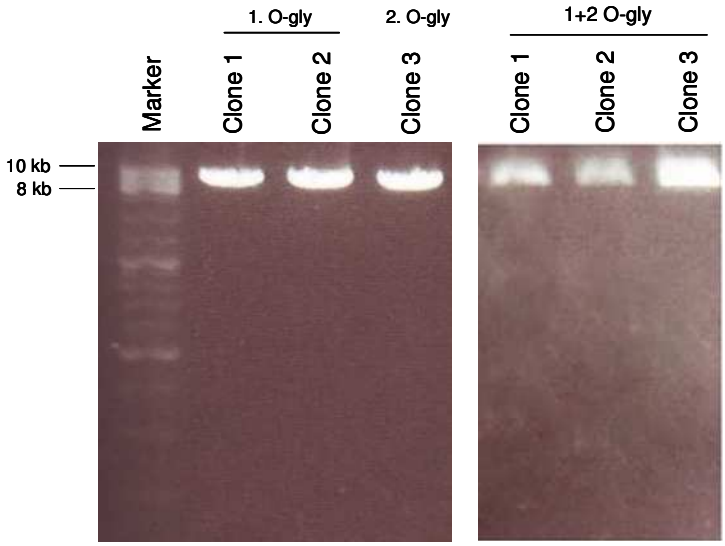


Figure 14: Verification of DNA plasmid presence.

After DNA plasmid isolation the linearization was performed using Not I restriction enzyme and the presence of plasmid was verified by gel electrophoresis. The bands correspond to the size of 10 328 bp (10 328 bp of fibronectin, 2875 bp of pmax vector).

1.O-gly indicates the mutation in NH_2-terminus, 2.O-gly in variable domain and 1+2 O-gly indicates the both mutations together.

5.2.1 Sequencing

The fibronectin insert was sequenced to confirm that selected clones contain the desired mutation. The sequencing was performed by GATC Biotech Company.

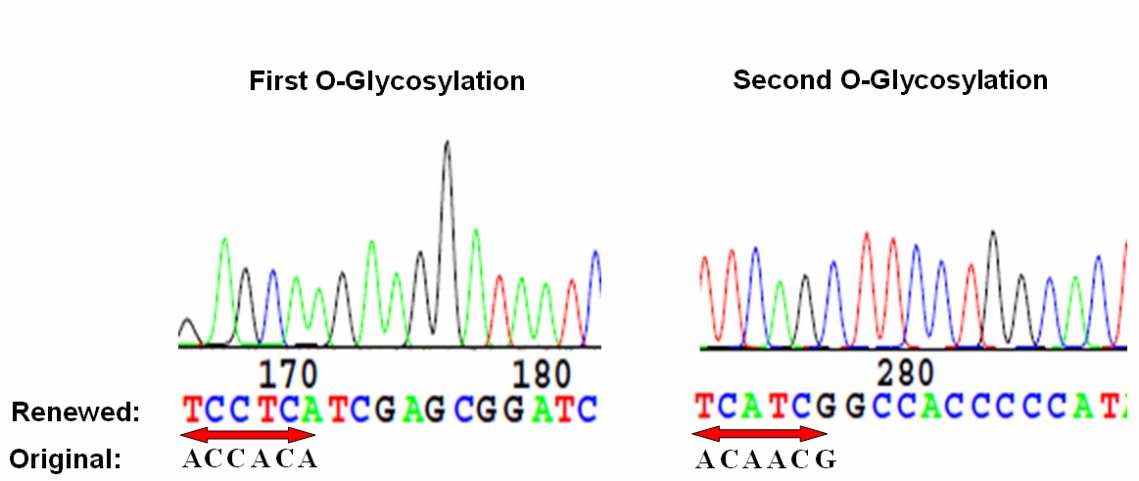


Figure 15: Sequencing.
The O-glycosylation sites in NH_2-terminus (first O-glycosylation) and variable domain (second O-glycosylation) were changed.

Testing of mutated fibronectin isoforms

To examine the functionality of all three mutated fibronectin isoforms *in vitro*, we performed the transient transfection. The knockdown HuH-7 cells were transfected with mutated fibronectin in NH_2-terminus (indicated as 1), in variable domain (indicated as 2) and fibronectin without O-glycosylation (indicated as 1+2) and non mutated cFN. The amount of fibronectin produced by cells was detected in cell lysates, in culture medium and fibrillar matrix using ELISA three days after transfection. The proportion of mutated fibronectin was compared with cells transfected with cFN, non transfected original HuH-7 cells and knockdown cells (negative control).

As show the figure 16 a,b the amounts of detected fibronectin by transfected cells exceed the negative control (except sample 1+2 where no increase is observed). The values are similar to cFN. Therefore we can assume that the transfection was successful. We sought that cells transfected with separately mutated O-glycosylation are able to form fibronectin (fig. 16 a) and release it into the culture media (fig. 16 b).

The original HuH-7 cells produce fibronectin in media probably faster than transfected knockdown cells. Thus the measured levels of fibronectin are lower in cell lysate (fig. 16 a), but higher in medium (fig.16 b) than by transfected cells.

We investigated that the fibronectin forms not containing one of the O-glycosylations are able to assemble into a fibrillar matrix, whereas the fibronectin with 1+2 mutation was completely absent as well as negative control (fig. 16 c).

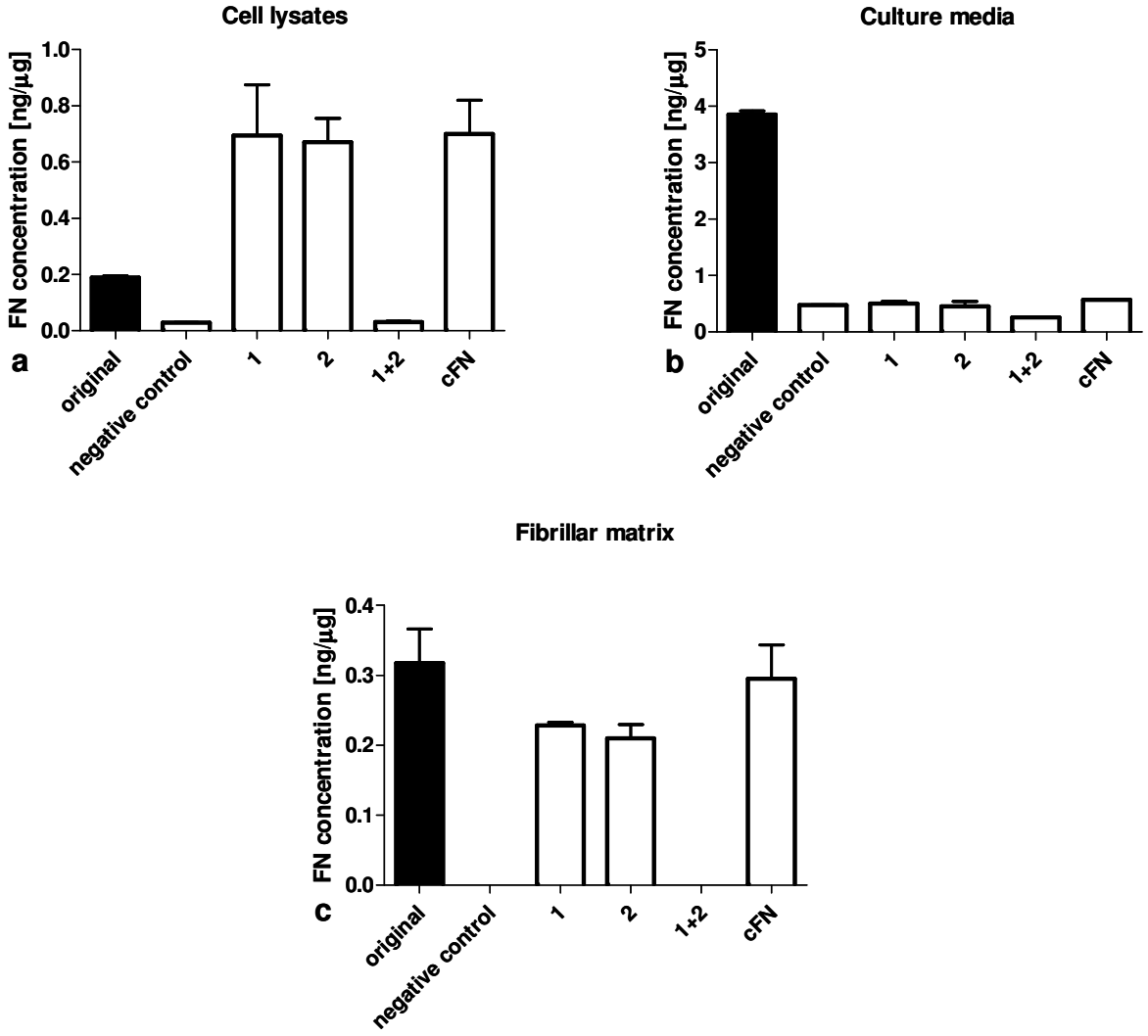


Figure 16: FN ELISA of cell lysates (a), culture media (b) and fibrillar matrix (c).

FN levels in material isolated from the transfected cells. Levels of fibronectin are correlated to protein amount and compared to non silenced HuH-7 (original), knockdown HuH-7 (negative control) and knockdown cells transfected with cFN. Number 1 represents mutation in the NH_2-terminus, number 2 in the variable domain and 1+2 fibronectin without O-glycosylation.

6 Discussion

Fibronectin is one of the key molecules having a role in the bone formation. It is important for osteoblasts differentiation and collagen matrix production (Moursi et al. 1996; Sottile and Hocking 2002). Total fibronectin knockout leads to defective vessel formation and even to early embryonic letality (George et al. 1993).

There are different fibronectin isoforms with characteristic features and sites of actions (Hynes 1990). The specific form oncofetal fibronectin is associated with the bone loss. An increase in the production of this oncofetal domain correlates with hepatic osteodystrophy (Kawelke et al. 2008). This special form is characteristic by O-glycosylation in the variable domain. Another O-glycosylation is found in NH_2-terminus (Tajiri et al. 2005).

To confirm that the oncofetal fibronectin has an influence on the hepatic osteodystrophy, we generated fibronectin knockdown mammalian cells and fibronectin lacking O-glycosylation.

6.1 Fibronectin knockdown in mammalian cells

The silence of fibronectin gene expression was performed using RNA interference. To increase the probability of high knockdown we used three different shRNA sequences target to untranslated region. After transduction we prepared the single cell clones. The fibronectin knockdown was measured using ELISA in FCS free culture medium and cell lysates after three days in culture. We detected the minimum concentration of fibronectin by clone transduced with shRNA number 207 about 94% compared to original cells (the reduction was by 94.7% in culture medium and 93.9 % in cell lysate). Low concentrations of fibronectin were also determinated by other clones in media but it was not confirmed by the lysates. Different detected levels of fibronectin can be caused by several reasons. The RNA interference can be mediate through translational repression or mRNA cleavage, depend on the degree of complementarity of shRNA to the target mRNA (Matzke and Birchler 2005). It is possible that fibronectin is formed despite the knockdown, but it is not fuctional. Therefore the levels of fibronectin could be higher in cell lysates, but lower in media, because only functional fibronectin can pass from the cells. The polyclonal antibody used for detection is able to bind to multiple sequences of fibronectin. And thus although the functional fibronectin is not formed it can be measured, because the antibody can recognize a functionless fibronectin part.

6.2 Generating of fibronectin mutated in O-glycosylation sites and its functionality

Both O-glycosylations of fibronectin are located at threonine (Tajiri et al. 2005). The tri-nucleotide sequences coding this amino acid were mutated using site directed mutagenis method. We decided to replace threonine by serine, because it is structural similar to threonine. We generated three different plasmids with fibronectin insert mutated in O-glycosylation: one in NH_2-terminus, second in variable domain and the last one with mutated both parts. The presence of codon coding serine was verified by sequencing performed by GATC Biotech Company.

We demonstrated the functionality of mutated fibronectin performing the transient transfection and fibronectin concentration measurement in cell lysates, culture medium and in fibrillar matrix.

Measured concentrations of fibronectin mutated either in NH_2-terminus (called 1) or in variable domain (called 2) were comparable to concentration measured by cells transfected with non mutated template cFN. Therefore fibronectin mutated in one of the O-glycosylation sites proved functional.

The fibronectin overexpression seen by sample 1, 2 and cFN in cell lysates in comparison to original (fig. 16 a) can be explained by slower transfer of fibronectin into the medium and incorporation into the matrix. On the contrary by original HuH-7 we could observe relatively low fibronectin levels in cell lysates, but higher in medium and matrix. This indicates a faster release of physiological fibronectin from the cells compared to mutated isoforms.

Fibronectin mutated in both O-glycosylation sites failed to show the ability to assembly into fibrillar network, but also very low fibronectin concentrations were measured in cell lysate and in medium. It is possible that the transient efficiency was low in this case. This could be caused by lower purity of plasmid DNA compared to other trasfection samples. Another reason that this type of mutated fibronectin was not found in the matrix could be a possible change in conformation as a result of complete absent of O-linked glycans.

A small amount of fibronectin detected by knockdown cells in cell lysate and medium was not demonstrated in the analysis of matrix, which proved the malfunction of residual fibronectin. This can also confirm the successful inhibition of fibronectin gene expression.

Further experiments are needed to find out whether the fibronectin without O-glycosylation losts the negative effect of the oncofetal fibronectin on the nodule formation, which means functional production of bone matrix.

To support this hypothesis the future *in vitro* plans are required: firstly to perform stabile transduction of silenced HuH-7 cells with prepared fibronectin isoforms inserted to a viral vector and generating stable cell lines due to antibiotic selection. The transduced cells will express the mutated fibronectin into the medium, from which it can be purified. The next step will be osteoblasts cultivation with adding of isolated fibronectin to culture media and von Kossa nodules staining that can show, whether the mineral deposition is or is not influenced.

7 Conclusion

In summary, our experiments provided the fibronectin knockdown of mammalian cells and three different mutated isoforms of fibronectin gene which are partially or completely lacking O-glycosylation.

8 Abbreviations

BMD	Bone mineral density
Bp	Base pair
BSA	Bovine serum albumin
cFN	Cellular fibronectin
dH_2O	Distilled water
ddH_2O	Double distilled water
DMEM	Dulbecco´s modified eagle medium
DMSO	Dimethylsulfoxid
DNA	Dioxyribonucleic acid
DTP	Deoxynucleotide triphosphates
DPBS	Dulbecco´s phosphate buffered saline
dsRNA	Double stranded ribonucleic acid
E.coli	Escherichia coli
ECM	Extracellular matrix
EDA	Extra domain A
EDB	Extra domain B
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FN	Fibronectin
Fig.	Figure
Fwd	Forvard
HEK cells	Human Embryonic Kidney cells
HuH-7 cells	Human Hepatoma cells
IGF-1	Insulin like growth factor 1
INF	Interferon
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KD	knockdown
kDa	kilo Dalton
LB	Luria Bertani
LDV	Leucin-Asparaginsäure-Valin
M-CSF	Macrophage colony-stimulating factor

MEM	Minimum essential medium
Min	Minute
MOI	Multiplicity of infection
mRNA	Messenger RNA
O-gly	O-glycosylation
oFN	Oncofetal fibronectin
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PBC	Primary biliary cirrhosis
PCR	Polymerase chain reaction
PDGF	Platelet derived grwth factor
pFN	Plasmafibronektin
PSC	Primary Sclerosing Cholangitis
PHSRN	Prolin-Histidin-Serin-Arginin-Asparagin
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	Receptor activator of nuclear factor kappa-B ligand
Rev	Reverse
RGD	Arginin-Glycin-Asparaginsäure
RISC	Ribonucleid acid induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT	Room temperature
Rpm	Rounds per minute
s	Second
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin ribonucleid acid
siRNA	Short interfering ribonucleid acid
Tab.	Tabule
Taq	*Thermus aquaticus* Polymerase
TGF-β	Transforming growth factor beta
TBE	Tris-Boric Acid-EDTA
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethan
TU	Transduction unit
UV	Ultraviolet light

V	Volt
VTHPGY	Valin-Threonin-Histidin-Prolin-Glycin-Tyrosin
X-gal	Bromo-chloro-indolyl-galactopyranoside

9 References

Adami, S., Zivelonghi, A., Braga, V., Fracassi, E., Gatti, D., Rossini, M., Ulivieri, F.M., and Viapiana, O. 2009. Insulin-like growth factor-1 is associated with bone formation markers, PTH and bone mineral density in healthy premenopausal women. *Bone* **46**(1): 244-247.

Bentmann, A., Kawelke, N., Moss, D., Zentgraf, H., Bala, Y., Berger, I., Gasser, J.A., and Nakchbandi, I.A. 2010. Circulating fibronectin affects bone matrix, whereas osteoblast fibronectin modulates osteoblast function. *J Bone Miner Res* **25**(4): 706-715.

Beuers U., Boberg K., Chapman R., Chazouille`res O., Invernizzi P., Jones D., Lammert F., Pare`s A., and Trauner M. 2009. EASL Clinical Practice Guidelines: management of cholestatic liver diseases. *J Hepatol* **51**(2): 237-267.

Borsi, L., Castellani, P., Risso, A.M., Leprini, A., and Zardi, L. 1990. Transforming growth factor-beta regulates the splicing pattern of fibronectin messenger RNA precursor. *FEBS Lett* **261**(1): 175-178.

Boyce, B.F., Li, P., Yao, Z., Zhang, Q., Badell, I.R., Schwarz, E.M., O'Keefe, R.J., and Xing, L. 2005. TNF-alpha and pathologic bone resorption. *Keio J Med* **54**(3): 127-131.

Boyce, B.F. and Xing, L. 2007. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res Ther* **9 Suppl 1**: S1.

Brown, L.F., Dubin, D., Lavigne, L., Logan, B., Dvorak, H.F., and Van de Water, L. 1993. Macrophages and fibroblasts express embryonic fibronectins during cutaneous wound healing. *Am J Pathol* **142**(3): 793-801.

Caetano-Lopes, J., Canhao, H., and Fonseca, J.E. 2009. Osteoimmunology--the hidden immune regulation of bone. *Autoimmun Rev* **8**(3): 250-255.

Castellani, P., Viale, G., Dorcaratto, A., Nicolo, G., Kaczmarek, J., Querze, G., and Zardi, L. 1994. The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int J Cancer* **59**(5): 612-618.

Clarke, B. 2008. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol* **3 Suppl 3**: S131-139.

Collier, J. 2007. Bone disorders in chronic liver disease. *Hepatology* **46**(4): 1271-1278.

Cooke, A.M. 1955. Osteoporosis. *Lancet* **268**(6871): 929-937.

Crosignani, A., Battezzati, P.M., Invernizzi, P., Selmi, C., Prina, E., and Podda, M. 2008. Clinical features and management of primary biliary cirrhosis. *World J Gastroenterol* **14**(21): 3313-3327.

de la Mata, J., Uy, H.L., Guise, T.A., Story, B., Boyce, B.F., Mundy, G.R., and Roodman, G.D. 1995. Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo. *J Clin Invest* **95**(6): 2846-2852.

Erlebacher, A., Filvaroff, E.H., Ye, J.Q., and Derynck, R. 1998. Osteoblastic responses to TGF-beta during bone remodeling. *Mol Biol Cell* **9**(7): 1903-1918.

Evans, J.F., Yeh, J.K., and Aloia, J.F. 2000. Osteoblast-like cells of the hypophysectomized rat: a model of aberrant osteoblast development. *Am J Physiol Endocrinol Metab* **278**(5): E832-838.

Felsenberg, D. 2001. [Supporting function of collagen and hydroxyapatite. Structure and function of bone]. *Pharm Unserer Zeit* **30**(6): 488-494.

Ffrench-Constant, C. 1995. Alternative splicing of fibronectin--many different proteins but few different functions. *Exp Cell Res* **221**(2): 261-271.

Ffrench-Constant, C., Van de Water, L., Dvorak, H.F., and Hynes, R.O. 1989. Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J Cell Biol* **109**(2): 903-914.

Floreani, A., Zappala, F., Fries, W., Naccarato, R., Plebani, M., D'Angelo, A., and Chiaramonte, M. 1997. A 3-year pilot study with 1,25-dihydroxyvitamin D, calcium, and calcitonin for severe osteodystrophy in primary biliary cirrhosis. *J Clin Gastroenterol* **24**(4): 239-244.

Franz-Odendaal, T.A., Hall, B.K., and Witten, P.E. 2006. Buried alive: how osteoblasts become osteocytes. *Dev Dyn* **235**(1): 176-190.

Gallego-Rojo, F.J., Gonzalez-Calvin, J.L., Munoz-Torres, M., Mundi, J.L., Fernandez-Perez, R., and Rodrigo-Moreno, D. 1998. Bone mineral density, serum insulin-like growth factor I, and bone turnover markers in viral cirrhosis. *Hepatology* **28**(3): 695-699.

George, E.L., Georges-Labouesse, E.N., Patel-King, R.S., Rayburn, H., and Hynes, R.O. 1993. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119**(4): 1079-1091.

Georges-Labouesse, E.N., George, E.L., Rayburn, H., and Hynes, R.O. 1996. Mesodermal development in mouse embryos mutant for fibronectin. *Dev Dyn* **207**(2): 145-156.

Globus, R.K., Doty, S.B., Lull, J.C., Holmuhamedov, E., Humphries, M.J., and Damsky, C.H. 1998. Fibronectin is a survival factor for differentiated osteoblasts. *J Cell Sci* **111 (Pt 10)**: 1385-1393.

Goral, V., Simsek, M., and Mete, N. 2010. Hepatic osteodystrophy and liver cirrhosis. *World J Gastroenterol* **16**(13): 1639-1643.

Guanabens, N. and Pares, A. Liver and bone. *Arch Biochem Biophys* **503**(1): 84-94.

Hadjidakis, D.J. and Androulakis, II. 2006. Bone remodeling. *Ann N Y Acad Sci* **1092**: 385-396.

Hauser S. C., Pardi D. S., and Poterucha J. J. 2008. *Mayo Clinic Gastroenterology and Hepatology Board Review*. MAYO CLINIC SCIENTIFIC PRESS.

Herman, S., Kronke, G., and Schett, G. 2008. Molecular mechanisms of inflammatory bone damage: emerging targets for therapy. *Trends Mol Med* **14**(6): 245-253.

Hill, P.A. 1998. Bone remodelling. *Br J Orthod* **25**(2): 101-107.

Hill, T.P., Spater, D., Taketo, M.M., Birchmeier, W., and Hartmann, C. 2005. Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev Cell* **8**(5): 727-738.

Holt, R.I.G., Hanley, N.A., and Brook, C.G.D. 2007. *Essential endocrinology and diabetes*. Blackwell Publishing, United Kingdom.

Horton, M.A. and Davies, J. 1989. Perspectives: adhesion receptors in bone. *J Bone Miner Res* **4**(6): 803-808.

Hughes, D.E., Salter, D.M., Dedhar, S., and Simpson, R. 1993. Integrin expression in human bone. *J Bone Miner Res* **8**(5): 527-533.

Hynes, R.O. 1990. *Fibronectins*. Springer-Verlag, New York.

Chalker, J.M. and Davis, B.G. 2010. Chemical mutagenesis: selective post-expression interconversion of protein amino acid residues. *Curr Opin Chem Biol* **14**(6): 781-789.

Chiang, C.Y., Kyritsis, G., Graves, D.T., and Amar, S. 1999. Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide. *Infect Immun* **67**(8): 4231-4236.

Jo, J.S., Hong, S.B., Shin, H.I., and Choi, J.Y. 1991. Homologous fibronectin enhances healing of excised wounds in rats. *J Korean Med Sci* **6**(3): 197-205.

Johansson, S., Svineng, G., Wennerberg, K., Armulik, A., and Lohikangas, L. 1997. Fibronectin-integrin interactions. *Front Biosci* **2**: d126-146.

Karsenty, G. and Wagner, E.F. 2002. Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* **2**(4): 389-406.

Kawelke, N., Bentmann, A., Hackl, N., Hager, H.D., Feick, P., Geursen, A., Singer, M.V., and Nakchbandi, I.A. 2008. Isoform of fibronectin mediates bone loss in

patients with primary biliary cirrhosis by suppressing bone formation. *J Bone Miner Res* **23**(8): 1278-1286.

Krolo, M., Vilovic, K., Sapunar, D., Vrdoljak, E., and Saraga-Babic, M. 1998. Fibronectin expression in the developing human spinal cord, nerves, and ganglia. *Croat Med J* **39**(4): 386-391.

Liao, Y.F., Gotwals, P.J., Koteliansky, V.E., Sheppard, D., and Van De Water, L. 2002. The EIIIA segment of fibronectin is a ligand for integrins alpha 9beta 1 and alpha 4beta 1 providing a novel mechanism for regulating cell adhesion by alternative splicing. *J Biol Chem* **277**(17): 14467-14474.

Liu, J., Cao, Z., and Li, C. 2009. Intermittent PTH administration: a novel therapy method for periodontitis-associated alveolar bone loss. *Med Hypotheses* **72**(3): 294-296.

Manabe, R., Oh-e, N., and Sekiguchi, K. 1999. Alternatively spliced EDA segment regulates fibronectin-dependent cell cycle progression and mitogenic signal transduction. *J Biol Chem* **274**(9): 5919-5924.

Manabe, R., Ohe, N., Maeda, T., Fukuda, T., and Sekiguchi, K. 1997. Modulation of cell-adhesive activity of fibronectin by the alternatively spliced EDA segment. *J Cell Biol* **139**(1): 295-307.

Mao, Y. and Schwarzbauer, J.E. 2005. Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. *Matrix Biol* **24**(6): 389-399.

Matsuura, H., Greene, T., and Hakomori, S. 1989. An alpha-N-acetylgalactosaminylation at the threonine residue of a defined peptide sequence creates the oncofetal peptide epitope in human fibronectin. *J Biol Chem* **264**(18): 10472-10476.

Matsuura, H. and Hakomori, S. 1985. The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma. *Proc Natl Acad Sci U S A* **82**(19): 6517-6521.

Matuskova, J., Chauhan, A.K., Cambien, B., Astrof, S., Dole, V.S., Piffath, C.L., Hynes, R.O., and Wagner, D.D. 2006. Decreased plasma fibronectin leads to delayed thrombus growth in injured arterioles. *Arterioscler Thromb Vasc Biol* **26**(6): 1391-1396.

Matzke, M.A. and Birchler, J.A. 2005. RNAi-mediated pathways in the nucleus. *Nat Rev Genet* **6**(1): 24-35.

Mauss S., Berg T., Rockstroh J., Sarrazin C., and H., W. 2009. *Hepatology 2009 A clinical textbook*. Flying Publisher.

Midwood, K.S., Valenick, L.V., Hsia, H.C., and Schwarzbauer, J.E. 2004. Coregulation of fibronectin signaling and matrix contraction by tenascin-C and syndecan-4. *Mol Biol Cell* **15**(12): 5670-5677.

Mounach, A., Ouzzif, Z., Wariaghli, G., Achemlal, L., Benbaghdadi, I., Aouragh, A., Bezza, A., and El Maghraoui, A. 2008. Primary biliary cirrhosis and osteoporosis: a case-control study. *J Bone Miner Metab* **26**(4): 379-384.

Moursi, A.M., Damsky, C.H., Lull, J., Zimmerman, D., Doty, S.B., Aota, S., and Globus, R.K. 1996. Fibronectin regulates calvarial osteoblast differentiation. *J Cell Sci* **109 (Pt 6)**: 1369-1380.

Muro, A.F., Moretti, F.A., Moore, B.B., Yan, M., Atrasz, R.G., Wilke, C.A., Flaherty, K.R., Martinez, F.J., Tsui, J.L., Sheppard, D., Baralle, F.E., Toews, G.B., and White, E.S. 2008. An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *Am J Respir Crit Care Med* **177**(6): 638-645.

Murray, R., Granner, D., Mayes, P., and Rodwell, V. 2002. *Harperova biochemie*. H & H.

Nageotte, M.P., Casal, D., and Senyei, A.E. 1994. Fetal fibronectin in patients at increased risk for premature birth. *Am J Obstet Gynecol* **170**(1 Pt 1): 20-25.

Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., and Sato, J. 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res* **42**(9): 3858-3863.

Nakchbandi, I.A. and van der Merwe, S.W. 2009. Current understanding of osteoporosis associated with liver disease. *Nat Rev Gastroenterol Hepatol* **6**(11): 660-670.

Ng, F., Boucher, S., Koh, S., Sastry, K.S., Chase, L., Lakshmipathy, U., Choong, C., Yang, Z., Vemuri, M.C., Rao, M.S., and Tanavde, V. 2008. PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. *Blood* **112**(2): 295-307.

Nguyen, D.L., Juran, B.D., and Lazaridis, K.N. Primary biliary cirrhosis. *Best Pract Res Clin Gastroenterol* **24**(5): 647-654.

Ni, H., Yuen, P.S., Papalia, J.M., Trevithick, J.E., Sakai, T., Fassler, R., Hynes, R.O., and Wagner, D.D. 2003. Plasma fibronectin promotes thrombus growth and stability in injured arterioles. *Proc Natl Acad Sci U S A* **100**(5): 2415-2419.

Orimo, H. 2010. The mechanism of mineralization and the role of alkaline phosphatase in health and disease. *J Nippon Med Sch* **77**(1): 4-12.

Ormarsdottir, S., Ljunggren, O., Mallmin, H., Olofsson, H., Blum, W.F., and Loof, L. 2001. Circulating levels of insulin-like growth factors and their binding proteins in patients with chronic liver disease: lack of correlation with bone mineral density. *Liver* **21**(2): 123-128.

Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K., and Sekiguchi, K. 1989. Deregulation of alternative splicing of fibronectin pre-mRNA in malignant human liver tumors. *J Biol Chem* **264**(18): 10331-10334.

-. 1990. Oncodevelopmental regulation of the alternative splicing of fibronectin pre-messenger RNA in human lung tissues. *Cancer Res* **50**(4): 1075-1078.

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16**(8): 948-958.

Pankov, R. and Yamada, K.M. 2002. Fibronectin at a glance. *J Cell Sci* **115**(Pt 20): 3861-3863.

Peters, J.H., Maunder, R.J., Woolf, A.D., Cochrane, C.G., and Ginsberg, M.H. 1989. Elevated plasma levels of ED1+ ("cellular") fibronectin in patients with vascular injury. *J Lab Clin Med* **113**(5): 586-597.

Pfeilschifter, J., Chenu, C., Bird, A., Mundy, G.R., and Roodman, G.D. 1989. Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclastlike cells in vitro. *J Bone Miner Res* **4**(1): 113-118.

Plow, E.F., Haas, T.A., Zhang, L., Loftus, J., and Smith, J.W. 2000. Ligand binding to integrins. *J Biol Chem* **275**(29): 21785-21788.

Rennard, S.I. and Crystal, R.G. 1982. Fibronectin in human bronchopulmonary lavage fluid. Elevation in patients with interstitial lung disease. *J Clin Invest* **69**(1): 113-122.

Rennard, S.I., Hunninghake, G.W., Bitterman, P.B., and Crystal, R.G. 1981. Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. *Proc Natl Acad Sci U S A* **78**(11): 7147-7151.

Rodés, J., Benhamou, J., and Rizzetto, M. 2007. *Textbook of hepatology: from basic science to clinical practice*. Wiley-Blackwell.

Ruffoni, D., Fratzl, P., Roschger, P., Klaushofer, K., and Weinkamer, R. 2007. The bone mineralization density distribution as a fingerprint of the mineralization process. *Bone* **40**(5): 1308-1319.

Sambrook, P. and Cooper, C. 2006. Osteoporosis. *Lancet* **367**(9527): 2010-2018.

Sano, K., Asahi, M., Yanagibashi, M., Hashii, N., Itoh, S., Kawasaki, N., and Ogawa, H. 2008. Glycosylation and ligand-binding activities of rat plasma fibronectin during

liver regeneration after partial hepatectomy. *Carbohydr Res* **343**(13): 2329-2335.

Shi, F., Harman, J., Fujiwara, K., and Sottile, J. 2010. Collagen I matrix turnover is regulated by fibronectin polymerization. *Am J Physiol Cell Physiol* **298**(5): C1265-1275.

Schiebler, T.H. and Korf, H.-W. 2007. *Anatomie*. Steinkopff Verlag, Darmstadt.

Schwarzbauer, J.E., Spencer, C.S., and Wilson, C.L. 1989. Selective secretion of alternatively spliced fibronectin variants. *J Cell Biol* **109**(6 Pt 2): 3445-3453.

Simonet, W.S., Lacey, D.L., Dunstan, C.R., Kelley, M., Chang, M.S., Luthy, R., Nguyen, H.Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H.L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T.M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, J., Derby, P., Lee, R., and Boyle, W.J. 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**(2): 309-319.

Sottile, J. and Hocking, D.C. 2002. Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. *Mol Biol Cell* **13**(10): 3546-3559.

Steele D.G. and Bramblett C.A. 1994. *The Anatomy and Biology of the Human Skeleton*. Texas A&M University Press.

Stepp, M.A., Daley, W.P., Bernstein, A.M., Pal-Ghosh, S., Tadvalkar, G., Shashurin, A., Palsen, S., Jurjus, R.A., and Larsen, M. Syndecan-1 regulates cell migration and fibronectin fibril assembly. *Exp Cell Res* **316**(14): 2322-2339.

Sutherland, A.E., Calarco, P.G., and Damsky, C.H. 1988. Expression and function of cell surface extracellular matrix receptors in mouse blastocyst attachment and outgrowth. *J Cell Biol* **106**(4): 1331-1348.

Sylvester, F.A. 2001. Bone abnormalities in gastrointestinal and hepatic disease. *Rev Endocr Metab Disord* **2**(1): 75-80.

Tajiri, M., Yoshida, S., and Wada, Y. 2005. Differential analysis of site-specific glycans on plasma and cellular fibronectins: application of a hydrophilic affinity method for glycopeptide enrichment. *Glycobiology* **15**(12): 1332-1340.

van der Merwe, S.W., Attfield, D., Fevery, J., and Bogaerde, J.B. 2000. Hepatic osteodystrophy: the influence of liver disease and portal hypertension on cytokine activation. *Med Hypotheses* **54**(5): 842-845.

Vartio, T., Laitinen, L., Narvanen, O., Cutolo, M., Thornell, L.E., Zardi, L., and Virtanen, I. 1987. Differential expression of the ED sequence-containing form of cellular fibronectin in embryonic and adult human tissues. *J Cell Sci* **88 (Pt 4)**: 419-430.

Velling, T., Risteli, J., Wennerberg, K., Mosher, D.F., and Johansson, S. 2002. Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins alpha 11beta 1 and alpha 2beta 1. *J Biol Chem* **277**(40): 37377-37381.

Wang, X.Q., Chen, L., Pan, R., Zhao, J., Liu, Y., and He, R.Q. 2008. An earthworm protease cleaving serum fibronectin and decreasing HBeAg in HepG2.2.15 cells. *BMC Biochem* **9**: 30.

Wierzbicka-Patynowski, I., Mao, Y., and Schwarzbauer, J.E. 2004. Analysis of fibronectin matrix assembly. *Curr Protoc Cell Biol* **Chapter 10**: Unit 10 12.

Wilson, C.L. and Schwarzbauer, J.E. 1992. The alternatively spliced V region contributes to the differential incorporation of plasma and cellular fibronectins into fibrin clots. *J Cell Biol* **119**(4): 923-933.

Xia, P. and Culp, L.A. 1994. Adhesion activity in fibronectin's alternatively spliced domain EDa (EIIIA) and its neighboring type III repeats: oncogene-dependent regulation. *Exp Cell Res* **213**(1): 253-265.

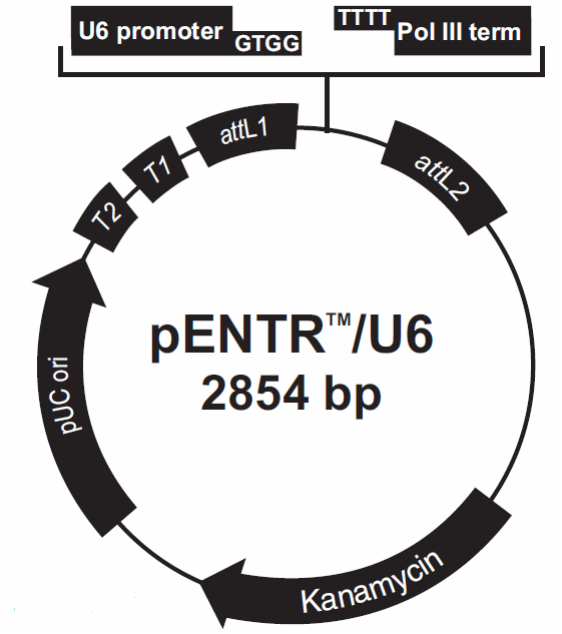
Xu, G., Niki, T., Virtanen, I., Rogiers, V., De Bleser, P., and Geerts, A. 1997. Gene expression and synthesis of fibronectin isoforms in rat hepatic stellate cells. Comparison with liver parenchymal cells and skin fibroblasts. *J Pathol* **183**(1): 90-98.

Yakar, S., Rosen, C.J., Beamer, W.G., Ackert-Bicknell, C.L., Wu, Y., Liu, J.L., Ooi, G.T., Setser, J., Frystyk, J., Boisclair, Y.R., and LeRoith, D. 2002. Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest* **110**(6): 771-781.

Yu, H., de Vos, P., and Ren, Y. Overexpression of osteoprotegerin promotes preosteoblast differentiation to mature osteoblasts. *Angle Orthod* **81**(1): 100-106.

10 Appendix

10.1 Map and features of pENTR™/U6 vector



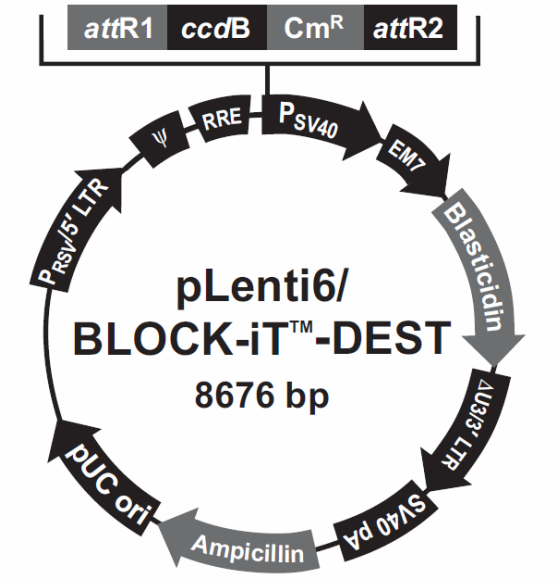
Comments for pENTR™/U6
2854 nucleotides

*rrn*B T2 transcription terminator: bases 268-295 (C)
*rrn*B T1 transcription terminator: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (C)
U6 promoter: bases 705-968
U6 forward priming site: bases 890-909
5′ overhang: bases 965-968 (C)
5′ overhang: bases 969-972
Pol III transcription terminator: bases 969-974
*att*L2: bases 979-1078
M13 reverse priming site: bases 1119-1135
Kanamycin resistance gene: bases 1248-2057
pUC origin: bases 2178-2851

(C) = complementary strand

(BLOCK-iT ™ U6 RNAi Entry Vector Kit, Invitrogen; Karlsruhe, GE)

10.2 Map and features of pLenti6/Block-iT™-DEST

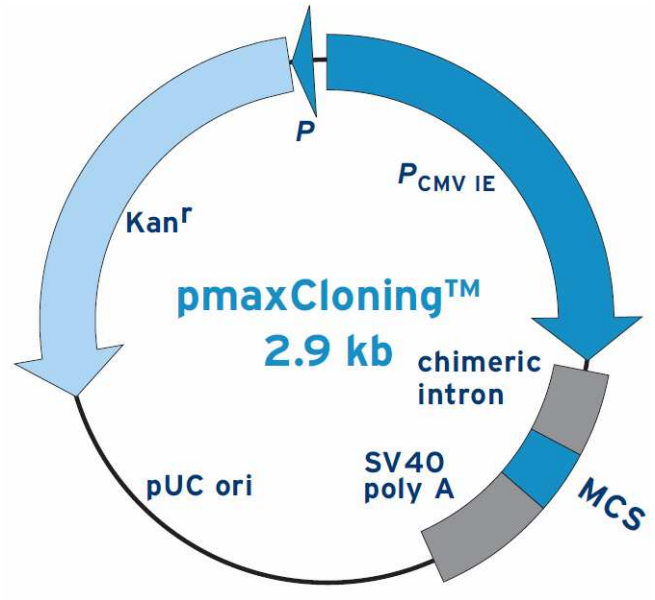


Comments for pLenti6/BLOCK-iT™-DEST
8676 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
RSV promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
*att*R1 site: bases 1868-1992
*ccd*B gene: bases 2421-2726 (C)
Chloramphenicol resistance gene (Cm^R): bases 3068-3727 (C)
*att*R2 site: bases 4008-4132
SV40 early promoter and origin: bases 4281-4590
EM7 promoter: bases 4645-4711
Blasticidin resistance gene: bases 4712-5110
ΔU3/3′ LTR: bases 5196-5430
ΔU3: bases 5196-5249
3′ LTR: bases 5250-5430
SV40 polyadenylation signal: bases 5502-5636
bla promoter: bases 6492-6590
Ampicillin (*bla*) resistance gene: bases 6591-7451
pUC origin: bases 7596-8269

(BLOCK-iT ™ Lentiviral RNA Expression System Kit, Invitrogen; Karlsruhe, GE)

10.3 Maps and features of pmaxCloning™ vector



Vector description

pmaxCloning™ (1,2) is an eukaryotic expression vector to promote constitutive expression of cloned DNA inserts in mammalian cells. The pmaxCloning™ vector backbone contains the immediate early promoter of cytomegalovirus ($P_{CMV\ IE}$) for protein expression, a chimeric intron for enhanced gene expression and the pUC origin of replication for propagation in *E. coli*. The bacterial promoter (*P*) provides kanamycin resistance gene expression in *E. coli*. The multiple cloning site (MCS) is located between the CMV promoter and the SV40 polyadenylation signal (SV40 poly A).

The pmaxCloning™ vector can be used for both transient and stable expression of genes. For stable expression the pmaxCloning™ vector must be co-transfected with an expression vector containing a selectable gene for mammalian cells.

(from http://www.biocenter.hu/pdf/pmax.pdf, date 3.5.2011)

10.4 Amino acid codes

A	Ala	Alanine	M	Met	Methionin
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

10.5 Nucleoid codes

A = Adenine T = Thymine G = Guanine C = Cytosine

10.6 Standart genetic codes

Amino acid	**Triplet**	**Amino acid**	**Triplet**	**Amino acid**	**Triplet**	**Amino acid**	**Triplet**
TTC	Phe	TCT	Ser	TAT	Tyr	TGT	Cys
TTC	Phe	TCC	Ser	TAC	Tyr	TGC	Cys
TTA	Leu	TCA	Ser	TAA	Stop	TGA	Stop
TTG	Leu	TCG	Ser	TAG	Stop	TGG	Trp
CTT	Leu	CCT	Pro	CAT	His	CGT	Arg
CTC	Leu	CCC	Pro	CAC	His	CGC	Arg
CTA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CTG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
ATT	Ile	ACT	Thr	AAT	Asn	AGT	Ser
ATC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
ATA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
ATG	Met/Start	ACG	Thr	AAG	Lys	AGG	Arg
GTT	Val	GCT	Ala	GAT	Asp	GGT	Gly
GTC	Val	GCC	Ala	GAC	Asp	GGC	Gly
GTA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GTG	Val	GCG	Ala	GAG	Glu	GGG	Gly

10.7 Amino acid sequence of fibronectin (Homo sapiens)

MLRGPGPGLLLLAVQCLGTAVPSTGASKSKRQAQQMVQPQSPVA

VSQSKPGCYDNGKHYQINQQWERTYLGNALVCTCYGGSRGFNCESKPEAEETCFDKYT

GNTYRVGDTYERPKDSMIWDCTCIGAGRGRISCTIANRCHEGGQSYKIGDTWRRPHET

GGYMLECVCLGNGKGEWTCKPIAEKCFDHAAGTSYVVGETWEKPYQGWMMVDCTCLGE

GSGRITCTSRNRCNDQDTRTSYRIGDTWSKKDNRGNLLQCICTGNGRGEWKCERHTSV

First O-glycosylation

QTTSSGSGPFTDVRAAVYQPQPHPQPPPYGHCVTDSGVVYSVGMQWLKTQGNKQMLCT

ACCACA

CLGNGVSCQETAVTQTYGGNSNGEPCVLPFTYNGRTFYSCTTEGRQDGHLWCSTTSNY

EQDQKYSFCTDHTVLVQTRGGNSNGALCHFPFLYNNHNYTDCTSEGRRDNMKWCGTTQ

NYDADQKFGFCPMAAHEEICTTNEGVMYRIGDQWDKQHDMGHMMRCTCVGNGRGEWTC

IAYSQLRDQCIVDDITYNVNDTFHKRHEEGHMLNCTCFGQGRGRWKCDPVDQCQDSET

GTFYQIGDSWEKYVHGVRYQCYCYGRGIGEWHCQPLQTYPSSSGPVEVFITETPSQPN

SHPIQWNAPQPSHISKYILRWRPKNSVGRWKEATIPGHLNSYTIKGLKPGVVYEGQLI

SIQQYGHQEVTRFDFTTTSTSTPVTSNTVTGETTPFSPLVATSESVTEITASSFVVSW

VSASDTVSGFRVEYELSEEGDEPQYLDLPSTATSVNIPDLLPGRKYIVNVYQISEDGE

QSLILSTSQTTAPDAPPDPTVDQVDDTSIVVRWSRPQAPITGYRIVYSPSVEGSSTEL

NLPETANSVTLSDLQPGVQYNITIYAVEENQESTPVVIQQETTGTPRSDTVPSPRDLQ

FVEVTDVKVTIMWTPPESAVTGYRVDVIPVNLPGEHGQRLPISRNTFAEVTGLSPGVT

YYFKVFAVSHGRESKPLTAQQTTKLDAPTNLQFVNETDSTVLVRWTPPRAQITGYRLT

VGLTRRGQPRQYNVGPSVSKYPLRNLQPASEYTVSLVAIKGNQESPKATGVFTTLQPG

SSIPPYNTEVTETTIVITWTPAPRIGFKLGVRPSQGGEAPREVTSDSGSIVVSGLTPG

VEYVYTIQVLRDGQERDAPIVNKVVTPLSPPTNLHLEANPDTGVLTVSWERSTTPDIT

GYRITTTPTNGQQGNSLEEVVHADQSSCTFDNLSPGLEYNVSVYTVKDDKESVPISDT

IIPEVPQLTDLSFVDITDSSIGLRWTPLNSSTIIGYRITVVAAGEGIPIFEDFVDSSV

GYYTVTGLEPGIDYDISVITLINGGESAPTTLTQQTAVPPPTDLRFTNIGPDTMRVTW

APPPSIDLTNFLVRYSPVKNEEDVAELSISPSDNAVVLTNLLPGTEYVVSVSSVYEQH

ESTPLRGRQKTGLDSPTGIDFSDITANSFTVHWIAPRATITGYRIRHHPEHFSGRPRE

DRVPHSRNSITLTNLTPGTEYVVSIVALNGREESPLLIGQQSTVSDVPRDLEVVAATP

TSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVY

AVTGRGDSPASSKPISINYRTEIDKPSQMQVTDVQDNSISVKWLPSSSPVTGYRVTTT

PKNGPGPTKTKTAGPDQTEMTIEGLQPTVEYVVSVYAQNPSGESQPLVQTAVTNIDRP

KGLAFTDVDVDSIKIAWESPQGQVSRYRVTYSSPEDGIHELFPAPDGEEDTAELQGLR

PGSEYTVSVVALHDDMESQPLIGTQSTAIPAPTDLKFTQVTPTSLSAQWTPPNVQLTG

YRVRVTPKEKTGPMKEINLAPDSSSVVVSGLMVATKYEVSVYALKDTLTSRPAQGVVT

TLENVSPPRRARVTDATETTITISWRTKTETITGFQVDAVPANGQTPIQRTIKPDVRS

YTITGLQPGTDYKIYLYTLNDNARSSPVVIDASTAIDAPSNLRFLATTPNSLLVSWQP

PRARITGYIIKYEKPGSPPREVVPRPRPGVTEATITGLEPGTEYTIYVIALKNNQKSE

PLIGRKKTDELPQLVTLPHPNLHGPEILDVPSTVQKTPFVTHPGYDTGNGIQLPGTSG

Second O-glycosylation

QQPSVGQQMIFEEHGFRRTTPPTTATPIRHRPRPYPPNVGEEIQIGHIPREDVDYHLY

ACAACG

PHGPGLNPNASTGQEALSQTTISWAPFQDTSEYIISCHPVGTDEEPLQFRVPGTSTSA

TLTGLTRGATYNIIVEALKDQQRHKVREEVVTVGNSVNEGLNQPTDDSCFDPYTVSHY

AVGDEWERMSESGFKLLCQCLGFGSGHFRCDSSRWCHDNGVNYKIGEKWDRQGENGQM

MSCTCLGNGKGEFKCDPHEATCYDDGKTYHVGEQWQKEYLGAICSCTCFGGQRGWRCD

NCRRPGGEPSPEGTTGQSYNQYSQRYHQRTNTNVNCPIECFMPLDVQADREDSRE

(http://www.ncbi.nlm.nih.gov/nuccore/47132556?from=1&to=8815&report=gbwithparts, date 3.5.2011)