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**Eva Balážiová**

**Definice expresního vzorce „DASH systému“ v transformovaných gliálních buňkách,  
koexpresie proteinu aktivovaných fibroblastů a dipeptidylpeptidázy-IV.**

**Definition of the expression pattern of DASH system in transformed glial cells, the  
coupled expression of fibroblast activation protein and dipeptidyl peptidase-IV.**

PhD Thesis

Supervisor: Professor Aleksi Šedo, MD, PhD, DSc

Praha, 2012

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## Abstrakt

Dipeptidylpeptidáza-IV (DPP-IV) je multifunkční transmembránový glykoprotein odštěpující X-Pro dipeptid z N-konce peptidového řetězce. Tato evolučně konzervovaná sekvence chrání řadu biologicky aktivních peptidů před nespecifickým štěpením. DPP-IV patří do skupiny "Dipeptidylpeptidáze-IV Aktivitou a/nebo Strukturou Homologních" molekul (DASH), do které mimo ní patří například fibroblastový aktivační protein- $\alpha$ /sepráza (FAP) a několik dalších molekul. I když většina z těchto molekul jsou primárně enzymy, je známo, že alespoň některé své biologické funkce mohou vykonávat nezávisle na své vnitřní enzymové aktivitě. DASH molekuly, spolu s molekulami, s nimiž funkčně interagují, představují „DASH systém“, který se patrně významně uplatňuje v řadě patologických procesů, například tumorogeneze. O DPP-IV a jejím strukturálně nejbližším protějšku FAP se předpokládá, že jsou zapojeny do regulace mnoha biologických procesů, významných mimo jiné pro vznik a progresi maligních gliálních nádorů.

V této práci popisujeme expresi a kolokalizaci DPP-IV a FAP v nádorově transformovaných gliálních buňkách *in vitro* a v bioptickém materiálu astrocytárních tumorů. Kromě DPP-IV/FAP dvojité pozitivních buněk jsme v glioblastomu též našli kvantitativně významnou populaci FAP pozitivních mesenchymálních buněk přítomných ve vaskulárním kompartmentu. Dále popisujeme korelativní expresi DPP-IV a FAP v primárních buněčných kulturách odvozených z glioblastomu a asociaci dynamiky změn exprese obou molekul v permanentních astrocytárních buněčných liniích. Absence vztahu exprese endogenního FAP v buňkách exprimujících transgenní, a tudíž v nefyziologickém genomickém kontextu kódovanou DPP-IV, svědčí spíše pro koregulaci exprese obou molekul na transkripční než posttranskripční úrovni. Naše experimenty věnované studiu funkčního významu DPP-IV a FAP v nádorové progresi prokázaly, že exprese obou molekul negativně ovlivňuje adhezi transformovaných gliálních buněk ke komponentám extracelulární matrix, přičemž pro dosažení tohoto efektu je třeba jejich hydrolytická aktivita.

Poznání expresního vzorce DPP-IV a FAP a jejich funkční koordinace v nádorovém mikroprostředí může pomoci pochopit jejich biologickou roli v maligních gliomech.

## Abstract

Dipeptidyl peptidase-IV (DPP-IV) is a multifunctional transmembrane glycoprotein removing X-Pro dipeptide from the amino-terminus of the peptide chain. This evolutionary conserved sequence protects a number of biologically active peptides against the unspecific proteolytic cleavage. DPP-IV belongs into the group of “Dipeptidyl peptidase-IV Activity and/or Structure Homologues” (DASH), which, except the canonical DPP-IV, comprises fibroblast activation protein- $\alpha$ /seprase (FAP), and several other molecules. However several of DASH molecules are the enzymes, they execute at least some of their biological functions by non-proteolytic protein-protein interactions. DASH molecules, their substrates and binding partners are parts of “DASH system” which is affected in several pathological process including a cancer. Specifically DPP-IV and its closest structural relative FAP are among others expected to be involved in the development and progression of malignant glioma.

In this study we showed the expression and colocalization of DPP-IV and FAP in glioma cells *in vitro* and in human high grade gliomas. In addition to the DPP-IV/FAP double positive transformed glial cells, we also identified a subpopulation of FAP positive mesenchymal cells located in the perivascular compartment. Moreover we described the correlative expression of DPP-IV and FAP in the glioblastoma-derived primary cell cultures and the associated expression dynamics of both molecules in astrocytoma cell lines. Uncoupled expression of the endogenous FAP and DPP-IV transgene, placed into the non-physiological genomic context argues for the joint control of DPP-IV and FAP genes expression rather than the indirect reciprocal regulation, involving the changes of their mRNA and/or protein. Our experiments focused on the functional relevance of DPP-IV and FAP to cancer progression demonstrate that the overexpression of both molecules impaired the cell adhesion to proteins of extracellular matrix.

Understanding of the DPP-IV and FAP expression pattern and their functional coordination in the tumour microenvironment may help to clarify their biological role and molecular mechanisms in the malignant gliomas.

## Abbreviations

AEBSF – 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride

ADA – Adenosine deaminase

APCE – Antiplasmin cleaving enzyme

BEHAB – Brain-enriched hyaluronan binding/brevican

BSA – Bovine serum albumin

CD – Cluster of differentiation

CEP – Chromosome enumeration probe

CXCL – CXC ligand

CXCR – CXC receptor

DASH – Dipeptidyl peptidase-IV activity and/or structure homologues

DPL – Dipeptidyl peptidase like

DPP – Dipeptidyl peptidase

DMEM – Dulbecco's modified Eagle's medium

E.C. – Enzyme Commission number

ECM – Extracellular matrix

ELISA – Enzyme-linked immunosorbent assay

ERK – Extracellular signal-regulated kinase

FAP – Fibroblast activation protein  $\alpha$

f.c. – final concentration

FCS – Foetal calf serum

FISH – Fluorescence in situ hybridization

FGF – Fibroblast growth factor

GCP – Glutamate carboxypeptidase II

GFAP – Glial fibrillary acidic protein

h – Hour

IgG – immunoglobulin G

M – mol/l

mAb – Monoclonal antibody

min – Minute

MMP – Matrix metalloproteinase

MW – Molecular weight

NAALADase – N-acetylated- $\alpha$ -linked acidic dipeptidase



NPY – Neuropeptide Y  
PBS – Phosphate buffered saline  
PEP – Prolyl endopeptidase  
QPP – Quiescent cell proline dipeptidase  
RANTES – Regulated upon activation, normal T-cell expressed and secreted  
RT-PCR – Reverse transcriptase polymerase chain reaction  
SDF-1 $\alpha$  – Stromal cell-derived factor-1 $\alpha$   
SMA –  $\alpha$ -smooth muscle actin  
SP – Substance P  
SPARC – Secreted protein acidic and rich in cysteine  
TEM-1 – Tumor endothelial marker 1, endosialin  
TSSP – Thymus-specific serine protease  
uPA/uPAR – Urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor  
vW – von Willebrand factor  
w.c. –working concentration  
WHO – World health organisation

## 1. Introduction

Cancer is one of the leading causes of death in the world. Brain tumours belong to devastating neoplasm, causing a significant disability, decreasing quality of life and ultimately leading to death. Gliomas are the most frequent primary brain tumours, with an annual incidence of 5-6 cases per 100,000 populations. Within them, glioblastoma multiforme is the most malignant and the most common tumour. Due to its aggressive growth, less than half of the patients survive more than one year (Louis 2007).

Aberrant response of the malignant cells to humoral growth regulators leads to the tumour progression. Most of the growth regulators are peptides and their turnover is a result of their balanced production and degradation. The amino acid, proline in the penultimate position of the amino-terminal of the peptide chain is evolutionary conserved and protects peptides against unspecific proteolytic cleavage (Vanhoof et al. 1995). Peptides containing this conserved proline residue that are involved in the tumour growth and progression comprise neuropeptide Y (NPY); peptide YY; growth hormone releasing hormone; substance P (SP); glucagon-like peptide 1,2; gastrin releasing peptide; Regulated upon activation, normal T-cell expressed and secreted (RANTES); eotaxin; stromal derived factor-1 $\alpha$  (SDF); Monokine induced by interferon-gamma, interleukins-2 and -6, etc. (Busek et al. 2004; Gorrell 2005).

The plasma membrane-bound serine protease dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) was originally described by Hopsu-Havu and Glenner on the basis of its unique substrate specificity processing a chromogenic substrate glycyl-prolyl-beta-naphthylamide (Hopsu-Havu and Glenner 1966). DPP-IV was believed to be the only enzyme cleaving X-Pro dipeptides from the amino-terminus of peptides. Subsequently, several other molecules possessing the DPP-IV-like enzymatic activity and bearing a variable degree of structural similarity to DPP-IV were described. This led to a definition of a group of “Dipeptidyl peptidase-IV Activity and/or Structure Homologues” (DASH). In addition to the canonical DPP-IV, fibroblast activation protein- $\alpha$ /seprase (FAP; EC 3.4.21.B28), dipeptidyl peptidase 8 and 9 (DPP8, DPP9; both not yet included in IUBMB nomenclature), dipeptidyl peptidase II (DPPII, identical to quiescent cell proline dipeptidase, DPP7; E.C. 3.4.14.2), dipeptidyl peptidase-like protein (DPL1, DPPX, DPP6), thymus-specific serine protease (TSSP) dipeptidyl peptidase IV- $\beta$  (DPP IV- $\beta$ ) and N-acetylated- $\alpha$ -linked acidic dipeptidase (NAALADase) have been included (Sedo and Malik 2001).

The regulated processing of bioactive peptides by the DPP-IV hydrolytic activity can lead to their degradation and to the termination of the relevant biological pathways. On the other hand, DPP-IV proteolytic activity can also change the receptor preference of its biological active substrate and thus may result in the switch of the cellular response to the given biologically active, autocrine/paracrine acting molecule. At least some of the DASH biological functions are executed by protein-protein interactions (e.g. interaction with proteins of the extracellular matrix (ECM)) while their intrinsic enzymatic activity is not required. Members of the DASH group, their substrates and non-hydrolytic partners were included into functionally defined “DASH system” known to be involved in numerous biological processes, involved also in cancer development and progression. Understanding of DASH expression pattern and its functional coordination with their interacting molecules may help to identify the role of “DASH molecules” in gliomagenesis.

### *1.1. Dipeptidyl peptidase-IV (DPP-IV)*

Human DPP-IV is a 220-240 kDa membrane-associated homodimeric serine protease, consisting of two 110 kDa subunits. In addition to this transmembrane form, a soluble isoform of DPP-IV was detected in human serum and body fluids (Hino et al. 1976; Durinx et al. 2000). The cellular source of the soluble DPP-IV is unclear, but it was suggested that it may be proteolytically shed from the cell surface of endothelial cells, hepatocytes and lymphocytes (Lambeir et al. 2003; Cordero et al. 2009). Due to its post-proline dipeptidyl aminopeptidase activity DPP-IV preferentially releases X-Pro or X-Ala dipeptides from the amino-terminus of the peptide chain. The DPP-IV gene is localized on the chromosome 2, locus 2q24.3. Several splicing forms were described or hypothesized, however, only some of these proteins seemed to possess hydrolytic activity

(<http://www.ncbi.nlm.nih.gov/IEB/Research/Acemby/av.cgi?db=human&c=Gene&l=DPP4>).

In humans, DPP-IV is almost ubiquitously expressed in the epithelial cells of the gastrointestinal and biliary tract, in the exocrine pancreas, kidney, uterus, placenta, prostate, endothelial cells of various organs, thymus, lymphatic nodes and activated lymphocyte subpopulation (reviewed in Gorrell et al. 2001). Altered DPP-IV expression and/or the change of DPP-IV blood plasma concentration are hallmarks of several pathologies including the rheumatoid arthritis, lupus erythematoses, multiple sclerosis, diabetes mellitus as well as cancer.

DPP-IV is known to execute multiple functions in the immune system, metabolism and cancer progression through the processing of the several biologically active peptides (Cordero et al. 2001; Busek et al. 2004; Cordero et al. 2009). They could be cleaved or clipped (Murphy et al. 2008), thus they lose, gain or change their biological function. Since DPP-IV acts as a binding molecule for adenosine deaminase (ADA), plasminogen 2 and several structural proteins (Hanski et al. 1988; De Meester et al. 1994; Abbott et al. 1999), it may also orchestrate the processes of cell growth, migration and invasion in a non-enzymatic manner. DPP-IV contains a fibronectin as well as a collagen binding domains. Indeed, DPP-IV was reported to increase the fibronectin-mediated cell adhesion in epithelial cell line (Wang et al. 2006), hepatocytes (Piazza et al. 1989) and fibroblasts (Hanski et al. 1985), but contrary to that, Löster did not observe binding of DPP-IV to fibronectin (Loster et al. 1995). Similarly, literature data DPP-IV-collagen interaction are also ambiguous. Actually, DPP-IV is suggested to serve as an auxiliary adhesive molecule.

### *1.2. Fibroblast activation protein- $\alpha$ (FAP)*

FAP, identical to seprase (**S**urface **E**xpressed **P**Rotease), is a 150-170 kDa integral transmembrane serine protease, consisting of two 97 kDa subunits (Rettig et al. 1986; Pineiro-Sanchez et al. 1997). DPP-IV and FAP proteins share 52% amino acid sequence identity (Abbott et al. 1999). Moreover, both genes are localized close to each other on the chromosome 2 (Mathew et al. 1995; Levy et al. 1999), thus some authors suggest them to be a product of gene duplication (Havre et al. 2008). In a murine model, three alternatively spliced forms of FAP (Niedermeyer et al. 1997) were found. Not all the proteins encoded by the alternatively spliced forms are enzymatically active according to the AveView database (<http://www.ncbi.nlm.nih.gov/IEB/Research/Asembly/av.cgi?db=human&c=Gene&l=FAP>). Additionally, Goldstein described a truncated FAP isoform in a human cell line derived from malignant melanoma (Goldstein and Chen 2000). Homodimerization of FAP is a condition of its proteolytic activity. Except to its DPP-IV-like exopeptidase activity, FAP is also endopeptidase with a gelatinase/collagenase activity (Rettig et al. 1994; Goldstein et al. 1997). FAP was detected in the bovine serum however it is not clear whether it represents a primary secreted isoform or a product of shedding form the plasma membrane (Collins et al. 2004). Later it was identified also in human blood plasma where it was previously known as an antiplasmin cleaving enzyme (APCE) (Lee et al. 2006).

FAP is typically expressed in the foetal mesenchymal tissue. In adults, it is transiently expressed on the activated fibroblast during wound healing, and FAP positive cells can be

also found in the pancreas (glucagon-producing  $\alpha$ -cells of pancreatic islets) and the endometrium (Rettig et al. 1988). Contrary to the restricted physiological distribution, FAP is abundantly expressed in the stromal cells in most of epithelial and mesenchymal tumours as well as in malignant elements of melanoma, bone and soft tissue tumours, breast, lung and ovarian carcinoma (reviewed in O'Brien and O'Connor 2008; Kotackova et al. 2009). Previously, we described FAP transcripts in glioblastoma (Stremenova et al. 2007); however the particular cell type expressing FAP remains still unknown.

Although FAP is known to cleave collagen type III and gelatine, the denatured form of the collagen type I, its function in the degradation of the extracellular matrix is not completely understood yet (Christiansen et al. 2007). On the other hand, soluble form of FAP is known to process  $\alpha$ 2-antiplasmin, a potent inhibitor of plasmin. Thus, via inhibition of plasmin, FAP/APCE may modify the plasmatic fibrinolytic system, but also may alter degradation of the extracellular matrix (Lee et al. 2006). On the top of its gelatinolytic activity participating on matrix-digestion, FAP was reported to efficiently hydrolyse NPY, B-type natriuretic peptide, SP and peptide YY by its DPP-IV-like exopeptidase enzymatic activity. However, the biological relevance of this phenomenon was not documented so far (Keane et al. 2011).

Upregulation of FAP is demonstrated to decrease the adhesion and migration of epithelial cells. Interestingly, enzymatically inactive FAP mutant has not such functional potential and thus it seems that the non-hydrolytic mechanism is involved (Wang et al. 2006).

Gherzi et al. studied the functional expression of DPP-IV and FAP together on the surface of migratory connective tissue cells (Gherzi et al. 2002). His results suggest the presence of oligomeric complexes formed from both DPP-IV and FAP molecules at the invasive edge of fibroblasts and on the surface of endothelial cells (Gherzi et al. 2006). Such molecular complexes exhibited exopeptidase as well as endopeptidase proline-specific enzymatic activity, the latter was even more potent than that of the homodimeric FAP/seprase. The monoclonal antibodies targeting the DPP-IV collagen binding domain as well as the serine-protease inhibitor (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, AEBSF) blocked the collagen degradation. Thus, the authors concluded, that there is a functional cooperation of DPP-IV and FAP, acting as a collagen binding protein and a gelatin cleaving protease respectively, on the matrix degradation. Moreover, the combination of antibodies targeting the DPP-IV collagen binding domain and inhibitors of matrix metalloproteinases (MMP) exhibited stronger inhibition of cell migration than each of the compounds alone. These results demonstrate that both, DPP-IV as well as FAP cooperate

with MMPs on the extracellular matrix degradation. Subsequently, MT1-MMP, MMP-2, MMP-9, DPP-IV and FAP were shown to be localized in the plasma membrane of invadopodia and membrane vesicles of the migrating endothelial cell (Salamone et al. 2006). These observations were supported also by Christiansen et al, who described the cooperation between FAP and MMPs in the degradation of collagen type I and III. And moreover, it was suggested that the products of FAP/MMP cooperated truncation may affect malignant cell invasion and migration (Christiansen et al. 2007) via degradation of the extracellular matrix proteins.

### 1.3. Other DASH molecules

DPP-IV and FAP are the most intensively studied members of the functional group of “Dipeptidyl-peptidase-IV Activity and/or Structure Homologues” so far (Sedo and Malik 2001). Recent data resulted into the reclassification of some molecules as the members of DASH group.

In 2000, Abbott et al described human **dipeptidyl peptidase 8** that is highly homologous to DPP-IV and FAP (Abbott et al. 2000). Using an in-silico approach, Olsen and coworkers later identified **dipeptidyl peptidase 9**, which possesses a serine peptidase motif, GWSYG, identical to that found in DPP-IV (Olsen and Wagtmann 2002). Although not originally included, DPP9, due to its structural homology with and similar enzymatic activity to DPP-IV, is now listed as a member of the DASH group. Both, DPP8 and DPP9, are intracellular enzymes, possessing the DPP-IV-like exopeptidase activity. Recently some authors are also claiming the endopeptidase postprolyl activity of DPP8 (Park et al. 2006). DPP8 and DPP9 were initially described as monomeric molecules (Abbott et al. 2000; Olsen and Wagtmann 2002), but further analysis revealed that they also form dimers. DPP8 and DPP9 are ubiquitously expressed e.g. in immune cells, epithelial cells of the gastrointestinal tract, spleen, liver, lung, brain, testis and muscle (Abbott et al. 2000). The physiological importance of the enzymatic activity of both enzymes remains unclear. Recent data demonstrated that DPP8 and DPP9 are able to cleave several bioactive peptides *in vitro* (glucagon-like peptide -1 and -2, NPY, peptide YY; DPP8 also SDF, INF- $\gamma$ -inducible protein 10, interferon-inducible T-cell chemo-attractant), but it is unclear whether intracellular DPP8 or DPP9 can reach physical contact with these molecules *in vivo* and thus have a potential to modify their biological effects (Yu et al. 2010).

**Dipeptidyl peptidase II** (identical to quiescent cell proline dipeptidase, DPP7, E.C.3.4.14.2) is a 100-130 kDa post-proline cleaving protease, consisting of two 58 kDa and 61 kDa subunits and is localized in intracellular vesicles and lysosomes. The primary structure of rat DPPII shows significant similarity to prolyl carboxypeptidase, but only low to DPP-IV. Similarly to DPP-IV, homooligomerisation (dimer, trimer) appears to be necessary for the DPPII post-proline dipeptidyl aminopeptidase activity. The pH optimum of DPPII is in a range 5.1-6.2 and its dipeptidyl peptidase-IV-like activity drastically decreases with increasing length of substrate chain (Mentlein and Struckhoff 1989). It has been shown that the brain DPPII is able to cleave several short neuropeptides such as substance P and its fragments, casomorphin and bradykinin (Mentlein and Struckhoff 1989), but the physiological significance of this remains dubious. Meas suggested association of DPPII expression with the astrocyte differentiation (Maes et al. 2007).

**N-acetylated- $\alpha$ -linked acidic dipeptidase II** (glutamate carboxypeptidase II/GCPII) is the best studied molecule of the NAALADase group comprising NAALADase I, II, III and NAALADase-like (Pangalos et al. 1999). NAALADases are metalloproteinases, belonging to the M28 enzyme family. They hydrolyze extracellular N-acetylaspartylglutamate to glutamate and thus increase its neurotoxicity, which is implicated in the pathophysiology of the neurodegenerative disorders and stroke. The native forms of NAALADase L as well as NAALADase II/GCPII were claimed to exhibit dipeptidyl peptidase-IV-like enzymatic activity (Pangalos et al. 1999). Based on this observation, they were formerly included into the DASH group. However, further research proved that NAALADases do not contain serine in the consensus sequence of the serine proteases (Abbott et al. 2000) and the recombinant form of NAALADase II/GCPII was shown to have no DPP-IV-like activity (Barinka et al. 2002). These contradictory observations make the inclusion of NAALADase into the DASH group is disputed.

**Dipeptidyl peptidase like 1** (formerly named DPP6 or DPPX) and **dipeptidyl peptidase like 2** (also known as DPP10 or DPY) are sequentially similar to DPP-IV but enzymatically inactive members of DASH group. They act as modulators of the voltage-gated potassium channels in neurons and play a role in the development of central nervous system and synaptic plasticity (McNicholas et al. 2009). Their role in cancer pathogenesis was not studied (Busek et al. 2008).

**Attractin** was originally included into the DASH group as a molecule possessing DPP-IV-like enzymatic activity but without structural similarity to DPP-IV (Sedo and Malik 2001). However, later research demonstrated that the DPP-IV-like activity in human blood

serum, originally attributed to attractin, is factually the activity of co-purified canonical DPP-IV (Friedrich et al. 2007).

**Thymus-specific serine protease (TSSP)**, a post proline acting lysosomal exopeptidase is involved in the pathogenesis of diabetes mellitus type I, however there is no known relation between TSSP and cancer so far (Viret et al. 2011).

**Dipeptidyl peptidase IV- $\beta$**  (DPP IV- $\beta$ ) is a 82 kDa monomeric cell-surface molecule possessing DPP-IV-like activity, but contrary to DPP-IV it is unable to bind adenosine deaminase (ADA) (Blanco et al. 1998). DPP IV- $\beta$  was described in human lymphoblastoid cell line (C8166), but its expression *in vivo* was not studied so far.

Although the involvement of other members of the DASH group in cancer is possible, this thesis focuses on the study of DPP-IV and FAP in pathogenesis of malignant glioma from the following reasons.

The plasma membrane localization and extracellular orientation of DPP-IV and FAP is an important prerequisite to their interaction with the components of the tumour microenvironment. Both DPP-IV and FAP have been reported to cleave the bioactive peptides relevant to the glioma progression such as SP, SDF and NPY etc. (Mentlein 1999; Bajetto et al. 2006; Keane et al. 2011). SP and SDF are known to affect the glioma cell proliferation, survival, migration and invasion (Sharif 1998; Palma and Maggi 2000; Barbero et al. 2003). NPY acts as a vasoconstrictor as well as an angiogenic factor, depending on the subtype of signaling receptor. By cleaving Tyr-Pro dipeptide from NPY, DPP-IV changes the affinity of resulting peptide to the particular NPY receptor subtype, and thus switches between both functions of NPY (Kitlinska et al. 2003). Finally, DPP-IV and FAP directly interact with an ECM; DPP-IV as a fibronectin and collagen binding molecule (Hanski et al. 1988), while FAP as an enzyme possessing the gelatinolytic activity.

#### *1.4. DPP-IV and FAP in malignant tissue, “a tale of contradictions”*

Expression of DPP-IV and FAP was described in numerous types of cancers so far (as e.g. melanoma, ovarian carcinoma, colorectal and breast cancer) (reviewed in Busek et al. 2004; Kotackova et al. 2009). However, the majority of the authors approached either DPP-IV or FAP individually and thus, there is just few parallel information about both molecules in one experimental model (Monsky et al. 1994; Wesley et al. 2004; Stremenova et al. 2007; Goscinski et al. 2008).



Recent knowledge argues for possible pro- as well as anti-oncogenic role of DPP-IV and FAP, which may depend on the particular cancer type (Table 1) (Sulda et al. 2006; Kotackova et al. 2009). Such seeming contradiction may be explained by tumour type-specific local microenvironment and by the cell-specific function(s) of DPP-IV and FAP in the given cell population within either the tumour stroma or parenchyma (Cheng et al. 1998; Iwata and Morimoto 1999; Chen and Kelly 2003; Wesley et al. 2005; Sulda et al. 2006).

Moreover, the heterooligomeric DPP-IV/FAP complexes observed on the surface of some cell types may influence the migratory and invasive potential by increasing the ability to degrade extracellular matrix and cleave bioactive peptides.

**Table 1. Dipeptidyl peptidase-IV (DPP-IV) and Fibroblast activation protein- $\alpha$  (FAP) in cancer tissues compared to control tissue (Kotackova et al. 2009).**

Type of cancer	DPP-IV or FAP- $\alpha$ expression	Notes
Lung cancer	↑ DPP-IV <sup>(l)</sup>	
Follicular cancer	↑ DPP-IV <sup>(f, o)</sup>	
Endometrial cancer	↓ DPP-IV <sup>(g)</sup>	Grade dependent
Squamous carcinoma of the esophagus	↑ DPP-IV <sup>(b)</sup> , ↑ FAP <sup>(c)</sup>	Associated with tumour progression
Colon adenocarcinoma	↓ ↑ DPP-IV <sup>(n)</sup> ↑ FAP <sup>(d, h)</sup>	Associated with tumour progression
Gastric cancer	↑ FAP <sup>(j)</sup>	
Ductal breast cancer	↑ FAP – cells of ductal breast cancer <sup>(a)</sup> ↑ FAP – cells of tumour stroma <sup>(a)</sup>	Associated with tumour progression Better survival of patients
Cervical cancer	↑ FAP <sup>(e)</sup>	
Melanoma	↓ DPP-IV <sup>(p)</sup> , ↓ FAP <sup>(k)</sup> ↑ FAP <sup>(i)</sup>	Stromal fibroblast expression
Epithelial skin cancer	↑ FAP <sup>(i)</sup>	Stromal fibroblast expression
Glioma	↑ DPP-IV, FAP <sup>(m)</sup>	Grade dependent

(a) (Ariga et al. 2001); (b) (Goscinski et al. 2008); (c) (Goscinski et al. 2008); (d) (Iwasa et al. 2005); (e) (Jin et al. 2003); (f) (Kehlen et al. 2003); (g) (Khin et al. 2003); (h) (Henry et al. 2007); (i) (Huber et al. 2006); (j) (Mori et al. 2004); (k) (Rettig et al. 1993); (l) (Sedo et al. 1991); (m) (Stremenova et al. 2007); (n) (Tan et al. 2004); (o) (Tanaka et al. 1995); (p) (Wesley et al. 1999)

### 1.5. DPP-IV, FAP and microenvironment of high grade gliomas

The invasiveness is one of the hallmarks of the transformed cells. The “three-steps model” of invasion includes subsequent detachment of the invading cell from the primary tumour mass, its adhesion to the extracellular matrix and degradation of surrounding ECM proteins, the last opening a migratory path into the adjacent tissue.

The glioma cells typically possess high invasive potential; they preferentially infiltrate along the blood vessels, the subpial glial space and along the white matter tracts. The brain parenchyma has a unique composition of extracellular matrix; the major ECM component is hyaluronan while the common fibrillar proteins such as collagen, fibronectin or laminin are restricted mainly into the perivascular compartment. The glioma progression is associated with the alteration of ECM composition in both parenchymal as well as perivascular compartment (Delpech et al. 1993; Bellail et al. 2004). The upregulation of hyaluronan, vitronectin, osteopontin, tenascin-C, secreted protein acidic and rich in cysteine (SPARC) and brain-enriched hyaluronan binding (BEHAB)/brevican is characteristic for the tumour parenchyma. Compared to the normal brain vessels, the tumour ones are characteristic by up regulation of tenascin C, thrombospondin and SPARC (Zagzag et al. 1995). Moreover, collagen, fibronectin and laminin are suggested to be actively involved in the processes of glioma cell adhesion and migration as well (Merzak et al. 1995; Chintala et al. 1996; Senner et al. 2008).

The interaction between the glioma cell and extracellular matrix is mediated by the adhesive molecules such as integrins and CD44, receptor for hyaluronan. The integrins  $\alpha 2\beta 1$ ,  $\alpha 5\beta 1$  and  $\alpha 6\beta 1$  are typically expressed in gliomas (Gingras et al. 1995). The aberrant repertoire of the adhesive molecules results in altered glioma cell adhesion.

The other important prerequisite of high potential of glioma cells to invade is the complex system of ECM processing enzymes. Among them, the MMPs, especially MMP-2 and MMP-9, cathepsin B and uPA/uPAR/plasmin are the most important. While MMP-9 exhibits restrictive pattern of expression mainly in regions of neovascularization (Forsyth et al. 1999), MMP-2 is expressed in parenchymal glioma cells and thus it is suggested to be involved in the invasiveness of glioma *in situ*. Plasmin as well as urokinase activate MMP-2 and MMP-9 (Baramova et al. 1997; Zhao et al. 2008). These proteases are localized on the invading edge of tumour and in perivascular compartment (Bellail et al. 2004).

In our previous work we demonstrated the expression of DPP-IV and FAP transcripts in tissues of the high grade gliomas (Stremenova et al. 2007). These observations well correspond to the later results of Mentlein et al., describing the major expression of DPP-IV

on the endothelial cells, and just the minor one on the glioma cells (Mentlein et al. 2011). Moreover, Mentlein's observations suggest the dominant presence of FAP in GFAP positive glioma cells within the glioblastoma tissue. Our further research demonstrated, that the transgenic overexpression of wild type as well as mutated, enzymatically inactive DPP-IV impairs the glioma cell migration and adhesion to fibronectin (Busek et al. 2012).

#### *1.6. DPP-IV and FAP, "a tale of hidden cooperation"*

In order to interpret properly the role of DPP-IV and FAP in processes of tumour progression, several facts have to be considered : (1) DPP-IV and FAP are structurally similar enzymes, thus their identification by using antibodies or specific substrates/inhibitors is difficult; (2) DPP-IV and FAP, due to their similar exopeptidase enzymatic activity and thus sharing similar set of biologically active substrates, can functionally overlap, at least in some of their functions; (3) despite both being enzymes, some of their biological effects are independent on their proteolytic activity; and finally (4) DPP-IV and FAP have been shown to be co-expressed and speculated to functionally cooperate with two major matrix degrading proteolytic systems, MMP (Salamone et al. 2006) and plasminogen/uPAR (Artym et al. 2002).

## 2. Hypothesis and aims

Our previous data confirmed significant correlation of DPP-IV and FAP transcripts and protein expression in astrocytic tumours (Stremenova et al. 2007). However the particular cell type or cell population expressing DPP-IV and/or FAP remains unknown. Moreover, although the co-expression of DPP-IV and FAP was observed in several biological systems and experimental models (Monsky et al. 1994; Wesley et al. 2004; Stremenova et al. 2007; Goscinski et al. 2008), their putative co-regulation was not noted so far. Thus, on the basis of ours and others results, suggesting functional association of both molecules in the biological processes, relevant to the glioma progression, we aimed to test the hypothesis of co-regulation of DPP-IV and FAP in transformed glial cells and to test their effect on the cell adhesion.

**Aim 1:** To characterise expression and staining pattern of DPP-IV and FAP in human glioblastoma multiforme.

**Aim 2:** To verify co-expression of DPP-IV and FAP in permanent glioma cell lines and in the primary cell cultures derived from human glioblastoma multiforme.

**Aim 3:** To approach mechanism of putative coupling of DPP-IV and FAP expression in glioma cell lines.

**Aim 4:** To determine the relevance of DPP-IV, FAP and their enzymatic activity for adhesion of glioma cells to the components of extracellular matrix *in vitro*.

### 3. Material and methods

#### 3.1. Cell lines, primary cell cultures and sample preparation

Cell lines U138MG (derived from human glioma WHO grade IV; ATCC, Teddington, UK), U87MG (derived from glioma WHO grade IV; ATCC), U373 (derived from glioma WHO grade III; ATCC), stable DPP-IV transfected U87MG and stable DPP-IV or FAP transfected U373 clones (see below) were cultured on the Nunc tissue plastic (Thermo scientific, Langensfeld, Germany) in the Dulbecco's modified Eagle's medium (DMEM; Sigma, Prague, Czech Republic) supplemented with 10% foetal calf serum (FCS; Sigma) under a humidified (90%) atmosphere of 5% CO<sub>2</sub> and 95% air, at 37°C. In some experiments, a serum-free DMEM (serum-free medium, SFM) was used to model growth factors deficiency conditions, inducing adaptive cell differentiation (Sedo et al. 1998). The cells were growing in serum free medium for 72 hours prior the experiments.

Primary cell cultures were derived from biopsies of human high grade astrocytic tumours. The study was approved by the Institutional ethic committee and was conducted in accordance with the Declaration of Helsinki. All patients signed informed consent before an operation. A fresh tissue sample was cut into small pieces and cultured in DMEM, supplemented with 20% FCS (Sigma), and streptomycin in f.c. 100 µg/ml and penicillin G in f.c. 100 U/ml (Sigma). Between the 5<sup>th</sup> - 7<sup>th</sup> day of explantation, when outgrowths were observed, the explants were removed and the medium was replaced with fresh DMEM supplemented with 10% FCS and the antibiotics (penicillin in f.c. 100 U/ml; streptomycin in f.c. 100 µg/ml).

Total cell lysates (cca 20 x 10<sup>6</sup> cells/ml) were prepared on ice in a lysis buffer (10 mM Tris-HCl pH 7.5, containing 1 mM EGTA, 1 mM Na<sub>2</sub>EDTA, 1% Triton X-100, 0.1% SDS, and 10% glycerol) supplemented with a mixture of protease inhibitors (pepstatin A 25 µM, AEBSF 200 µM, E-64 50 µM) and cleared by centrifugation at 27 000 g, 4°C for 30 min. For separation of the soluble and membrane fraction, the cells were first disrupted by sonication (three times at ice cold bath for 15 s in the total volume of 7 ml) and the homogenates were centrifuged at 250 g, 4°C for 10 min to remove the nuclei. The supernatant was then ultracentrifuged at 136 000 g, 4°C for 30 min, the supernatant formed the soluble fraction and was stored at -74°C until further analysis. The pellet was subsequently dispersed in the lysis buffer, 10-times passed through a 25 G needle and again ultracentrifuged at 136 000 g, 4°C

for 30 min. The resulting supernatant, i.e. the solubilized membrane fraction, was loaded onto the gel filtration column (see below).

### 3.2. *Fluorescent in situ hybridisation (FISH)*

FISH analyses were carried out on the suspensions of fixed cells. To identify chromosome 2, the commercially available centromeric DNA chromosome enumeration probe 2 (CEP 2; D2Z1) Spectrum Orange and a differently labelled DNA probe CEP 18 (D18Z1) Spectrum Green, serving as a control, were used according to the manufacturer's recommendations (Abbott Molecular, Des Plaines, USA). To localize the DPP-IV and FAP DNA sequences, respectively, the commercially available bacterial artificial chromosome probes were used (FAP - RP11-576I16 and DPP-IV - RP11-178A14, Pentagen, Kolin, Czech Republic).

### 3.3. *Construction of DPP-IV and FAP cDNA vector and cell transfection*

U87MG cells were transfected with human DPP-IV using the Mifepristone inducible Gene Switch System (Invitrogen, Life Technologies, Prague, Czech Republic). Glioma cells were transfected with the regulatory vector GeneSwitch and pGene vector containing the full-length cDNA of human DPP-IV by using the Lipofectamine<sup>TM</sup>2000 (Invitrogen) according to the manufacturer's instructions. Stable clones were selected with Zeocin (Invitrogen) and Hygromycin (Invitrogen). Mifepristone (Invitrogen) in concentrations of 0.25 nM was used to induce DPP-IV expression in transfected cells; cells not treated by Mifepristone were used in control experiments (Busek et al. 2008).

U373 cell line was transfected with human DPP-IV or FAP (U373onCD26, U373onMUTCD26 and U373onFAP, U373onMUTFAP) using the Tetracycline inducible pTet-On-Advanced system (Clontech, Saint-Germain-en-Laye, France). Glioma cells were transfected with the regulatory pTet-On-Advanced plasmid. Subsequently, the selected clones were co-transfected with the pTRE-Tight vector containing the wild-type or mutated, enzymatically inactive, full-length human DPP-IV or FAP and a linear Hygromycin marker (Clontech) by using Lipofectamine<sup>TM</sup>2000. Stable clones were subsequently selected with G418 (Sigma) and Hygromycin B (Invitrogen). Doxycycline (Invitrogen) in concentrations of  $10^{-6}$  mg/ml was used to induce DPP-IV or FAP expression in transfected cells; cells not treated by Doxycycline were used in control experiments (Busek et al. 2012).

### 3.4. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

To determine the expression levels of DPP-IV and FAP- $\alpha$  mRNAs, a coupled RT-PCR was used as described previously (Busek et al. 2008). Total RNA was isolated by TriZol Reagent (Invitrogen) according to the manufacturer's instructions. The expression of DPP-IV, FAP and  $\beta$ -actin transcripts was quantified by using gene coding region-specific oligonucleotide primers carrying fluorescent TaqMan probes by real time RT-PCR assay; ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Carlsbad, USA). The transcript expression was normalized to  $\beta$ -actin mRNA using  $\Delta$ Ct method (Livak and Schmittgen 2001).

### 3.5. Enzymatic activity assays and gel filtration chromatography

The cell surface and the total cellular enzymatic activities were determined using the continuous rate fluorimetric assay as described in detail previously (Sedo et al. 1998; Sedo et al. 2003; Lee et al. 2006). Briefly, the exopeptidase and endopeptidase enzymatic activities were determined with the fluorogenic substrates glycyl-L-prolyl-7-amidomethylcoumarine (H-Gly-Pro-AMC) and N-benzyloxycarbonyl-glycyl-L-prolyl-7-amidomethylcoumarine (Z-Gly-Pro-AMC) (both from Bachem, Bubendorf, Switzerland), respectively, in a final concentration of 50  $\mu$ M. The assays were performed in 1950  $\mu$ l of the prewarmed phosphate buffered saline (PBS) of pH 7.4 and 25 mM phosphate buffer containing 4% methanol and 1 mM EDTA of pH 7.4, respectively. The release of 4-amino-7-methylcoumarin was monitored at 380 nm excitation and 460 nm emission wavelengths respective (Perkin-Elmer, Ueberlingen, Germany). The assay was performed on suspension of viable cells (cell surface enzymatic activity) and after the permeabilization of the cells by 0.1% Triton X-100 (total enzymatic activity).

To describe the elution profile of DPP-IV-like exopeptidase and endopeptidase enzymatic activities of soluble and solubilized membrane cell fraction, gel filtration chromatography on a column of Sephacryl S-300 (Pharmacia, Uppsala, Sweden) was used as described previously (Sedo et al. 2003). The cell lysate was loaded onto the chromatographic column (1.5 x 95 cm) and eluted with equilibrating 30 mM phosphate buffer of pH 7.4 containing 0.1 Triton X-100 and 0.02% sodium azide. The 1.5 ml fractions were collected in 15 min intervals. The void volume of chromatographic system was assessed by Dextran Blue elution. The Gel filtration calibration kit HMW (GE Healthcare, Buckinghamshire, UK) containing thyroglobulin (MW 669 kDa), ferritin (MW 440 kDa), aldolase (MW 158 kDa), conalbumin (MW 75 kDa), ovalbumin (MW 43 kDa) was used to define the MW elution

profile. In the individual collected fractions, the exopeptidase and endopeptidase enzymatic activities were determined by using the above mentioned fluorogenic substrates in 180  $\mu$ l of 100 mM phosphate buffer of pH 7.4 and 25 mM phosphate buffer containing 4% methanol and 1 mM EDTA of pH 7.4, respectively.

Total protein concentration in samples was assayed according to Lowry (Lowry et al. 1951).

### 3.6. Western blot and zymography assay

The cell lysates were cleared by centrifugation at 14 000 g, 4°C for 20 min. The Tris-HCl sample buffer, pH 6.8, containing 1% sodium dodecyl sulphate (SDS), 10% glycerol and 0,005% bromphenol blue were added and 25  $\mu$ g of total protein without boiling were loaded per well onto SDS 7,5% polyacrylamid gel. The electrophoresis run in the conditions of 25 mM Tris buffer containing 1.92 M glycine and 0.1% SDS. Proteins were then transferred onto the PVDF membrane (Biorad, Prague, Czech republic) by semidry blotting, the membrane was blocked for 3 hours with 5% non-fat milk in 0,05% Tween 20 Tris-buffered saline and then incubated overnight at 4°C with primary monoclonal antibodies targeting DPP-IV (clone E19, dilution 1:1000) and FAP (clone D28, dilution 1:3000) diluted in 5% non-fat milk. The blots were subsequently developed with horseradish peroxidase conjugated secondary antibody (R1371HRP, Acris, Herford, Germany, 1:10 000 in 5% non-fat milk) and enhanced chemiluminiscence (2.5 mmol/l luminol in 0.1 mol/l Tris, pH 8.8 mixed with equal volume of 5.4 mmol/l H<sub>2</sub>O<sub>2</sub> and 2.25 mmol/l p-coumaric acid in 0.1 mol/l Tris pH 8.8). The blots were exposed to the photographic film (Hyperfilm<sup>TM</sup> ECL, Amersham).

The zymography was run on SDS 7.5% polyacrylamid gel containing 0.1% of gelatine under condition of 25 mM Tris buffer containing 1.92 M glycine and 0.1% SDS. The gels were incubated for 72 hours in 37°C under the condition of 2 mM EDTA or 2 mM CaCl<sub>2</sub> Tris-buffered saline. The gels were stained with Coomassie Blue R-250 for 30 min.

### 3.7. Enzyme-Linked ImmunoSorbent Assay (ELISA)

The DPP-IV and FAP proteins were assayed by DuoSet DPP-IV and DuoSet FAP ELISA kits (DY1180 and DY3715, R&D Systems, Abingdon, UK) according to the manufacturer's recommendations. Each step was performed in the room temperature and the samples as well as the standards were diluted in 1% bovine serum albumin in PBS. Briefly, the 96-well transparent plate was coated by capture antibodies diluted in PBS (anti DPP-IV, cat.No. 842127, w.c. 2 $\mu$ g/ml and anti FAP, cat.No. 842997, w.c. 1 $\mu$ g/ml; for both the



overnight incubation). The wells were washed three times, blocked by blocking buffer (1% bovine serum albumin in PBS) for one hour and then following the washing step, the samples were applied for two hours. We used the recombinant human FAP and DPP-IV proteins as the negative controls in the DPP-IV and FAP assays, respectively. The wells were washed again three times; and the biotinylated detection antibodies (w.c. 1 µg/well of anti DPP-IV and anti FAP) were applied (2 hours). The system of Streptavidin-horseradish peroxidase and 1:1 mixture of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine (DY998, R&D Systems) was used for the visualization. The reaction was terminated by adding 50 µl of 2 M sulphuric acid. The absorbance of samples at 450 nm was read by the microplate reader Sunrise (Tecan, Malmendorf, Switzerland). The measured absorbance values were corrected by subtracting the absorbance values obtained at a second wavelength of 570 nm. The resulting differential absorbance values were used for constructing the calibration curves and data evaluation.

### *3.8. Immunocytochemistry and immunohistochemistry*

All procedures of the immunocytochemistry and immunohistochemistry were held in a humidified chamber. In the staining controls, the primary antibodies were omitted from the immunostaining procedure.

The cells, cultured on the glass coverslips, were fixed by 4% paraformaldehyde and permeabilised by 0.1% Triton X-100 at a room temperature for 10 respectively 5 min. To reduce unspecific binding, the coverslips were pretreated in 3% heat-inactivated FCS for 20 min. The samples were incubated with the monoclonal primary antibodies, anti DPP-IV (clone MA 261) and anti FAP (clone D8) diluted in PBS of pH 7.4, overnight at 4°C. The mAb are listed in detail in Table 2. This step was followed by the incubation with the Alexa Fluor 488-labelled goat anti-mouse IgG and the Alexa Fluor 546-labelled goat anti-rat IgG, respectively. The coverslips were finally mounted in the anti-fading Gel/Mount medium (Biomedica Corp., Foster City, USA), observed and photographed using laser scanning microscope IX81 (Olympus, Prague, Czech Republic).

Specimens of tumour (20 bioptic samples of high grade gliomas – grade III and IV) and non-tumorous brain (4 bioptic samples of pharmacoresistant epilepsy) were stored in -70°C until they were used. The samples were cut into 10 µm-thick sections. The slides were fixed with 4% paraformaldehyde for 10 min at a room temperature, washed twice in PBS of pH 7.4 and permeabilised by 0.1% Triton X-100 for 5 min. After the preincubation in 10% FCS 0.1% BSA TBS for 60 min, the immunodetection of antigens was performed. In the Table 2, the antibodies, dilutions and the incubation time are summarised. Double

immunostainings were performed to identify the cell type expressing DPP-IV and FAP. The sequential protocol was introduced; the slides were incubated with first primary antibody overnight at 4 °C (anti DPP-IV or anti FAP) and followed by 1 hour incubation with the appropriate secondary antibodies. Subsequently, the second staining for the markers of differentiation was done (vW, GFAP, CD105, TEM-1, SOX-2, TE-7). The nuclei were stained by Hoescht (bisbenzimidazole, Sigma) in f.c. 0.1 µg/ml for 1 hour, at room temperature. The samples were mounted in the anti-fading Gel/Mount medium, observed and photographed using inverse fluorescent microscope IX70 (Olympus).

**Table 2. Antibodies used for the immunocytochemical and immunohistochemical analysis.**

Antibody (species, clone/cat.No, company)	Dilution	Incubation time
Anti DPP-IV (mouse, MA 261, Abcam, UK)	1:100	overnight
Anti DPP-IV (rat, E19, Vitatex, USA)	1:100	overnight
Anti FAP (rat, D8, Vitatex, USA)	1:100	overnight
Anti FAP (rat, D28, Vitatex, USA)	1:100	overnight
Anti CD105 (mouse, SN6h, DAKO, Denmark)	1:250	60 min
Anti GFAP (mouse, GP-01, Abcam, UK)	1:200	60 min
Anti Iba (rabbit, 019-19741, Wako, Japan )	1:250	60 min
Anti human fibroblast (mouse, TE-7, Chemicon, Germany)	1:100	60 min
Anti TEM-1 (rabbit, ab67275, Abcam, UK)	1:100	60 min
Anti SMA (mouse, 1A4, Abcam, UK)	1:200	60 min
Anti SOX-2 (rabbit, ab97959, Abcam, UK )	1:800	60 min
Anti vW (rabbit, A 0082, DAKO, Denmark)	1:200	60 min
Alexa Fluor 488 goat anti rat IgG (goat, A11006, Molecular Probes/Invitrogene, Paisley, UK)	1:500	60 min
Alexa Fluor 546 goat anti rat IgG (goat, A11081, Molecular Probes/Invitrogene, Paisley, UK)	1:500	60 min
Alexa Fluor 488 donkey anti mouse IgG (donkey, A21202, Molecular Probes/Invitrogene, Paisley, UK)	1:500	60 min
Alexa Fluor 546 donkey anti mouse IgG (donkey, A10036, Molecular Probes/Invitrogene, Paisley, UK)	1:500/600/700	60 min
Alexa Fluor 546 goat anti rabbit IgG (rabbit, A11010, Molecular Probes/Invitrogene, Paisley, UK)	1:250/350/450/900	60 min

### 3.9. Adhesion assay

Cell adhesion was assessed in collagen coated 96-well plates. The wells were coated with collagen type I or type IV (5µg/cm<sup>2</sup>) and subsequently blocked with 0.1% BSA in

DMEM for 15 min. Cells were grown for 24 hours in 10% FCS in DMEM. Subsequently, media were exchanged for 10% FCS in DMEM with or without Doxycycline in final concentration  $10^{-6}$  mg/ml. After 72 hours of DPP-IV or FAP induction, cells were harvested and resuspended in 0.1% BSA (Sigma) in DMEM. 50 000 cells were added to the wells and attached for 15 and 30 min at 37°C. Non-adherent cells were gently removed with three PBS washes. The adherent cells were fixed by 5% glutaraldehyde and stained with methylene blue. Cells were lysed with 1% SDS and the absorbance was read at 630 nm using 96-well plate reader (Sunrise, Tecane).

### *3.10. Spreading assay*

Cell spreading was assessed in the collagen type I or type IV coated 12-well plates. Wells were coated with collagen type I or type IV ( $5\mu\text{g}/\text{cm}^2$ ). FAP transfected U373 were grown for 48 hours in 10% FCS in DMEM. Media were subsequently exchanged for 10% FCS in DMEM with or without Doxycycline in final concentration  $10^{-6}$  mg/ml. After 72 hours of FAP induction, cells were trypsinized and resuspended in 0.1% BSA (Sigma) in DMEM. 50 000 cells were added to the wells that have been blocked with 0.1% BSA in DMEM for 15 min, and allowed to attach at 37 °C for indicated time. Subsequently the adherent cells were fixed by 5% glutaraldehyde and stained with methylene blue R-250. The cells were photographed using inverse fluorescent microscope Olympus IX70.

### *3.11. Statistical analysis*

The Mann-Whitney test was used to evaluate the differences between the investigated groups. Correlation of quantitative variables was assessed by computing the Spearman's correlation coefficient. For calculations, software package Statistica 8.0 (StatSoft, Tulsa, USA) was used.

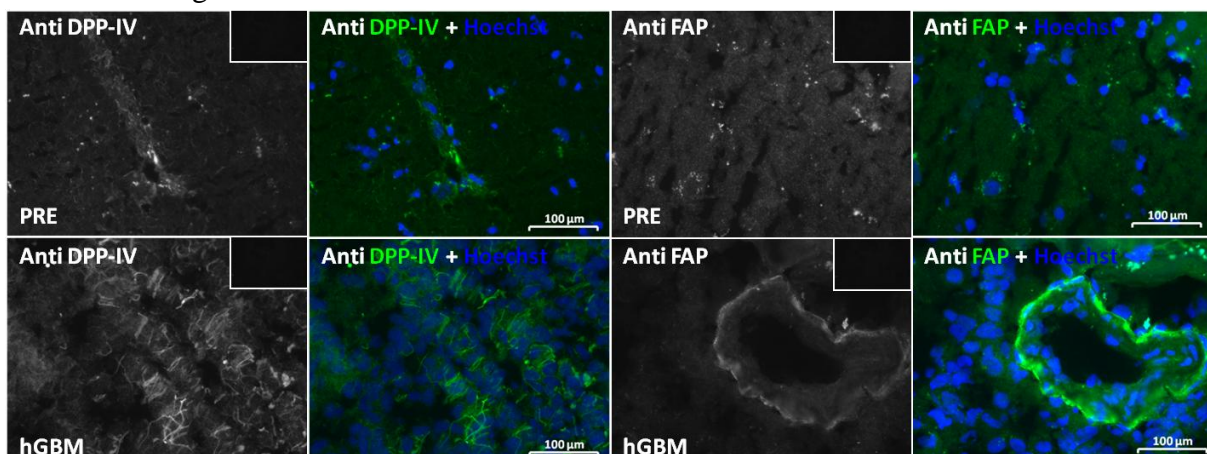
## 4. Results

### 4.1. *In situ* DPP-IV and FAP expression in human glioblastoma multiforme

To characterize the distribution of DPP-IV and FAP in the brain tumour microenvironment, immunohistochemistry analyses were performed by using mAb clone E19 or MA 261 (anti DPP-IV) and mAb clone D8 or D28 (anti FAP). Similarly to our previously published data (Stremenova et al. 2007), DPP-IV and FAP were detected mainly in tumorous tissue and were either absent or present only in traces in non-tumorous brain (Figure 1, 3). In tumours, DPP-IV was detected mainly in the parenchyma, it was also sporadically observed in the perivascular compartment. FAP expression was evident in both parenchymal as well as in the perivascular compartments. Both antibodies targeting FAP were able to identify both FAP positive cell subpopulations; however, mAb D8 preferentially bound to the perivascular FAP positive cells, while the mAb D28 stained mainly FAP positive cells localized in the parenchyma. The DPP-IV staining pattern was identical using the rat clone E19 as well as the mouse clone MA 261.

#### **Figure 1. Immunodetection of DPP-IV and FAP in the human glioblastoma multiforme and non-tumorous brain.**

DPP-IV (clone MA 261) and FAP (clone D8) in green; nuclei in blue (Hoechst). Human glioblastoma multiforme (hGBM), non-tumorous brain (PRE – pharmacoresistant epilepsy). Inserts: staining controls.



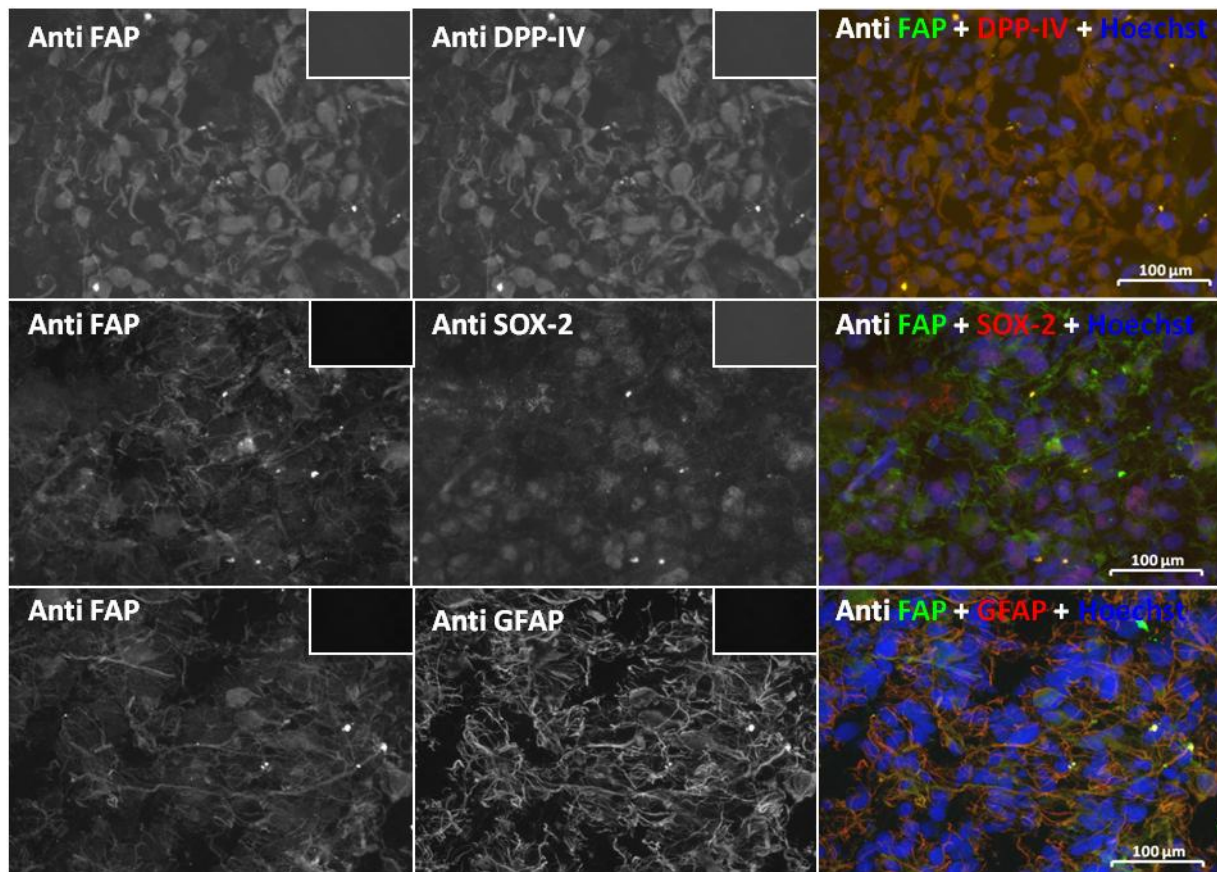
Double staining analysis revealed that the intraparenchymal FAP positive cell population co-expressed also DPP-IV, glial fibrillary acidic protein (GFAP) and the transcription factor SOX-2, a marker of the multipotent neural and glioma stem cell (Figure 2A). These cells were negative for the endothelial cell markers such as von Willebrand factor

(vW) or endoglin (CD105) and for the markers of mesenchymal elements such as  $\alpha$ -smooth muscle actine (SMA), microglial marker (Iba) and tumour endothelial marker 1 (TEM-1) (data not shown). Thus, these cells apparently represent the transformed elements of glial origin. On the other hand, perivascular FAP positive subpopulation was vW, GFAP, CD105 and SOX-2 negative, it sporadically expressed SMA, TEM-1 and the fibroblast antigen detected by TE-7 antibody (Figure 2B). These perivascular FAP positive cells were found in approximately 50 % (10/20) of the analysed samples. Interestingly, they typically surrounded dysplastic and hypertrophic vessels (Figure 2B). Since the FAP positive perivascular cells co-expressed several mesenchymal but lacked the glial markers, they highly probably belong among to the mesenchymal elements.

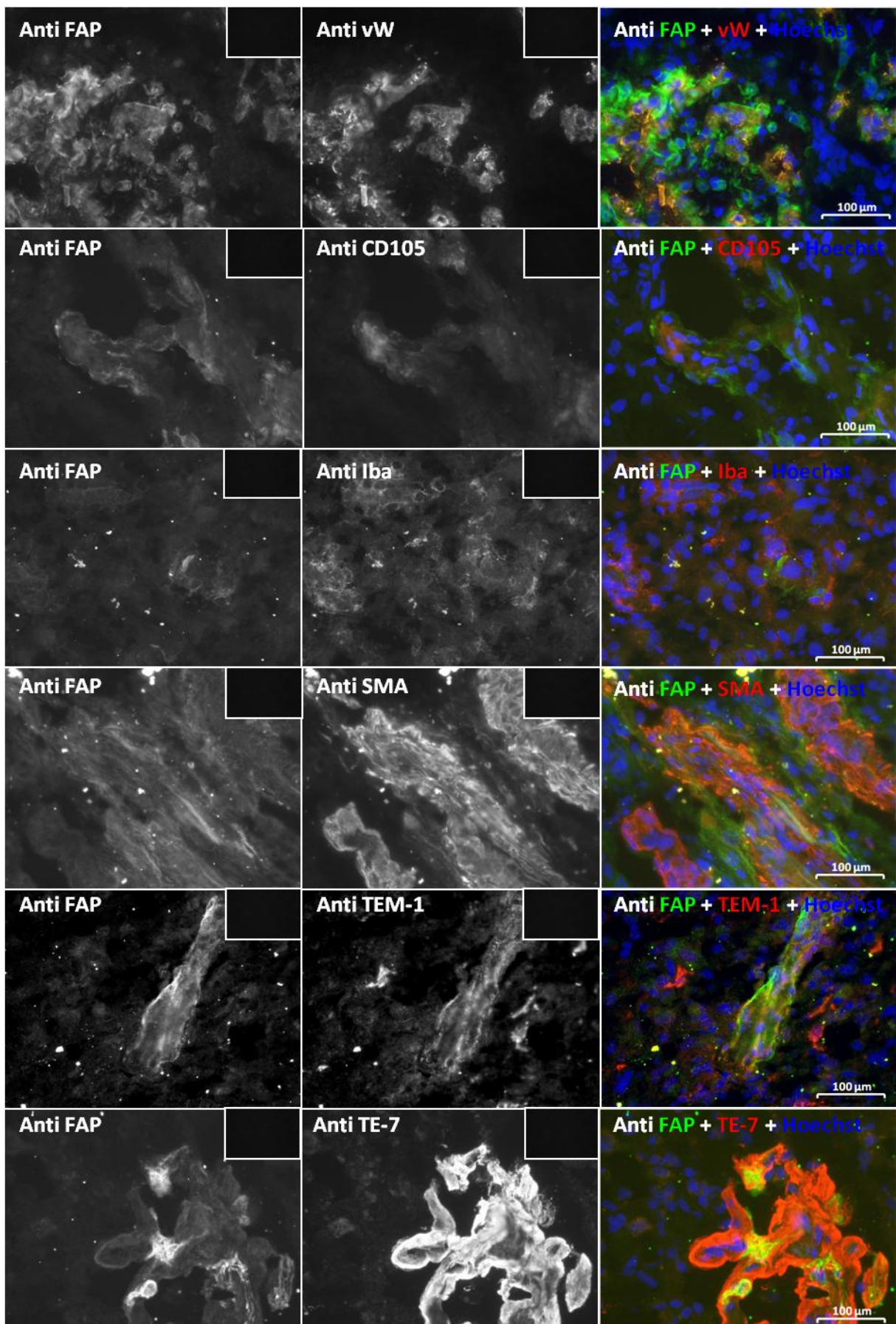
**Figure 2. Double-staining analysis of FAP positive cells in human glioblastoma multiforme.**

FAP positive cells localized (A) in the parenchyma and (B) in the perivascular compartment. FAP in green; DPP-IV, SOX-2, GFAP, vW, CD105, Iba, SMA, TEM-1 and TE-7 in red; nuclei in blue, stained by Hoechst. Inserts: staining controls.

**A**



**B**



#### *4.2. DPP-IV and FAP co-expression in primary astrocytic cell cultures*

Similarly as in the bioptic samples of glioblastoma multiforme (Stremenova et al. 2007), we observed a positive correlation between the expression of DPP-IV and FAP on the mRNA and protein level in the primary cell cultures (Figure 3A). We analyzed several primary cell cultures to identify active DPP-IV and FAP; (representative samples in Figure 3B, 3C, 3D). Immunoblotting using mAb D8 (anti FAP) and mAb E19 (anti DPP-IV) identified single band at about 150 kDa in non-denaturing conditions (Figure 3B). The possible antibody cross-reactivity was excluded using the DPP-IV and FAP transfected cells as controls. Membrane overlay assay using H-Gly-Pro-AMC as a substrate targeting post-proline-specific exopeptidase activity confirmed that the band contains the active enzyme possessing DPP-IV-like enzymatic activity (Figure 3C). Using the zymography in conditions of MMPs inhibition (+2 mM EDTA) we demonstrated the band of MW 150 kDa contained the gelatinolytic activity (Figure 3D). To summary, in primary cell culture, we identified active 150 kDa homodimers of DPP-IV and FAP.

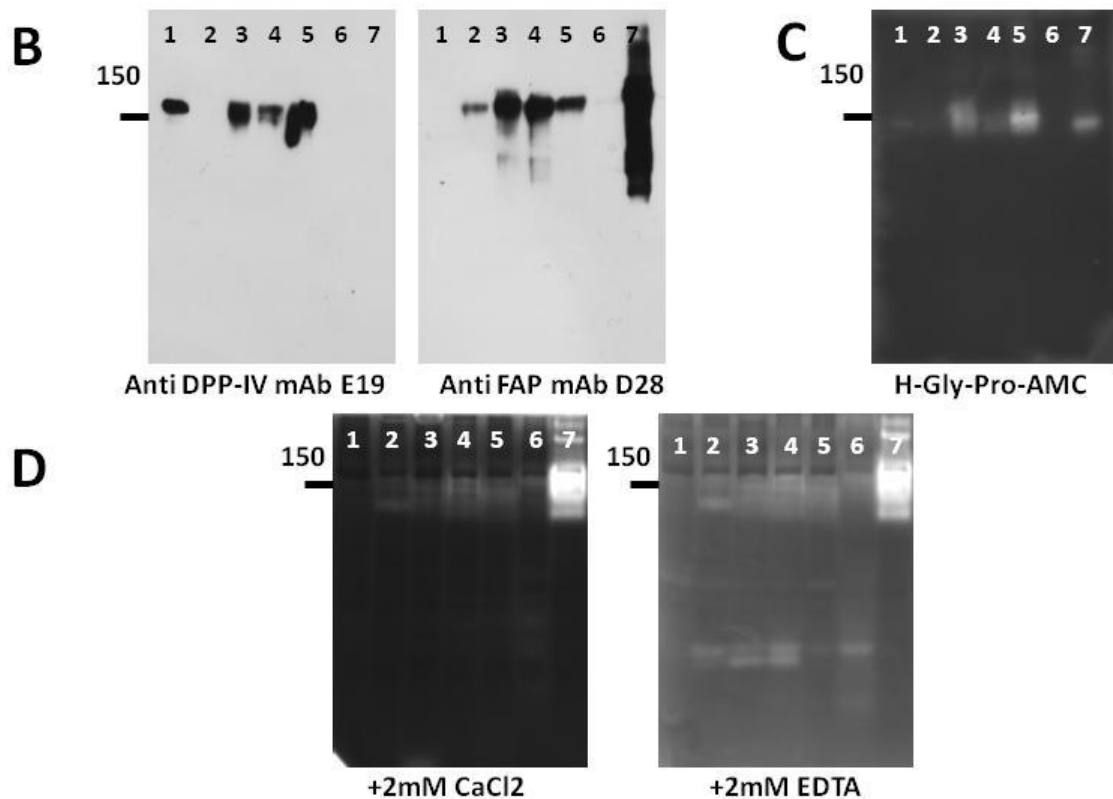
**Figure 3. DPP-IV and FAP in primary cell cultures derived from human glioblastoma multiforme.**

(A) correlation of DPP-IV and FAP transcripts and proteins; (B) immunodetection of DPP-IV (mAb E19) and FAP (mAb D28); (C) membrane overlay assay (H-Gly-Pro-AMC) of DPP-IV-like enzymatic activity; (D) zymography assay of gelatinolytic activity. Zymograms incubated in the buffers activating (+2mM CaCl<sub>2</sub>) and inhibiting (+2mM EDTA) matrix metalloproteinases.

Line 1: DPP-IV positive control; Line 2: primary cell culture 7; Line 3: primary cell culture 9; Line 4: primary cell culture 10-II; Line 5: primary cell culture 16-II; Line 6: primary cell culture 20; Line 7: FAP positive control

**A**

		Transcript		Protein	
		DPP-IV	FAP	DPP-IV	FAP
Transcript	DPP-IV	x	R=0,58; p<0.0001 N=47	R=0,67; p<0.004 N=16	n.s.
	FAP		x	n.s.	n.s.
Protein	DPP-IV			x	R=0,497; p<0.016 N=23
	FAP				x





### 4.3. DPP-IV and FAP co-expression during experimentally induced changes of DPP-IV-like enzymatic activity

To prove the dynamic association of DPP-IV and FAP expression, permanent glioblastoma cell lines U87MG and U138MG were used. Both cell lines co-express DPP-IV and FAP and exhibit the respective gene loci in the proper localization on the chromosome 2 (Figure 4A) as a necessary prerequisite for the natural biological regulation. Cell culture under the conditions of growth factor deficiency in serum-free medium substantially increases the DPP-IV-like enzymatic activity in these cell lines (Sedo et al. 1998). The upregulation of biochemically assayed DPP-IV-like enzymatic activity was associated with the increase of the expression of both DPP-IV and FAP mRNAs and also with the increase of DPP-IV and FAP proteins as determined by ELISA, immunoblotting and immunocytochemistry (Table 3, Figure 4B, 4C, 4D). Similarly to the primary cell cultures, we observed single band of about 145 kDa detected by the anti DPP-IV as well as by anti FAP antibody. This band corresponded to the DPP-IV-like exopeptidase enzymatic activity visualized by the membrane overlay assay (Figure 4C). Thus, similarly to the primary cell cultures, we identified active 150kDa homodimers of DPP-IV and FAP and moreover we demonstrated the co-expression of both the DPP-IV and FAP in permanent human glioma cell lines U87MG a U138MG.

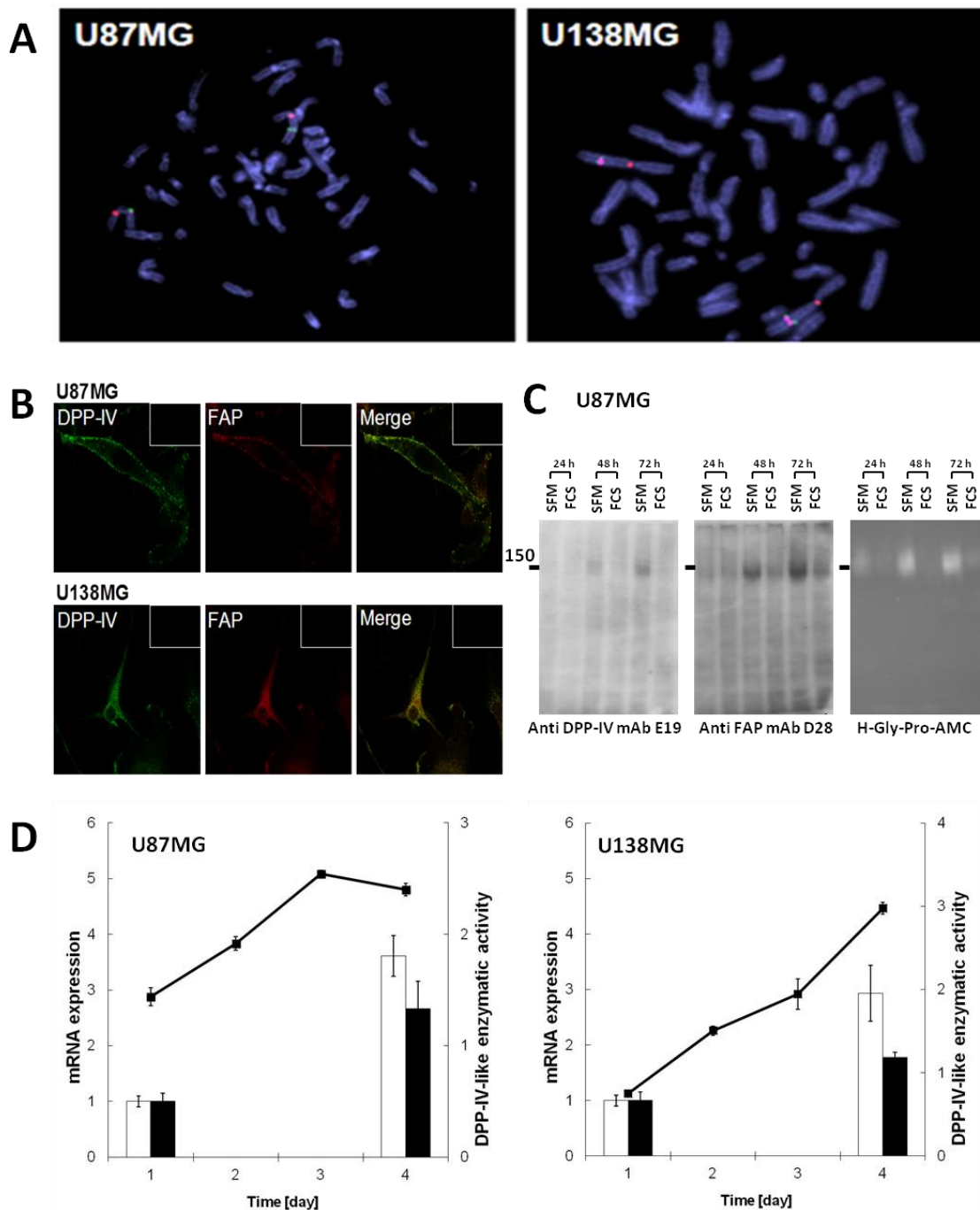
**Table 3. DPP-IV and FAP in permanent glioma cell lines, U87MG, U138MG and DPP-IV transfected U87MG.**

U87MG and U138MG cells cultured in serum free media (SFM) compared to the cells grown in 10% foetal calf serum supplemented medium (FCS). DPP-IV transfected U87MG cells stimulated to express transgenic DPP-IV by 0.25 nM Mifepristone (Mifepristone), untreated DPP-IV transfected U87MG cells (Controls).

Culture conditions		DPP-IV	FAP
		Mean $\pm$ S.D. [pg/ $\mu$ g protein]	
U87MG	SFM	56.1 $\pm$ 0.5	90.5 $\pm$ 12.3
	FCS	13.5 $\pm$ 6.7	23.3 $\pm$ 1.2
U138MG	SFM	66.4 $\pm$ 1.2	108.7 $\pm$ 0.7
	FCS	36.6 $\pm$ 3.3	88.4 $\pm$ 1.8
DPP-IV transfected U87MG	Mifepristone	105.4 $\pm$ 1.7	5.4 $\pm$ 1.7
	Controls	3.8 $\pm$ 0.7	8.4 $\pm$ 1.8

**Figure 4. DPP-IV and FAP in permanent glioma cell lines U87MG and U138MG.**

(A) genomic localization of DPP-IV (green) and FAP (rouge) loci on the chromosome 2 detected by FISH. Centromeric probe for chromosome 2 in red; (B) immunodetection of DPP-IV (mAb MA 261, green) and FAP (mAb D8, red); (C) immunoblotting and membrane overlay assay (H-Gly-Pro-AMC) of DPP-IV and FAP (150 kDa) derived from the U87MG cells cultured in serum free media (SFM) compared to the ones grown in 10% foetal calf serum supplemented medium (FCS) for 24, 48 and 72 hours (h); (D) relative increment of DPP-IV (white bars) and FAP (black bars) mRNA expression and plasma membrane DPP-IV-like enzymatic activity (■). U87MG and U138MG cells cultured in serum free media compared to the cells grown in 10% foetal calf serum supplemented.

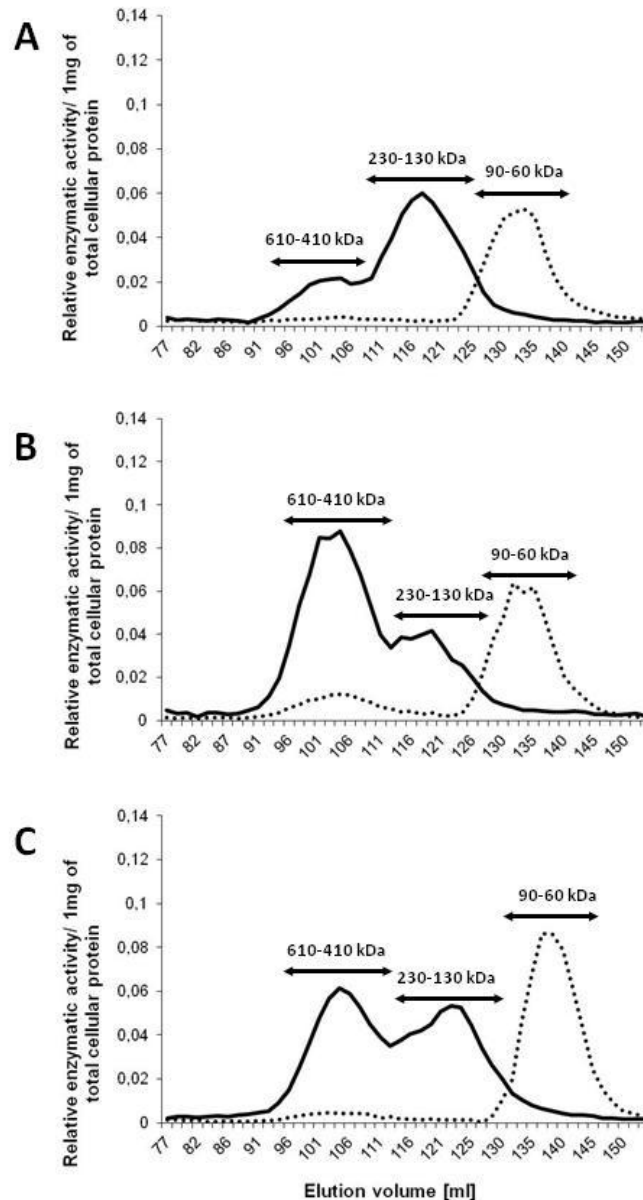


To distinguish the participation of DPP-IV and FAP within the whole DPP-IV-like enzymatic activity, the gel filtration step preceded the enzymatic activity assay. Elution profile of the exopeptidase DPP-IV-like enzymatic activity (using H-Gly-Pro-AMC as a substrate) demonstrated two molecular weight (MW) forms of about 410 - 610 kDa and 130 - 230 kDa MW respectively. Using Z-Gly-Pro-AMC as a substrate to detect the proline-specific endopeptidase enzymatic activity, two peaks corresponding to 410 - 610 kDa and 60 - 90 kDa were detected. In U87MG cells cultured in serum free media, the DPP-IV-like exopeptidase as well as the proline-specific endopeptidase enzymatic activity increased in the MW-region of about 410 - 610 kDa (Figure 5A, 5B).

The changes in DPP-IV-like exopeptidase and the proline-specific endopeptidase enzymatic activities induced by cultivation under the growth factors' deficiency conditions were fully reverted by the addition of 10% foetal calf serum into the culture media (Figure 5C). In line with that, further experiments demonstrated decrease of the DPP-IV and FAP protein expression associated with the replacement of serum free medium with 10% foetal calf serum (data not shown).

**Figure 5. Elution profile of the DPP-IV-like exopeptidase and proline-specific endopeptidase activity in the U87MG cell line.**

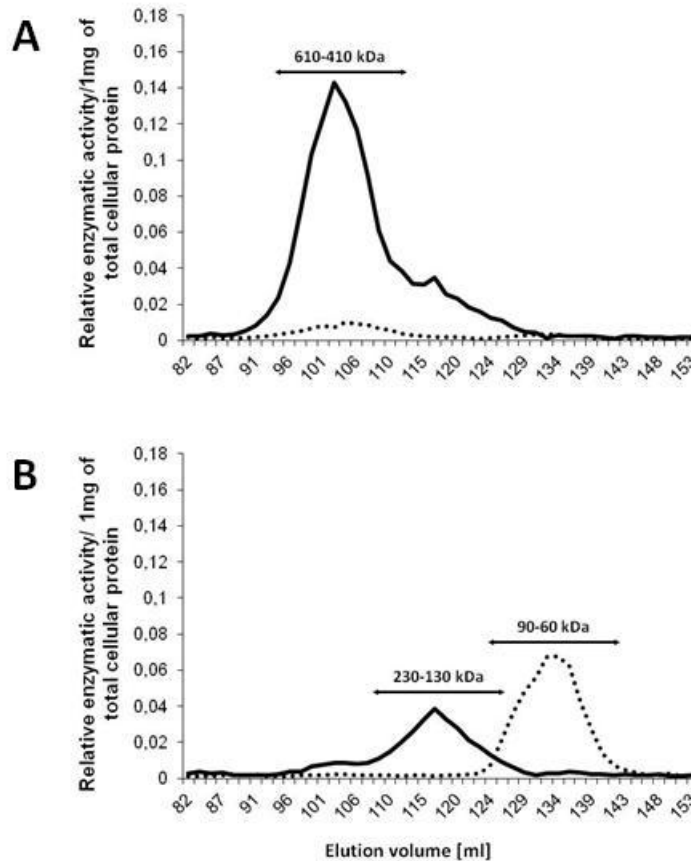
U87MG cells were cultured for (A) 72 hours in 10% foetal calf serum supplemented medium; (B) 72 hours in serum free medium; (C) 72 hours in serum free medium followed by 48 hours in 10% foetal calf serum supplemented medium. DPP-IV-like exopeptidase activity (full line); proline-specific endopeptidase activity (dotted line).



Further analysis of the elution profiles revealed that both DPP-IV-like exopeptidase and prolyl-endopeptidase enzymatic activities in the MW-region of about 410 - 610 kDa were detectable in the solubilized membrane fraction, but not in the soluble fraction. On the contrary, the 130-230 kDa DPP-IV-like exopeptidase and 90-60 kDa prolyl-endopeptidase activities were characteristic for the soluble fractions (Figure 6).

**Figure 6. Elution profile of the DPP-IV-like exopeptidase and proline-specific endopeptidase activity in U87MG cell fractions.**

(A) solubilized membrane fractions; (B) soluble fractions. U87MG cells were cultured in serum free medium for 72 hours. DPP-IV-like exopeptidase enzymatic activity (full line) and proline-specific endopeptidase enzymatic activity (dotted line).



Contrary to U87MG cells, the prolyl-endopeptidase activity in the MW-region of about 410 - 610 kDa was not detectable in U138MG cells under both, the growth factor proficiency and deficiency culture conditions, while the DPP-IV exopeptidase activity in U138MG cells rose similarly as in U87MG cells (data not shown).

In conclusion, in U87MG cell line, we identified membrane-associated specific DPP-IV-like exopeptidase and specific prolyl-endopeptidase enzymatic activity co-migrating in MW-region of about 410 – 610 kDa.

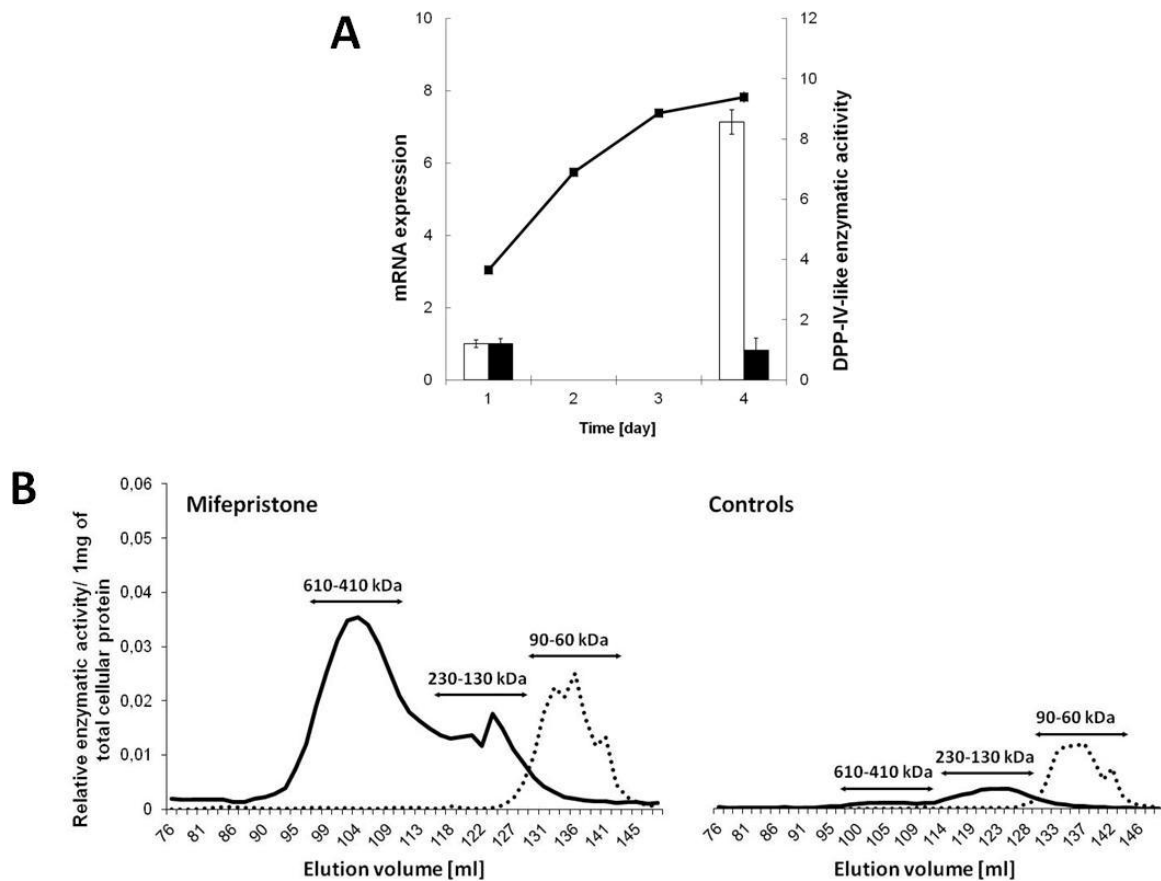
#### 4.4. Expression of FAP in DPP-IV transfected U87MG cell line

To reveal the mechanism of DPP-IV and FAP coupled expression in permanent cell lines the experiments with the DPP-IV transfected U87MG cells were done. In this model, the

DPP-IV transgene is highly probably localized in the aberrant genomic context and thus operated by different transcriptional factors. In these experiments, neither the upregulation of FAP mRNA and protein expression (Figure 7A, Table 3) nor the increment of the relevant proline-specific endopeptidase enzymatic activity (Figure 7B) were observed after the induction of DPP-IV overexpression.

**Figure 7. DPP-IV and FAP in the DPP-IV transfected U87MG cell line.**

(A) relative increment of DPP-IV (white bars) and FAP (black bars) mRNA expression and plasma membrane DPP-IV-like enzymatic activity (■); (B) elution profile of the DPP-IV-like exopeptidase (full line) and proline-specific endopeptidase (dotted line) activity. DPP-IV transfected U87MG cells were stimulated to express the DPP-IV transgene by 0.25 nM Mifepristone (Mifepristone) treatment. Controls = untreated DPP-IV transfected U87MG cells.



#### 4.5. The effect of DPP-IV and FAP expression on cell adhesion and spreading

To characterize the effect of DPP-IV and FAP on cell adhesion and spreading, we used the transgenic system of DPP-IV or FAP expressing U373 cell line. In this model,

neither the expression of transgenic DPP-IV nor FAP induced the expression of the other enzyme (Figure 3B). In adhesion assays, two different cell clones of DPP-IV as well as FAP transfected cells were used in experiments.

Upregulation of transgenic wild type DPP-IV impaired adhesion of U373 glioma cells on a plastic coated with collagen type I. The cells expressing DPP-IV exhibited about 30% less adhesion than the controls in first 15 min. However, the negative effect of DPP-IV on the cell adhesion became less evident in the following time intervals tested. The overexpression of mutated enzymatically inactive transgenic DPP-IV did not alter adhesion of U373 at all (Figure 8A).

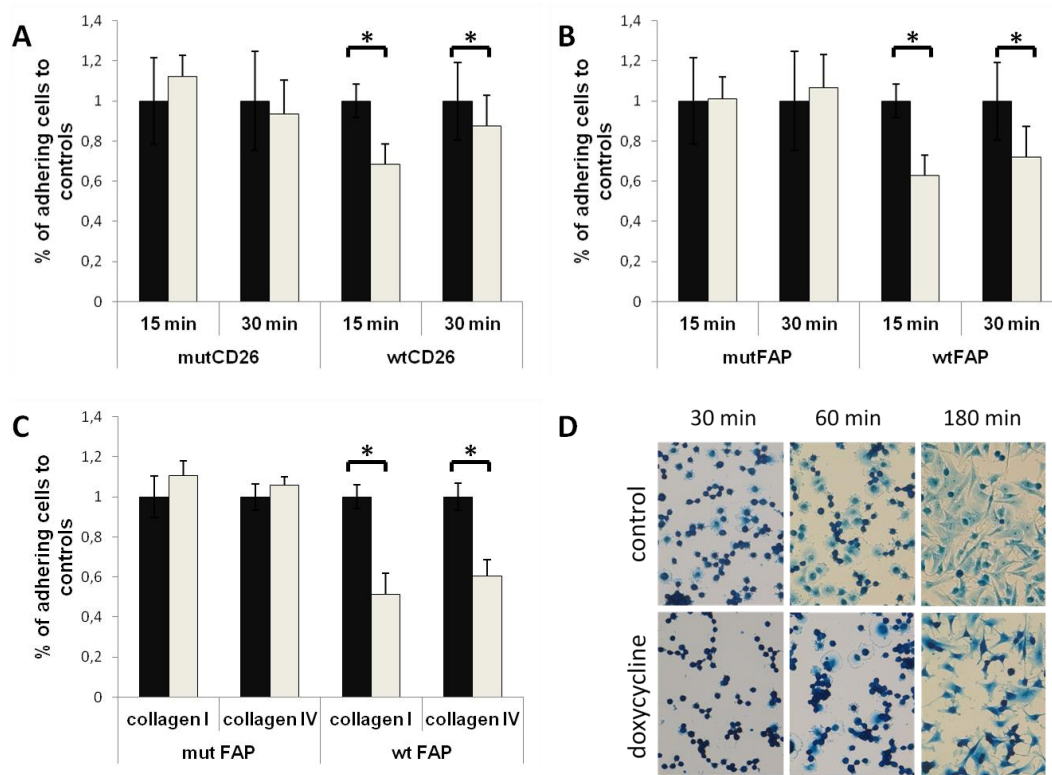
Similarly to the cells expressing wild type DPP-IV, transgenic expression of the wild type FAP negatively influenced adhesion and spreading of U373 glioma cells on collagen type I and IV (Figure 8B, 8C, 8D). The expression of mutated enzymatically inactive FAP did not induce the impairment of cell adhesion (Figure 8B, 8D).

In conclusion, enzymatic activity of both DPP-IV and FAP may act as a negative factor of glioma cell adhesion to the components of extracellular matrix.

**Figure 8. Effect of DPP-IV and FAP on glioma cell adhesion and spreading.**

(A) % of adhering DPP-IV overexpressing cells (white bars) to the controls (black bars) to the collagen I in 15 and 30 min (B) % of adhering FAP overexpressing cells (white bars) to the controls (black bars) to the collagen I in 15 and 30 min (C) % of adhering FAP overexpressing cells (empty bars) to controls (full bars) to the collagen I and IV in 15 min (D) Spreading of wild type FAP transfected U373 cells on collagen type I

DPP-IV or FAP transfected U373 cells were stimulated to express the given wild type (wt) or mutated (mut) transgene by  $10^{-6}$  M Doxycycline treatment. Controls = untreated transfected U373 cells.





## 5. Discussion

The group of “Dipeptidyl peptidase-IV activity and/or structure homologous” molecules was first defined in 2001 (Sedo and Malik 2001). Strong evidence about their contribution on multiple physiological and pathological processes, including the growth, migration and invasion of transformed cells has been brought in the last decade. The processing of the bioactive peptides by DPP-IV-like proteolytic activity results either in their functional termination (Proost et al. 1998), or change of their receptor subtype affinity (Kitlinska et al. 2003) and thus “switching” among relevant biological pathways. Moreover, at least some of the DASH biological functions are also based on non-hydrolytic protein-protein interactions. DASH molecules, their substrates and binding partners are seen by some authors as a “DASH system” which deregulation is involved into the pathogenesis of several diseases, including cancer. Due to the similar enzymatic activity, molecular structure and in some cases also co-localization in the same subcellular compartment (e.g. membrane-bound DPP-IV and FAP, intracellular soluble DPP8 and 9), individual DASH molecules might in some cases either substitute each other. Finally, DASH molecules (e.g. DPP-IV and FAP), although individually expressed on different cell populations, co-participate on the complex tumour microenvironment. Together, context of specific DASH patterns on the subcellular, cellular and tumour-tissue levels may determinate resulting overall pro- or anti- oncogenic effect of given DASH molecule (Busek et al. 2004; Sulda et al. 2006; Kotackova et al. 2009).

In this study, we describe DPP-IV and FAP expression pattern within the human glioblastoma microenvironment and demonstrate for the first time coupled DPP-IV and FAP expression in transformed glial cells. Finally, we analyze the possible implication of DPP-IV and FAP in the transformed cell adhesion.

### *5.1. Expression pattern and localisation of DPP-IV and FAP in human glioblastoma multiforme (Aim 1)*

In our study, we demonstrate the parenchymal and perivascular localisation of both DPP-IV and FAP. In the tumour parenchyma, but not in the perivascular compartment, we found dispersed double positive DPP-IV/FAP and FAP/GFAP glial cells. The sporadic expression of SOX-2 within these cells suggests that, at least part of this population, represents cells of glial origin possessing characteristics of malignant transformation (Fang et al. 2011). Since SOX-2 is also reported as a one of the markers of stemness, possible

pluripotent potential of these SOX-2 positive DPP-IV/FAP and FAP/GFAP double positive cell populations should be considered. Correlative expression of DPP-IV and FAP transcripts and antigens in human glioblastoma multiforme tissue has previously been noted by our team. However, in this prior work, the particular cell type(s) expressing both molecules has not been analysed (Stremenova et al. 2007). Our recent results correspond well to the very recent observations of the FAP/GFAP double positive cells in the malignant glioma tissue (Mentlein et al. 2011). In addition, we found FAP positive but DPP-IV and GFAP negative cell population in the perivascular localization. Such cells were tightly associated with the dysplastic and hyperproliferative tumour vessels. Because such FAP positive cells sporadically express the markers of mesenchymal cells, such as SMA, TEM-1, TE-7, but not antigens attributed to the endothelia, microglial and astrocytic elements (vW, CD105, Iba, GFAP), their mesenchymal origin seems probable. The pericytes, vascular smooth muscle cells and myofibroblast/fibroblast belong to the group of mural mesenchymal cells, surrounding endothelium and basal membrane (Kurz et al. 2004). Although several mesenchymal markers have been proposed to identify these cell types, however their specificity is not fully satisfactory (Sugimoto et al. 2006). Moreover, the potential of some cell types to transdifferentiate has been demonstrated (Rajkumar et al. 2005). Thus, strict identification of given cell population remains partially equivocal. FAP was originally described as a marker of activated fibroblasts. Such fibroblasts (also known as myofibroblasts) upregulate expression of contractile molecules to increase their migratory potential. Moreover, recent work of other groups reported the expression of FAP on the peripheral myofibroblast/pericytes in a rodent experimental model (Wang and Shi 2009). Together our results demonstrating co-expression of SMA and TEM-1, marker of glioblastoma-associated pericytes (Christian et al. 2008; Simonavicius et al. 2008), we conclude that the perivascular FAP positive cells in glioblastoma tissue are either the pericytes or activated fibroblast/myofibroblasts.

## *5.2. Co-expression of DPP-IV and FAP in glioma (Aim 2)*

The significant correlation of DPP-IV and FAP mRNA templates and the respective proteins is found in the glioblastoma-derived primary cell cultures (Table 4A). The correlation between the DPP-IV protein expression and DPP-IV-like enzymatic activity (Balaziova et al. 2006) suggests the canonical DPP-IV represents the substantial molecular source of the given exopeptidase activity. Moreover, further analysis of the putative molecular forms possessing DPP-IV enzymatic activity using immunoblotting revealed single

band of about 150 kDa, stained by both anti DPP-IV and anti FAP antibody respectively. This band was colocalizing with the one detected by fluorogenic substrate H-Gly-Pro-AMC membrane overlay assay as well as by gelatinolytic enzymatic activity visualized by the zymography assay. Similarly to that, DPP-IV and FAP immunopositive band of about 150 kDa comigrating with the DPP-IV-like enzymatic activity was found also in both tested permanent glioma cell lines, U87MG and U138MG. Molecular weight of DPP-IV and FAP, both naturally occurring as homodimers, varies depending on the source, typically around 220kDa and 170-150 kDa, respectively. However, Gorrell et al observed 140 – 160 kDa homodimers of DPP-IV in cirrhotic liver and lymphocytes (Gorrell et al. 2001) and group of Chen described 37 kDa enzymatically active FAP isoform in melanoma cells (Chen et al. 2006). The authors suggested that the lower MW of DPP-IV and FAP can either be product of an alternative splicing or can represent differentially glycosylated form, specific for the given cellular source. Together, we presume that the glioma cells most probably express enzymatically active DPP-IV and FAP, both of 145-150 kDa MW. However, existence of DPP-IV/FAP heterodimers, hypothesized by Ghersi et al., based on his experiments on fibroblasts and endothelial cells (Ghersi et al. 2003; Ghersi et al. 2006) might also partially explain conjunction of results of our immunodetection and enzyme activity assays. However, the existence of such “chimeric” heterodimers still awaits to be confirmed or denied.

### *5.3. Coupled expression of DPP-IV and FAP in glioma cells (Aim 3)*

To confirm the dynamic association of the DPP-IV and FAP expression, hypothesized on the basis of previous correlative observations, the model of permanent glioblastoma cell lines was introduced. For this purpose, we choose U87MG and U138MG cell lines, both spontaneously expressing DPP-IV and FAP. The respective DPP-IV and FAP gene loci in the proper localization on the chromosome 2, a necessary prerequisite for a proper biological regulation, were verified by FISH (Figure 4A). The upregulation of DPP-IV-like enzymatic activity, induced by the culture propagation in conditions of growth factors deficiency (Sedo et al. 1998; Sedo et al. 2004), is associated with the coupled upregulation of both DPP-IV and FAP transcripts and protein in both glioma cell lines (Figure 4; Table 3). Due to their similar hydrolytic properties, both molecules are probably participating on the overall assayed DPP-IV-like enzymatic activity. To assess the share of FAP on the total DPP-IV-like enzymatic activity, the proline-specific endopeptidase enzymatic activity exhibited by FAP, but not by the canonical DPP-IV, was measured using Z-Gly-Pro-AMC as a substrate (Barelli et al. 1999). Since the prolyl endopeptidase (PEP; EC 3.4.21.26), differing from FAP by the MW,

is in general responsible for the substantial part of cellular proline-specific endopeptidase activity, gel filtration chromatography preceded the biochemical enzyme activity assay in order to take apart FAP from PEP.

In U87MG cells cultured in the conditions of growth factors deficiency, the increment of both the DPP-IV-like exopeptidase and the proline-specific endopeptidase enzymatic activities are observed in the MW-region of about 410 - 610 kDa (Figure 5) and all observed changes were reverted by the replacement serum free condition with 10% FCS. Similar oligomeric complexes possessing DPP-IV-like enzymatic activity (480 - 900 kDa) are known to occur in the other cell types and are suggested to be a result of DPP-IV and FAP homodimers aggregation (Scanlan et al. 1994; Ghersi et al. 2002). 80 kDa proline-endopeptidase activity peak (Figure 7) present in the elution profiles of the soluble, but not solubilized membrane cell fraction corresponds well with the cytosolic PEP with expected molecular weight (Polgar 2002).

Upregulation of FAP mRNA and protein transcription following elevation of DPP-IV induced by growth factor deficiency was observed also in U138MG cells. However, contrary to U87MG, we did not observe expected increment of proline-specific endopeptidase enzymatic activity in the MW region of about 410 - 610 kDa attributed to FAP in U138MG (data not shown). Thus, an abundant expression of FAP transcript accompanied by a high FAP protein concentration, together with the absence of characteristic endopeptidase activity may argue for the expression of enzymatically inactive FAP alternatively spliced variant(s) in these cells. According to the recent data, at least eleven alternatively spliced variants of the FAP primary transcript have been proposed, some of them encoding putative enzymatically inactive FAP isoforms (Niedermeyer et al. 1997; Goldstein and Chen 2000).

To further approach the mechanism of observed association of DPP-IV and FAP expressions, a model of DPP-IV transfected glioma cells was used, but the coupled expression of transgenic DPP-IV and endogenous FAP was not observed there (Balaziová et al. 2011).

Together, we demonstrated for the first time the coupled expression of DPP-IV and FAP transcripts and proteins in the transformed glial cells. Uncoupled expression of transgenic (exogenous) DPP-IV and the intrinsic (endogenous) FAP in the DPP-IV transfected glioma cells suggests that the coupling of expression of both molecules is more likely a result of a joint control of their expression, rather than a consequence of an indirect reciprocal posttranscriptional regulation involving changes of their mRNA and/or protein expression (Balaziová et al. 2011). In contrast, Wesley and colleagues described the induction

of FAP expression following the experimental restoration of DPP-IV in primarily DPP-IV negative non small cell lung cancer cell line (Wesley et al. 2004). However, this observation was surprisingly neither further analyzed nor discussed in their paper.

#### 5.4. DPP-IV and FAP in adhesion of glioma cells to the components of extracellular matrix (Aim 4)

Demonstrated co-regulation of DPP-IV and FAP in glioma elements implies their functional cooperation in the glioma cells biology. In order to test the effect of DPP-IV and FAP molecules on the cell adhesion and spreading, we used U373 cell line, naturally expressing extremely low quantities of both enzymes and prepared clones with inducible expression of either DPP-IV or FAP transgenic proteins.

Considering significant accumulation of FAP positive cells to the perivascular compartment observed in bioptic material, our *in vitro* adhesion assays were performed on plastic coated with collagen type I and IV. Overexpression of transgenic FAP significantly decreased the cell adhesion to both types of collagen. Moreover, this effect was dependent on the FAP enzymatic activity. Preliminary results of our laboratory also suggest participation of FAP in the processes of glioma cell migration and invasion (not shown, see Enclosure 6). In view of these observations, we hypothesize that in our model of the glioma cells, FAP alone or in cooperation with other matrix degrading system processes collagen, which then serves as worse adhesion substrate for the cells. Interestingly, the FAP role in cellular adhesion and migration might be cell type specific. Similarly to our results, Wang et al observed decrease of cell adhesion and migration in a model of HEK 293 epithelial cell line. In contrast to that, the same authors noted an opposite effect of FAP in LX-2 human stellate cell line (Wang et al. 2006). Although FAP is known to cooperate on the cleavage of extracellular matrix components with the matrix metalloproteinases and plasmin (Chen and Kelly 2003; Christiansen et al. 2007), Wang et al. demonstrated that the FAP effect in their experiments was independent on its hydrolytic activity at all. Based on these results, Wang concluded that the impairment of cell adhesion might be caused by the FAP-associated downregulation of integrin- $\beta$ 1 and upregulation of matrix metalloproteinase 2 and CD44.

Similarly to FAP, overexpression of enzymatically active, but not mutant, transgenic DPP-IV impaired the adhesion of the glioma cell to the collagen type I. These results fit well with previously observed potential of DPP-IV to hinder glioma cell migration and adhesion to fibronectin (Busek et al. 2012). The collagen binding domain of DPP-IV is presumed to be localized in its cystein-rich region (Loster et al. 1995). However, whether the DPP-IV binds

collagen directly or via fibronectin interaction remains disputable (Hanski et al. 1985; Cheng et al. 2003). Wang demonstrated positive effect of overexpression of both enzymatically active and inactive DPP-IV on the cell adhesion to fibronectin, but not to collagen, and the negative effect on migration of HEK 293 epithelial cells (Wang et al. 2006). Similarly, transgenic DPP-IV expression increased the adhesion of ovarian carcinoma cells to fibronectin (Kikkawa et al. 2003). Considering all of that, here we report for the first time observations suggesting a DPP-IV enzymatic activity may hinder cell adhesion to collagen. The presence of collagen binding domain within the DPP-IV sequence primarily implies its adhesive function. However, it might also be speculated that the hydrolytic effect of the enzyme on the locally present bioactive peptides, controlling processes of cell adhesion and migration, may, cell-system specifically, weaken or even revert the assumed straightforward pro-adhesive potential of DPP-IV molecule.

Together, we suggest that DPP-IV and FAP might represent important players in complex molecular machinery moderating cell adhesion and migration. Demonstrated coupled expression of DPP-IV and FAP, their proven potential to affect balances of bioactive substrates as well as structural proteins of extracellular matrix, together with their non-hydrolytic functions argue for contextual role of the “DASH system” in biological processes.

## 6. Conclusion

In this study, we demonstrated:

- In human glioblastoma multiforme, DPP-IV and FAP are expressed on the GFAP and SOX-2 positive cells, which represent probably the transformed glioma elements.
- In human glioblastoma multiforme, FAP is also expressed on the mesenchymal cell population, which is tightly associated with dysplastic and hyperproliferative vessels.
- In human glioblastoma multiforme, DPP-IV and FAP are co-expressed, at least, at some of glioma cell populations. Using *in vitro* model of the permanent glioma cell lines, we demonstrate that the coupled of their expression is likely a result of a joint DPP-IV and FAP transcriptional control.
- Individual expression of both transgenic, enzymatically active DPP-IV and FAP has the negative effect on cell adhesion.

These observations argue for putative involvement of DPP-IV and FAP into the malignant glioma progression and suggest the role in glioma vascularisation.

## 7. Further perspectives

In bioptic material, we have identified cell subpopulation characterized by FAP positivity and GFAP negativity, with varying positivity for mesenchymal markers, localized closely to the dysplastic vessels. Biological significance of these non-glioma cells for glioma vascularization remains unclear. The isolation and further characterization of the FAP positive cell populations would help to identify FAP function and putative alternative FAP splicing isoforms with presumed differing functional roles in transformed and non-transformed cell subpopulations. Additionally, *in vitro* co-cultivation experiments with glioma and glioma-derived endothelial cells would help to explain the contextual role of FAP in the glioma vascularization.

Our further results demonstrate that DPP-IV and FAP are coexpressed by a population of transformed glioma cells and the DPP-IV and FAP coupled expression is most probably a result of their transcriptional regulation. However, molecular mechanism and the biological consequences of this coregulation in glioma cells remain to be identified.

Finally, we approached the individual effects of DPP-IV and FAP on glioma cell adhesion. In respect to the fact that DPP-IV and FAP are co-expressed at least in some glioma cells, their functional crosstalk should be considered. To further the substance of such crosstalk, the double DPP-IV/FAP transfected cells with the constitutive expression of the one molecule and regulated expression of the second one respectively might serve as an experimental tool. In parallel, spontaneously DPP-IV and FAP expressing primary cell cultures might serve to analyze further molecular context characteristic for such double positive cells and to study biological consequences of experimental modification of expression of both enzymes and their candidate functional partners.

Mechanism of functional cooperation of DPP-IV and FAP in cell migration and invasion as well as their role on the level of cancer microenvironment awaits for experimental confirmation. Herein, we hypothesize the model mechanism of functional cooperation and participation of both DPP-IV and FAP in the tumour progression via modification of the extracellular matrix (Figure 9).

DPP-IV and FAP are present together with MMP2, MMP9, MT-MMP, uPA in the vesicles derived from the membrane of endothelial cells (Salamone et al. 2006). Close molecular proximity of FAP and uPAR on the membrane of melanoma cells has been shown

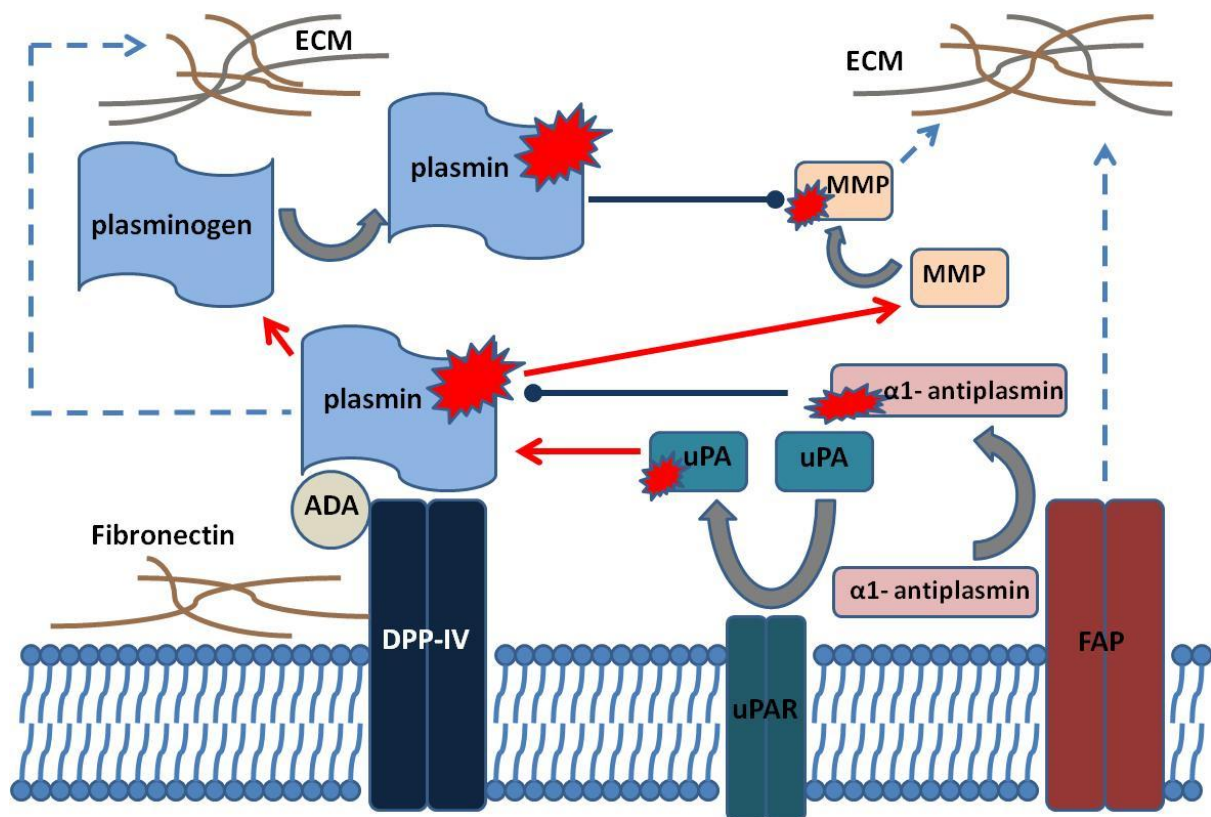


by Artym et al (Artym et al. 2002). Such intimate subcellular co-localizations argue for mutual engagement of DPP-IV, FAP and uPAR in biological functions.

DPP-IV is described as a receptor of plasminogen 2 $\gamma$ , 2 $\delta$ , 2 $\epsilon$  and ADA acts as a cofactor for this interaction (Gonzalez-Gronow et al. 2004). By means of that, plasminogen is immobilized to the plasma membrane close to its activation system of uPA/uPAR/urokinase. The conversion of plasminogen into the active plasmin results in (1) the degradation of proteins of the extracellular matrix, (2) self-activation in a positive feedback manner and (3) activation of some of the MMPs e.g. MMP-9 and MMP-2 (Baramova et al. 1997; Rao 2003; Bellail et al. 2004). It is worth to mention that the soluble plasmin can inactivate at least some of MMPs and therefore to control their proteolytic activity (Carmeliet et al. 1997). Thus, DPP-IV as a plasminogen receptor can help to modulate several important enzymes related to the matrix degradation.

FAP alone, or in cooperation with MMPs, is able to degrade collagen, and hence to act as an extracellular matrix degrading protease (Christiansen et al. 2007). On the contrary, FAP can also activate  $\alpha$ 2-antiplasmin, a potent inhibitor of plasmin (Lee et al. 2006), present in blood plasma but also secreted by gliomastoma in primary organ culture (Keohane et al. 1990). This seeming contradiction can indicate the role of FAP in the fine tuning of the local extracellular matrix turnover.

**Figure 9. Hypothetical model of DPP-IV and FAP cooperation in the process of extracellular matrix degradation.**



ADA – adenosine deaminase; DPP-IV – dipeptidyl peptidase IV; ECM – extracellular matrix; FAP – fibroblast activation protein; MMP – matrix metalloproteinases; uPA – urokinase; uPAR – urokinase receptor; → activation; \* activated form of enzyme; —• inhibition/deactivation; -> degradation

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## 8. Literature

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The AceView Database;

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## 9. Enclosure

### 1.1. Original papers and review related to the thesis

- 1.1.1. **ENCLOSURE 1:** Busek, P., Stremenova, J., Sromova, L., Hilser, M., Balaziova, E., et al. (2012) Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity. *International journal of biochemistry and cell biology*. 2012, 44: 738-747. **IF 4.7**
- 1.1.2. **ENCLOSURE 2:** Balaziova, E., Busek, P., Stremenova, J., Sromova, L., Krepela, E., et al. (2011) Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein- $\alpha$  in transformed astrocytic cells. *Molecular and cellular biochemistry*. 354: 283-289. **IF 2.0**
- 1.1.3. **ENCLOSURE 3:** Kotackova, L., Balaziova, E., Sedo, A. (2009) Expression Pattern of Dipeptidyl Peptidase IV Activity and/or Structure Homologues in Cancer. *Folia biologica*. 55: 77-84. **IF 1.1**

### 1.2. Posters related to the thesis

- 1.2.1. **ENCLOSURE 4:** Balaziova, E., Busek, P., Fejfarova, E., Sedo A. Expression of fibroblast activation protein- $\alpha$  in human glioblastoma and its effect on glioma cell adhesion and invasion. (Poster) Podosomes, invadopodia and focal adhesion in physiology and pathology, 2011, Madrid, Spain.
- 1.2.2. **ENCLOSURE 5:** Balaziova, E., Kotackova L., Sedo A. Regulation of co-expression of Dipeptidyl peptidase-IV and Fibroblast activation protein in glioma cell lines. (Poster) 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, 2009, Loutraki, Greece.

## **ENCLOSURE 1**

Busek, P., Stremenova, J., Sromova, L., Hilser, M., Balaziova, E., et al. (2012) Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity. *International journal of biochemistry and cell biology*. 2012, 44: 738-747. **IF 4.7**

## **ENCLOSURE 2**

Balaziová, E., Busek, P., Stremenová, J., Sromová, L., Krepela, E., et al. (2011) Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein- $\alpha$  in transformed astrocytic cells. *Molecular and cellular biochemistry*. 354: 283-289. **IF 2.0**

### **ENCLOSURE 3**

Kotackova, L., Balaziová, E., Sedo, A. (2009) Expression Pattern of Dipeptidyl Peptidase IV Activity and/or Structure Homologues in Cancer. *Folia biologica*. 55: 77-84. **IF 1.1**

#### **ENCLOSURE 4**

Balaziová, E., Busek, P., Fejfarová, E., Sedo A. Expression of fibroblast activation protein- $\alpha$  in human glioblastoma and its effect on glioma cell adhesion and invasion. **(Poster)**  
Podosomes, invadopodia and focal adhesion in physiology and pathology, 2011, Madrid, Spain.

# Expression of fibroblast activation protein- $\alpha$ in human glioblastoma and its effect on glioma cell adhesion and invasion



Eva Balaziová, Petr Busek, Edita Fejfarová, Aleksi Sedo  
 Laboratory of Cancer Cell Biology, Institute of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague, Czech Republic



## Introduction

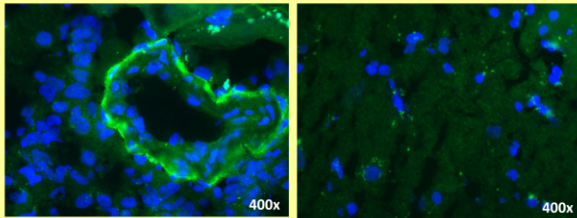
Malignant gliomas are aggressive tumours with invasive growth due to aberrant interactions with extracellular matrix and characteristic spreading along the white matter tracts, blood vessels and the subpial space. Fibroblast activation protein- $\alpha$  (EC 3.4.21.-; FAP) is a dual specificity serine protease with a post proline dipeptidyl exopeptidic and collagenolytic

endopeptidic activity. FAP, processing both structural and regulatory peptides is suggested to participate in the degradation of the extracellular matrix, tissue remodelling and cancer cell invasion. Increased FAP mRNA expression was previously demonstrated in human glioblastoma [1], but its role in glioma progression remains largely unknown.

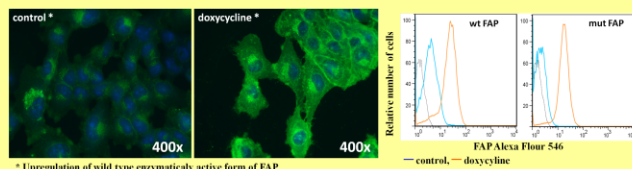
The aim of the study is to analyze the staining pattern of FAP in human glioblastoma compared to the non-malignant brain, and determine the effect of FAP on glioma cell adhesion, migration and invasion.

## Results

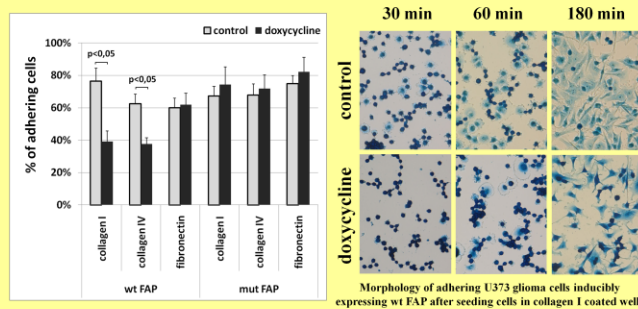
### I. Localization of FAP positive cells predominantly around blood vessels in human glioblastomas (A) compared to non-malignant brain (B).



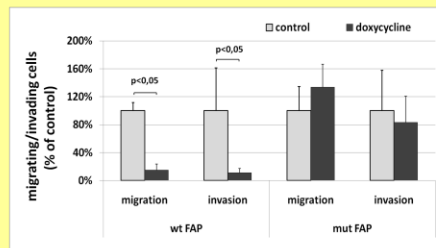
### II. Doxycycline inducible expression of transgenic wild type (wt FAP) and enzymatically inactive mutant FAP (mut FAP) in the human glioma cell line U373.



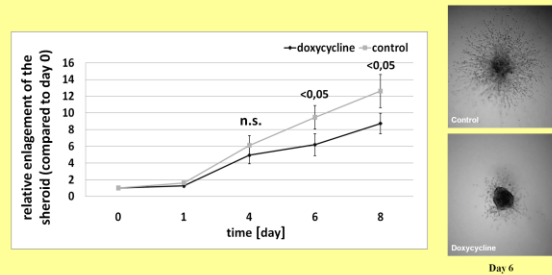
### III. Transgenic wild type FAP negatively influences the adhesion and spreading of U373 glioma cell on collagen I and IV.



### IV. Transgenic wild type FAP decreases the migration and invasion through matrigel in U373 glioma cells.



### V. Transgenic wild type FAP negatively influences the ability of U373 to invade collagen I in a spheroid invasion model.



## Conclusions

- FAP is predominantly localized in the close vicinity of blood vessels in the human glioblastoma.
- Overexpression of transgenic wild type FAP in U373 glioma cells leads to decreased migration and invasion, as well as cell adhesion and spreading on collagen I and IV, but not fibronectin.
- FAP may influence the interaction of glioma cells with specific components of the surrounding extracellular matrix via its enzymatic activity.

## Material and Methods:

U373 cells (ATCC, Middlesex, UK) were transfected with the wild type and mutant (active site Ser624→Ala) FAP using the doxycycline inducible TetOn system (Clontech, USA) according to the manufacturer's instructions. The cells were cultured under standard conditions.

Immunodetection of FAP was performed using the rat monoclonal anti human FAP antibodies (clones D8 and D28, Vitatec, USA).

Adhesion assays were performed in 96WP coated with collagen I, IV or fibronectin (Sigma, CR). Cell migration and invasion was evaluated using the modified Boyden chamber assay using cell culture inserts with 8  $\mu$ m pores.

Spheroids were formed using the „hanging drop” method and embedded in collagen I. Cell invasion was assessed by measuring the area covered with invading cells.

## References:

Stremova, J., Krejela, E., Mares, V., Trm, J., Dhub, V., Marek, J., Vanickova, z., Liso, V., Yea, C., Sedo, A. (2007) Expression and enzymatic activity of dipeptidyl peptidase-IV in human astrocytic tumours are associated with tumour grade. *Int. J. Oncol.* 31, 785-792.

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**Contact:** A.S. Laboratory of Cancer Cell Biology, U Nemocnice 5, 128 53, Prague 2, Czech Republic Tel/Fax: +420 224 965 826, E-mail: aleksi@cesnet.cz, www.ifi.cmm.cz/lcb



## ENCLOSURE 5

Balaziova, E., Kotackova L., Sedo A. Regulation of co-expression of Dipeptidyl peptidase-IV and Fibroblast activation protein in glioma cell lines. **(Poster)** 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine, 2009, Loutraki, Greece.

# Co-expression of Dipeptidyl peptidase-IV and fibroblast activation protein in glioma cell lines



Balaziová Eva, Kotacková Lenka, Sedo Aleksi  
 Laboratory of Cancer Cell Biology, Department of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague, Czech Republic



## Introduction

Since a discovery of dipeptidyl peptidase-IV (DPP-IV), post-proline specific hydrolase, a several proteases possessing similar enzymatic activity or structure have been described and classified as - "Dipeptidyl peptidase-IV activity and/or structure homologues" (DASH) (Sedo et al., 2001). Along with canonical DPP-IV, this group comprises DPP-IV $\beta$ , DPP-II, DPP6, DPP8, DPP9, DPP10 and Fibroblast activation protein- $\alpha$  (FAP- $\alpha$ ). DASH molecules are suggested to be involved in regulation of a number of biologically active peptides as neuropeptide Y, substance P, chemokines etc.

Our previous data suggest possible involvement of DPP-IV and FAP in pathogenesis of human astrocytic tumors, where significant correlation of DPP-IV and FAP expression has been observed (Stremenořva et al 2007). Furthermore, correlative expression of both molecules was found in primary cell cultures derived from astrocytic tumors. Since co-expression of DPP-IV and FAP was recently observed also in several other human tumours, possible functional coordination of both molecules should be considered.

The aim of this work is to confirm DPP-IV and FAP co-expression and to demonstrate their coordination in glioma cells.

## Results:

**I.** Up-regulation of DPP-IV-like exopeptidase enzymatic activity in glioma cell lines (U87, U138) in a model of growth factor deficiency (serum free medium) was observed. While in U138 cells the increment of DPP-IV-like exopeptidase activity has been proportional in both plasma membrane and intracellularly (Fig. 1A), the increase of enzymatic activity in U87 cell line was localized dominantly in plasma membrane (Fig. 1B).

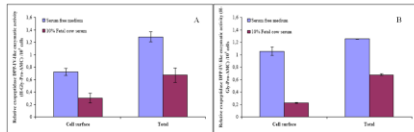


Fig. 1 Relative exopeptidase DPP-IV-like enzymatic activity of U138MG (A) and U87MG (B).

**II.** Gel chromatography revealed varying patterns of MW forms of the exopeptidase DPP-IV-like (610-410kDa and 230-130kDa) and endopeptidase (610-410kDa, 230-130kDa and 90-60kDa) activities further characterized by substrate preference (Fig. 2). In U87 in a model of growth factor deficiency (Fig. 2A) an increment of 610-430kDa MW form of the exopeptidase DPP-IV-like activity associated with an augmentation of the endopeptidase activity in the same MW ranges (Fig. 2B).

On the contrary, in U138 cell line, the up-regulation of exopeptidase DPP-IV-like activity was due to an increment of both 610-410kDa and 230-130kDa MW forms (Fig. 2C), however, an increment of the endopeptidase activity was detected only in 90-60kDa MW form (Fig. 2D).

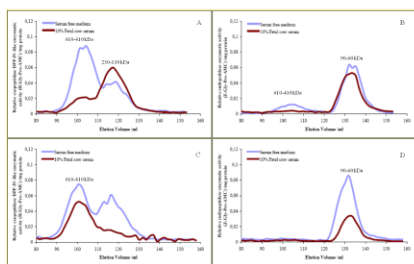


Fig. 2 Elution profile of exopeptidase DPP-IV-like (H-Gly-Pro) (A,C) and endopeptidase (Z-Gly-Pro) (B,D) enzymatic activity of U87MG (A,B) and U138 (C,D) cell lines

**III.** In model of regulated expression of DPP-IV in transfected T98 cells, the increment of exopeptidase DPP-IV-like enzymatic activity (Fig. 3A) was not associated with any up-regulation of the endopeptidase activity of 610-410kDa MW forms (Fig. 3B).

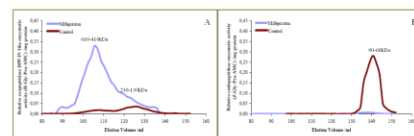


Fig. 3 Elution profile of exopeptidase DPP-IV-like (H-Gly-Pro) and endopeptidase (Z-Gly-Pro) enzymatic activity of DPP-IV transfected cells T98G

**IV.** In U87 cells, the 610-410kDa MW form of both exopeptidase DPP-IV-like and endopeptidase enzymatic activities is localized in plasma membrane, whilst the enzymatic activity of 230-130kDa MW forms of exopeptidase DPP-IV-like and 90-60kDa of endopeptidase enzymatic activity is distributed intracellularly (data not shown).

**V.** Considering MW of individual enzymatic activity isoforms, their subcellular localization and inhibition studies, 610-410kDa MW form of exopeptidase DPP-IV-like and endopeptidase activity most probably represent canonical DPP-IV and FAP. Contrary, 230-130kDa MW peak of exopeptidase DPP-IV-like activity could be attributed to the intracellular DASH molecules: intracellular form of DPP-IV as well as DPP8 and/or DPP9.

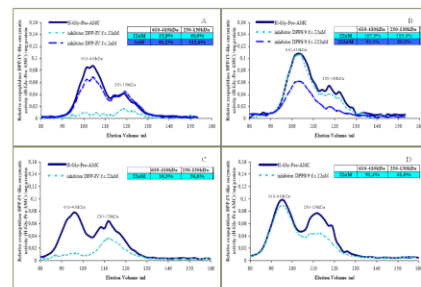


Fig. 4 Inhibition profiles of exopeptidase DPP-IV-like enzymatic activity of U87 (A,B) and U138 (C,D) in model of growth factor deficiency using DPP-IV (A,C) and DPP-9 (B,D) specific inhibitors. Insets: Respective residual activities (%)

**VI.** Immunomicroscopy confirmed plasma membrane localization of both DPP-IV and FAP in U87 cells (Fig. 4A). In contrary, in U138 cells, DPP-IV and FAP were detected mainly intracellularly (Fig. 4B).

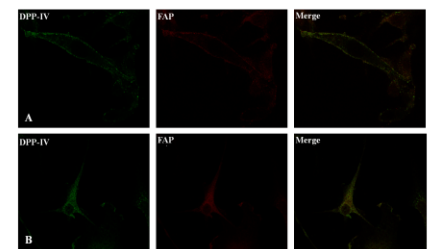


Fig. 5 Localization of DPP-IV and FAP in U87 (A) and U138 cell lines (B).

## Conclusions:

Growth factor deficiency leads to the increase of DPP-IV-like activity in U87 and U138 glioma cell lines.

Considering MW, subcellular localization and inhibition studies, an increment of DPP-IV-like activity in U87 cell line is dominantly attributed to the canonical DPP-IV and is associated with upregulation of FAP.

The overexpression of DPP-IV in DPP-IV transfected T98 cells was not associated with the increase of FAP.

We conclude that the coregulation of DPP-IV and FAP expression results from coordinated transcription of both molecules rather than to be a consequence of DPP-IV protein function eventually leading to the control of FAP expression.

## Material and Methods:

Cell lines (U87MG, U138MG, T98G) were cultured under standard conditions (ATCC, Middlesex, UK). In one experiment, serum free medium was used as model of growth factor deficiency. Glioma cell line T98G was transfected with DPP-IV/C268 using the *mifepristone* inducible Gene Switch system (Invitrogen).

Enzyme activities were measured by a continuous fluorimetric assay with H-Gly-Pro-AMC, Z-Gly-Pro-AMC as a substrate at pH 7.4 after lysing the cells with 1% Triton X-100.

Expression pattern of DASH was analysed using gel filtration, subcellular fractionation was performed by ultracentrifugation.

Detection of DPP-IV and FAP was performed by using the monoclonal rat human DPP-IV (cloneE19, Vitex, USA) and FAP (clone D8, Vitex, USA).

## References:

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 Stremenořva J, Krepeřa E, Mareš V, Trnř J, Dřubř V, Mareš J, Vanřkova Z, Lisa V, Ÿa C, Sedo A. (2007) Expression and enzymatic activity of dipeptidyl peptidase-IV in human astrocytic tumours are associated with tumour grade. *Int. J. Oncol.* 31, 785-792.

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**Contact:** AS Laboratory of Cancer Cell Biology, U Nemocnice 5, 128 53, Prague 2, Czech Republic Tel/Fax: +420 224 965 826, E-mail: aleksi@cesnet.cz, www.lli.com.cz/llsb