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The role of fibroblast activation protein (FAP) in glioma cell growth and invasiveness

Role fibroblastového aktivačního proteinu (FAP) v růstu a invazivitě gliomových buněk

Edita Fejfarová

Supervisor: Petr Bušek, MD, PhD

Institute of Biochemistry and Experimental Oncology

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I hereby declare that this thesis is my own work and to the best of my knowledge it contains no material previously published or written by another person. This thesis was prepared under the supervision of Petr Bušek, MD, PhD. This work or a substantial part has not been used to reach another educational degree.

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Abstract

High grade astrocytomas are very progressive brain tumors. Glioblastoma multiforme is the most frequent and the most malignant type with very infiltrative phenotype of the tumor cells. Fibroblast activation protein FAP is a predominantly membrane bound prolyl peptidase bearing exo- and endopeptidase hydrolytic activities. FAP is known to play a role in wound healing, cell migration and invasion and its expression is linked to the pathogenesis of several malignancies. mRNA expression of FAP is upregulated in 48% of glioblastomas according to The Cancer Genome Atlas microarray data. The involvement of FAP in the pathogenesis of astrocytic tumors is largely unknown.

The aims of this work are to analyse the expression of FAP in primary cell cultures derived from high grade gliomas and to analyse the influence of FAP on the growth, migration and invasion of glioma cells.

Our ELISA and western blot results showed heterogenous expression of FAP in the studied glioma primary cell cultures and cell lines. Both enzymatic activities characteristic of FAP were detected in the primary glioma cell culture P11 with high expression of FAP. In these cells, FAP was present not only in the typical plasma membrane localization, but also in the cytoplasm as demonstrated by immunofluorescence staining. The P11 cells were tumorigenic in immunodeficient mice and retained FAP expression *in vivo*. In three primary glioma cell cultures, high expression of FAP was associated with slow growth rate *in vitro*. No such effect was observed on transfected cells expressing either enzymatically active or enzymatically inactive form of transgenic FAP. Therefore, FAP might be rather a marker of slower proliferation than a molecule directly influencing growth rate. Interestingly, the migration in the transwell assay and invasion of glioma cells from spheroids into the collagen gels was decreased in the transfected cells expressing the enzymatically active form of FAP. These effects were dependent on the enzymatic activity of FAP as we did not observe the decreased migration or invasion in glioma cells expressing the enzymatically inactive form of FAP. Although, FAP is presented in many studies as a proinvasive molecule, our data show transgenically expressed FAP is not sufficient to increases invasion of the glioma cells normally negative for this protein.

Key words: FAP, invasion, migration, glioblastoma

Abstrakt

Astrocytární tumory vysokého stupně malignity jsou velice progresivní mozkové nádory. Glioblastoma multiforme je nejčastější a nejmalignější typ s infiltrativním fenotypem nádorových buněk. Fibroblastový aktivační protein (FAP) je prolyl peptidáza převážně vázaná na membránu a vykazující exo- a endopeptidázovou hydrolytickou aktivitu. Je známo, že se FAP podílí na hojení ran, buněčné migraci a invazi a jeho xprese je spojována s patogenezí řady malignit. Exprese FAP mRNA je podle microarray dat z The Cancer Genome Atlas zvýšena ve 48% glioblastomů. Vliv FAP na patogenezi astrocytárních nádorů není objasněn.

Cíly této práce je analyzovat expresi FAP v primárních gliomových kulturách odvozených od gliomů vysokého stupně malignity a analýza vlivu FAP na růst, migraci a invazi gliomových buněk.

Naše výsledky z ELISA a wester blotu ukázaly heterogenní expresi napříč studovanými gliomovými primárními buněčnými kulturami a permanentími liniemi. Obě enzymové aktivity charakteristické pro FAP byly detekovány v primární gliomové kultuře P11 s vysokou expresí FAP. Pomocí imunofluorescenčního značení se ukázalo, že v těchto buňkách se FAP nacházel nejen na plasmatické membráně, kde je typicky lokalizován, ale také v cytoplazmě. Buňky z kultury P11 byly tumorigenní v immunodeficientních myších a udržely si expresi FAP *in vivo*. U třech primárních gliomových kultur byla vysoká exprese FAP spojena s pomalejší rychlostí růstu *in vitro.* Na transfekovaných buňkách exprimujících enzymaticky aktivní či enzymaticky neaktivní formu FAP podobný efekt nebyl pozorován. FAP je tedy spíše možným markerem pomalejšího růstu, než molekulou přímo ovlivňující rychlost růstu. Zajímavé je, že se snížíla migrace gliomových buněk skrze membránu (transwell assay) i jejich invaze ze sféroidů do kolagenových gelů, a to u transfekovaných buněk exprimujících enzymaticky aktivní formu FAP. Jelikož jsme nepozorovali snížení migrace či invaze u gliomových buněk exprimujících enzymaticky neaktivní formu FAP, je tento vliv závislý na enzymové aktivitě. Ačkoli řada prací uvádí FAP jako proinvazivní molekulu, naše data ukazují, že transgenní FAP exprimovaný v buňkách přirozeně negativních pro tento protein není dostačující pro zvýšení invaze gliomových buněk.

Klíčová slova: FAP, invaze, migrace, glioblastoma

Abbreviations:

ADAMs – disintegrin-and-metalloproteases

AFC – 7-amino-4-trifluoromethylcoumarin

AMC – 7-Amido-4-methylcoumarin

APCE – antiplasmin cleaving enzyme

ATCC – American Type Culture Collection

bHLH – basic helix-loop-helix

DPP – dipeptidyl peptidase

ECM – extracellular matrix

EMT – epithelial-mesenchymal transition

EGFR – epidermal growth factor receptor

FAK – focal adhesion kinase

FAP – fibroblast activation protein

f.c. – final concentration

FGF 2 – fibroblast growth factor 2

GFAP – glial fibrillary acidic protein

GIP – gastric inhibitory polypeptide

GPI – glycophosphatidylinositol

GLP–1 - glucagon like peptide 1

HGF – hepatocyte growth factor

IDH1 – isocitrate dehydrogenase 1

IGFR – insulin growth factor receptor

MT–MMP – membrane type matrix metalloproteinase

MMP – matrix metalloproteinase

NF1 – neurofibromatosis 1

PAI-1 – plasminogen activator inhibitor 1

PCR – polymerase chain reaction

PDGFRA – platelet-derived growth factor receptor-A

PRE – pharmacoresistant epilepsy

Pro – proline

PTEN – phosphatase and tensin homology

TF – transcription factor

TGF (β/α) – transforming growth factor

TNF-α – tumor necrosis factor α

uPA – urokinase activator/urokinase plasminogen activator

uPAR – urokinase receptor/urokinase plasminogen activator receptor

VEGF – vascular endothelial growth factor

Table of contents

1.1. Astrocytic tumors

According to the World Health Organization (WHO) astrocytic brain tumors are classified on four grade scale. Grade I (pilocytic astrocytoma) and Grade II (diffuse astrocytoma) are named low-grade astrocytomas. Grade III (anaplastic astrocytoma) and Grade IV (glioblastoma multiforme) are called high-grade astrocytomas. The classification is made on the basis of histopathological features, for GBM are characteristic mitotic activity, nuclear atypia, cellular polymorphism and microvascular proliferation and/or necrosis (*Louis, Ohgaki, 2007*).

Glioblastoma multiforme (GBM, synonymously called glioblastoma) is the most frequent brain tumor. Its incidence in Europe is 3,55 new cases per 100 000 population per year (*Ohgaki, Kleihues, 2007*), with almost equal sex distribution. Glioblastoma occurs at any age, but the most threatened age group is between 45 and 75 years. GBM can develop from lower grade astrocytic tumor (secondary glioblastoma) but more than 90% of glioblastomas arise without longer clinical history (primary glioblastoma) and show great invasive growth and very poor prognosis (*Brandes et al., 2008*). Bad prognosis of GBM patients (survival time vary mostly between 6 and 18 months) is mainly due to the high rate of the tumor recurrence and aggressive spreading of glioma cells. The aim of recent studies is to recognize elements responsible for invasive phenotype of these cells.

Gliomas are a heterogeneous group, as multiple genetic alterations linked to the disease. Genomic analyses identified four glioblastoma types (TCGA; *Verhaak et al., 2010*): Classical, Mesenchymal, Proneural and Neural. The Classical was classified by chromosome 7 amplification and chromosome 10 loss. Typical for the Classical subtype is *EGFR* alteration that either leads to fourfold higher expression of EGFR or to mutated constitutively active form EGFRvIII. EGFR is a transmembrane receptor of epidermal growth factor (EGF) or TGF-α, transducing these pro-proliferative signals via PI3K/PKB/PTEN pathway or activates MAPKKK or JAK/STAT signalling. Other characteristic of Classical class is deletion of *CDKN2A*, where are encoded e.g. *p16INK4A* and *p14ARF* which are involved in regulation of cell cycle

progression. The Mesenchymal class, typically expressing mesenchymal markers, is carrying deletion in 17q11.2, where is localized *NF1.* NF1 (neurofibromatosis 1) is less expressed or mutated in the Mesenchymal subtype and also PTEN (phosphatase and tensin homology) is often mutated. For the Proneural class are characteristic *PDGFRA* alterations leading to PDGFRA (platelet-derived growth factor receptor-A) higher expression, together with mutated isocitrate dehydrogenase 1 gene *(IDH1)* and TP53 mutations/loss of heterozygosity. *IDH1* encodes isocitrate dehydrogenase catalyzing the production of nicotinamide adenine dinucleotide phosphate. The IDH1 mutation in GBM affect arginine 132 localized to the substrate binding site (*Parsons et al., 2008*). Proneural subtype is associated with younger patients. The Neural class expresses neuronal markers similarly to normal brain samples (*Verhaak et al., 2010*). In all subtypes the 10q23 region is frequently deleted, where PTEN is localized. PTEN amino terminal domain can for example directly dephosphorylate (inactivate) focal adhesion kinase, which is important in regulation of cell movement and adhesion (*Tamura et al., 1999*).

1.2. Molecules involved in cell migration/invasion of high grade astrocytic tumors

Migration is natural process which is of a great importance during embryo's development, in an adult tissue during regeneration, wound-healing or immune defence. Migration is also prerequisite of tumor generalization - tumor metastases formation or rapid tumor spreading in primary tissue as it happens in gliomas (*Yamahara et al., 2010*).

There are two known mechanisms of cell movement in 3D space: mesenchymal and amoeboid-like migration (*Pankova et al., 2010*).

Prototype of the mesenchymal migration is fibroblasts or fibroblast-like migration. Cells using the mesenchymal pattern are polarized and spindle shaped. These cells form protrusions at leading edge called invadopodia. Invadopodia are stabilized by contact with extracellular components and the cell moves forward by means of actinomyosin contraction. Leading protrusion is F-actin rich and adheres to extracellular proteins; smaller side protrusions are enriched in collagenases and clusters of β1 integrin and generate ECM degradation (*Friedl & Wolf, 2009*).

1.2.1. Matrix metalloproteinases (MMPs)

The cells express collagenases which degrade ECM components and so facilitate locomotion throughout the network of extracellular proteins. Matrix metalloproteinases (MMP) are mainly responsible for ECM remodelation. MMP either secreted or with a transmembrane domain (referred as MT-MMP) are zinc atom dependent endopeptidases. Most of them are expressed as zymogens activated by proteolytic truncation. Activating cleavage can be performed by plasmin, active MMP or they can be autoactivated. MMP-1, -8, -13 start degradation of several collagens to gelatin, which is a substrate for MMP-2 and -9. MMP-9 expression is induced during development, wound healing and tumor invasion (*Stamenkovic, 2000*). MMP-2 interacts with αuβ3 integrin on the cell surface of endothelial invadopodia (*Brooks et al., 1996*). MMP-3, -10 and -11 are expressed in epithelial cells, their substrates are e.g. collagens, fibronectin, laminin, gelatin or precursors of some MMP. MMPs also liberate growth factors bound to the ECM proteins: transforming growth factor-β (TGF-β), insulin-like growth factors I and II, fibroblast growth factor 2 (FGF 2). MMPs activate tumor necrosis factor α (TNF-α) and TGF-α while cleaving their inactive form (*Bergers and Coussens, 2000*).

Four MT-MMPs anchored in a membrane are known so far. MT1-MMP activates proMMP2, degrades collagen I, laminin or fibronectin. MT1-MMP is localized on invadopodia of tumor cells. Similar substrates have also MT2-MMP and MT3-MMP. MT4-MMP substrate specifity remains unknown (*Stamenkovic, 2000*).

In 90% of primary glioblastomas was detected MMP9 zymogen and in 50% of the samples was detected its active form. MMP9 expression can be activated via EGFR signalling which is altered in primary glioblastoma *(Choe et al., 2002; Forsyth et al., 1998).* MT1-MMP expression varies between glioma cell lines. In D54MG, LG11, T98, U87MG it is high compare to the lower expression in A173, Hs683, U373MG and U251MG. MT1-MMP mRNA is overexpressed in high grade glioma brain tissues and it is not detectable in normal brain *(Fillmore et al., 2001)*.

1.2.2. Urokinase and its receptor

Urokinase receptor is bound to invadopodia membrane by GPI-anchoring. This receptor binds urokinase (uPA) that converts proenzyme plasminogen into plasmin (*Ploug, 2003*). Plasmin is a serine protease able to degrade glycoproteins of basement membranes and many components of ECM, such as fibrinogen,

fibronectin and vitronectin. It can activate latent collageneases degrading plasmin-resistant collagenous elements. Its increased activity is believed to play an important role in tumor cell invasiveness (*Stoppelli, 2003*). uPA system can not only release growth hormones such as FGF2, VEGF (vascular endothelial growth factor), HGF (hepatocyte growth factor), TGF-β from ECM, but also activates a cascade leading to positive feedback on uPA and uPAR expression. Plasmin can be inhibited by α2-antiplasmin. uPA in complex with its receptor can be inhibited by plasminogen activator inhibitor 1 (PAI-1), endocytosed and degraded in lysosomes (*Tang & Wei, 2008; Yamamoto et al., 1994*).

Amounts of uPAR mRNA are 15-fold higher in glioblastoma compared to normal adult brain. Its expression is localized on the tumor cells near vascular proliferation sites and within the necrotic tissue *(Yamamoto et al., 1994; Lakka et al., 2005)*. The mRNA of uPAR along with plasminogen activator inhibitor 1 (PAI-1) mRNA is upregulated by interleukin 1 and sphingosine-1-phosphate and enhances invasion in glioma cells (*Bryan et al., 2008)*. uPAR knockdown by RNA interference alters invasion of glioma cells and increases α3β1 integrin expression (*Fukushima et al., 1998*). Furthermore, glioblastoma cells show higher level of TNFα receptors in response to the uPAR silencing, and so the cells can be more sensitive for apoptotic stimuli *(Lakka et al., 2005)*.

1.2.3. Cathepsins

Cathepsins are a group containing 11 members of cystein proteases responsible for protein degradation in lysosomes. Cathepsins are synthesized as inactive precursors activated in lysosomes by acidic pH. In cancer cells they can localize also on the cell membrane. Together with cleaving of ECM components (laminin, fibronectin, collagen IV) cathepsins activate other proteases such as MMP1, MMP3 or uPA, thus contributing to cell invasion (*Gocheva and Joyce, 2007*). Cathepsin B is a lysosomal enzyme with exo- and endopeptidase activity, e.g. on E-cadherin. In many cancers has been observed its altered localization and secretion.

In gliomas cathepsin B mRNA, protein and activity are higher compared to normal brain (*Rempel et al., 1994; Demchik et al., 1999)*. Glioma cells invade into a cell aggregates more while they have higher cathepsin B expression (*Demchik et al., 1999*). Also a clinical invasion measured by magnetic resonance imaging correlates with elevated cathepsin B (*Rempel et al., 1994*). Cathepsin B is not only localized to

the lysosomes in glioma cells but it is expressed on the cell membrane as well (*Demchik et al., 1999*). Similarly cathepsin H is upregulated in glioma tumor tissue and its inhibition decreases invasion of glioma cells *(Sivaparvathi et al., 1996)*.

1.2.4. Integrins

Focal contact crucial for the mesenchymal type of migration is formed by clusters of integrins. Integrins form heterodimers from subunits alpha and beta. 8β and 18α subunits have been characterized so far. Both subunits cross the plasma membrane once and via adaptor proteins interact with cytoskeleton or enzymes, and outside the cell with extracellular proteins. 24 integrins with diverse ligand specifity have been described. Integrins have docking function for focal adhesion kinase (FAK; *Flier et al., 2001*), proteolytic enzymes such as fibroblast activation protein (*Mueller et al., 1999*), MMP2 (*Brooks et al., 1996*) and urokinase receptor (uPAR; *Tang and Wei, 2008*). Contact between integrins and actin cytoskeleton is provided by adaptor proteins (e.g. talin, vinculin, paxilin and α-actin), and influences cell architecture and regulation of migration (*D´Abaco et al, 2007*).

Laminin-5 receptor, also abbreviated as α3β1 integrin is a major receptor for contact with ECM in glioblastomas. Laminin-5 stimulates adhesion and migration and it is expressed in tumor parenchyma of glioma *(Fukushima et al., 1998)*.
β1 subunit is able to heterodimerize with many α. Its expression is enhanced in high-grade gliomas and glioma cell lines together with its pairing partners α2, α3, α5, α6. When β1 is neutralised by antibodies, glioma cell migration is reduced. Also αυ subunit, reported to interact with MMP-2, is upregulated in gliomas. αυ expression was shown to be associated with GBM tumor vasculature in contrast to normal or low-grade astrocytomas *(D´Abaco et al.;2007; Lakka et al., 2005)*.

The extracellular matrix of brain influences glioma cell spreading and actually can be modulated by glioma cells. Glioma cells change brain extracellular matrix composition, expressing fibrous proteins normaly located only in a basement membrane of blood vessels (*Gladson, 1999; Wiranowska and Rojiani, 2011*). In addition, chemoattractants (e.g. EGF and related factors, TGF-β, SDF1) and chemorepellents secreted by cells surrounding the tumor or by glioma cells itself have an effect on glioma cell invasion (*Viapiano and Lawler, 2009*).

Beside the already mentioned and most known proteases, family of proteases called disintegrin-and-metalloproteases (ADAMs) and ADAMs with thrombospondin motif *(reviewed in Viapiano and Lawler, 2009*) and other molecules such as fibroblast activation protein are considered to participate in the processes of glioma cell invasion (*Mentlein et al., 2011*).

1.3. Fibroblast activation protein (FAP) as a member of the Dipeptidyl peptidase IV gene family

The dipeptidyl peptidase (DPP) IV gene family comprises four members bearing the same enzymatic activity and similar structure as dipeptidyl peptidase IV together with two nonenzyme members. DPPIV, FAP, DPP8 and DPP9 possess enzymatic activity (sometimes called "DPPIV-like" hydrolytic activity) cleaving the N-terminal dipeptide from substrates with proline at penultimate position. DPP6 and DPP10 are structurally similar to DPPIV, but lack the enzymatic activity (*Yu et al., 2010*).

1.3.1. Dipeptidyl peptidase IV (DPPIV, CD26, EC 3.4.14.5.)

DPPIV is almost ubiquitously expressed (*Gorrell, 2005*) and is the most widely studied member of the family.

Gene encoding DPPIV is localized on the long arm of chromosome two (2q.24.3; *Abbott et al., 1994*), close to *FAP* (2q23; *Mathew et al., 1995*).

DPPIV is the closest homologue of FAP, while its C-terminal terminal sequence containing catalytic site is identical from 68% (*Goldstein et al., 1997*). In C-terminal sequence there are nine potential N-glycosylation sites. DPPIV is a serine protease with exopeptidase activity on a variety of biological active substrates such as hormones, chemokines, neuropeptides and peptide hormones (*Mentlein, 1999*). DPPIV is active as a 120kDa homodimer (*Bednarczyk et al., 1991*). DPPIV also cleaves efficiently artificial substrates (H-Gly-Pro-AMC, Ala-Pro-AMC, Gly-Pro-AFC). The main difference in enzymatic activities and preferred substrates of FAP and DPPIV is caused by Asp^{663} of DPPIV. This Asp is pushing Glu^{206}, which is important for binding to a substrate, deeper into the active site. As a result the pocket in DPPIV is more negatively charged than in FAP. In FAP is Ala^{657} responsible for the cleavage of internal peptide bonds of substrates and corresponds to Asp^{663} residue of DPPIV (*Aertgeerts et al., 2003 and 2005*). DPPIV – as a transmembrane protein - is typically

localized on the cell surface. According to some authors DPPIV and FAP can form heterodimeric complexes (*Scanlan et al., 1994*). Soluble form of DPPIV, lacking the transmembrane and intracellular domains, is present in serum (*Durinx et al., 2000*).

1.3.2. Dipeptidyl peptidases 8 and 9 (DPP8 and 9)

Genes for DPP8 and DPP9 are localized on 15q22 and 19p13.3 respectively.
Both molecules have dipeptidyl peptidase activity as monomers. DPP8 and 9 are cytosolic proteins, with no glycosylations. The molecular weight of DPP8 is 82kDa (*Abbott et al., 2000*), and of DPP9 98kDa (*Olsen, 2002*).

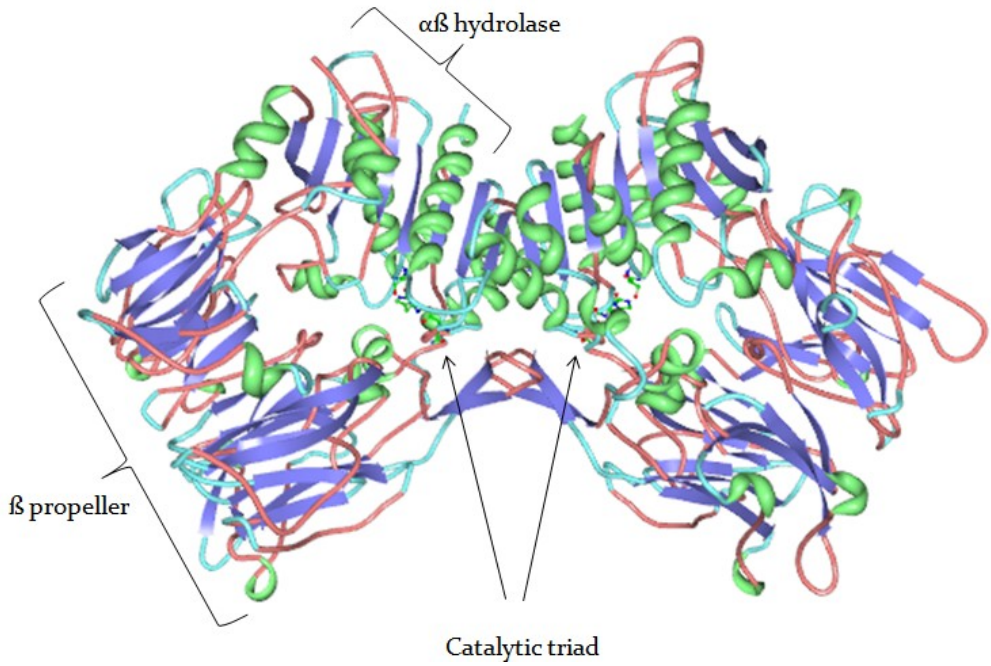
1.3.4. Fibroblast activation protein (FAP, seprase, EC 3.4.21.B28)

Fibroblast activation protein is a serine protease firstly recognized by monoclonal antibody F19 and called F19 Cell Surface Antigen (*Rettig et al., 1988*). It is a 120kDa/95kDa integral plasma membrane glycoprotein expressed on fibroblasts that was later named fibroblast activation protein. It is the same protein as a seprase (surface expressed protease), which was firstly described as a 170kDa gelatinolytic transmembrane glycoprotein on melanoma cell line LOX (*Aoyama, Chen, 1990*).

Human gene for FAP is localized on the long arm of chromosome two (2q23; *Mathew et al., 1995*). *FAP* orthologues have been identified in *Mus musculus*, *Xenopus laevis* and many other organisms. The gene size is 72,8kb and contains 26 exons (*Niedermeyer er al., 1998*). *FAP* expression is partly driven by bHLH transcription factor TWIST1 and by zinc finger transcription factor EGR1; the binding sites for HOXA4 and E2F1 were also described in the FAP promoter (*Mikheeva et al., 2010; Zhang et al., 2010*).

The transmembrane 170 kDa homodimer is composed from N-glycosylated 97kDa monomers. Each monomer contains 760 amino acids: long C-terminal extracellular domain that includes the active site contains 734 amino acids, hydrophobic transmembrane domain contains 20 amino acids and short cytoplasmic tail only 6 amino acids (*Goldstein et al., 1997*). Soluble extracellular form is probably shed from the transmembrane domain *(Collins et al., 2004; Lee et al., 2006).* Moreover, alternatively spliced variant of FAP generating short 27kDa isoform localized intracellularly and possessing C-terminal catalytic region was also observed *(Goldstein et al., 2000)*.

The tertiary structure of the FAP monomer solved with X-ray crystallography contains β propeller (residues 54-492) and α/β-hydrolase (residues 27-53 and 493-760) domains (Scheme 3). The active site consists of conserved serine catalytic triad Ser^{624}, Asp^{702}, His^{734} and is located on the interface of the two domains accessible through the cavity between them. β propeller is a domain composed of 8 blades, each containing three or four-stranded antiparallel β sheet and forms central hole that is narrower access to the active site. β propeller domain presents a gate to the active site and determinates smaller size substrates. Four N-glycosylation sites are in β propeller domain on asparagine residues and one N-glycosylation site is in the hydrolyse domain *(Aertgerts et al., 2005)*. Glycosylations are required for the enzymatic activity *(Sun et al., 2002)*.



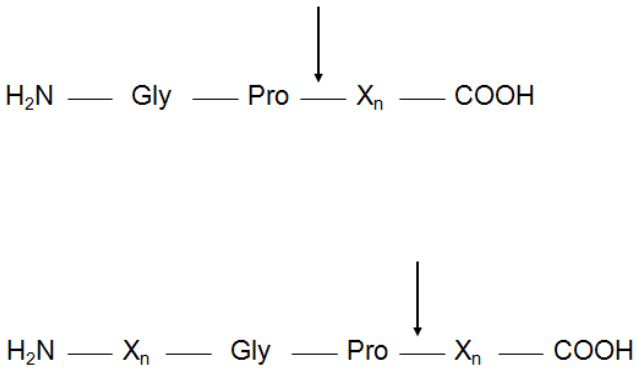
Scheme 3: The structure of FAP dimer: The active site residues Ser^{624}, Asp^{702} and His^{734} are represented as ball and sticks (Prepared in Jmol according to Aertgerts et al., 2005)

1.3.4.1. Enzymatic features of FAP

FAP is a prolyl peptidase with exopeptidase activity on short peptides. So far described substrates are neuropeptide Y, B-type natriuretic peptide, substance P

and peptide YY; incretins GLP-1 (glucagon like peptide 1) and GIP (gastric inhibitory polypeptide) are cleaved with lower efficiency *(Keane et al., 2011).*

Furthermore, FAP has an EDTA resistant endopeptidase enzymatic activity on denaturated collagen I and III *(Christiansen et al., 2007). S*oluble form of FAP cleaves alfa2-antiplasmin and it is identical to antiplasmin cleaving enzyme (APCE; *Lee et al., 2006)*. Another substrate of FAP is an intracellular inhibitor of tyrosine kinases SPRY2 (*Huang et al., 2011a; Table*). Both enzymatic activities of FAP are mediated by Ser^{624} (*Park et al., 1999*) and they require homodimerization (*Aertgerts et al., 2005*). Biological relevance of FAP enzymatic activities is now being investigated. The effort to indentify FAP substrates is related to design of specific FAP inhibitor with assumed medical relevance (*Lee et al., 2009; Edosada et al., 2006; Gilmore et al., 2006*).



FAP substrates	
exopeptidase activity	
Neuropeptide Y	
Substance P	
Peptide YY	
B-type natriuretic peptide	
Glucagon like peptide 1	
Gastric inhibitory polypeptide	*(Keane et al., 2001)*
endopeptidase activity	
denaturated Collagen I	
denaturated Collagen III	*(Christiansen et al., 2007)*
alfa2-antiplasmin	*(Lee et al., 2006)*
SPRY2	*(Huang et al, 2011a)*

Table: List of the natural substrates of FAP

1.3.4.2. Molecules interacting with FAP

Beside the dipeptidyl peptidase IV, which is described to form complexes with FAP on invadopodia (*Ghersi et al., 2002*), other molecules are known to interact with FAP and modulate cell movement. Proximity of FAP and urokinase plasminogen activator receptor (uPAR) was shown by means of fluorescence resonance energy transfer (*Artym et al., 2002*). These FAP-uPAR complexes were localized on invadopodia of adhered cells and occurred in a parallel with expression of integrins, specifically β1 integrin *(Artym et al., 2002)*. The association of FAP with α3β1 integrin on invadopodia is stimulated by collagen I *(Mueller et al., 1999)*. Moreover, FAP

induced invasion and migration in hepatic stellate cells seems to be dependent on β1 integrin *(Wang et al., 2005).*

1.3.4.3. FAP expression during development, tissue remodelling and in tumors

Fibroblast activation protein is not expressed in most of adult tissues except healing wounds and tumors (*Rettig et al., 1993; Dolznig et al., 2005*).

FAP is actively expressed during processes of tissue remodelling such as cirrhotic liver (hepatic stellate cells; *Levy et al., 1999*) as well as during embryo development in remodelating tissues, dermomyotome region of somites, myotubes and perichondral mesenchyme from cartilage primordia *(Niedermeyer et al., 2001)*. In *Xenopus laevis* FAP homologue is absent in embryos, but it is expressed during tail resorption during metamorphosis (*Brown et al.,* 1996).

FAP can be expressed in cancer stroma as well as on cancer cells with different influence.

1.3.4.3.1. FAP expression in cancer stroma

FAP expression pattern changes with malignancy, for example in epithelial, mezenchymal and neuroectodermal tissues (*Dolznig et al., 2005*). Its expression has been shown in stromal fibroblasts of more than 90% of epithelial tumors, including colorectal, breast, ovarian, and lung carcinomas *(Garin-Chesa et al., 1990)*.

FAP expression at myofibroblasts, fibroblasts and partly endothelial cells was linked to the lymph node metastasis of colorectal cancer (*Iwasa et al., 2003*). Stromal cells positive for FAP were shown as an immune-suppressive component of a tumor mass in mouse model *(Kraman et al., 2010).* Furthermore, targeting of FAP in lung and colon cancers leads to inhibition of tumor growth in mice (*Santos et al., 2009*).

In breast cancer expression of FAP on stromal fibroblasts is speculated as a marker of better prognosis *(Ariga et al., 2001)*. Recent work of Lee et al. demonstrates possible involvement of FAP in functional and structural modulation of extracellular matrix. FAP positive fibroblasts produce extracellular matrices richer in fibronectin and collagen I compared to FAP negative/inhibited cells. Interestingly, cell movement within the matrices from the FAP positive fibroblasts was facilitated because of parallelized collagen and fibronectin fibres in matrix (*Lee et al., 2011*).

1.3.4.3.2. FAP expression on cancer cells

FAP expression is not restricted only to the tumor stroma but it is expressed also on transformed cells e.g. on melanoma LOX cell line, where presence of FAP correlates with higher invasiveness on gelatine *(Aoyama & Chen, 1990; Monsky et al., 1994).* FAP expression in ovarian tumor cells was associated with greater peritoneal metastasis in xenograft model (*Kennedy et al., 2009).* In contrast to the results on melanoma LOX cell line *(Aoyama & Chen, 1990)*, FAP was described as a tumor suppressor in other melanoma cell lines *(Ramirez-Montagut et al., 2004)*.

1.3.4.4. FAP as a target of anti cancer treatment

However differing, tumor specific role of FAP in cancer has not been fully understood yet, restriction of FAP in human adult to tumor microenvironment makes it a tempting target for anti-tumor therapy.

FAP can be used for both visualization and targeting of the tumors (*Scott et al., 2003*). Immunotherapy against FAP leads to induction of immunity response and inhibits tumor growth in mice (*Fassnacht, 2005; Lee, 2005; Wen, 2010*). FAP-targeted delivery of a lipid coated nanoparticle containing tumor necrosis factor, which supports apoptosis, is under investigation (*Messerschmidt et al., 2009*). Another possibility is based on the FAP exopeptidase activity - protoxin made from melittin (peptide toxic for prokaryotic and eukaryotic cells from venom of honeybee *Apis milefera)* is activated in FAP positive tumor mass and inhibits tumor growth in mice (*LeBeau et al., 2009*).

1.4. Aims of the thesis

Aims of this work are:

- To analyse the expression of FAP in primary cell cultures derived from high grade astrocytic tumors.
- To analyse the role of FAP in glioma growth, migration and invasion.

2. Material

2.1. Laboratory equipment

Biohazard Safety Cabinet, Clean Air Systems

Cassette for developing films, Amersham Bioscience

Centrifuges, Hettich Zentrifugen Universal 320R and Universal 16R

Confocal microscope IX81, Olympus

Coulter Counter Z2, Beckman Coulter with 100µm capilary

CO_2 termostat, Sanyo

Electrphoretic aparature, BioRad

Microscope IX70, Olympus

Multilabel Victor^3 Counter, Perkin Elmer

Nitrocellulose membrane, Schleider and Scuel, Microscience

Plastic films welder, ETA

Spectrofluorimeter Fluoro Max-4, Horiba Jobin Yvon

Spectrophotometer Helios, Thermo Scientific

Hamilton Syringe, Hamilton Company

Termostat Heraeus, Kendro

Transluminator TFX-20LM, Vilber Lourmat

Vortex-Genie2, Scientific Industries

Water bath, JULABO

2.2. Solutions

- Primary culture cultivating medium: fetal calf serum (FCS, 10%, Sigma) in Dulbecco's modified Eagle's medium (D-MEM, Sigma), 100U/ml Penicillin G and 100µg/ml Streptomycin (Sigma).
- Cell line cultivating medium 10% FCS (Sigma) in D-MEM (Sigma)
- Sample medium for tissue transport D-MEM (Sigma), 100U/ml Penicillin G and 100µg/ml Streptomycin (Sigma).
- Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; Sigma).
- Methylene Blue 5g/l in 50% v/v ethanol/water.
- Phosphate buffer (PBS) – 137 mM NaCl, 4 mM Na_2HPO_4, 2,68 mM KCl, 1,76 mM KH_2PO_4, pH 7,4
- 0,1 M phosphate buffer: 0,1 M Na_2HPO, 0,1 M NaH_2PO_4, pH 7,5

- PBS/EDTA: 0,02% EDTA in phosphate buffer saline pH 7,4
- Lysate buffer with inhibitors of proteases - Tris-HCL 10 mM pH 7,5; 1% Triton X-100; 0,1% SDS; NaCl 100 mM; EDTA 1 mM; EGTA 1 mM; 10% glycerol; added just prior to use: Pepstatin A 25 μM (Calbiochem); E64 50 μM (Sigma Aldrich); AEBSF 200 μM (Sigma Aldrich)
- H-Gly-Pro-AMC (Bachem AG), final concentration (f.c.) 100 μM
- Z-Gly-Pro-AMC (Bachem AG) f.c. 50 μM
- Sitagliptin Phosphate Monohydrate, BioVision
- 1% Triton X-100 (DPPIV-like hydrolytic activity measurment)
- 0,1% Triton X-100 (immunostaining)
- Wash buffer for ELISA – 0,05% Tween in PBS
- Stop Solution for ELISA - 2 N H_2SO_4
- Tris-buffer saline (TBS) 100mM Tris-HCl, NaCl (0,9% w/v), pH 7,5
- TBS with 0,03% Triton X-100
- 1% BSA in TBS
- Rat Tail Collagen type I 4,05mg/ml, f.c. 0,85mg/ml (BD Bioscience)
- Doxycycline - diluted in deionised sterile water to concentration 1mg/ml and filtrated (Clontech-Takara Bio, USA) f.c.10^{-6} g/ml
- 5% glutaraldehyde (25%glutaraldehyde 1:4 with PBS)
- TTBS: Tween 20 (0,05% v/v) in TBS
- Blocking TTBS: non fat dry milk (5% w/v) v 0,05% TTBS
- ECL reagents: 2,5 mM Luminol in 0,1 M TRIS, pH 8,8; 90 mM p-coumaric acid in DMSO; 5,4 mM H_2O_2 in 0,1 M TRIS, pH 8,8
- SDS solution for determination of protein concentration (2% Na_2CO_3, 0,4% NaOH, 0,16% Natrium tartaricum, 1% SDS);
- Folin & Ciocalteu's reagent (Sigma)
- Electrode buffer: 125 mM TRIS, 192 mM glycin, pH 8,3; 0,1%SDS
- Sample buffer: 63 mM TRIS, 10% glycerol, bromphenol blue (1% w/v), 2% SDS
- Marker Kaleidoscope (BioRad)
- Transfer buffer with 20% methanol: 48 mM TRIS, 39 mM glycin, methanol (20% v/v)
- Developer and fixer (Foma, CZ)

- Stop bath (films developing): 2% acetic acid solution
- Wash buffer for zymography: 0,025% Triton-X 100, 0,02% NaN_3
- Inhibiting buffer for zymography: Tris 50mM pH 7,5, EDTA 5mM, 0,02% NaN_3, 1,5%TritonX-100
- Activating buffer for zymography: Tris 50mM pH 7,5, 2mM $CaCl_2$ x 2 H2O, 0,02% NaN_3, 1,5% TritonX-100
- Coomassie brilliant blue (0,8% w/v) 0,88g CBB diluted in 110ml 5:5:1 methanol : deionised water : 99% acetic acid
- Destining solution for zymography: 5:5:1 100% methanol : deionised water : 99% acetic acid

2.3. Antibodies

- Immunohistochemistry
  Rat Monoclonal Anti-human FAP Antibody D8, Vitatex MABS1001
  1:300, 90 minutes, room temperature (RT)
  Alexa Fluor 488 Donkey Anti-Rat IgG (H+L), Invitrogen A-21208
  1:100, 1 hour, RT, dark
- Immunocytochemistry
  Rat Monoclonal Anti-human FAP Antibody D8, Vitatex MABS1001
  1:100, over night (ON), 4°C
  Alexa Fluor 488 Donkey Anti-Rat IgG (H+L), Invitrogen A-21208
  1:300, 1 hour, RT, dark
- Western blot
  F19; AntiHuman Mouse FAP isolated from hybridoma F19 (ATCC)
  f.c. 14 µg/ml ON, 4°C
  Sheep Anti-Mouse IgG - HRP, GE Healthcare Life Sciences NA931
  1:20 000, 1 hour, RT
  Rat Monoclonal Anti-human FAP Antibody D8, Vitatex MABS1001
  1:10 000, ON, 4°C
  Goat F(ab')2 polyclonal Aniti-Rat IgG - HRP, pre-adsorbed, Abcam ab6257
  1:20 000, 1 hour, RT

3. Methods

3.1. Derivation of primary cell cultures

Primary cell cultures were derived from tissue samples collected from patients undergoing high astrocytoma tumor resection at Neurosurgery, Hospital Na Homolce in Prague. The samples were immediately put into a sterile Falcon tube with sterile sample medium and transported on ice to our laboratory.

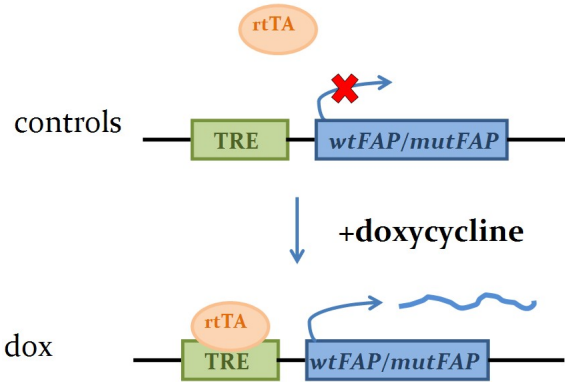
Vascularisation and necrosis were removed from the fresh tissue samples. Sample was dissected to small pieces with sterile scalpel and cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% FCS, 100U/ml Penicillin G and 100µg/ml Streptomycin. When cells grew out, the pieces were removed. The cultures were harvested and expanded after reaching confluence. In most cases the analysis were done after fifth passage.

3.2. Permanent cell cultures

Cell cultures from high astrocytic tumors were used: U87MG (glioblastoma multiforme, Grade IV), U138MG (glioblastoma multiforme, Grade IV), U373MG (Astrocytoma, Grade III), Hs683 (malignant glioma) all obtained from American Type Culture Collection (ATCC, USA). Permanent cell lines were cultured under standard conditions: 37°C, 5% CO_2 and 95% air, in D-MEM supplemented with 10% FCS.

3.3. Transfected cell culture

Cell line U373MG (ATCC, USA) was transfected with human FAP gene (*wtFAP*) or with mutated form of FAP gene (*mutFAP*) using pTet-On system (Clontech; see scheme 1) in our laboratory during previous projects (performed by Dalibor Košek, MSc and Jana Trylčová, MSc). The expression of transgenic FAP in this system was induced by tetracycline derivative doxycycline in final concentration 10^{-6} g/ml. *mutFAP* encodes mutated enzymatically inactive form, which has substituted catalytic Ser^{624} for Ala (*Park et al., 1999*). Cells transfected with a respective vector not treated with inducing agent (doxycycline) were used as a control.



Scheme 1: Inducible expression of transgenic FAP. TetOn Advanced Inducible Expression System (Clontech) was used for transfection of cell line U373. In this system cells were transfected with regulatory vector carrying rtTA gene and construct coding wtFAP or mutated form of FAP. rtTA is expressed constitutively and while heterodimerize with doxycycline bind the promoter of Tre-Tight construct and expression of inserted gene starts (Clontech).

3.4. Growth rate analysis

100µl of cell suspension (40 000 cells/ml in D-MEM with 10% FCS, 100U/ml Penicillin G and 100µg/ml Streptomycin) per well was seeded into 96 well plate. Cells were harvested by Trypsin/EDTA every 1 – 4 days and counted (Coulter Counter Z2). The population doubling time was determined from the regression fit of the exponential part of the growth curve.

For analysis of growth rate of the transfected cells, 100µl of cell suspension (40 000 cells/ml in DMEM with 10% FCS) per well was seeded into 96 well plate. Cells were fixed and stained by methylene blue in EtOH for 20 minutes regularly each 1-2 days. Than it was washed twice by destilled water, dried and dissolved in 100µl of 1% SDS, optical density was measured on Microplate reader Sunrise, Tecan, at a wavelenght of 630nm.

3.5. Cell suspension concentration

Cell suspension was diluted 1:200 in Coulter Izoton II Diluent and counted by Coulter Counter Z2, Beckman Coulter. Objects between 10 – 27 µm were counted.

3.6. Preparation of cell lysates

Cells were harvested from Petri dishes by PBS/EDTA, after 15 min in 37°C were centrifuged in 15ml Falcon tube 4min, 5°C, 1800 rpm. Pellet was resuspended in lysate buffer with inhibitors of proteases to concentration 10^7 cells/ml and incubated shaking, 4°C. After 20min it was centrifuged 30min, 4°C, 12 000 rpm, supernatant was aliquoted and stored at -20°C. Cell lysates were prepared on ice.

3.7. Enzyme-linked immunosorbent assay (ELISA)

DuoSet ELISA development human FAP kit or DuoSet ELISA DDPIV/CD26 kit (R&D Systems, UK) was used for quantitative analysis of human fibroblast activation protein (FAP) or dipeptidyl peptidase IV (DPPIV) respectively according to the manufacturer's instructions. Standards or cell lysates were diluted in reagent diluent to final volume 100µl and applied into a 96 well plate with capture antibody. After 2 hours incubation at room temperature the wells were washed and incubated two more hours with biotinylated detection antibody. Then it was washed and incubated for 20 minutes in dark with 100µl of diluted Streptavidin-HRP (1:200 with reagent diluent), washed again and incubated for 20 minutes in dark with 100µl of diluted substrate (H2O2 and tetramethylbenzidine 1:1), the reaction was stopped by 50µl 2N H2SO4. Optical density was measured at wavelength of 450nm at Microplate Reader Sunrise; correction reading of 540nm was subtracted.

3.8. Immunocytochemistry and immunohistochemistry

The detection of FAP was performed on cells grown on coversplis or in tissue sections, after fixation by 4% paraformaldehyde and permeabilization by 0,1% Triton X-100 (both followed by rinsing twice in PBS) were incubated with 10% FCS with 1% BSA in TBS for 60 min. Primary Rat Monoclonal Anti-human FAP Antibody D8 was applied and incubated with the samples at 4°C, overnight. The sample was rinsed twice in TBS with 0,03% Triton X-100 before incubation with secondary antibody Alexa Fluor 488 Donkey Anti-Rat. The antibodies were diluted in solution of 1% BSA in TBS. Negative control was prepared the same way but incubated in primary antibody-free solution of 1% BSA in TBS. For nuclei staining Hoechst was added at concentration 1µM at the same time as secondary antibody.

The samples were rinsed three times in TBS, twice in PBS and finally in distilled water and let dried. The preparates were mounted in antifading Gel and viewed on fluorescence microscopy (Olympus IX70 equipped with the DP30BW camera) or on the confocal microscope (Olympus IX81, UPlan Sapo 60x, laser Ar488, Olympus, Czech Republic).

3.9. Spheroid formation and cell invasion into collagenous matrices

Spheroids were formated according to the modified hanging drop method (based on *Kelm et al., 2003*). Confluent grown cells were harvested with Trypsin-EDTA. The suspension was centrifuged 4 min, 4°C, 900 rpm and pellet resuspended to the final concentration $2*10^5$ cells/ml in D-MEM supplemented with 10% FCS, with or without the induction agent (doxycycline f.c. 10^{-6} g/ml). Spheroids were made by hanging a 20µl drops from the suspension on a Petri dish lid (4000 cells per drop).

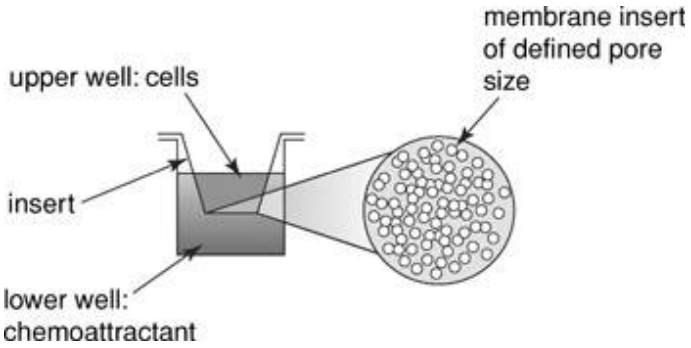
After three days at 37°C, 5% CO_2 and 95% humidity, spheroids from the drops were implanted into Rat Tail Collagen type I. DMEM supplemented with 10% FCS, 1N NaOH (2,3% of collagen volume) and Rat Tail Collagen type I 4,05mg/ml were mixed to the final concentration 7,9% FCS and 0,85mg/ml of collagen, with or without doxycycline. All preparations were done on ice to prevent gelatination. 400µl of collagen solution was applied into 24 well plate and let gelatinise at 37°C, 5% CO_2 and 95% humidity for 30 minutes. Spheroids were put onto the firm collagen and another 200µl of collagen solution was added.

Each 1-2 days spheroids were documentated (Olympus IX70 40x magnification, camera Olympus). Pictures were analysed in UTHSCSA Image Tool, where the invaded area was defined manually. Cell invasion was assessed as an area of the invading cells over time.

3.10. Transwell migration assay

72 hours prior to the experiment, cells cultured to confluence were induced with doxycycline. Transfected cells not treated with the induction agent were used as controls. Cells harvested by Trypsin/EDTA were centrifuged 4min, 4°C, 900 rpm., and diluted in D-MEM with 10% FCS to concentration $2*10^5$ ml. Inserts (BD Falcon, USA) with 8µm pores in polyethylene terephthalate (PET) membranes were placed to the 24 well plate containing 750 µl of DMEM (supplemented with 10% FCS or not). 300µl of the cell suspension ($6*10^4$ cells) was pippeted into the inserts. After the incubation time (12 hours in D-MEM with 10% FCS or 24 hours in serum free media) under standard cultivation conditions (37°C, humidified atmosphere of 5% CO_2 and 95% air) the cells were removed with cotton swab from the upper part of the inserts (*Scheme 2*). Migrated cells on the lower part of the membrane were fixed with 5% glutaraldehyde 15 min and stained with methylene blue over night, rinsed twice in

distilled water. The cells were counted manually in five vision fields per insert on microscope Olympus IX70 using the 200x magnification.



Scheme 2: The Transwell migration assay: Inserts with membrane having 8μm pores on the bottom are placed into the well containing culture medium. Cells are seeded into the upper well and allowed to migrate through the pores to the other side of the membrane. After defined incubation time, rest cells from upper well are removed; migrated cells are fixed, stained and counted. (figure from currentprotocols.com)

3.11. Polyacrylamide-gel electrophoresis (PAGE)

Discontinual electrophoresis was run on polyacrylamide gel (thickness 0,75 mm or 1,5 mm) as Laemmli *(Laemmli, 1970)*. 4% stacking gel and 7,5% running gel, containing 0,1% SDS were used. Samples were cell lysates diluted in sample buffer, without denaturation. Electrophoresis run at 60V till samples reached the end of stacking gel (45 minutes), than voltage was increased to 140V till the samples reached the end of running gel (90 minutes).

3.12. Electrotransfer and immunodetection of FAP

After the electrophoresis the gel was incubated for 30 min in transfer buffer with 20% methanol. Prior to use PVDF membrane was moisten in 100% methanol to become transparent and it was equilibrated in transfer buffer for 30 min. From the gel to the PVDF membrane the proteins were transferred in “sandwich” ordering (*Kyhse-Andersen, 1984*) in constant voltage 10V for 30min (0,75 mm gels) and 60min (1,5 mm gels).

The PVDF membranes were washed in deionised water (3x15min) and equilibrated for 20min in 0,05% TTBS buffer and blocked for 1 hour in 5% non-fat dry milk in 0,05% TTBS. Primary antibody F19/D8 was diluted in blocking solution and applied

in volume 0,1ml per 1 cm^2 of membrane and incubated over night, 4°C. The PVDF membrane was washed in 0,05% TTBS (4x15min) and incubated with secondary antibody for 60min, RT.
Immunodetection was realised by chemiluniscent method (ECL; *Whitehead et al., 1979*). The membranes laid foil were incubated with ECL solutions applied in volume 0,125ml per 1 cm^2 of membrane, packed into the foil and closed into a developing casette. The film was exposed for 5 min (transfected cells) or 15min (primary cell cultures), developed and fixed.
Intensity of the positive zones was measured on scanned film in programme Kodak1D and background intensity was subtracted.

3.13. Gelatin zymography

The same 4% stacking gel was used as in PAGE, 7,5% running gel contained 6mg of gelatine/ml. Sample preparation and electrophoresis was in the same arrangement as for PAGE, two gels in same ordering were needed. The gels were washed in wash buffer, one gel was incubated in inhibiting and second gel in activating buffer for 72 hours, 37°C Both were stained with 0,8% CBB. For detection of enzymatic activity the gels were destined and scanned.

3.14. H-Gly-Pro-AMC/Z-Gly-Pro-AMC substrate membrane overlay assay

After electrophoretic separation, gels were equilibrated in phosphate buffer, pH 7,5. Substrate H-Gly-Pro-AMC in f.c. 100 µM respective Z-Gly-Pro-AMC in f.c. 50µM in 2 ml of phosphate buffer was applied on the gel. Nitrocellulose membrane was laid on the gel and incubated in 37°C for 30 – 60 min. Hydrolytic activity was detected on the membrane on transluminator Vilber Lourmat at wavelength of 365 nm, as a fluorescence of released AMC.

3.15. Measurement of H-Gly-Pro-AMC cleavage and DPPIV inhibition

Enzymatic activity on substrate H-Gly-Pro-AMC was assayed at room temperature using a continuous fluorimetric assay. Each well of 96 well plate, Perkin Elmer contained 5µl of sample in 193µl of filtered PBS. The reaction was started by addition of 2 µl of substrate H-Gly-Pro-AMC in f.c. 100 µM. AMC release was measured on Multilabel Victor^3 Counter, Perkin Elmer at excitation wavelength of 380nm and

emission wavelength of 460nm. The activity was measured again after adding specific DPPIV inhibitor Sitagliptin in f.c. 0,3μM.

3.16. Total protein concentration

The total protein concentration was assayed according to Lowry (*Lowry et al., 1951*). Samples of 5μl of cell lysates were filled to volume 400 μl and 1,2 ml of SDS solution was added (100:1 with 4% $CuSO_4$). After 10 min incubation 120μl of Folin & Ciocalteu's reagent (diluted 1:1 with deionised water) was added, the samples were mixed and incubated for 45 min in dark, room temperature. Absorption was measured at wavelength of 660 nm at spectrophotometer Helios.

3.17. Statistical analysis

The Statistica 10.0 software (StatSoft, USA) was used for statistical analyses. Differences between the groups were evaluated with the Mann–Whitney U test; correlations were assessed by Spearman's correlation coefficient. The Tukey post-hoc test was used for multivariate analysis of variance (MANOVA).

4. Results

4.1. Derivation of primary cell cultures from human brain tissue samples

Nineteen primary cell cultures were derived from Grade IV (glioblastoma multiforme) tumor tissue samples. Cell explants outgrowing from the tissue were visible after 3-5 days then the tissue was removed and cells were grown as adherent cultures. Cells were expanded and analysed for FAP expression and growth rate at early passage. The cultures displayed diverse morphology (Fig.1) – some cells were long and spindle shaped, some were slim with small cellular bodies, others were large flat polygonal cells and in some cultures they were highly confluent or grew in clusters.

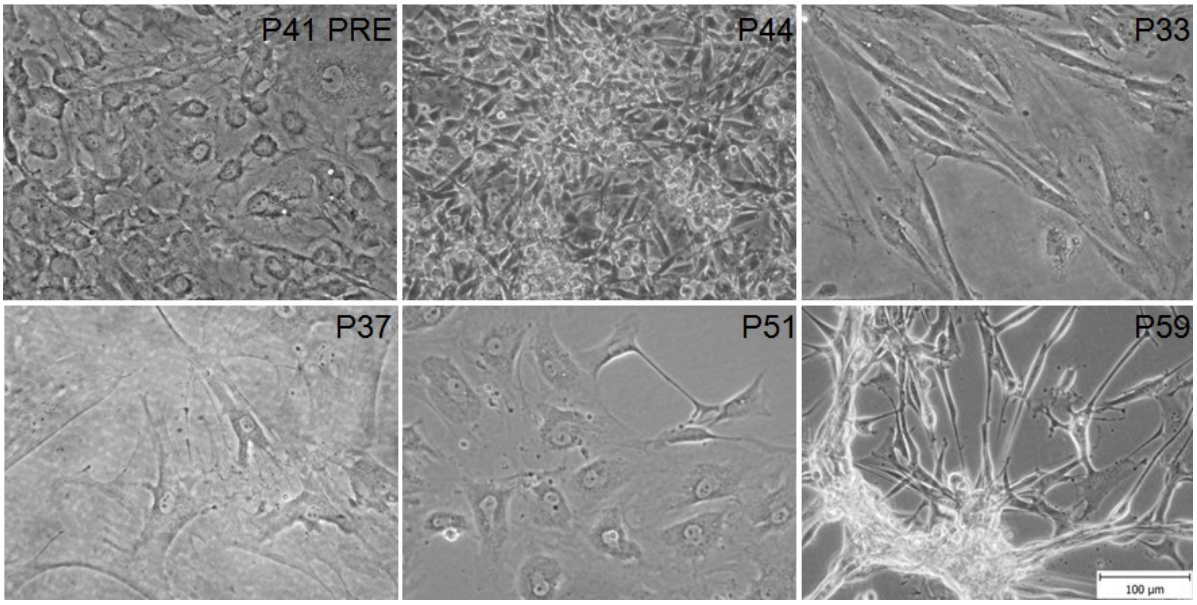


Figure 1: Primary cell cultures in early passage present heterogeneous morphology

4.2. Variable expression of FAP in early passage and long term propagated primary cell cultures and glioma cell lines

The expression of FAP was quantified in twenty-two primary cell cultures, three of them were derived previously and long-term cultured. Additionally five permanent glioma cell lines were analysed using ELISA (Fig. 2). Sample of blood mononuclear cells (BMNC), where FAP is not expected (*Sromova et al., 2010; Maes et al., 2007*) was used as a negative control of the method. In contrast, cancer associated fibroblasts (CAF) are known to express FAP (*Lebret et al., 2007)*. Also in dermal fibroblast (DF) can be FAP expressed, while culturing *in vitro* *(Rettig et al.,1993;* Fig. 2).

Expression of FAP is diverse among the high grade glioma permanent cell lines (U138, Hs683, U87, T98G, U373MG): very low in U373MG, T98, low in U138 and U87, high in Hs683 cell line (Fig. 2).
Primary glioma cell cultures, similarly to the permanent glioma cell lines, showed very heterogeneous expression of FAP - from no detectable (e.g. P44) to very high amounts of the protein (e.g. P37, P55; Fig. 2).

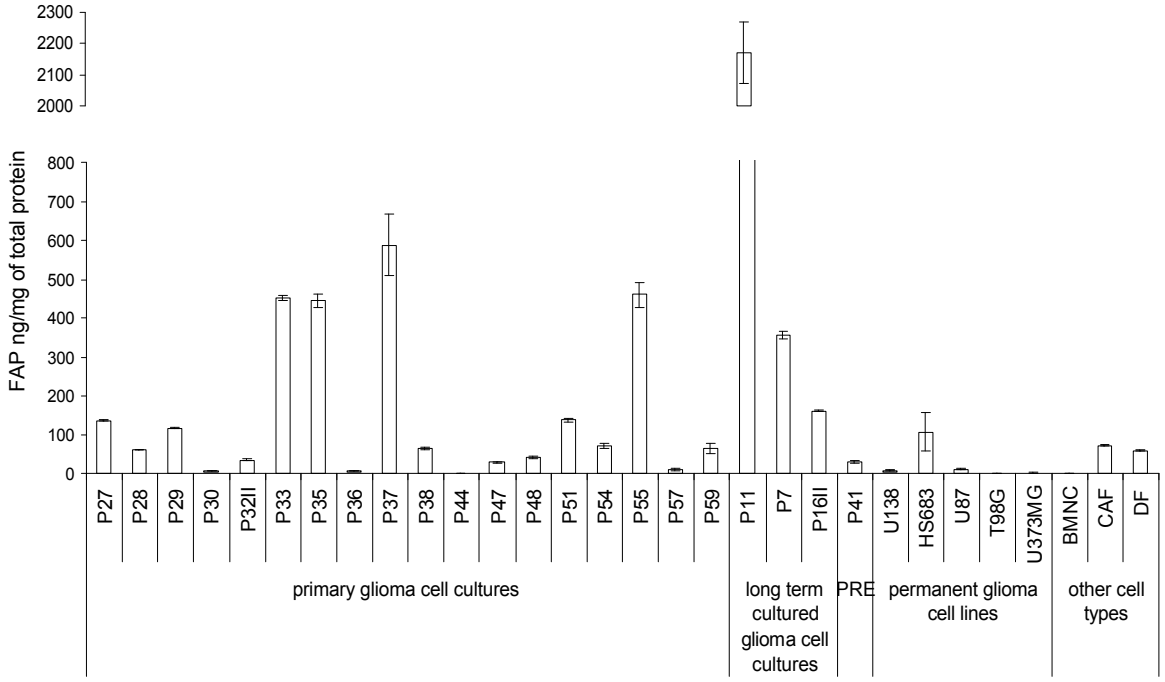


Figure 2: The expression of fibroblast activation protein according to ELISA: primary glioma cell cultures derived from glioma tissues short or long term cultured, primary cell culture derived from pharmacoresistant epilepsy tissue (PRE), permanent glioma cell lines, blood mononuclear cells (BMNC), cancer associated fibroblasts (CAF) and dermal fibroblasts (DF).

To confirm the data from ELISA we performed also western blot analysis (Fig. 3). Native samples were loaded at 5µg of total protein per well and detected with the F19 FAP antibody. One major band with an apparent molecular weight of 144kDa was detected in the majority of primary cell cultures expressing FAP according to ELISA (correlation between WB densitometric data and the protein quantification according to ELISA Spearman's r=0.567, p=0.003).

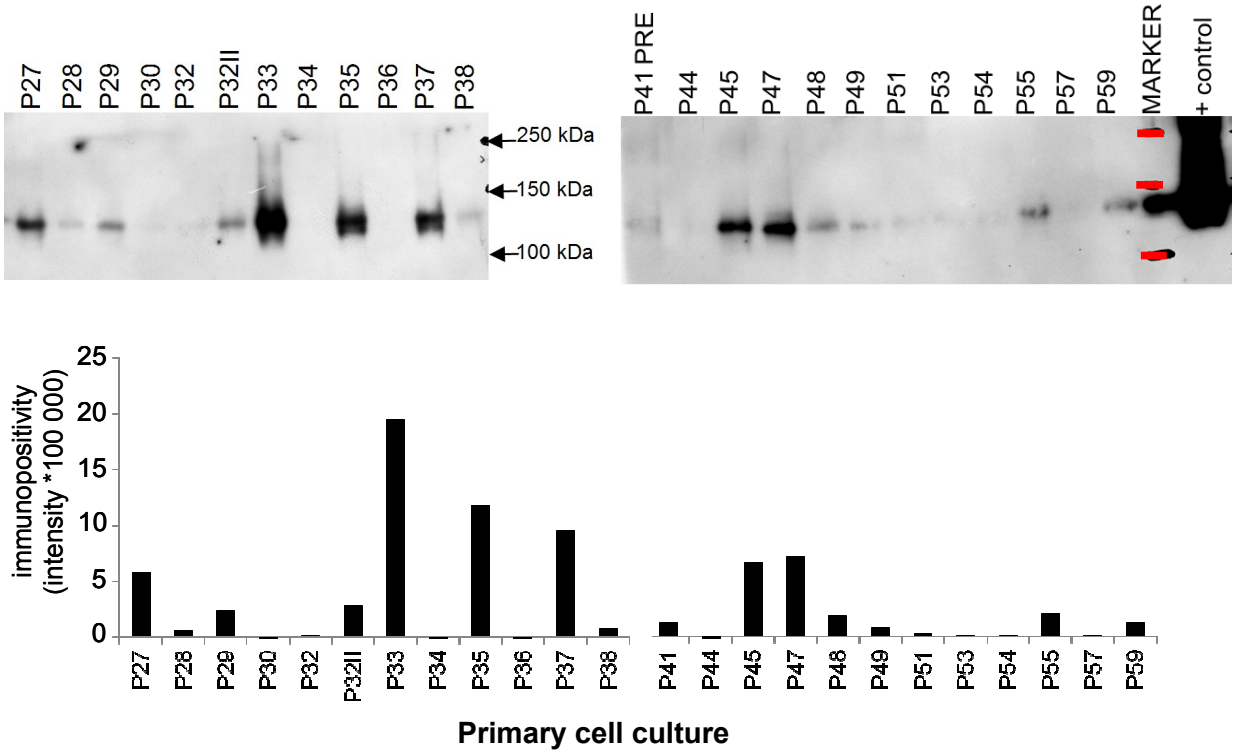


Figure 3: Immunodetection of FAP in primary cell cultures. Cultures P34, P45 and P49 were derived from oligodenrogliomas and were not used in further analysis. Cultures P32 and P32II were derived from the same tissue sample, in other analysis only culture P32II was used. P41 is a primary cell culture derived from pharmacoresistant epilepsy (PRE) tissue. The rest of the samples were cultures originated from glioblastoma multiforme. 5µg of total protein per well were loaded, non-reducing conditions, detected by AntiHuman FAP antibody F19. As a positive control was used lysate from cells expressing transgenic FAP. Densitometric quantification of immunodetected bands (B).

The expression of FAP mRNA was analyzed in the primary cell cultures (performed by Z.Vaníčková, MD and E.Křepela, MD) and the data corresponded well with the protein amounts according to ELISA (Fig.4)

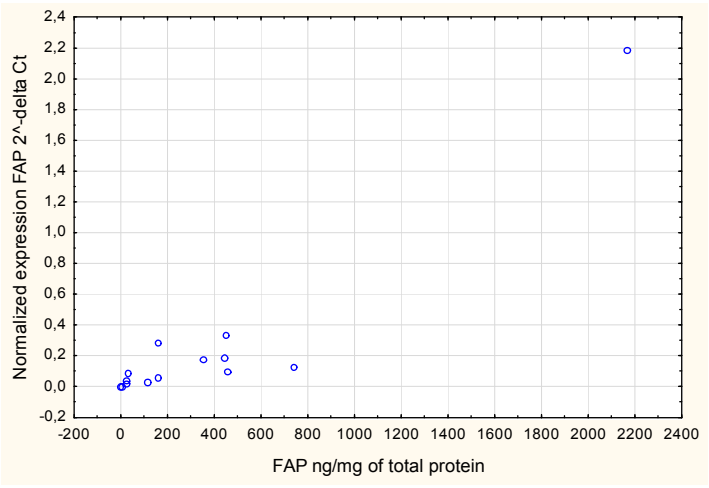


Figure 4: Correlation between relative FAP mRNA level and the FAP protein measured by ELISA, Spearman's r= 0.859, p<0.001.

One primary cell culture was successfully derived from pharmacoresistant epilepsy (PRE) brain tissue sample. FAP was also detected in these cells (Fig. 2), which indicates the presence of FAP expression also in non-malignant cells cultured *in vitro*.

4.2.1. FAP is localized in plasma membrane and in perinuclear area in P11 cells

The long term cultured P11 expressed high amounts of FAP and was analysed further. FAP positive P11 cells were used to visualize FAP cellular localization by immunocytochemistry with AntiHuman FAP antibody D8. The cells showed positivity not only in their cell membrane but in the perinuclear area as well and some cells were positive overall in the cytoplasm (Fig. 5A).

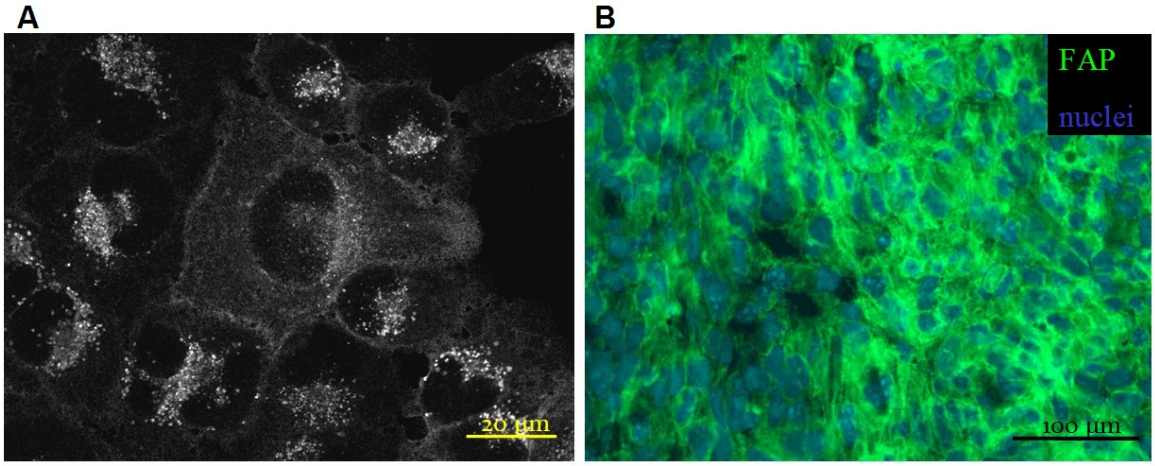


Figure 5: Immunopositivity for FAP in P11 cells cultured in vitro *(A) and in xenotransplanted tumor mass (B).*

4.2.2. Primary glioma cell culture highly positive for FAP retain FAP expression while transplanted into the mouse brain

The P11 primary glioma culture cells were orthotopically xenotrasplanted (performed by Marek Hilšer, MD) into the immunodefficient (NOD.129S7(B6)-Rag1tm1Mom/J) mice and gave rise to tumors. High immunopositivity of FAP was observed in the tumor mass (Fig. 5B).

4.2.3. Analysis of the DPP-IV-like and FAP endopeptidase activities

FAP is known to have exo- and endopeptidase activity on peptide bonds after proline. H-Gly-Pro-AMC was used as a substrate for the exopeptidase activity and Z-Gly-Pro-AMC was used as a specific substrate for the endopeptidase activity. Using the P11

culture expressing high amounts of endogenous FAP both enzymatic activities were detectable (Fig. 6).

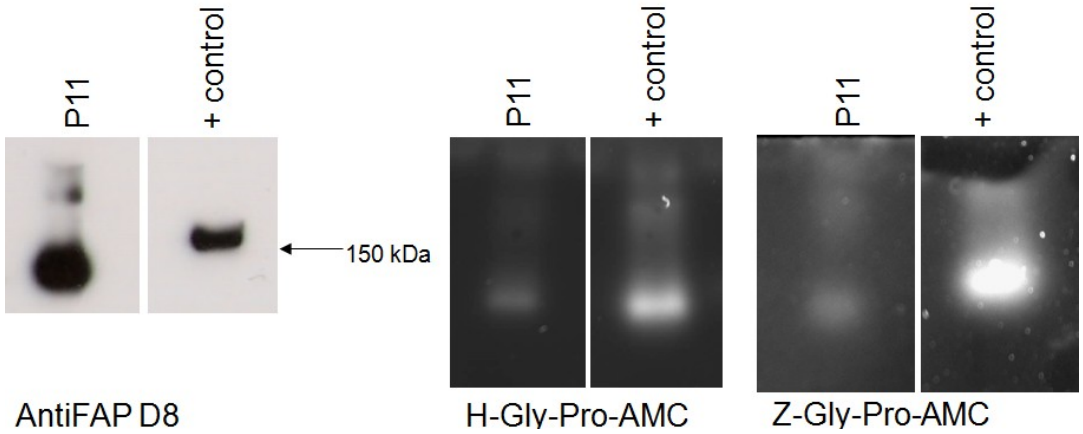


Figure 6: Detection of FAP in culture P11 samples on western blot and substrate membrane overlay assay. The substrates H-Gly-Pro-AMC (exopeptidase enzymatic activity) and Z-Gly-Pro-AMC (endopeptidase enzymatic activity) were used. Lysate from the cells expressing transgenic FAP was used as a positive control. The samples were run in non-consecutive lanes but on the same gel.

The substrate H-Gly-Pro-AMC was used for the analysis of the DPPIV-like hydrolytic activity in the rest of the primary glioma cell cultures. The activity was detected on the gels after the electrophoresis (Fig. 7) and for the quantification purposes it was measured biochemically using the same substrate (Fig. 8).

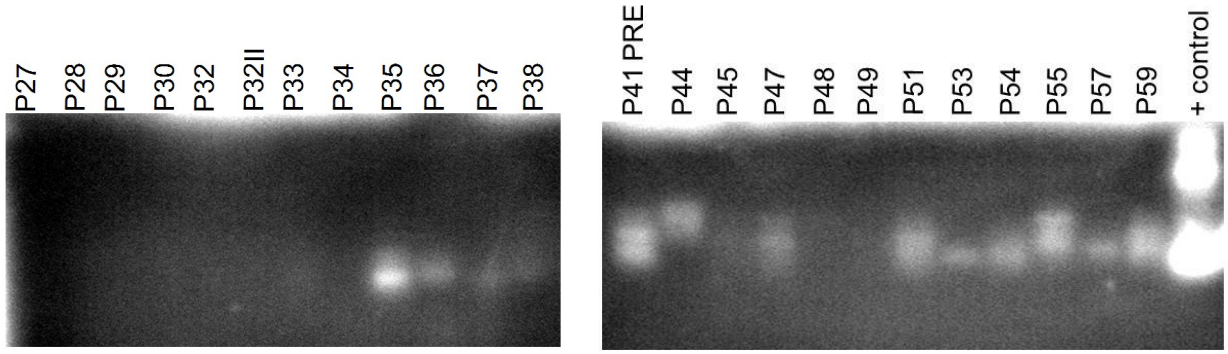


Figure 7: H-Gly-Pro-AMC substrate membrane overlay assay in the primary cell cultures. P41 is a primary cell culture derived from pharmacoresistant epilepsy (PRE) tissue. Lysate from the cells expressing transgenic FAP was used as a positive control.

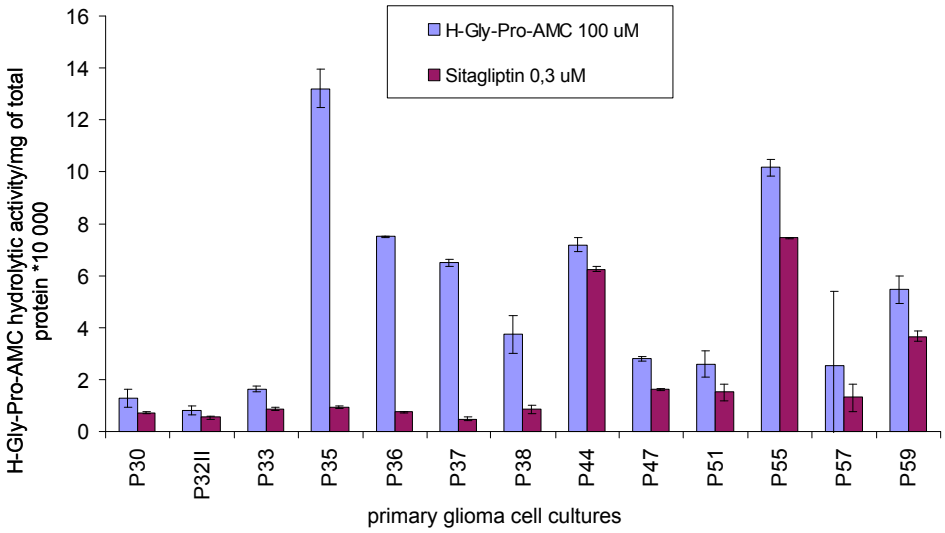


Figure 8: Hydrolytic activity on substrate H-Gly-Pro-AMC in primary glioma cell cultures and inhibition of DPPIV activity by its specific inhibitor sitagliptin measured using a continuous fluorimetric assay.

The absence of correlation (Spearman´s r= -0.0549; p= 0.858) of the results of biochemical assay with the expression of FAP determined by ELISA is most probably caused by the coincidence of FAP with other molecules processing the substrate. The closest homologue of FAP bearing the same dipeptidyl peptidase enzymatic activity is the canonical DPPIV. Its expression correlates (Spearman´s r= 0.728, p=0.008) with the cleavage of H-Gly-Pro-AMC (Fig. 9A). Thus, we used DPPIV specific inhibitor for further analysis of the given enzymatic activity. By means of that DPPIV activity was inhibited in several samples (Fig. 8). The value of enzymatic activity after subtraction of residual activity correlated (Spearman´s r= 0.818, p=0.001) to the DPPIV expression (Fig. 9B).

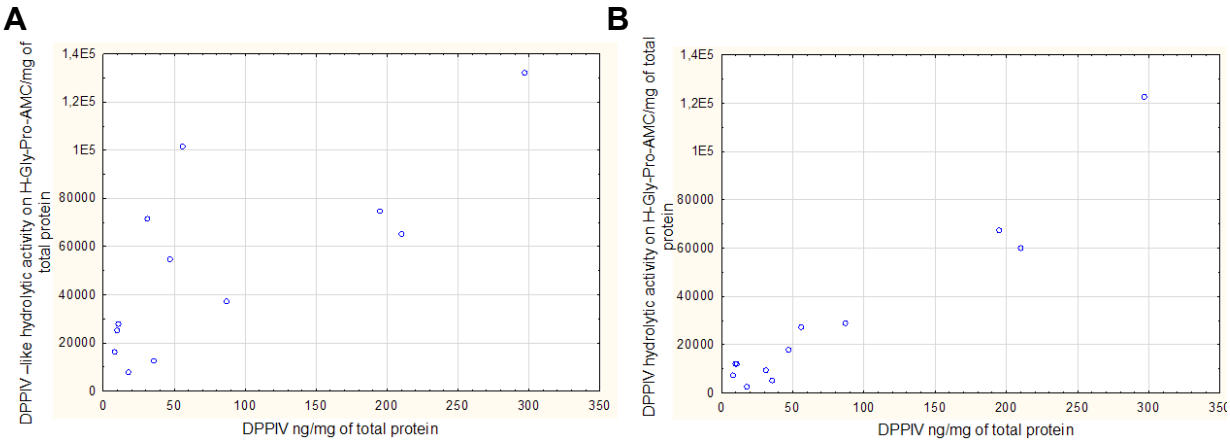


Figure 9: DPPIV-like hydrolytic activity measured with substrate H-Gly-Pro-AMC, Spearman´s r= 0.728, p=0.008 (A) and DPPIV H-Gly-Pro-AMC hydrolytic activity Spearman´s r= 0.818, p=0.001 (B) correlated with the DPPIV protein amounts according to ELISA.

Endopeptidase activity on substrate Z-Gly-Pro-AMC is detectable only on samples with high positivity of FAP, so we tried to analyse FAP endopeptidase activity in set of primary glioma cell cultures by zymography. Probably due to the low sensitivity of the method the activity was not detected.

4.3. Growth rate analysis of primary glioma cell cultures

We examined the growth rate of primary cell cultures derived from glioblastoma at early passage. The growth rates were variable in the individual primary cell cultures with the population doubling time ranging from 20 to 420 hours. Although a positive correlation (Spearman´s r=0.627, p=0.038) between the doubling times and the FAP expression determined by ELISA was observed (Fig. 10), the correlation seemed to be based only on few slowly growing FAP highly positive cultures.

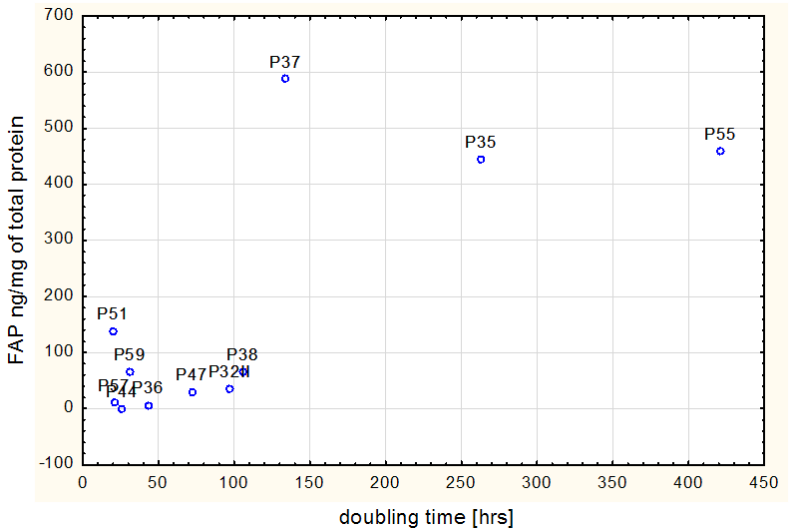


Figure 10: Correlation between the doubling times and the expression of FAP (Spearman´s r=0.627, p=0.038) in primary glioma cell cultures analysed in fifth passage.

4.4. Transgenic FAP expressed in U373 cell line is localized on plasma membrane and in perinuclear area

In order to assess the role of FAP in migration and invasion of glioma cells, we used transfected cells inducibly expressing the gene for human enzymatically active

(wtFAP) and enzymatically inactive (mutFAP) FAP (Fig. 11A, B). Based on the comparable expression of transgenic FAP, two clones wtFAP 5AA and mutFAP 33 (Fig. 11B) were used for further experiments.

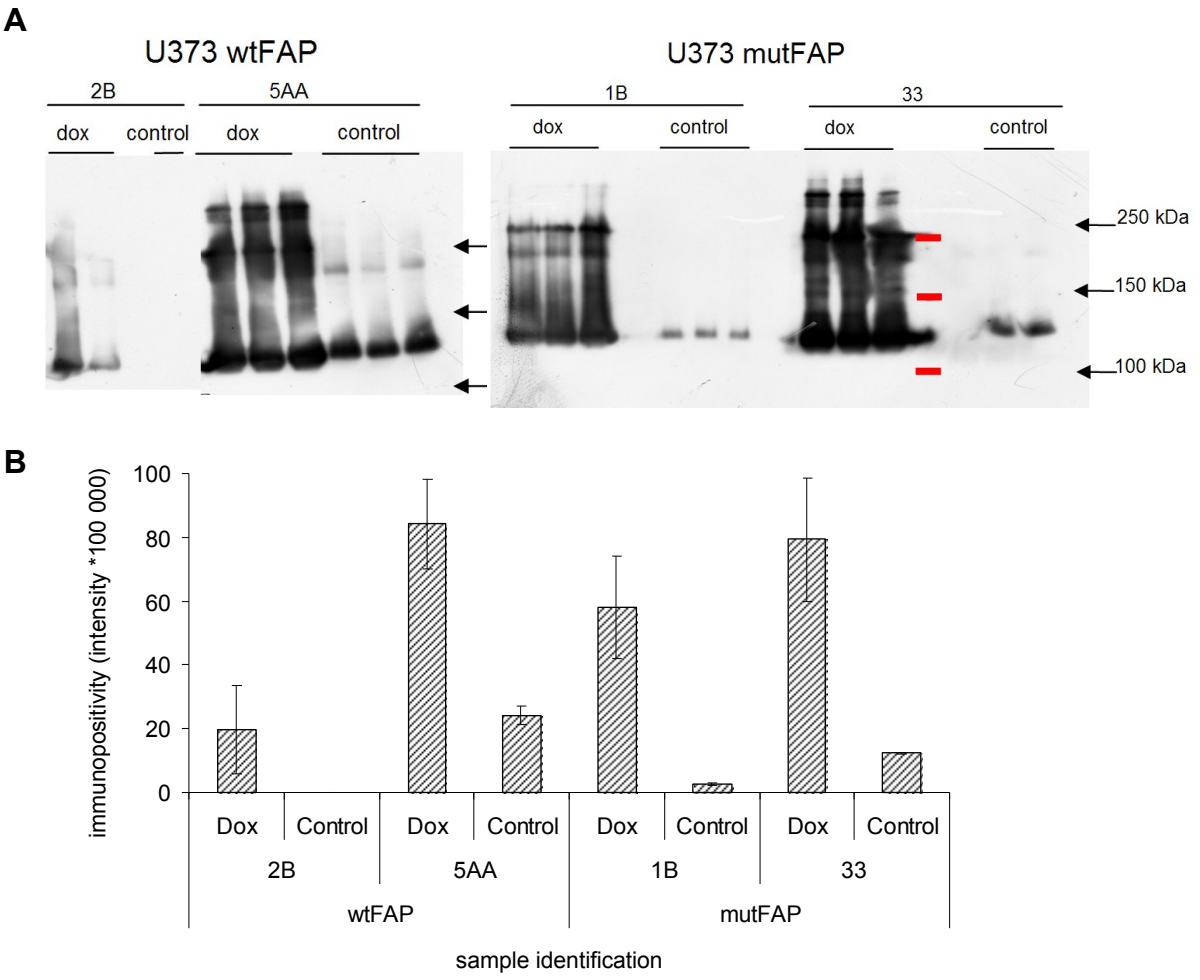


Figure 11: The expression of enzymatically active wtFAP and enzymatically inactive mutFAP in U373 cell line: Immunodetection of FAP in individual cell clones (A). Observed molecular weight corresponded well to expected values. Immunodetected positivity densitometricaly quantified (B). Duplicates or triplicates were from different biological samples.

Cellular localization of FAP was determined by immunocytochemistry and confocal microscopy (Fig. 12). The presence of transgenic FAP was observed in wt as well as mutFAP in the plasma membrane as well as intracellularly. FAP was also detected in control cells, probably as a result of leakage activity, which is expectable in this expression system (Fig. 12).

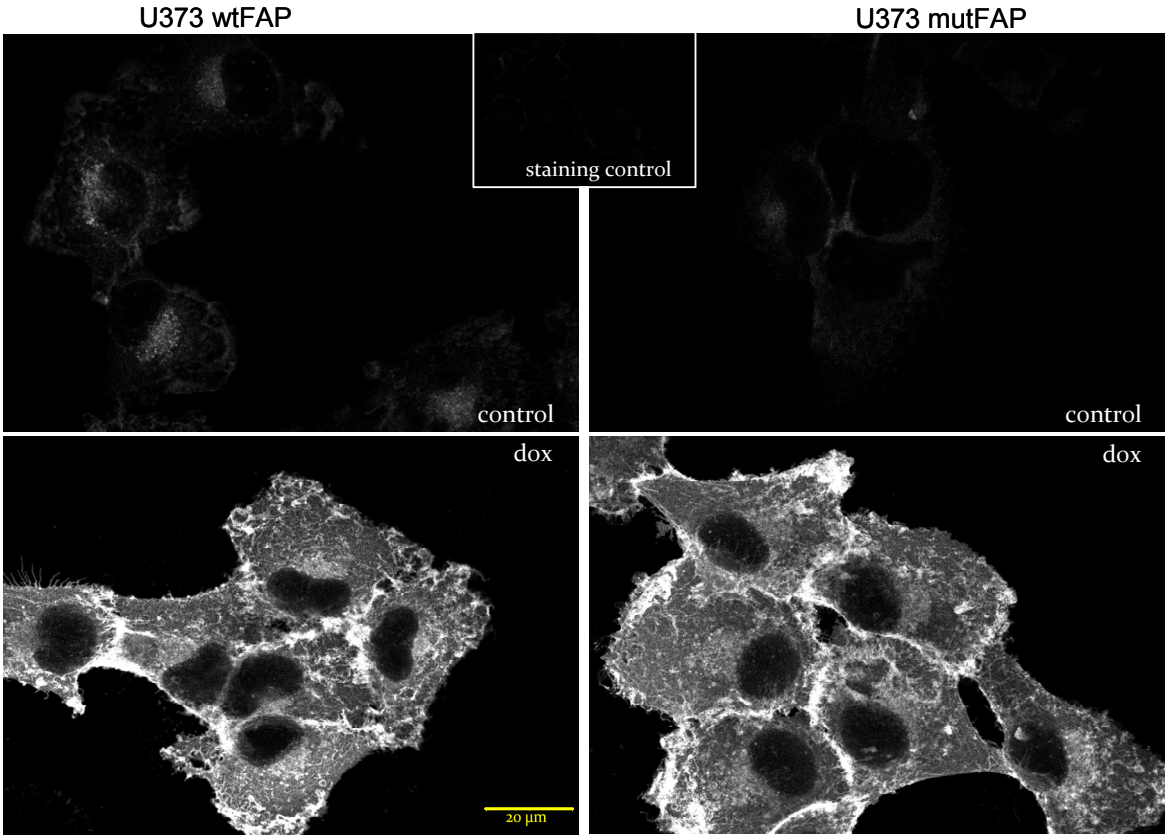


Figure 12: Confocal microscopy detection of inducible transgenic expression of enzymatically active wtFAP and enzymatically inactive mutFAP in the transfected U373 cells. FAP expression was induced by doxycycline (dox), wtFAP/mutFAP transfected cells without the induction agent were used as controls.

4.5. Enzymatic activities of the inducibly expressed transgenic FAP

Active site Ser^{624} is responsible for both the exopeptidase and the endopeptidase enzymatic activities (*Park et al., 1999*). As expected, both enzymatic activities were observed in the cells expressing transgenic wtFAP but not in the mutFAP cells (Fig. 13A, B) expressing the enzyme with Ser^{624} to Ala^{624} substitution.

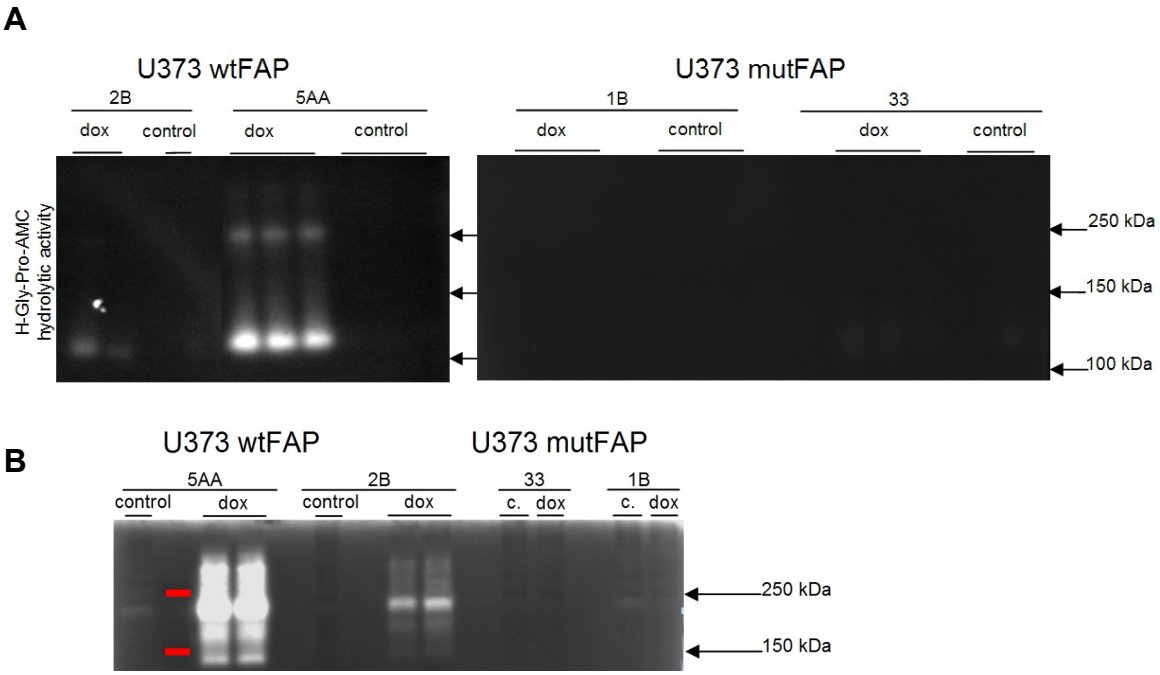


Figure 13: The exopeptidase enzymatic activity of transgenic FAP assayed with H-Gly-Pro-AMC on gels (A). The endopeptidase enzymatic activity of transgenic FAP analysed by means of gelatin zymography (B). Duplicates or triplicates were from different biological samples.

4.6. The growth rate of the glioma cells inducibly expressing FAP is not influenced in vitro

The growth rates of transfected cell clones were analysed. We did not observed any effect on the growth rate in both enzymatically active wtFAP and enzymatically inactive mutFAP cell clones (Fig. 14).

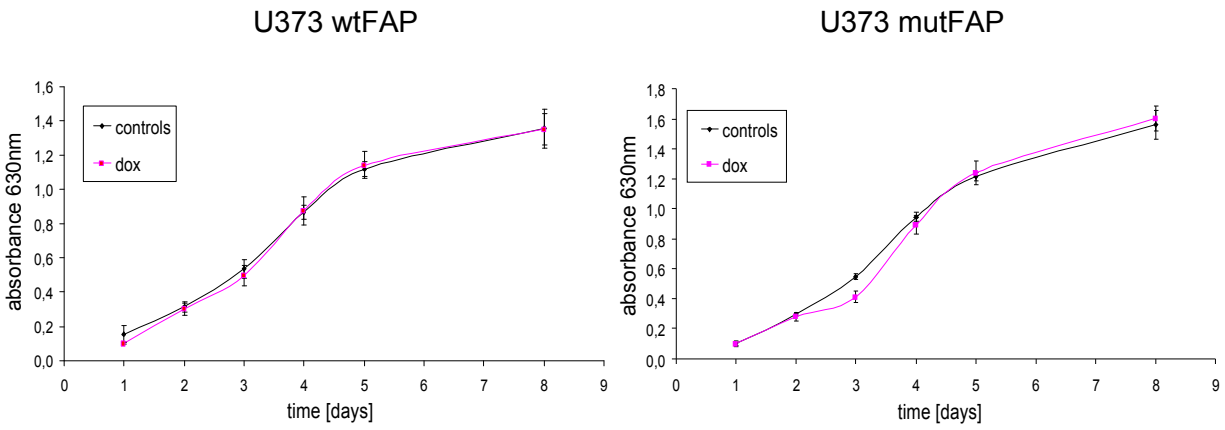


Figure 14: Growth rate analysis of cell clones transfected with enzymatically active wtFAP and enzymatically inactive mutFAP, with (dox) or without (controls) the induction agent doxycycline.

4.7. Enzymatically active transgenic FAP decreases glioma cell migration

We used the transwell migration assay to analyse the migration of glioma cells expressing enzymatically active wtFAP or enzymatically inactive mutFAP.

The cells expressing enzymatically active wtFAP migrated 3,4x less than the control cells. The assay was also performed in the serum-supplemented media (10% FCS), with the same results (Fig. 15)

Interestingly, the expression of enzymatically inactive mutFAP did not affect glioma cell migration (Fig. 15).

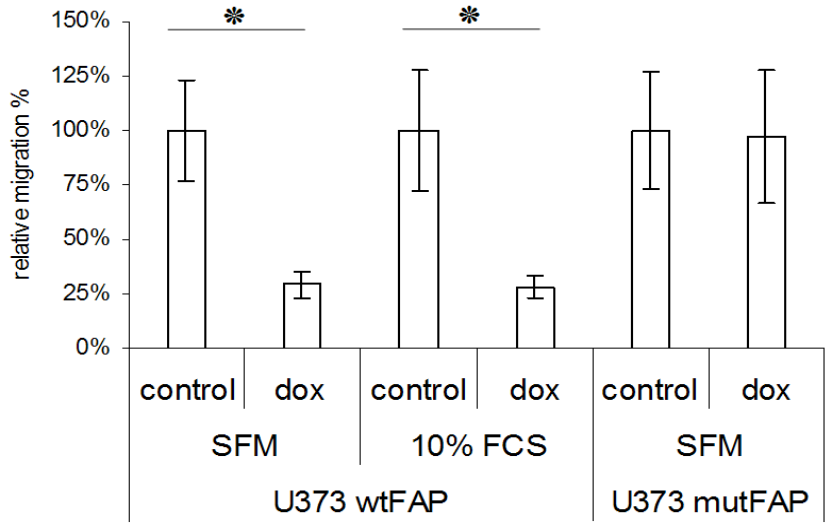


*Figure 15: Migration of FAP transfected cells with the induction agent doxycycline (dox) or without (controls) in the Transwell migration assay. The assay was performed 2 times in tetraplicates. Man Whitney U test, * p<0,05.*

4.8. Enzymatically active transgenic FAP decreases glioma cell invasion into collagen I

To examine the invasion abilities of glioma cells, spheroids were placed into collagen I gels. The cells invading from the spheroids were visible the first day after spheroid implantation. Invasion was lower in the cells expressing enzymatically active FAP compared to the control cells (Fig. 16A, B). This difference in invasiveness was not observed in the cells expressing enzymatically inactive form of FAP (Fig. 16A).

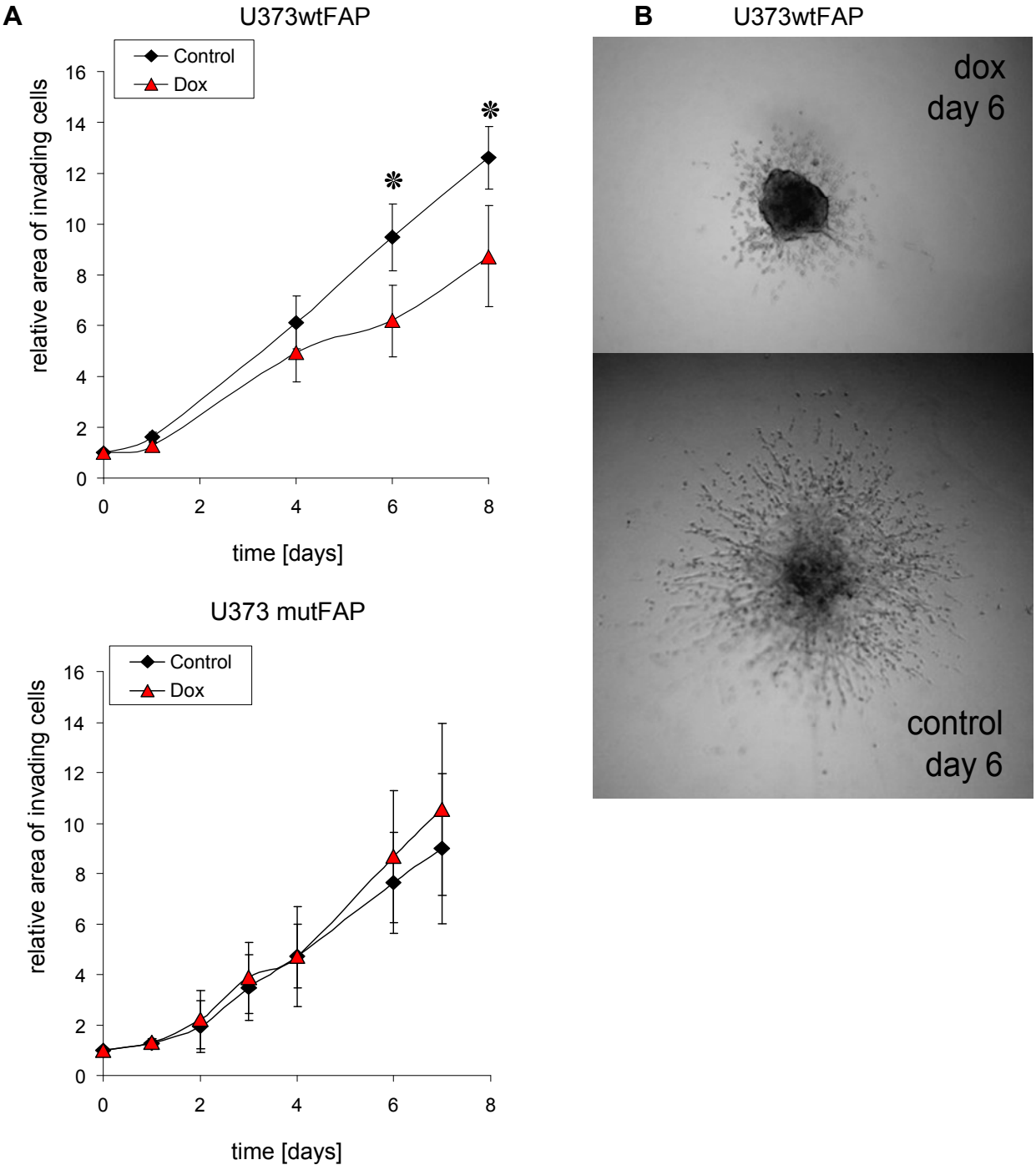


*Figure 16: The effect of transgenic FAP expression on glioma cell invasion. FAP transfected cells (inducibly expressing enzymatically active wtFAP or ezymatically inactive mutFAP) with the induction agent doxycycline (dox) or without (controls) were used for the assay. Statistical analysis was done by multivariate analysis of variance (MANOVA) using the Tukey post-hoc test, * p<0,05 (A). Representative phase contrast images of spheroids of induced and control wtFAP cells (B).*

Similar results were obtained using the clone wtFAP 2B with lower transgenic FAP expression (Fig. 17).

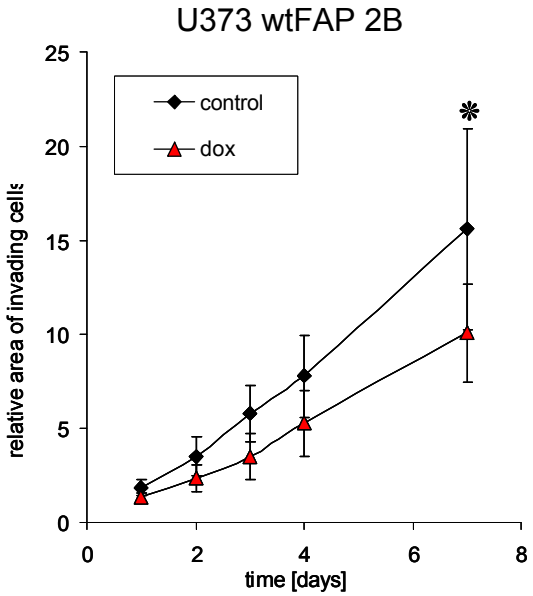


*Figure 17: Invasion of the cells with lower expression of transgenic FAP (wtFAP 2B) into collagen I. FAP transfected cells inducibly expressing enzymatically active wtFAP with the induction agent doxycycline (dox) or without (controls) were used for the assay. The statistical analysis was done by multivariate analysis of variance (MANOVA) using the Tukey post-hoc test, * p<0,05.*

5. Discussion

High grade astrocytic tumors and especially glioblastoma multiforme are brain malignancies with very poor prognosis mainly because of infiltrative phenotype of the tumor cells that evade currently available therapies. Therefore investigation of molecules participating in spreading of the tumor cells within the brain is aimed at identification of potential therapeutic targets. One molecule of interest is the fibroblast activation protein (FAP).

FAP was identified on invadopodia of migrating melanoma cells and has been suggested as marker of invasiveness (*Monsky et al., 1994*). FAP promotes invasive phenotype of ovarian tumor cells (*Kennedy et al., 2009; Chen et al., 2009*) and enhances invasion of breast cancer cells (*Huang et al., 2011b*). Expression of FAP on the tumor cells is also associated with lymph node metastasis in colorectal cancer (*Iwasa et al., 2003*). Its expression in the tumor environment is connected with more aggressive phenotype of diseases and worse clinical outcome (*Henry et al., 2007; Cohen et al., 2008; Levy et al., 2002, Okada et al., 2003*). These reports are in contrast with the results showing stromal expression of FAP as a factor associated with better prognosis in breast cancer (*Ariga et al., 2001*). Similarly, decreased expression of FAP was observed during transformation of mouse melanocytes to melanoma. In this model FAP acted as a tumor suppressor (*Ramirez-Montagut et al., 2004*). In osteosarcoma cells, FAP positivity was connected with nontumorigenic clones growing to lower density compare to FAP negative, highly tumorigenic clones. Similar observation was done in FAP^- fibrosarcoma and FAP^+ liposarcoma cell lines (*Rettig et al., 1993*). In summary, the precise mechanisms by which FAP participates on the pathogenesis of human tumors are not understood and are likely dependent on the tissue specific microenvironment.

Previous studies *(Dolznig et al., 2005; Stremenova et al., 2007; Mentlein et al., 2011; Mikheeva et al., 2010)* demonstrated increased expression of FAP mRNA in glioblastoma tissue compared to normal brain and lower grade astrocytomas. Mentlein et al. showed FAP mRNA expression also in glioma cell lines (*Mentlein et al., 2011*). The data on the expression of FAP on the protein level and its function in glioma cells are rather scarce. Rettig et al. (1986) detected FAP expression in the majority of analyzed astrocytoma cell lines by immunocytochemistry, whereas our ELISA data on a panel of glioma cell lines showed rather heterogeneous expression

of the FAP protein. The expression of FAP ranged from very low to high amounts according to ELISA and WB data in primary cell cultures derived from GBM samples. Such heterogeneity may be explained by the origin of the samples: glioblastomas are highly heterogeneous tumors and the derived primary cell cultures may reflect this heterogeneity. In addition, individual tumors contain a mixture of different cells (*Shapiro et al., 1981*).

Normal adult brain tissue was described to be FAP-negative by Rettig et al. (1986); similarly FAP was not detected in pharmacoresistant epilepsy tissue (*Balaziova et al., unpublished data*). Surprisingly, we detected FAP protein in the primary cell culture derived from pharmacoresistant epilepsy tissue. Cellular composition of cultures derived from human adult brain is controversial (*Lue et al., 1996; Gibbons et al., 2007* and references therein). The decreasing expression of the glial cell marker – glial fibrillary acidic protein (GFAP) was consistently observed during in vitro propagation and one explanation is that cultures derived from epilepsy tissues tend to be overgrown by fibroblast-like cells. This conclusion is supported by immunocytochemistry detection of fibroblast markers in the cultured cells (*Gibbons et al., 2007*). Since fibroblasts were shown to express FAP in response to *in vitro* culture (*Rettig et al., 1993*), they are a possible source of the FAP expression in our primary cell culture derived from pharmacoresistant epilepsy tissue.

The apparent molecular weight of FAP in primary glioma cell cultures and FAP transfected glioma cells as detected by western blot was 144kDa, which corresponds to the enzymatically active dimer. Although a truncated 50-70 kDa soluble form of FAP isolated from ovarian carcinoma cells was shown to carry enzymatic activity *(Chen et al., 2006)* the majority of authors describe the molecular weight of FAP dimer ranging from 120 to 200kDa (*Rettig et al., 1986,1987; Pineiro-Sánchez et al., 1997; Monsky et al., 1994; Levy et al., 1999).*

FAP has dipeptidyl peptidase activity on substrates with Pro at penultimate position. Nevertheless the measured H-Gly-Pro-AMC cleaving activity in the glioma primary cell culture samples did not correlate with FAP expression. Dipeptidyl prolyl peptidase activity is carried by a group of molecules. In addition to FAP, DPPIV, DPP8 and DPP 9 can cleave H-Gly-Pro-AMC substrate in our experimental setting. Indeed, the protein levels of DPPIV correlated with the hydrolytic activity on H-Gly-Pro-AMC substrate. Furthermore, this hydrolytic activity could be inhibited in many samples by a specific DPPIV inhibitor sitagliptin (*Kim et al., 2005*). Neither the

residual activity after the sitagliptin inhibition correlated with FAP expression (not shown), suggesting that DPP8 and/or DPP9 probably also participate on the H-Gly-Pro-AMC cleavage. Together, the H-Gly-Pro-AMC cleaving activity in primary glioma cell cultures is linked to DPPIV rather than to FAP.

FAP was localized to the perinuclear area and to the plasma membrane in the primary glioma cell culture P11 with high expression of FAP *in vitro*. Although FAP is predominantly described as a type II plasma membrane protein (*O´Brien and O´Connor, 2008*), several authors show that FAP is localized also intracellularly. Intracellular localization similar to that in the P11 cells was observed in the human hepatic stellate cells (*Wang et al., 2005; Levy et al., 1999*), ovarian cancer cells (*Chen et al., 2009*), permanent glioma cells (*Balaziova et al., 2011*) or in the human fibroblasts (*Waster et al., 2011*). FAP, as a transmembrane glycoprotein, could be stored/waiting for hodimerization or modification in endoplasmic reticulum and Golgi. In addition, the FAP immunopositivity in the cytoplasm could correspond to the intracellular, soluble isoform of FAP (*Goldstein et al., 2000*).
The P11 cells were tumorigenic in nude mice and the xenotransplantated cells expressed FAP and GFAP (not shown) *in situ.* Thus P11 are transformed glial cells and their expression of FAP is not just an artefact of *in vitro* culturing.

FAP expression in breast cancer cells was associated with faster proliferation compared to FAP–silenced cells, but only in serum starvation conditions (*Goodman et al., 2003*). In contrast, we observed slower growth in three primary glioma cell cultures with high FAP expression. However, the cell growth was not affected in the transfected cells inducibly expressing FAP. Therefore FAP itself may be a marker of more slowly proliferating cells rather than a molecule directly affecting the proliferation of glioma cells.

FAP is linked with migration and invasion in various cell types (*Aoyama & Chen, 1990; Monsky et al., 1994*), can act independent of its enzymatic activities (*Huang et al, 2011b; Wang et al., 2005*), and is promoting (*Kennedy et al., 2009; Wang et al., 2005; Mentlein et al., 2011*), or inhibiting invasion (*Wang et al., 2005*) probably depending on the cell type studied. To clarify the influence of FAP and its enzymatic activity on migration and invasion of glioma cells, we used a glioma cell line transfected with a vector carrying *FAP* or its mutant form encoding a protein with abrogated enzymatic activity. Substantial part of the transgenic FAP was translocated into the plasma membrane as expected. Such localization is probably

important to execute its proposed role in modulating invasion via its endopeptidase activity on pre–digested collagen (*Christiansen et al., 2007).*

Cells expressing enzymatically active transgenic FAP migrated through the porous membrane three-time less than controls in our experimental conditions. Such decreased migration was not observed in cells producing comparable amounts of mutant, enzymatically inactive FAP. In invasion assay, FAP expressing cells were less efficient in invasion compare to controls, whereas cells expressing mutant form of FAP invaded comparable to control cells. Our data showed decreased migration and invasion of glioma cells overexpressing enzymatically active FAP.

Our model enables inducible expression of exogenous FAP, but has also limitations, which include leakage expression in control cells and also possible expression of FAP over the physiological range. High gelatinolytic activity of FAP might than result in over-digestion of collagen and thus artificially disable the formation of focal contact necessary for attachment and formation of the leading edge. Similarly, decreased migration of glioma cells expressing FAP in our model may be linked to decreased adhesion of the cells caused by uncontrolled cleavage of the extracellular matrix, possibly produced by the cells *(Gladson, 1999).* Interestingly, FAP deletion in mice led to the increased Tyr^{397} phosphorylated focal adhesion kinase (*Santos et al., 2009*), which promotes cell motility and invasion (*Hsia et al., 2003*).

Although some data show that FAP might contribute to the invasive phenotype of glioma cells (*Mentlein et al., 2011, Mikheeva et al., 2010; Wang et al., 2003;Tatenhorst et al., 2004*), our results suggest that FAP by itself is not sufficient to increase the migration/invasion and may even have an opposite effect.

6. Conclusion

- Expression of FAP varies among studied primary glioma cell cultures and permanent cell lines.
- Although primary glioma cell cultures with high expression of FAP grew more slowly, overexpression of transgenic FAP did not influence cell growth rate.
- Transgenic expression of FAP decreased migration and invasion of glioma cells.
- The effect of transgenic FAP was dependent on its enzymatic activity.

7. Future perspectives

Silencing of FAP in cells naturally expressing FAP together with functionally related molecules (e.g. uPAR, DPPIV, ß1 integrin; *Artym et al., 2002; Ghersi et al., 2002; Wang et al., 2005*), especially in the xenotransplantation model would improve our understanding of its role in glioma cells *in vivo*. Experiments using selective FAP inhibitor should be implemented to assess the effects of enzymatic activity of FAP. Such inhibitors are under investigation but still not available.

The data from studies of FAP biological function clearly demonstrate that its role may strongly dependent on the cell type and specific tissue environment. To better understand biological relevance of FAP it is necessary to explore particular biological pathways with FAP participation. Moreover, just few studies have been focused on the mechanisms regulating *FAP* expression. Two transcriptional factors *(Zhang et al., 2010; Mikheeva et al., 2010 Lal et al., 2002)* and some indirect features (*Kennedy et al., 2009; Waster et al., 2011; Wang et al., 2003*) were shown to partly drive FAP transcription. Deeper insight into the pathways leading to the FAP expression may suggest the role of FAP not only in astrocytic tumors.

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9. Appendix

Part of the data of this work was presented as a poster on the congres Proteolytic Enzymes & Their Inhibitors, June 17-22, Barga, Italy.