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Multifunctional proteases dipeptidyl peptidase-IV and fibroblast activation protein as possible pathogenetic factors, biomarkers and therapeutic targets in cancer

HABILITATION THESIS

Medical chemistry and biochemistry

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Contents

1. Preface and acknowledgements

Dipeptidyl peptidase IV (DPP-IV) and Dipeptidyl peptidase-IV activity and/or structure homologues such as fibroblast activation protein (FAP) and their possible involvement in the pathogenesis of malignant tumors have sparked my interest during medical studies and I have continued working in the field since then. In this habilitation thesis I first provide a short introduction of these "moonlighting" molecules that are involved in various aspects of physiological and pathological processes. Subsequently, I summarize the results of our work published over the past few years which focused among others on DPP-IV and FAP in the context of two recalcitrant human malignancies. In the first section of the results, an overview of our studies in pancreatic ductal adenocarcinoma is provided. The second part sums up our research on DPP-IV and FAP in glioblastoma, the most common and the most deadly form of a primary brain tumor. Detailed information on the experimental approaches and results can be found in our published papers that are included in this habilitation thesis (see attachments). Attachments also contain our review articles and book chapters that give a more general overview of proteases in brain tumors, discuss approaches to early detection of sporadic pancreatic cancer and summarize the information regarding the role and therapeutic targeting of DPP-IV and FAP in cancer.

The results presented in this work could not have been obtained without the contribution of many people with whom I had the opportunity and the privilege to collaborate. I would especially like to thank the members of the Laboratory of Cancer Cell Biology at the First Faculty of Medicine of the Charles University, who provided help during experimental work and were a constant source of support and inspiration. My big thanks go to Aleksi Sedo, who introduced me to the field of "Dipeptidyl peptidase IV activity and/or structure homologues", was a mentor during my studies and provided support throughout the work. I would also like to thank Evzen Krepela for always being a source of expertise and critical comments and Premysl Fric, who introduced me to the field of pancreatic cancer.

Finally, I would like to thank my wife Vendula for patience and understanding and my whole family for lifelong support.

2. List of publications included in the habilitation thesis

First or corresponding author:

Attachment 1: Busek, P., R. Mateu, M. Zubal, L. Kotackova, A. Sedo. "Targeting fibroblast activation protein in cancer – Prospects and caveats." Frontiers in Bioscience, in press.

Attachment 2: Busek, P. and A. Sedo (2013). "Dipeptidyl Peptidase-IV and Related Proteases in Brain Tumors." Book chapter in: Evolution of the Molecular Biology of Brain Tumors and the Therapeutic Implications, Ed. T. Lichtor, InTech. ISBN 978-953-51-0989-1.

Attachment 3: Busek, P., P. Hrabal, P. Fric and A. Sedo (2015). "Co-expression of the homologous proteases fibroblast activation protein and dipeptidyl peptidase-IV in the adult human Langerhans islets." Histochem Cell Biol **143**(5): 497-504.

Attachment 4: Busek, P., Z. Vanickova, P. Hrabal, M. Brabec, P. Fric, M. Zavoral, J. Skrha, K. Kmochova, M. Laclav, B. Bunganic, K. Augustyns, P. Van Der Veken and A. Sedo (2016). "Increased tissue and circulating levels of dipeptidyl peptidase-IV enzymatic activity in patients with pancreatic ductal adenocarcinoma." Pancreatology **16**(5): 829-838.

Attachment 5: Busek, P., M. Prevorovsky, E. Krepela and A. Sedo (2014). "Glioma associated proteases." Book chapter in: Glioma cell biology. Eds. A. Sedo and R. Mentlein, Elsevier, ISBN 978-3-7091-1430-8.

Attachment 6: Busek, P., J. Stremenova, L. Sromova, M. Hilser, E. Balaziova, D. Kosek, J. Trylcova, H. Strnad, E. Krepela and A. Sedo (2012). "Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity." Int J Biochem Cell Biol **44**(5): 738-747.

Attachment 7: Busek, P., E. Balaziova, I. Matrasova, M. Hilser, R. Tomas, M. Syrucek, Z. Zemanova, E. Krepela, J. Belacek and A. Sedo (2016). "Fibroblast activation protein alpha is expressed by transformed and stromal cells and is associated with mesenchymal features in glioblastoma." Tumour Biol **37**(10): 13961-13971.

Attachment 8: Dvorakova, P., P. Busek, T. Knedlik, J. Schimer, T. Etrych, L. Kostka, L. Stollinova Sromova, V. Subr, P. Sacha, A. Sedo and J. Konvalinka (2017). "Inhibitor-Decorated Polymer Conjugates Targeting Fibroblast Activation Protein." J Med Chem **60**(20): 8385-8393.

Co-author:

Attachment 9: Fric, P., A. Sedo, J. Skrha, P. Busek, M. Laclav, P. Skrha and M. Zavoral (2017). "Early detection of sporadic pancreatic cancer: time for change." Eur J Gastroenterol Hepatol **29**(8): 885-891.

Attachment 10: Skrha J, P. Fric, P. Busek, P. Skrha, A. Sedo. "Sporadic Pancreatic Cancer: Glucose Homeostasis and Pancreatogenic Type 3 Diabetes". Book chapter in: Pancreatic Cancer, Ed. L Rodrigo. Intech, ISBN 978-953-51-6222-3, in press.

Attachment 11: Skrha, J., P. Busek, J. Uhrova, P. Hrabal, K. Kmochova, M. Laclav, B. Bunganic and P. Fric (2017). "Lower plasma levels of glucose-dependent insulinotropic peptide (GIP) and pancreatic polypeptide (PP) in patients with ductal adenocarcinoma of the pancreas and their relation to the presence of impaired glucoregulation and weight loss." Pancreatology **17**(1): 89-94.

Attachment 12: Krepela E., P. Busek, A. Sedo. "Nádorové mikroprostředí glioblastomu." Book chapter in: Gliomy. Současná diagnostika a léčba- druhé vydání. Ed. P. Šlampa, in press.

Attachment 13: Matrasova, I., P. Busek, E. Balaziova and A. Sedo (2017). "Heterogeneity of molecular forms of dipeptidyl peptidase-IV and fibroblast activation protein in human glioblastomas." Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **161**(3): 252-260.

Attachment 14: Krepela, E., P. Busek, M. Hilser, Z. Vanickova and A. Sedo (2017). "Species-specific real-time RT-PCR analysis of expression of stromal cell genes in a tumor xenotransplantation model in mice." Biochem Biophys Res Commun **491**(1): 126-133.

Attachment 15: Trylcova, J., P. Busek, K. Smetana, Jr., E. Balaziova, B. Dvorankova, A. Mifkova and A. Sedo (2015). "Effect of cancer-associated fibroblasts on the migration of glioma cells in vitro." Tumour Biol **36**(8): 5873-5879.

3. List of abbreviations

ADA = Adenosine deaminase

Akt = Protein Kinase B

BNP = Brain natriuretic peptide

CARMA1 = Caspase recruitment domain-containing protein 11

CCL = Chemokines belonging to the CC family

CD = Cluster of differentiation

CXCL = Chemokines belonging to the CXC family

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

DM = Diabetes mellitus

DPP = Dipeptidyl peptidase

EC = Enzyme Commission

ELISA = Enzyme-Linked ImmunoSorbent Assay

EGFR = Epidermal Growth Factor Receptor

FAP = Fibroblast activation protein, seprase

FGF21 = Fibroblast growth factor 21

G-CSF = Granulocyte colony-stimulating factor

GFAP = Glial fibrillary acidic protein

GHRH = Growth hormone releasing hormone

GIP = Gastric inhibitory polypeptide/ glucose-dependent insulinotropic peptide

GLP = Glucagon-like peptide

GM-CSF = Granulocyte-macrophage colony-stimulating factor

GRP = Gastrin-releasing peptide

GSC= Glioma stem-like cells

HCV = Hepatitis C virus

HIV = Human immunodeficiency virus

IL = Interleukin

MERS = Middle East respiratory syndrome

NHE3 = Sodium–hydrogen exchanger 3

NPY = Neuropeptide Y

PACAP = Pituitary adenylate cyclase-activating peptide

PAR = Protease activated receptor

PDAC = Pancreatic ductal adenocarcinoma

PHM = Peptide Histidine-Methionine

PYY = Peptide YY

qRT-PCR = Real-Time Quantitative Reverse Transcription PCR

SOX-2 = Transcription Factor SOX-2

SP = Substance P

SPRY2 = Sprouty (Drosophila) homolog 2

TAT = Trans-activator of transcription

TCGA = The Cancer Genome Atlas

uPAR = Urokinase-type plasminogen activator (UPA) receptor

VIP = Vasoactive intestinal peptide

4. Introduction

Dipeptidyl peptidase-IV (DPP-IV, DPP4, CD26, adenosine deaminase complexing protein 2, EC 3.4.14.5) and fibroblast activation protein (FAP, Surface Expressed Protease [seprase], antiplasmin-cleaving enzyme [APCE], EC 3.4.2.1.B28), are non-classical serine proteases belonging to S9B family and to "Dipeptidyl peptidase IV activity and/or structure homologues" (DASH) (Sedo and Malik 2001, Busek, Malik et al. 2004). The genes encoding these proteases are highly evolutionary conserved, are localized in close proximity on the long arm of chromosome 2 in humans and have similar organization, suggesting that they may be a product of gene duplication. The proteins encoded by these genes share several common characteristics, but exhibit substantial differences in tissue distribution and possible pathophysiological functions. DPP-IV and FAP proteins consist of 766 and 760 amino acid residues respectively, and exhibit 52% identity (Darmoul, Lacasa et al. 1992, Goldstein, Ghersi et al. 1997). Both DPP-IV and FAP are type II transmembrane proteins that contain a short cytoplasmic tail, a transmembrane region and a large glycosylated extracellular domain with the catalytic triad comprised in humans of Ser^{630} Asp^{708} His^{740} in DPP-IV and Ser^{624} Asp^{702} His^{734} in FAP (Goldstein, Ghersi et al. 1997, Lambeir, Durinx et al. 2003). DPP-IV is broadly expressed, although in a cell differentiation/activation-dependent manner, in lungs, liver, proximal convoluted tubules in the kidney, epithelial cells of the intestine, pancreas and prostate, placenta, and in certain types of endothelial and immune cells (for a review see (Lambeir, Durinx et al. 2003, Klemann, Wagner et al. 2016)). In contrast, FAP is only scarcely present under physiological conditions in adults, but is upregulated in states of tissue remodeling such as during embryonic development, in healing wounds, chronic inflammation and cancer (for a review see **Attachment 1**, (Jacob, Chang et al. 2012, Kelly, Huang et al. 2012)). Interestingly, a soluble form of both proteins lacking the intracellular part and the transmembrane region is present in body fluids (Durinx, Lambeir et al. 2000, Lee, Jackson et al. 2006). Co-expression and possible co-regulation of both proteases was reported by us and others in some types of cancer cells, migrating endothelial cells and fibroblasts (Wesley, Albino et al. 1999, Ghersi, Dong et al. 2002, Wesley, Tiwari et al. 2004, Ghersi, Zhao et al. 2006, Goscinski, Suo et al. 2008, Balaziova, Busek et al. 2011). Although the implications of these observations are currently largely speculative, it should be noted that both proteases share an overlapping set of substrates and likely act in concert during the remodeling of extracellular matrix.

DPP-IV and FAP are enzymatically active as dimers and cleave peptides and proteins that contain proline or alanine in the penultimate position at the N-terminus. In addition to this dipeptidyl aminopeptidase enzymatic activity, FAP also exhibits a post-proline endopeptidase activity (Aertgeerts, Levin et al. 2005, Edosada, Quan et al. 2006). Due to the unique structure of proline, most proteases do not cleave the peptide bonds adjacent to it and proline thus often prevents protein degradation or cleavage (Vanhoof, Goossens et al. 1995). Several chemokines, neuropeptides and incretins have been shown to be cleaved by DPP-IV and/or FAP with their resulting inactivation, activation or change in receptor preference (Figure 1, for a review see **Attachment 2**, (Mentlein 1999, Busek, Malik et al. 2004, Klemann, Wagner et al. 2016)).

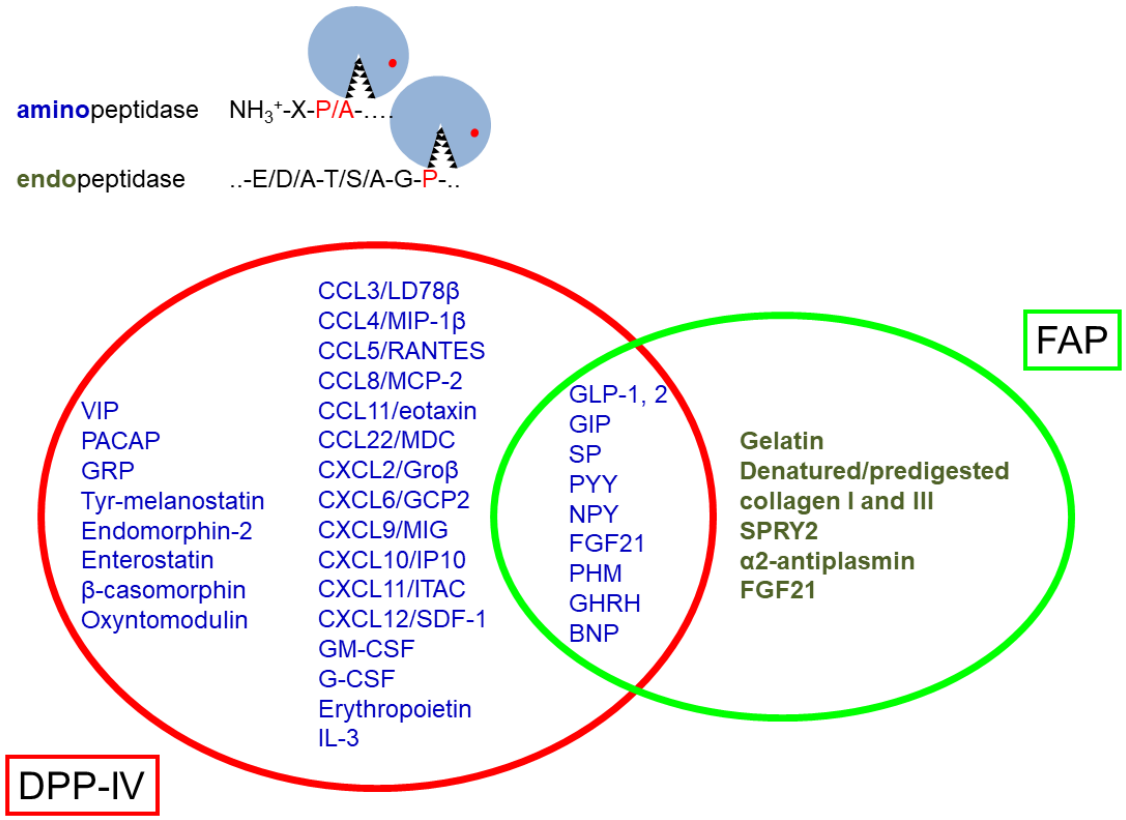

Figure 1. *Substrate specificity and biologically active peptides cleaved by DPP-IV and/or FAP.*
BNP = Brain natriuretic peptide, CCL/CXCL = Chemokines belonging to the CC and CXC family respectively, FGF21 = Fibroblast growth factor 21, G-CSF = Granulocyte colony-stimulating factor, GHRH = Growth hormone releasing hormone, GIP = Gastric inhibitory polypeptide/ glucose-dependent insulinotropic peptide, GLP-1, 2 = Glucagon-like peptide-1, 2, GM-CSF = Granulocyte-macrophage colony-stimulating factor, GRP = Gastrin-releasing peptide, IL-3 = Interleukin 3, PACAP = Pituitary adenylate cyclase-activating peptide, PHM = Peptide Histidine-Methionine, SP = Substance P, PYY = Peptide YY, NPY = Neuropeptide Y, SPRY2 = Sprouty (Drosophila) homolog 2, VIP = Vasoactive intestinal peptide. Modified from ***Attachment 2****.*

In addition to proteolytically modifying several biopeptides, both proteases are known to bind other proteins outside of their catalytic site. The proteins interacting with DPP-IV include human adenosine deaminase 1 (ADA), the protein phosphatase CD45, mannose 6-phosphate/insulin-like growth factor II receptor, caveolin-1, CARMA1, the chemokine receptor CXCR4, the HIV TAT and gp-120 proteins, the extracellular matrix proteins vitronectin, collagen and fibronectin, plasminogen receptor, or the Na^+/H^+ exchanger isoform NHE3 (see (Klemann, Wagner et al. 2016) and references therein). In the case of FAP, formation of complexes with DPP-IV, caveolin-1, erlin-2, stomatin (Knopf, Tholen et al. 2015) and with Urokinase-type plasminogen activator receptor (uPAR) and alpha3 beta1 integrin (Artym, Kindzelskii et al. 2002) was reported in cancer associated fibroblasts and melanoma invadopodia, respectively.

The "moonlighting" characteristics of both proteases, mediated by the cleavage of a wide variety of biologically active peptides and by "non-proteolytic" interactions with various molecules, lead to their involvement in various physiological and pathological states and processes (Figure 2).

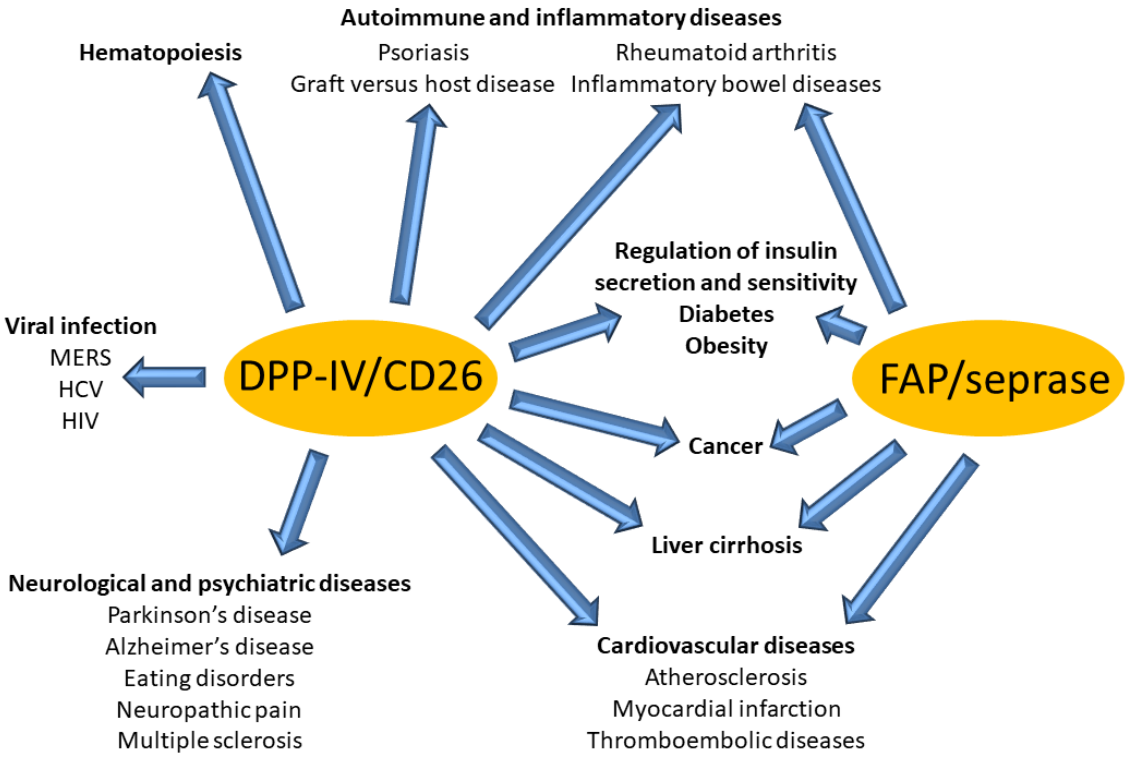

Figure 2. *Physiological and pathological conditions and processes associated with deregulated expression or functional involvement of DPP-IV and/or FAP.*
MERS = Middle East respiratory syndrome, HCV = Hepatitis C virus, HIV = Human immunodeficiency virus.

Perhaps the best explored is the ability of DPP-IV to cleave and thereby inactivate several insulinotropic peptides (e.g. GLP-1, GIP, PACAP, NPY). Research in this area has led to the discovery that the use of DPP-IV resistant GLP-1 analogs or low-molecular weight DPP-IV inhibitors (gliptins) improves insulin secretion and glucose homeostasis. Although our understanding of the exact underlying mechanisms is still incomplete (Omar and Ahren 2014, Mulvihill, Varin et al. 2017, Yabe, Seino et al. 2018), both approaches are currently routinely used in treating patients with type 2 diabetes mellitus (DM) (Deacon, Mannucci et al. 2012). Together with its effect on insulinotropic peptides, DPP-IV appears to have a negative influence on other mechanisms of glucose homeostasis regulation, particularly in obesity and metabolic syndrome. In an enzyme activity dependent manner, soluble DPP-IV impairs insulin sensitivity on the level of Akt (Protein Kinase B) phosphorylation in adipocytes and muscle cells (Lamers, Famulla et al. 2011). Recently, it has also been shown that soluble DPP-IV in combination with protease activated receptor 2 (PAR2) activation by plasma factor Xa promotes inflammation in the visceral adipose tissue probably by binding caveolin-1 in macrophages (Ghorpade, Ozcan et al. 2018). Inhibition of DPP-IV enzymatic activity has been shown to affect a broad spectrum of other processes in addition to glucoregulation. In preclinical studies, DPP-IV inhibitors seem to prevent the progression of atherosclerosis, which was recently confirmed in a clinical trial (Fadini and Avogaro 2013, Mita, Katakami et al. 2016, Duan, Rao et al. 2017). DPP-IV also participates on the pathogenesis of liver diseases and its inhibition alleviates hepatic steatosis and cirrhosis (Itou, Kawaguchi et al. 2013, Baumeier, Schluter et al. 2017, Nakamura, Fukunishi et al. 2017, Amano, Tsuchiya et al. 2018). Most likely by modulating the activity of various biopeptides, DPP-IV seems to participate in the regulation of food intake and energy expenditure and in a preclinical study its inhibition was shown to have anti-obesity effects (Lee, Kim et al. 2014). Nociception, especially in the setting of inflammatory and neuropathic pain, seems to be influenced by DPP-IV. DPP-IV inhibition has analgesic and anti-inflammatory effects in several models (Ujhelyi, Ujhelyi et al. 2014, Kiraly, Kozsurek et al. 2018). In animal studies, DPP-IV inhibition was shown to have beneficial effects in neurological disorders such as Parkinson's and Alzheimer's disease and multiple sclerosis, possibly by promoting the neurotrophic effects of various DPP-IV substrates and by immunoregulatory mechanisms (see (Al-Badri, Leggio et al. 2018) for a review). Case reports have been published describing improvement of psoriasis in patients using sitagliptin (van Lingen, Poll et al. 2008, Nishioka, Shinohara et al. 2012, Lynch, Tobin et al. 2014). By

increasing the bioavailability of GLP-1, CXCL12 (stromal cell derived factor 1) and other biopeptides, DPP-IV inhibition may have beneficial effects in renal and cardiovascular diseases including myocardial infarction (for a review see (Hocher, Reichetzeder et al. 2012)). Recently, soluble DPP-IV has been demonstrated to mediate endothelial dysfunction by activating protease activated receptors (PAR) in endothelial cells and its inhibition may thus preserve endothelial function in cardiometabolic diseases (Romacho, Vallejo et al. 2016). By modulating the bioactivity of GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), erythropoietin and CXCL12, DPP-IV seems to play an important role in the regulation of hematopoiesis. Inhibition of DPP-IV is therefore being tested as an approach to improve hematopoietic recovery after irradiation, chemotherapy, or hematopoietic stem cell transplantation (Broxmeyer, Hoggatt et al. 2012). DPP-IV is well known to be involved in T cell activation and function (Ohnuma, Hosono et al. 2011, Klemann, Wagner et al. 2016), and changes in DPP-IV expression and/or activity have been reported by us and others in various autoimmune diseases including rheumatoid arthritis (Ellingsen, Hornung et al. 2007, Sromova, Busek et al. 2015), inflammatory bowel diseases (Hildebrandt, Rose et al. 2001), multiple sclerosis (Tejera-Alhambra, Casrouge et al. 2014) or graft versus host disease (Ohnuma, Hatano et al. 2015).

DPP-IV is also implicated in the pathogenesis of viral diseases. DPP-IV mediated cleavage of CXCL10 contributes to viral persistence in hepatitis C infection (Riva, Laird et al. 2014). In HIV infection, DPP-IV is thought to influence the entry of the virus into target cells possibly by serving as a co-receptor and cleaving chemokines that prevent HIV interaction with its other co-receptors (see (Lambeir, Durinx et al. 2003) for a review). Recently, DPP-IV has been identified as a receptor for the Middle East respiratory syndrome (MERS) coronavirus (Lu, Hu et al. 2013, Raj, Mou et al. 2013) and antibodies blocking the interaction between spike protein S1 and DPP-IV are being developed as possible new treatments of this disease (Ohnuma, Haagmans et al. 2013).

Targeting DPP-IV thus seems an attractive approach in various pathologies. Nevertheless, the multitude of functions of DPP-IV itself and of its substrates raises some concerns regarding the safety of gliptins (Matteucci and Giampietro 2009, Stulc and Sedo 2010). For example, we and others have recently reported that sitagliptin treatment may cause changes of the proportion of lymphocyte subpopulations in patients with type 2 DM (Aso, Fukushima et al. 2015, Sromova, Busek et al. 2016).

Although the rate of side effects of gliptins is generally considered to be comparable to placebo, several reports have described cases with gliptin-associated arthritis (Yokota and Igaki 2012, Saito, Ohnuma et al. 2013, Crickx, Marroun et al. 2014) and skin diseases (Skandalis, Spirova et al. 2012, Attaway, Mersfelder et al. 2014, Bene, Jacobsoone et al. 2015, Keseroglu, Tas-Aygar et al. 2017). Furthermore, the question of possible role of gliptins in the development of malignancies remains currently unresolved (Raz, Bhatt et al. 2014, Tseng 2016, Overbeek, Bakker et al. 2018).

The functions of FAP under physiological conditions are largely unknown. In mice, its absence during embryonic development does not lead to developmental defects, mice are fertile and have no significant differences in any of the major organs or biochemistry parameters (Niedermeyer, Kriz et al. 2000, Niedermeyer, Garin-Chesa et al. 2001). Recent reports demonstrating that FAP cleaves fibroblast growth factor 21 (FGF21), a protein controlling energy metabolism and insulin sensitivity (Dunshee, Bainbridge et al. 2016, Zhen, Jin et al. 2016), nevertheless point to its possible involvement in the regulation of metabolism. Indeed, FAP knockout mice are protected against diet-induced obesity and pharmacological inhibition of FAP enhances levels of FGF21 and has several metabolic benefits in obese mice (Sanchez-Garrido, Habegger et al. 2016). The soluble form of FAP further cleaves and thereby converts alpha-2-antiplasmin into a more potent inhibitor of plasmin (Lee, Jackson et al. 2004). FAP may thus participate on the pathogenesis of thromboembolic diseases and its inhibition could enhance the thrombolytic activity of plasmin (Lee, Jackson et al. 2011, Uitte de Willige, Malfliet et al. 2013, Uitte de Willige, Malfliet et al. 2015). FAP is strongly upregulated in disease states associated with tissue remodeling such as liver cirrhosis, pulmonary fibrosis, arthritis, remodeling heart tissue after myocardial infarction, advanced atherosclerotic lesions, strictured regions in Crohn's disease and a variety of cancers. In these conditions, FAP seems to contribute to the remodeling of extracellular matrix, induction of immunosuppression and promotion of the malignant phenotype of cancer cells (reviewed in **Attachment 1**).

DPP-IV and FAP are deregulated in several types of cancers, participate on their pathogenesis and represent possible biomarkers and therapeutic targets. This topic has been reviewed by us (**Attachment 1, 2**) and others (Yu, Yao et al. 2010, Beckenkamp, Davies et al. 2016), and therefore only selected examples are mentioned here. DPP-IV participates on the pathogenesis of melanoma and malignant mesothelioma. Normal melanocytes express DPP-IV (Houghton, Albino et al. 1988). DPP-IV is downregulated

by promoter methylation in melanoma cell (McGuinness and Wesley 2008) and its re-expression induces profound phenotypic changes including loss of tumorigenicity, anchorage-independent growth and a reversal in a block in differentiation and an acquired dependency on exogenous growth factors, in part through its enzymatic activity (Wesley, Albino et al. 1999). In contrast to this tumor suppressor role in melanoma, DPP-IV seems to be important for the malignant phenotype of mesothelioma cells. DPP-IV mediates adhesion of the cells to extracellular matrix, enhances their invasion and is expressed in a subset of mesothelioma stem-like cells (Inamoto, Yamada et al. 2007, Ghani, Yamazaki et al. 2011, Okamoto, Iwata et al. 2014). FAP is in general believed to promote the malignant phenotype of cancer cells (well documented e.g. in oral squamous cell carcinoma (Wang, Wu et al. 2014)). Nevertheless, it was reported that it acts as a tumor suppressor in melanoma (Wesley, Albino et al. 1999, Ramirez-Montagut, Blachere et al. 2004). Regarding the role of DPP-IV and FAP as biomarkers, absence of DPP-IV was proposed to discriminate malignant melanomas from deep penetrating nevi (Roesch, Wittschier et al. 2006), while its presence may be useful in predicting the malignant character of thyroid lesions (Tanaka, Umeki et al. 1995, Hirai, Kotani et al. 1999, Aratake, Umeki et al. 2002, de Micco, Savchenko et al. 2008, Zheng, Liu et al. 2015). Expression of FAP in several tumor types is associated with more aggressive disease (reviewed in **Attachment 1**). Interestingly, lower plasma level of FAP in combination with other serum markers was proposed to be useful for the early detection of colorectal cancer (Wild, Andres et al. 2010, Werner, Krause et al. 2016).

The role of FAP as a possible therapeutic target in cancer has been documented by a number of preclinical and phase I clinical studies using low molecular weight inhibitors, FAP activated prodrugs, anti-FAP antibodies and their conjugates, FAP-chimeric antigen receptor (CAR) T cells, and FAP vaccines (reviewed in **Attachment 1**). Targeting of DPP-IV by humanized monoclonal antibodies is being tested as a novel treatment for malignant mesothelioma (Inamoto, Yamada et al. 2007, Raphael, Le Teuff et al. 2014) and a recent phase I trial suggests that it is well tolerated and may stabilize disease in patients with advanced/refractory mesothelioma (Angevin, Isambert et al. 2017). Preclinical studies suggest that low molecular weight DPP-IV inhibitors may inhibit metastatic spread of cancer cells in colorectal carcinoma (Jang, Baerts et al. 2015). In addition, DPP-IV inhibition can enhance antitumor immune response by preserving biologically active CXCL10, thereby increasing the infiltration of lymphocytes expressing the CXCR3 receptor into the tumor. That study also

demonstrated that inhibition of DPP-IV may have a synergistic effect with currently used immunotherapeutic approaches (Barreira da Silva, Laird et al. 2015).

The few abovementioned examples clearly illustrate that both proteases can act – by various mechanisms and in a tumor type-dependent manner – as either tumor promoters, or tumor suppressors. Therefore detailed analysis of their possible role and potential diagnostic or therapeutic applicability in particular tumor types is necessary. Below I summarize our studies that evaluated the possible pathophysiological role of DPP-IV and FAP and their potential use as biomarkers and therapeutic targets in pancreatic adenocarcinoma and gliomas.

5. Summary of published papers

5.1 DPP-IV and FAP in pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is the main histological tumor type originating in the pancreas. PDAC mostly occurs as sporadic; similarly to other tumor types only 5 to 10% cases are estimated to have an inherited component (Ryan, Hong et al. 2014). The incidence of PDAC in the Czech Republic is one of the highest in the world, increases steadily and currently reaches 20 cases per 100 000 (http://www.svod.cz/). A complete surgical removal of the tumor is the only potentially curative therapy. Most patients are however diagnosed with an advanced stage disease and less than 20% are candidates for surgery. The prognosis of PDAC is thus grim – the 5-year survival rate is less than 6% and most of the patients die within one year from diagnosis. Due to its increasing incidence and dismal prognosis, PDAC is estimated to become the second leading cause of cancer-related deaths in the United States by 2020 (Chari, Kelly et al. 2015). Absence of effective screening tools is an important contributor to the late diagnosis of these tumors. Approaches to identify persons with potentially curable premalignant states and early stage PDAC are needed as reviewed by us (**Attachment 9**) and others (Hanada, Okazaki et al. 2015, Chari, Kelly et al. 2015).

Diabetes is present in more than 50% of PDAC patients (Chari, Leibson et al. 2005) and newly diagnosed impairment of glucose homeostasis, in particular when accompanied by weight loss, is an early sign of PDAC. The differentiation from the initial stage of the much more prevalent "common" type 2 DM is currently not possible and the pathogenetic mechanisms underlying the increased insulin resistance and beta cell dysfunction in PDAC are incompletely understood (**Attachment 10**, (Cui and Andersen 2012, Sah, Nagpal et al. 2013)).

Both the circulating and cell membrane bound DPP-IV has been shown to be involved in various aspects of glucoregulation, in general reducing insulin secretion from beta cells and increasing insulin resistance in target tissues (Lamers, Famulla et al. 2011, Deacon, Mannucci et al. 2012, Omar and Ahren 2014, Mulvihill, Varin et al. 2017, Ghorpade, Ozcan et al. 2018, Yabe, Seino et al. 2018). Similarly, FAP seems to be involved in the regulation of energy metabolism through FGF21 cleavage (Sanchez-Garrido, Habegger et al. 2016).

In a collaborative project we therefore analyzed the role of DPP-IV and FAP in PDAC with a special focus on their possible association with an impaired glucose homeostasis.

Attachment 3 contains our published findings revealing the co-expression of the two proteases in Langerhans islets. By catalytic histochemistry and by using a highly selective DPP-IV inhibitor sitagliptin, we detected the enzymatic activity of DPP-IV in ducts of exocrine pancreas, which was in accord with previously published data. Somewhat surprisingly, we also observed strong DPP-IV positivity in the Langerhans islets in PDAC patients. We confirmed that this reflects the physiological expression of DPP-IV in an adult human pancreas using morphologically normal tissue samples from nondiabetic patients with a benign serous cystadenoma. Using immunohistochemistry, we proved that DPP-IV is expressed in pancreatic alpha cells and is co-expressed with FAP in more than 90% of the alpha cells.

Changes in plasma and tissue levels of DPP-IV and FAP in PDAC and their possible association with impaired glucoregulation in PDAC were the focus of the next paper (**Attachment 4**)**.** Impaired glucoregulation was present in 79% of the patients in our study and was diagnosed within 2 years before the diagnosis of PDAC in the majority of them. Using paired samples of tumorous and non-tumorous tissue from PDAC patients, we demonstrated that DPP-IV-like enzymatic activity was increased in the tumorous tissue due to an increase of both canonical DPP-IV (CD26) and other DPP-IV-like enzymatic activity exhibiting molecules. Concordantly with previous reports, we observed increased FAP expression in PDAC tissues, nevertheless abundant FAP expression was also observed in several non-tumorous samples from PDAC patients, particularly when the morphological features of chronic pancreatitis were present. Using immunohistochemistry we showed that DPP-IV and FAP expression was localized in cancer cells and also in stromal cells. There was a trend for more pronounced expression in stromal cells in the proximity of cancer cells, suggesting that paracrine signaling from cancer cells is responsible for DPP-IV and FAP upregulation in the stroma (see **Attachment 1** for a review of FAP expression regulation). In part of the samples, Langerhans islets surrounded by DPP-IV and FAP expressing cancer associated stroma were observed. We further evaluated whether the levels of circulating DPP-IV and FAP are changed in PDAC patients with a special focus on the possible association of these changes with the early symptoms of PDAC, i.e. new onset diabetes (duration less than 2 years) and weight loss greater than 2kg. DPP-IV enzymatic activity was highest in PDAC patients exhibiting both of these early symptoms and the difference was statistically significant compared to patients with type 2 diabetes without PDAC. A similar trend was observed for healthy controls, but the difference did not reach statistical significance. Contrary to this, detection of DPP-IV (CD26) by ELISA

revealed highest levels in patients with type 2 diabetes without PDAC. Consequently, the ration between DPP-IV enzymatic activity and DPP-IV protein concentration (specific enzymatic activity of DPP-IV) was significantly higher in PDAC compared to patients with type 2 diabetes without PDAC. Plasma FAP levels were lower in PDAC, which was consistent with previous studies in PDAC and other cancers (Wild, Andres et al. 2010, Javidroozi, Zucker et al. 2012), and rose after tumor removal suggesting a close association between the presence of the tumor and plasma FAP levels.

We evaluated the possible utility of plasma DPP-IV and FAP as biomarkers of PDAC associated impairment of glucoregulation using semiparametric logistic regression based on a generalized additive model. Of the analyzed variables, a specific DPP-IV (CD26) enzymatic activity (i.e. the ratio between measured DPP-IV enzymatic activity and CD26 antigen concentration) was the best predictor for discriminating PDAC patients with recently diagnosed diabetes/prediabetes from type 2 DM without PDAC and improved the predictive ability of CA19-9.

The abovementioned studies contribute to our understanding of the potential role of DPP-IV and FAP in the pathogenesis of PDAC. In addition, they suggest that both proteases may have an important role in regulating the signaling of biopeptides directly within human Langerhans islets under physiological conditions. There are substantial differences in the cytoarchitecture of Langerhans islets (e.g. (Bosco, Armanet et al. 2010)) as well as in DPP-IV expression in individual animal species (Schrader and West 1985, Dinjens, ten Kate et al. 1989, Dorrell, Grompe et al. 2011). Previous reports suggested that DPP-IV is present in the secretory granules of alpha cells in pigs (Poulsen, Hansen et al. 1993), but a study in human autoptic material (Dinjens, ten Kate et al. 1989) detected DPP-IV immunoreactivity in the epithelial cells of intra- and interlobular pancreatic ducts, but not in the Langerhans islets. Our data regarding DPP-IV expression in human pancreatic alpha cells were independently confirmed by concurrently published studies in autoptic material and isolated pancreatic islets (Liu, Omar et al. 2014, Omar, Liehua et al. 2014, Augstein, Naselli et al. 2015). Omar et al. has shown that isolated human islets exposed to a DPP-IV inhibitor exhibited increased secretion of intact GLP-1 and insulin (Omar, Liehua et al. 2014), further supporting the role of DPP-IV in the local regulation of insulin secretion. Interestingly, intrapancreatic GLP-1 production may be increased in type 2 DM (Marchetti, Lupi et al. 2012) and the intrapancreatic effects of DPP-IV inhibitors may thus play an important role in their glucose lowering action. The possible physiological role of FAP in human pancreas

remains to be determined. GLP-1 and GIP, the main incretins, are rather inefficient FAP substrates (Keane, Nadvi et al. 2011). On the other hand, the pleiotropic metabolic regulator FGF21 has recently been demonstrated to be efficiently cleaved by FAP (Coppage, Heard et al. 2016, Dunshee, Bainbridge et al. 2016, Zhen, Jin et al. 2016). FGF21 seems to play an important role in regulating pancreatic endocrine cells under physiological and pathological states (Wente, Efanov et al. 2006, So, Cheng et al. 2015, Singhal, Fisher et al. 2016) and has pleiotropic metabolic effects in other organs (Degirolamo, Sabba et al. 2016). It remains to be established, to what extend this is affected by FAP mediated FGF21 cleavage. Mouse and rat FGF21 is resistant to FAP due to an amino acid substitution at the cleavage site (Dunshee, Bainbridge et al. 2016), disease models using these animal species will thus have to be used cautiously.

In PDAC, tissue levels of DPP-IV and FAP are increased. Possible consequences of their upregulation remain largely speculative. The proteases may participate on the fibrotic reaction characteristic for PDAC. FAP positive cells are an important source of extracellular matrix. In addition, FAP together with other proteases produced by these cells contributes to the remodeling of the matrix and a creation of an environment facilitating tumor cell dissemination. FAP positive cells are further known to promote immunosuppression (see **Attachment 1**), an important contributor to PDAC progression (Sideras, Braat et al. 2014).

The high prevalence of impaired glucoregulation in our cohort of PDAC patients (**Attachment 4**) is consistent with the findings in other studies (see (Andersen, Korc et al. 2017) for a review). The pathogenetic mechanisms leading to the development of diabetes in PDAC are only poorly understood (Sah, Nagpal et al. 2013). Production of S-100A8 N-terminal peptide (Basso, Greco et al. 2006), adrenomedullin (Aggarwal, Ramachandran et al. 2012, Javeed, Sagar et al. 2015) and vanin-1 (Kang, Qin et al. 2016) may lead to beta cell dysfunction and increased insulin resistance. Our results raise an interesting possibility that DPP-IV and possibly FAP may be contributing factors to the development of impairment of glucoregulation in PDAC. In our cohort we observed changes in the plasma concentrations of neuroendocrine mediators, several of which are potential or proven DPP-IV substrates (**Attachment 11**). Mean fasting plasmatic levels of GIP and PP were decreased in PDAC compared to patients with type 2 diabetes without PDAC, and GIP concentrations rose after surgical removal of the tumor. Interestingly, GIP is known to be efficiently cleaved by DPP-IV. Concentrations of other DPP-IV and/or FAP substrates (GLP-1, NPY, PYY) were however not

changed. A limitation of our approach was that the assays could not differentiate between the cleaved and intact forms of the peptides. It therefore remains unclear, whether and to what extent the proteolytic cleavage may contribute to the observed decrease of GIP and PP in PDAC. Perhaps even more importantly, changes in systemic circulation may only poorly reflect the impact of the proteases on pancreatic beta cells. In this respect, the frequently observed close proximity of the Langerhans islets to FAP and/or DPP-IV expressing stromal cells may have a more important effect on the endo/para/autocrine loops regulating beta cell functions. For example, the impact of substantially increased tissue levels of FAP in PDAC on FGF21 signaling remains to be determined. In addition to increased enzymatic activity in the PDAC tissues, the systemic levels of DPP-IV enzymatic activity were increased in PDAC with impaired glucoregulation. This may not only lead to increased systemic degradation of biopeptides regulating insulin secretion, but soluble DPP-IV has also been shown to attenuate insulin signaling in adipocytes and skeletal muscle cells (Lamers, Famulla et al. 2011) and trigger inflammation in the visceral adipose tissue (Ghorpade, Ozcan et al. 2018).

In conclusion, our results suggest that DPP-IV and FAP may be implicated in the regulation of endocrine pancreas under physiological conditions. The increased expression of both proteases observed in patients with PDAC suggests that they may be involved in the pathogenesis of PDAC and may possibly contribute to the frequent co-occurrence of PDAC and newly diagnosed diabetes mellitus.

Summary of the main results of our work in PDAC:

- DPP-IV and FAP are co-expressed in human alpha cells in Langerhans islets under physiological conditions.
- Expression of DPP-IV and FAP is increased in PDAC tissues compared to matched paired non-tumorous pancreatic tissue.
- DPP-IV and FAP are expressed by cancer and stromal cells in PDAC, including stromal cells in close proximity to Langerhans islets.
- Plasma levels of DPP-IV enzymatic activity are increased in PDAC with recent onset DM and weight loss compared to patients with type 2 diabetes without PDAC. Plasma concentrations of GIP and PP are decreased in these patients.
- DPP-IV and FAP may be contributing factors to the pathogenesis of PDAC-associated impairment of glucose homeostasis.

5.2 DPP-IV and FAP in glioblastoma

Gliomas are the most common primary intraaxial brain tumors. According to the recently updated WHO classification (Louis, Perry et al. 2016), several entities are distinguished based on the histopathological and molecular characteristics of this heterogeneous group of tumors. Glioblastoma multiforme (GBM, WHO grade IV glioma) is the most common type of astrocytic tumors affecting mostly adults between the ages of 45 and 70 years. Although GBMs are relatively rare in terms of annual incidence (two to three per 100,000 adults per year), they represent an important cause of mortality and morbidity. The tumors are incurable- most patients die within one to two years from diagnosis (median survival of 15 months) and less than 5% patients survive 5 years despite multimodality treatment including maximal-safe surgical resection, adjuvant radiation therapy with concurrent and adjuvant temozolomide treatment (Weller, van den Bent et al. 2017).

Localization of the tumors in a vital organ with a limited reparatory capacity is an important factor contributing to the dismal prognosis of this disease. In addition to that, several biological characteristics of the tumors play an important role. Extensive infiltration into the surrounding tissue precludes curative removal of the tumors and the dispersed glioma cells are highly resistant to conventional therapies. Glioblastomas are highly heterogeneous tumors with various molecular subtypes defined by specific molecular aberrations and transcriptional profiles being present in individual patients (Karsy, Gelbman et al. 2012, Aldape, Zadeh et al. 2015), and even within individual tumors (Sottoriva, Spiteri et al. 2013). Undifferentiated, self-renewing and highly tumorigenic glioblastoma stem-like cells (GSC) are thought to importantly contribute to several of these characteristics including tumor recurrence and therapeutic resistance (Lathia, Mack et al. 2015, Nakano 2015).

In addition to cell-autonomous mechanisms operating in malignant cells, clues from their surrounding importantly contribute to glioblastoma progression (Charles, Holland et al. 2012, Quail and Joyce 2017). As we recently reviewed (**Attachment 12**), glioblastoma tissue contains various non-transformed elements such as microglia/macrophages, lymphocytes, neural precursor cells, neurons, pericytes/vascular smooth muscle cells, reactive astrocytes and endothelial cells. Together with extracellular matrix and the intratumoral fluid, these components constitute the tumor microenvironment. Intercellular interactions through cell-cell

contacts as well as secreted molecules and vesicles play an important role in promoting the malignant phenotype of cancer cell (**Attachment 12**, (Quail and Joyce 2017)).

Attachment 5 provides a comprehensive overview of proteases that are deregulated in the glioblastoma microenvironment, and summarized their presumed role in disease pathogenesis. The deregulation of proteolytic balance in gliomas is a result of a complex set of processes including aberrant activation of several signaling pathways, physico-chemical changes in the tumor microenvironment and therapeutic interventions such as radiotherapy. Our bioinformatic analysis of the TCGA (The Cancer Genome Atlas) data revealed that several proteases are consistently up- or downregulated in glioblastomas and for a number of them (including DPP-IV and FAP) most marked upregulation was observed in the mesenchymal subtype of glioblastoma. Proteases importantly contribute to glioma progression and participate on various hallmarks of gliomas (see **Attachment 5** for detailed information and references). For example, both extracellular (e.g. matrix metalloproteinases, urokinase type plasminogen activator and cathepsin B) and intracellular (e.g. calpain-2) proteases contribute to the increased invasiveness of glioma cells. This is not only due to their role in the breakdown and modification of extracellular matrix, but also due to their direct effect on cell adhesion molecules and cytoskeleton, and activation of motility promoting signal transduction cascades. Several proteases (e.g. presenilins, ADAMs, deubiquitinating enzymes, proteasome) play an important role in regulating the proliferation, self-renewal and apoptosis of glioma cells including stem-like cells. By activating protease activated receptor 2 (PAR2), proteases promote the formation of pseudopalisades, a typical morphological feature of glioblastomas (Dutzmann, Gessler et al. 2010). A number of proteases are also involved in the excessive neovascularization of glioblastomas acting as angiogenesis activators or inhibitors.

Previous work in our laboratory showed that DPP-IV-like enzymatic activity is increased in glioblastomas, possibly due to the overexpression of DPP-IV and FAP in these tumors (Stremenova, Krepela et al. 2007). This was consistent with literature data and strongly suggested that both proteases may be involved in glioma pathogenesis (see **Attachment 2** for a review).

Mechanistic studies on the possible role of DPP-IV and its enzymatic activity in glioma cells (**Attachment 6**) were a continuation of my PhD work. We derived several primary cell cultures from glioblastomas using an explant technique in serum containing media.

Cell surface DPP-IV-like enzymatic activity, which is mostly derived from DPP-IV, negatively correlated with the colony forming capacity and proliferation of these cultures. We further confirmed that DPP-IV overexpression in three different permanent glioma cell lines led to a decreased cell growth *in vitro* due to a cell cycle block. Overexpression of transgenic DPP-IV also impaired glioma cell migration and adhesion. Using whole genome expression profiling we identified that a number of genes linked to cell proliferation, cell adhesion, migration and regulation of cell development and neuron differentiation were deregulated upon DPP-IV overexpression and may contribute to the observed phenotype changes. Forced expression of DPP-IV also reduced the growth of glioma cells in an orthotopic mouse xenotransplantation model. Interestingly, when an enzymatically inactive mutant DPP-IV carrying an active site Ser630Ala substitution was overexpressed in glioma cells, a similar phenotype was observed suggesting that the observed effects are in large part independent of the intrinsic enzymatic activity of the protease. These data are consistent with the tumor suppressive effects of DPP-IV reported in ovarian (Kajiyama, Kikkawa et al. 2002), prostate (Wesley, McGroarty et al. 2005) as well as non-small cell lung cancer cells (Wesley, Tiwari et al. 2004) and also in the tumor cells derived from neuroectoderm such as melanoma (Wesley, Albino et al. 1999) and neuroblastoma (Arscott, LaBauve et al. 2009). The growth inhibitory effects of DPP-IV in glioma cells might seemingly be in contradiction to the observation of higher DPP-IV expression and activity in glioma tissue homogenates (Stremenova, Krepela et al. 2007). The "net" pro- or anti-oncogenic effects of proteases nevertheless seems to represent an outcome of several factors including their differing functions in individual cell populations of both the tumor parenchyma and stroma, and varying (in)dependence of these functions on the intrinsic enzymatic activity. For example, forced expression of MT1-MMP was described to cause glioma cell death, although its presence in the tumor microenvironment promoted tumor expansion (Markovic, Vinnakota et al. 2009). Thus, DPP-IV may – independent of its enzymatic activity – negatively influence the proliferation of glioma cells, slowing their growth possibly as a part of an adaptive response to the limited nutrition supply or hypoxia (Dang, Chun et al. 2008), yet support angiogenesis or promote intratumoral deregulation of immune response through the proteolytic processing of neuropeptides and chemokines. By degrading the chemokines such as CXCL12 (SDF-1), DPP-IV might also impair the recruitment of tumor suppressive neural precursor cells (Chirasani, Sternjak et al. 2010, Charles, Holland et al. 2012) and as a result promote glioma progression.

Literature data indicated that DPP-IV is expressed in cancer stem-like cells in mesothelioma (Ghani, Yamazaki et al. 2011, Yamazaki, Naito et al. 2012) and is characteristic for highly metastatic cancer stem-like cells in colorectal carcinoma (Pang, Law et al. 2010). We have therefore analyzed whether DPP-IV is expressed in glioma stem-like cells (GSC). The derivation of primary cell cultures using serum containing media is known to lead to a preferential isolation of more differentiated cells. In addition, cell propagation in serum containing media induces substantial changes of the glioma cells so that the resulting cell lines only distantly represent the original tumor (Lee, Kotliarova et al. 2006). We isolated and characterized primary cell cultures from several GBMs which were propagated in defined serum-free medium favoring the expansion of GSCs. The majority of the GSC cultures exhibited expression of a stem cell marker CD133 as determined by flow cytometry and could undergo differentiation into GFAP and beta III tubulin expressing cells when transferred into serum containing media (Sana, Busek et al. 2018). We detected DPP-IV expression in part of the GSC cultures, the remaining cultures exhibited low or absent DPP-IV expression. There was a trend for decreased DPP-IV expression in cells differentiated using 10% serum (Busek et al. unpublished data), but this may be linked to the negative effect of serum on DPP-IV expression in glioma cells (Balaziova, Busek et al. 2011). The GSC-cultures established in this study are being utilized in other projects of our laboratory, but our current results do not provide strong support for the possible role of DPP-IV in glioma stem-like cells.

Attachment 7 contains our publication analyzing FAP expression in human gliomas and preclinical glioma models and its association with patient survival. As we have recently reviewed (**Attachment 1**), FAP may be expressed by transformed and stromal cells and has tumor specific and cell-type-dependent functions. FAP is predominantly expressed by tumor associated fibroblasts (CAF), but the presence of these cells in the unique tumor microenvironment of human gliomas is rather controversial. Several studies indicate that in some, but not all tumor types FAP overexpression is associated with a worse disease prognosis (reviewed in **Attachment 1**). Using mRNA quantification, previous reports including ours (Stremenova, Krepela et al. 2007, Mikheeva, Mikheev et al. 2010, Mentlein, Hattermann et al. 2011) suggested that FAP is upregulated in glioblastomas. We and others showed that FAP is expressed in glioma cells *in vitro* (**Attachment 6,** (Balaziova, Busek et al. 2011, Mentlein, Hattermann et al. 2011)), nevertheless the quantity of FAP protein, its presence in various constituents of the glioma microenvironment and its relation to glioma patient survival were unknown.

In our patient cohort comprising 56 patients with newly diagnosed glioma, FAP expression was increased in glioblastomas compared to non-tumorous brain tissue (pharmacoresistant epilepsy, n = 15), in particular on the protein level. Our results revealed the presence of two to three molecular forms of FAP with an alkaline pI in GBM tissues, whereas glioma cell lines predominantly expressed forms with an acidic pI (**Attachment 13**). The amount of FAP protein as determined by ELISA and western blotting was highly heterogeneous in GBM, presumably reflecting the differences in FAP expression observed in individual molecular subtypes of GBM. Based on the TCGA data, FAP expression was highest in the mesenchymal subtype of glioblastoma with over 70 % of the mesenchymal tumors exhibiting at least a twofold upregulation compared to the controls. We further performed a gene set enrichment analysis of the transcripts that significantly positively correlated with FAP. Genes encoding various extracellular matrix proteins and factors involved in inflammation and wound healing were overrepresented and FAP expression also positively correlated with the transcripts for several proteases including DPP-IV.

FAP expression was reported as a possible negative prognostic factor in several malignancies. We therefore analyzed the association between FAP expression and survival in our patient cohort and publicly available datasets. FAP expression was associated with patient survival in a dataset comprising both low-grade and high-grade tumors. Nevertheless as FAP expression is typical for glioblastomas as compared to low-grade tumors, the observed effect on survival is most likely due to the larger proportion of glioblastomas in the subgroups with higher FAP expression. In our patient cohort, the median survival was 48 weeks for glioblastoma (n = 42) and at the time of last follow-up, 60 % of grade III (n = 5) and 100 % of grade II (n = 7) patients were alive. Using Cox regression analysis we identified tumor grade, age at operation, and tumor volume as factors most strongly predicting patient survival in the whole experimental cohort. In glioblastoma, the combination of patient age and the presence of residual tumor had the highest predictive power. FAP expression as determined by qRT-PCR, or western blotting neither alone nor in combination with the clinical variables was associated with patient survival in the whole patient cohort or in glioblastoma. Similarly, our analysis of two different publicly available microarray datasets revealed no association between FAP mRNA expression and survival in grade IV tumors (glioblastomas).

Using immunohistochemistry with a panel of monoclonal FAP antibodies, we observed three patterns of FAP expression in gliomas: fibrillary intraparenchymal positivity, predominantly perivascular positivity and the presence of both intraparenchymal and perivascular positivity. The fibrillary intraparenchymal FAP immunopositivity most likely corresponds to the presence of FAP in malignant cells – it colocalizes with the astrocytic marker GFAP and part of these intraparenchymal FAP^+ also express SOX-2, a marker of multipotent neural and glioma stem cells. We further observed the presence FAP^+GFAP^- cells in the majority of glioblastomas. These cells were absent in non-malignant brain tissue, were predominantly localized around dysplastic blood vessels and lacked EGFR amplification, a molecular aberration typical for glioma cells. FAP expression in these cells colocalized with typical mesenchymal markers (smooth muscle actin (SMA) and TE-7) and regions with these cells contained substantial amounts of the mesenchymal extracellular matrix protein fibronectin. FAP expression was not detected in microglia or endothelial cells. We further investigated whether hematopoietic cells originating in the bone marrow may contribute to the FAP^+ stromal subpopulation in human glioblastomas by determining the coexpression of CD45 in FAP^+ cells. Although the CD45 and FAP staining patterns were largely non-overlapping, sporadic $CD45^+FAP^+$ cells could be detected in several glioblastomas. Collectively these data suggest that in addition to glioma cells, FAP is expressed by several other cellular types within the microenvironment of human glioblastomas.

We further characterized FAP expression in glioma models that could be utilized in preclinical testing of FAP targeting therapies. For this purpose, a panel of permanent glioma cell lines, primary cell cultures derived from bioptic material and xenotransplants generated from these cell lines were utilized. Similarly to human bioptic material, FAP expression in glioma cells was variable in these preclinical glioma models. It was characteristically observed in serum derived primary cell cultures, U87 cells and corresponding xenotransplants, whereas FAP expression in glioma stem-like cells (GSC) and U373 cells was low (**Attachment 7** and unpublished data). The mouse xenotransplantation model also allowed assessment of FAP expression in the mouse stromal cells using a species specific real-time RT-PCR assay developed by us (**Attachment 14**). Stromal FAP mRNA expression was upregulated in the tumors compared to the contralateral hemisphere. These results were supported by immunohistochemical detection of FAP protein in mouse cells in close proximity of the tumors. Part of these cells coexpressed the CXCR4 chemokine receptor suggesting that these cells may be recruited to the tumors by chemotactic mediators released in the

tumor microenvironment. Collectively, FAP expression is upregulated in a subgroup of glioblastomas and FAP is expressed in both glioma cells and various types of stromal cells. FAP positive stromal cells in glioblastomas seem to have several characteristics of cancer associated fibroblasts (CAF) and mechanisms contributing to the tumor promoting effects of these cells seem to be conserved among various tumor types. In a collaborative project with the group of Prof. Smetana (Institute of Anatomy, First Faculty of Medicine Charles University), we have shown that cancer associated fibroblasts enhance the migration of glioma cells by soluble mediators and increase their growth by promoting cell proliferation (**Attachment 15**). An ongoing project in our laboratory evaluates whether FAP expressing stromal cells isolated from glioblastomas have similar effects.

Existing treatment options in glioblastoma are limited and largely palliative. Selective expression of FAP in glioblastomas and putative tumor promoting effects of FAP expressing stromal cells may offer a possibility to develop new therapeutic approaches that would exploit FAP as a means to selectively deliver cytotoxic compounds into the tumors. This approach would allow simultaneous targeting of several components of the tumor microenvironment. In an effort to design and test new FAP targeting approaches we are collaborating with the group of Jan Konvalinka (Institute of Organic Chemistry and Biochemistry of The Czech Academy of Sciences). Inhibition of FAP enzymatic activity with a highly selective low molecular weight inhibitor prepared by Jansen et al. (Jansen, Heirbaut et al. 2014) did not affect glioma cell growth or invasiveness (Busek et al. unpublished data). Nevertheless, a specific interaction between this inhibitor and the highly conserved active site of the FAP protein can be used to create highly specific FAP targeting compounds. **Attachment 8** contains our publication describing the synthesis and evaluation of an N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer containing a FAP-specific inhibitor as the targeting ligand (an anti-FAP iBody). iBodies are novel tools for the targeting of proteins with a known ligand (Sacha, Knedlik et al. 2016). These biocompatible synthetic compounds offer several advantages compared to antibodies. iBodies are highly modular and versatile, and conjugates containing virtually any desired compound can be easily prepared. Synthetic HPMA conjugates have long been used as carriers for drug delivery to solid tumors, often making use of the enhanced permeability and retention (EPR) effect. In addition, the molecular weight of the HPMA backbone can be easily adjusted to specifically tailor the pharmacokinetic properties. An anti-FAP iBody exhibited high specificity for FAP, with minimal binding to the related proteases DPP-IV, DPP9 and prolyl

oligopeptidase. We have tested the utility of the anti-FAP iBody in a variety of biochemical assays that traditionally use antibodies such as pull-down, western blotting, ELISA, flow cytometry and immunohistochemistry. Interestingly, conjugation of the reversible inhibitor to a polymeric scaffold led to a very stable interaction between FAP and the anti-FAP iBody with a remarkably low dissociation rate. The iBody could be utilized to detect FAP expression in living cells using flow cytometry and in tissue sections using histochemistry. In living cells, binding of an anti-FAP iBody triggered FAP internalization, suggesting its suitability for targeted delivery of cytotoxic compounds. As expected, the anti-FAP iBody interacted with both human and mouse FAP due to the high conservation of the enzyme active site. Anti-FAP iBodies thus provide a tool that can be easily translated into preclinical mouse cancer models. In summary we designed, synthesized, and characterized a novel type of a highly selective FAP targeting agent, an iBody based on an HPMA copolymer decorated with a FAP inhibitor. The specificity, modularity, and versatility of the anti-FAP iBody make it suitable for a broad spectrum of biochemical and biomedical applications, including its use for *in vivo* imaging and selective drug delivery into the tumor microenvironment.

Summary of the main results of our work in gliomas:

- Several DPP-IV homologs are expressed in primary cell cultures derived from high-grade gliomas and the DPP-IV-like enzymatic activity is associated with their lower clonogenic capacity and slower proliferation.
- Forced expression of DPP-IV in glioma cells decreases their proliferation by inducing a G2/M cell cycle block, and inhibits cell adhesion and migration.
- Forced DPP-IV expression reduces glioma growth in an orthotopic xenograft model.
- The observed anti-oncogenic effects of DPP-IV in glioma cells are in large part independent of its enzymatic activity.
- A protocol for the derivation and propagation of glioblastoma stem-like cells (GSC) from bioptic material was successfully established in our laboratory. DPP-IV is variably expressed in GSC, FAP expression in these cultures is low.
- FAP expression is increased in glioblastoma though with high inter-tumoral variability, possibly due to its differential expression in individual molecular subtypes of glioblastoma.

- FAP expression in glioblastoma is associated with the mesenchymal subtype of glioblastoma and correlates with the expression of genes encoding extracellular matrix proteins and several proteases.
- FAP is expressed in malignant, presumably non-stem cells in glioblastoma and in several types of stromal cells including perivascularly localized mesenchymal cells and a small subset of $CD45^+$ cells.
- The overall quantity of FAP is not associated with patient survival in glioblastoma.
- Prototype FAP targeting compounds based on a low molecular weight inhibitor (iBodies) were developed in collaboration with the group of Jan Konvalinka (Institute of Organic Chemistry and Biochemistry of The Czech Academy of Sciences).
- Anti-FAP iBodies are highly specific towards FAP and can be used in a variety of biochemical and cell biology systems.
- Using the iBody concept, we are currently developing and in preclinical models testing potential novel anticancer FAP targeting compounds.

6. Concluding remarks and future perspectives

Proteases constitute 2–3% of all known human genes and play an important role under physiological conditions and in the pathogenesis of the human malignancies. The view of these molecules in the field of cancer has shifted from their perception as mere "bulldozers" paving the road for cancer cell invasion to important regulators of various biological functions in the tumor microenvironment. In addition to the extracellular matrix remodeling, proteases cleave several regulatory biopeptides, cell surface receptors, ion channels and adhesion molecules, cytoskeletal proteins, components of the intracellular signaling cascades, and regulators of the cell cycle (reviewed in **Attachment 5**). In addition to their proteolytic activity, several proteases have non-proteolytic functions which broaden their functional potential.

In the presented thesis I summarized our studies which showed that DPP-IV and FAP may participate on the pathogenesis of a paraneoplatic form of diabetes (T3cDM) that accompanies pancreatic ductal adenocarcinoma (PDAC). From a clinical standpoint, differentiation of patients who develop diabetes as an early symptom of PDAC from the much more prevalent patients presenting with initial stage of a "typical" type 2 DM holds a promise for a more timely diagnosis and thus an improved outcome of this dismal cancer. Unfortunately, none of the parameters evaluated in our studies had sufficient discriminatory power for distinguishing PDAC from type 2 DM. Recently reported approaches utilizing e.g. detection of circulating tumor DNA and a panel of protein biomarkers (Cohen, Javed et al. 2017) could be beneficial in this setting. We further demonstrated that DPP-IV and FAP are co-expressed in pancreatic alpha cells under physiological conditions. Mechanistic studies testing their possible function in Langerhans islets however need to be performed in order to understand their role in regulating insulin secretion.

Our studies in glioblastoma revealed that, notwithstanding its upregulation in the tumor tissue, DPP-IV inhibits proliferation, adhesion and migration of glioma cells and reduces glioma growth in an orthotopic xenograft model independent of its enzymatic activity. This effect is consistent with reports in other tumor types demonstrating that DPP-IV is a tumor suppressor and illustrates that several proteases act as negative regulators of tumor progression (Lopez-Otin and Matrisian 2007). Our results also serve as an example of the fact that several proteases may execute their functions through non-proteolytic mechanisms (Del Rosso, Fibbi et al. 2002).

Upregulation of FAP in a subset of glioblastomas and its expression in transformed and stromal cells suggest that similarly to extracranial malignancies, this protease is overexpressed in primary brain tumors. Whether FAP contributes to glioma progression remains to be determined. Our study did not reveal association between overall levels of FAP in the glioblastoma tissue and patient survival. A more detailed analysis using bioptic material is currently ongoing together with experiments assessing the *in vitro* and *in vivo* effects of FAP overexpression or downmodulation in glioma cells and the role of FAP positive stromal cells derived from glioblastomas. Although there are several challenges (see **Attachment 1** for a review), the possible use of FAP as a therapeutic target in glioblastoma and possibly other tumors that overexpress FAP is the focus of our ongoing projects. In cooperation with our colleagues at the Institute of Organic Chemistry and Biochemistry of The Czech Academy of Sciences (J. Konvalinka, P. Sacha, T. Knedlik) we are developing and testing FAP targeting compounds based on the iBody concept. FAP expression is variable even in tumors known to be generally FAP-positive (e.g. 36% of PDAC in our study (**Attachment 4**) had low FAP expression according to immunohistochemistry). Non-invasive analysis of FAP expression in the tumors considered for the application of FAP targeting treatments is important and we are therefore testing FAP PET probes that would be suitable for this purpose.

In summary, in this thesis I have provided an overview of current studies on DPP-IV and FAP in cancer which together with our results suggest that these multifunctional molecules play a role in cancer pathogenesis. The available data also indicate that in selected cancers DPP-IV and FAP may represent useful biomarkers and potential therapeutic targets.

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8. Attachments – reprints of publications included in the habilitation thesis