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Fibroblastový aktivační protein v nádorovém mikroprostředí
glioblastomu a mozkových metastáz

Fibroblast activation protein in the tumour microenvironment
of glioblastoma and brain metastases

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Abstrakt

Fibroblastový aktivační protein (FAP) je serinová proteáza, která hraje klíčovou roli v širokém spektru patologických procesů, zejména v souvislosti s rakovinou. FAP je primárně exprimován ve „fibroblastech asociovaných s nádorem“ a v menší míře v nádorových buňkách v mnoha solidních nádorech. Předpokládá se, že se FAP podílí hlavně na remodelaci tkání, hojení ran a degradaci proteinů extracelulární matrix. V naprosté většině zdravých lidských tkání je exprese FAP minimální nebo zcela chybí, což z něj činí atraktivní cíl pro onkologický výzkum. Vzhledem k jeho specifické a zvýšené expresi v nádorovém mikroprostředí je FAP intenzivně zkoumán jako potenciální prognostický biomarker a jako cíl pro diagnostické zobrazování i cílenou protinádorovou léčbu.

V této disertační práci jsme prokázali, že exprese FAP je výrazně zvýšena v glioblastomech i mozkových metastázách solidních nádorů ve srovnání s nenádorovou mozkovou tkání. V tkáních rekurentních glioblastomů nedochází ke signifikantnímu zvýšení exprese FAP při srovnání s expresí v primárních glioblastomech. Naše analýzy naznačují, že FAP je převážně exprimován v mesenchymálních stromálních buňkách, které by mohli odpovídat fibroblastům asociovaným s nádorem v oblastech s depozity extracelulární matrix a se vztahem k vaskularizaci nádorů jak u glioblastomů, tak u mozkových metastáz. Exprese FAP ani zastoupení FAP+ stromálních buněk se signifikantně neliší při srovnání různých histologických subtypů mozkových metastáz. Dále jsme prokázali, že FAP je exprimován v části nádorových buněk v některých mozkových metastázách pocházejících z melanomu, plicních, prsních, gastrointestinálních, renálních a ovariálních karcinomů, z karcinomů neznámého origa, karcinomu slinných žláz a sarkomů. Podobná exprese FAP napříč různými histologickými subtypy mozkových metastáz spolu s jeho expresí ve stromálních a místy i nádorových buňkách, vytváří racionální základ pro další výzkum diagnostiky a terapie mozkových metastáz založené na cílení FAP, například prostřednictvím konjugátů s FAP inhibitory.

Naše výsledky ukazují, že exprese FAP pozitivně koreluje s expresí TGFβ1 jak u glioblastomů, tak i u mozkových metastáz. Funkční analýzy prokázaly, že TGFβ1 je významným regulátorem exprese FAP v glioblastomech. Naše předběžné výsledky naznačují, že fibroblasty asociované s nádorem z mozkových metastáz rakoviny plic mohou podporovat migraci nádorových buněk produkcí solubilních faktorů. Navíc jsme zjistili, že fibroblasty asociované s nádorem odvozené z glioblastomů a mozkových metastáz mají vyšší zastoupení senescentních buněk, což spolu s výsledky funkčních analýz naznačuje, že tyto fibroblasty asociované s nádorem mohou vykazovat tzv. „se senescencí asociovaný sekretorický fenotyp“. V této disertační práci jsme také hodnotili protinádorovou účinnost prototypu FAP-cílicího polymerního konjugátu v xenotransplantačním myším modelu FAP-exprimujících nádorů.

Tato práce rozšiřuje znalosti o expresi FAP v glioblastomech a mozkových metastázách a podporuje možné využití FAP v teranostických strategiích v léčbě těchto agresivních malignit.

Klíčová slova

glioblastom, mozkové metastázy, nádorové mikroprostředí, fibroblastový aktivační protein, s nádorem asociované fibroblasty, cílená léčba

Abstract

Fibroblast Activation Protein (FAP) is a serine protease that plays a crucial role in a broad spectrum of pathological processes, particularly in the context of cancer. FAP is primarily expressed in “cancer-associated fibroblasts” and, to a lesser extent, in tumour cells within many solid tumours. FAP is presumed to be involved in tissue remodelling, wound healing, and extracellular matrix protein degradation, while its expression in most healthy human tissues is minimal or absent. Due to its unique expression in the tumour microenvironment, FAP has been studied as a potential prognostic biomarker and as a target for diagnostic imaging and targeted cancer therapy

In this dissertation thesis, we demonstrated that FAP expression is significantly elevated in glioblastomas and brain metastases of solid tumours compared to non-cancerous brain tissue. There is no significant increase in FAP expression in recurrent glioblastomas in comparison to primary glioblastomas. Our analyses suggest that FAP is predominantly expressed in mesenchymal stromal cells, which may correspond to cancer-associated fibroblasts in areas with extracellular matrix deposits and in relation to tumour vascularisation, both in glioblastomas and brain metastases. FAP expression and the proportion of FAP⁺ stromal cells do not significantly differ among histological subtypes of brain metastases. Furthermore, we show that FAP is expressed in some tumour cells, but this was observed only in a subset of brain metastases from melanoma, lung, breast, gastrointestinal, renal and ovarian cancer, carcinoma of unknown primary, salivary gland cancer and sarcoma. Similar FAP expression across various histological subtypes of brain metastases, along with its presence in stromal and occasionally in tumour cells, provides a rationale for further research into the diagnostic and therapeutic approaches in brain metastases based on FAP targeting, for example, through conjugates with FAP inhibitors.

Our results indicate that FAP expression positively correlates with TGFβ1 expression in both glioblastomas and brain metastases. Functional analyses revealed that TGF-β1 is a crucial regulator of FAP expression in glioblastomas. Our preliminary findings suggest that cancer-associated fibroblasts from brain metastases of lung cancer may promote tumour cell migration by producing soluble factors. Additionally, we found that cancer-associated fibroblasts derived from glioblastomas and brain metastases exhibit a higher proportion of senescent cells, which, together with functional analysis results, suggests that these cancer-associated fibroblasts may display a “senescence-associated secretory phenotype”. In this dissertation, we also evaluated the anti-cancer efficacy of a FAP-targeting polymer conjugate prototype in a xenograft mouse model of FAP-expressing tumours.

This work expands the understanding of FAP expression in glioblastomas and brain metastases and supports its potential use in theranostic strategies for treating these aggressive tumours.

Key words

glioblastoma, brain metastases, tumour microenvironment, fibroblast activation protein, cancer associated fibroblasts, targeted therapy

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Abbreviations

- **α SMA** – Alpha smooth muscle actin
- **ADAM10** – A Disintegrin and metalloproteinase domain-containing protein 10
- **ADORA2A** – Adenosine A2A receptor
- **ALCAM** – Activated leukocyte cell adhesion molecule
- **AMPA** – α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- **ANGPTL4** – Angiopoietin-like 4
- **BBB** – Blood-brain barrier
- **BDNF** – Brain-derived neurotrophic factor
- **BiTEs** – Bispecific T-cell engagers
- **BMP4** – Bone morphogenetic protein 4
- **BSA** – Bovine Serum Albumin
- **CAFs** – Cancer-associated fibroblasts
- **CAMs** – Cell adhesion molecules
- **CAR-T cells** – Chimeric antigen receptor T cells
- **CD** – Cluster of Differentiation
- **CCL2** – C-C motif chemokine ligand 2
- **CEMIP** – Cell migration-inducing and hyaluronan-binding protein
- **cMASCs** – CNS Metastasis-associated Stromal Cells
- **CNS** – Central nervous system
- **Col1a1** – Collagen I
- **CSF-1** – Colony-stimulating factor-1
- **CT** – Computed Tomography
- **CUP** – Cancer of unknown primary origin
- **CXCL5** – C-X-C motif chemokine 5
- **CXCL12** – C-X-C motif chemokine ligand 12
- **Cx** – Connexin protein
- **DCs** – Dendritic cells
- **DM1** – Mertansine
- **DMSO** – Dimethyl Sulfoxide
- **DMEM** – Dulbecco's Modified Eagle Medium
- **DOTA** – Dodecane Tetraacetic Acid
- **DPP-IV** – Dipeptidyl peptidase IV
- **DDR2** – Discoidin domain receptor 2
- **ECM** – Extracellular matrix
- **ECM-1** – Extracellular matrix protein 1
- **EGF** – Epidermal growth factor
- **EGFR** – Epidermal growth factor receptor
- **EGR-1** – Early growth response protein 1
- **ELISA** – Enzyme-linked immunosorbent assay

- **EMT** – Epithelial-mesenchymal transition
- **EpCAM** – Epithelial Cell Adhesion Molecule
- **EPR** – Enhanced Permeability and Retention Effect
- **EZH2** – Enhancer of zeste homolog 2
- **FAK** – Focal adhesion kinase
- **FAK-c-Src-JAK2** – Focal adhesion kinase-c-Src-Janus kinase 2
- **FAP** – Fibroblast activation protein
- **FAPI** – Fibroblast Activation Protein Inhibitor
- **FCS** – Fetal Calf Serum
- **FDCs** – Follicular dendritic cells
- **FGF21** – Fibroblast growth factor 21
- **FOXP3** – Forkhead box P3
- **FSP1/S100A4** – Fibroblast-specific protein 1
- **GAMs** – Glioma-associated macrophages
- **GABA** – Gamma aminobutyric acid
- **GFAP** – Glial fibrillary acidic protein
- **G-CSF** – Granulocyte colony stimulating factor
- **GIT** – Gastrointestinal tract
- **GSC** – Glioblastoma Stem Cell
- **GSK-3 β** – Glycogen synthase kinase 3 beta
- **HGF** – Hepatocyte growth factor
- **HIF** – Hypoxia-inducible factors
- **HuNu** – Human nuclei
- **HUVECs** – Human umbilical vein endothelial cells
- **ICAM-1** – Intercellular adhesion molecule 1
- **IDH** – Isocitrate dehydrogenase
- **IFN1** – Interferon 1
- **IFNAR1** – Interferon-alpha/beta receptor alpha chain
- **IGF-1** – Insulin-like growth factor 1
- **IGFBP-2** – Insulin-like Growth Factor Binding Protein-2
- **IGFBP-3** – Insulin-like Growth Factor Binding Protein-3
- **IL** – Interleukin
- **IL-6** – Interleukin-6
- **iCAFs** – Inflammatory CAFs
- **JAK** – Janus kinase
- **LIF** – Leukemia inhibitory factor
- **MACS** – Magnetic-activated cell sorting
- **MAP2K1/2** – Mitogen-activated protein kinase kinase 1/2
- **MCTs** – Monocarboxylate transporters
- **MGMT** – Methylguanine-DNA methyltransferase
- **miR** – Micro RNA

- **MMPs** – Matrix metalloproteinases
- **MRI** – Magnetic Resonance Imaging
- **MSCs** – Mesenchymal stem cells
- **mGluRs** – Metabotropic glutamate receptors
- **mTOR** – Mammalian target of rapamycin
- **MDSCs** – Myeloid-derived suppressor cells
- **NLGN3** – Neuroligin 3
- **NF** – Negative fraction
- **NF- κ B** – Nuclear factor kappa-light-chain-enhancer of activated B cells
- **NK cells** – Natural killer cells
- **NLR** – Neutrophil-to-lymphocyte ratio
- **OS** – Overall survival
- **PBS** – Phosphate-buffered Saline
- **PEG** – Polyethylene Glycol
- **PET** – Positron Emission Tomography
- **PDGFR** – Platelet-derived growth factor receptor
- **PDGF** – Platelet-derived growth factor
- **PD-L1** – Programmed cell death ligand 1
- **PD-1** – Programmed cell death 1
- **PDPK1** – 3-phosphoinositide-dependent protein kinase-1
- **PF** – Positive fraction
- **PFS** – Progression-free survival
- **PHGDH** – 3-phosphoglycerate dehydrogenase
- **PHD/VHL** – Prolyl hydroxylase domain / Von Hippel–Lindau
- **PHD2** – Prolyl hydroxylase 2
- **PI3K** – Phosphatidylinositol 3-kinase
- **PLGF** – Placental growth factor
- **PRE** – Pharmacoresistant epilepsy
- **PRKDC** – Protein kinase, DNA-activated, catalytic subunit
- **P/S** – Penicillin/Streptomycin
- **PTEN** – Phosphatase and tensin homolog
- **RNA-seq** – RNA sequencing
- **SASP** – Senescence-associated secretory phenotype
- **scRNA-seq** – Single-cell RNA sequencing
- **SHH** – Sonic hedgehog
- **SMO** – Smoothed
- **SPECT** – Single-Photon Emission Computed Tomography
- **SR-A** – Scavenger receptor-A
- **TAMs** – Tumour-associated macrophages
- **TANs** – Tumour-associated neutrophils
- **TERT** – Telomerase reverse transcriptase

- **TGFbeta** – Transforming growth factor beta
- **TLS** – Tertiary lymphatic structures
- **TILs** – Tumour-infiltrating lymphocytes
- **TME** – Tumour microenvironment
- **TNF- α** – Tumour necrosis factor α
- **TrkB** – Tropomyosin receptor kinase B
- **VLA-4** – Very late antigen-4
- **VEGF** – Vascular endothelial growth factor
- **WHO CNS5** – The fifth edition of the World Health Organization Classification of Tumours of the Central Nervous System
- **WNT5 α** – Wnt family member 5A
- **ZO-1** – Zonula occludens-1

1. Introduction

1.1 Brain tumours

According to the fifth edition of the World Health Organization Classification of Tumours of the Central Nervous System (WHO CNS5) (Louis et al. 2021), brain tumours are divided into:

1. Gliomas, glioneuronal tumours, and neuronal tumours
 - a. Adult-type diffuse gliomas
 - i. Astrocytoma, isocitrate dehydrogenase (IDH)-mutant
 - ii. Oligodendroglioma, IDH-mutant, and 1p/19q-codeleted
 - iii. Glioblastoma, IDH-wildtype
 - b. Paediatric type diffuse low-grade gliomas
 - c. Paediatric type diffuse high-grade gliomas
 - d. Circumscribed astrocytic gliomas
 - e. Glioneuronal and neuronal tumours
 - f. Ependymal tumours
2. Choroid plexus tumours
3. Embryonal tumours
4. Pineal tumours
5. Cranial and paraspinal nerve tumours
6. Meningiomas
7. Mesenchymal, non-meningothelial tumours
8. Melanocytic tumours
9. Hematolymphoid tumours
10. Germ cell tumours
11. Tumours of the sellar region
12. Metastases to the central nervous system (CNS)

Tumours which originate within the brain or its surrounding tissues are referred to as primary brain tumours. Primary brain tumours occur at a rate of approximately 10.82 cases per 100,000 people annually, with about one-third of these being malignant (De Robles et al. 2015). Primary brain tumours can develop at any age but are more common in older adults. Secondary/metastatic brain tumours originate from cancers in other parts of the body. Brain metastases are the most common intracranial malignancy, affecting about 10 to 40% of patients with metastatic tumours (Lamba et al. 2021). The incidence of brain metastases is estimated to be ten times higher than the incidence of primary brain tumours (Ostrom et al. 2018b).

1.1.1 Glioblastoma

Glioblastoma, an aggressive type of grade 4 astrocytic glioma, is characterised by a diffusely infiltrative growth pattern with nuclear atypia, high mitotic activity, necrosis and microvascular proliferation. Glioblastomas are molecularly defined by the presence of telomerase reverse transcriptase (TERT) promoter mutations, epidermal growth factor receptor (EGFR) amplification or a combination of chromosome 7 gain and chromosome 10 loss. These tumours lack mutations in isocitrate dehydrogenase (IDH) 1 and 2, and histone H3 genes. According to the WHO CNS5 classification, these gliomas are currently categorised as Glioblastoma IDH wild type (Louis et al. 2021). Several studies have identified two to six glioblastoma subtypes using various molecular analyses (Le Mercier et al. 2012; Verhaak et al. 2010; Zhang et al. 2020c; Ensenyat-Mendez et al. 2021). Common subtypes include the proneural, classical and mesenchymal subtypes, characterised by specific genetic alterations and gene expression patterns (Verhaak et al. 2010). These subtypes exhibit different prognoses and treatment responses, with the proneural subtype generally associated with better prognosis (Le Mercier et al. 2012). In contrast, the mesenchymal subtype is associated with worse outcomes (Kim et al. 2021).

Glioblastomas comprise 57% of all gliomas and 48% of all primary malignant tumours in the CNS. In the U.S., the annual incidence rate of glioblastoma is 3.21 per 100,000 people, with the highest incidence in those aged 75-84 years. Glioblastoma is more common in males, with an incidence rate of 4.00 per 100,000 compared to 2.53 per 100,000 in females (Ostrom et al. 2018a).

Standard treatment for glioblastoma includes maximal safe surgical resection, followed by radiotherapy and administration of temozolomide, known as the “Stupp’s regimen.” This approach has improved median overall survival (OS) to 14.6 months compared to 12.1 months with radiotherapy alone (Stupp et al. 2005). Despite treatment, glioblastoma almost always recurs, with median progression-free survival (PFS) typically ranging from 1.5 to 6 months and median OS from 5 to 11 months, depending on the used treatment (Weller et al. 2013; Audureau et al. 2018). Treatment options for recurrent glioblastoma are varied and lack a standard approach. Options include additional surgical resection, reirradiation, systemic therapies (e.g., lomustine, bevacizumab, regorafenib), combined approaches, or supportive care. Eligibility for second-line therapy is often limited due to factors such as age and performance status (Nava et al. 2014; Gramatzki et al. 2018; Weller and Le Rhun 2020). Median survival of glioblastoma patients is less than four months with supportive care alone (Keime-Guibert et al. 2007).

Therapeutic advancements have extended the median survival of glioblastoma patients to over 15 months, with 1-year survival rates improving from 34.4% to 44.6%. Despite this, the prognosis for glioblastoma patients remains poor and the 5-year survival rate remains low at 5.8% after the diagnosis of glioblastoma (Ostrom et al. 2018a).

1.1.2 Brain metastases of solid tumours

Primary cancers that commonly spread to the brain include lung, breast, melanoma, and gastrointestinal cancers (Le Rhun et al. 2021). Brain metastases significantly impact the quality of life and increase morbidity and mortality in patients with advanced-stage cancer (Gavrilovic and Posner 2005). The treatment of brain metastases requires a multidisciplinary approach, incorporating surgery, radiation therapy, and systemic treatments, including targeted therapy and immunotherapy. Targeted therapy and immunotherapy have been shown to be initially effective in subsets of patients with brain metastases originating from breast, renal, non-small cell lung cancer and melanoma. However, tumour cell plasticity and changes in the tumour microenvironment often lead to resistance to these systemic treatments (Aldea et al. 2021; Vogelbaum et al. 2022).

Early detection and monitoring of metastatic spread are crucial. Advanced diagnostic methods combining anatomical and functional imaging (MRI, CT, SPECT, PET) are being developed (Palumbo et al. 2014; Langen et al. 2017). Despite improving diagnostic and therapeutic modalities, the prognosis of patients with brain metastases remains poor, with overall survival ranging from 3 to 47 months after diagnosis (Sperduto et al. 2020).

1.2 Tumour microenvironment and carcinogenesis

Tumour formation involves a complex interplay between neoplastic cells, local and migrating stromal cells, the extracellular matrix, tumour vasculature, and the immune system. Therefore, tumours are viewed as complex organs rather than mere clusters of transformed cells. The tumour microenvironment (TME) encompasses a range of immune cells, cancer-associated fibroblasts, endothelial cells, pericytes, and other resident cells from the surrounding tissue. The cellular makeup and functional state of the TME can vary significantly depending on factors such as the organ where the tumour develops, the inherent properties of cancer cells, tumour stage, and individual patient characteristics (De Visser and Joyce 2023).

In healthy tissue, the stroma acts as a barrier to tumour development, but the presence/establishment of transformed cells alters this environment to support cancer progression. These changes include the recruitment of fibroblasts, immune cells, remodelling of the extracellular matrix, and the development of tumour vasculature. In addition, regional differences in the tumour, such as hypoxia, acidity, and growth factors, shape its development (Junttila and De Sauvage 2013).

1.2.1 Tumour microenvironment of glioblastomas

The tumour microenvironment in glioblastoma comprises various cell types, including endothelial cells, neurons, astrocytes, oligodendrocytes, resident immune cells like microglia, and immune cells derived from circulating precursors such as tumour-associated macrophages and tumour-infiltrating lymphocytes (TILs). Noncellular components such as signalling molecules, exosomes, extracellular matrix (ECM), and ECM remodelling enzymes are crucial (Martinez-Lage et al. 2019). This environment is dynamic, with constant changes in cellular composition, interactions, and metabolic factors like pH and oxygen levels (**Fig. 1**). Glioblastoma cells reprogram their microenvironment, supporting rapid proliferation, invasion, and resistance to treatment (Sharma et al. 2023).

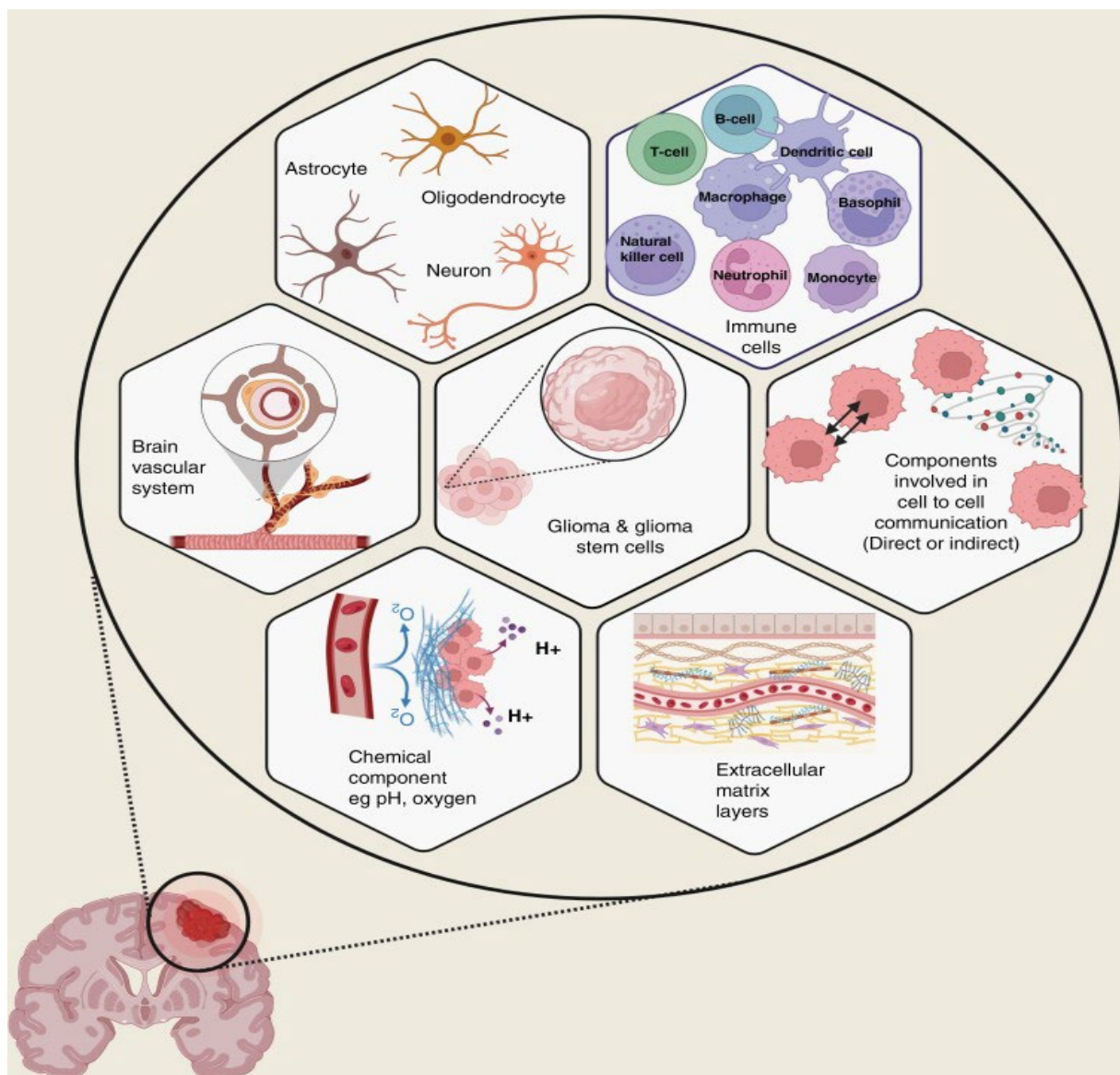


Figure 1. A schematic representation of the components within the glioma microenvironment. This complex, heterogeneous, and interactive system comprises glioma and glioma stem cells, immune cells, the nervous system, the brain's vascular system, and extracellular matrix. Factors that facilitate direct and indirect cell communication and elements of the chemical tumour microenvironment, such as pH and oxygen levels, play significant roles in modulating the glioma tumour microenvironment (Sharma et al. 2023).

Glioblastoma cells are the orchestrators of the TME, playing a central role in directing the behaviour of both cellular and noncellular elements. They do so through a network of signalling mechanisms involving cytokines, chemokines, growth factors, and matrix remodelling enzymes. Their various communication mechanisms, including exosomes, gap junctions, tunnelling nanotubes, and cell-free DNA, further underscore their pivotal role in shaping the TME (Taiarol et al. 2021; Baghban et al. 2020).

Recent discoveries have challenged the traditional view of the central nervous system as an immune-privileged site due to the blood-brain barrier (BBB) limiting immune cell infiltration. The discovery of the glymphatic system and meningeal lymphatic compartments suggests that adaptive immune responses can be initiated within the CNS (Louveau et al. 2015). The role of the meningeal lymphatic compartment in human gliomas is an area that needs further exploration. Extracranial metastases of primary gliomas are rare, with lymphatic spread being uncommon (Noch et al. 2021). Tertiary lymphatic structures (TLS), which are ectopic lymphoid aggregates that develop at sites of chronic inflammation, have also been found in preclinical models of glioblastomas (Van Hooren et al. 2021). TLS resemble secondary lymphoid organs and typically include a B cell follicle encircled by a T cell zone, mature dendritic cells (DCs), and a network of follicular dendritic cells (FDCs). The presence of TLS suggests the potential of immunotherapies in glioblastoma (Van Hooren et al. 2021).

The CNS depends heavily on innate immune mechanisms mediated by astrocytes and resident immune cells, particularly microglia, for defence against pathogens. This immune defence significantly influences the glioma microenvironment, where immune cells, which can make up to 50% of the tumour's cellular content, include microglia, glioma-associated macrophages (GAMs), monocytes, neutrophils, and TILs (Friebel et al. 2020; Klemm et al. 2020; Pombo Antunes et al. 2021). These immune cells interact multilaterally with cancer cells, neurons, glial cells, and vascular endothelial cells, shaping the glioma TME through direct and indirect interactions. Recent research has uncovered a complex interplay between tumour, immune, and host cells, resulting in a strongly immunosuppressive microenvironment, which can be exacerbated by metabolic stress, hypoxia, and damage-associated molecular patterns typical of high-grade gliomas (Sharma et al. 2023).

1.2.1.1 Cellular composition of the glioblastoma microenvironment

Microglia, glioma-associated macrophages, neutrophils

Microglia and GAMs are the most common immune cells found in the microenvironment of glioblastomas. Microglia and some macrophages originate from embryonic progenitors in the yolk sac. In contrast, bone marrow-derived macrophages are believed to arise from peripheral monocytes infiltrating the tumour microenvironment under pathological conditions. Recent research in mice suggests that a meningeal reservoir of monocytes and neutrophils, supplied directly from the adjacent

bone marrow of the skull, can infiltrate the brain parenchyma during CNS injury and inflammation (Cugurra et al. 2021). However, the relevance of this infiltration route in brain tumours remains uncertain.

The glioma microenvironment profoundly influences microglia and GAMs (Klemm et al. 2020), which are highly dynamic cell populations. In both cancerous and noncancerous contexts, cytokines and chemokines can polarise macrophages into either M1 or M2 states. M1 macrophages exhibit immune-supportive and cancer-inhibitory functions, while M2 macrophages typically promote tumour growth. However, this traditional phenotyping may not fully capture the functional diversity of GAMs (Müller et al. 2017). Generally, myeloid cells in the glioma microenvironment are immunosuppressive. Although the mechanisms are not entirely understood, GAMs can produce anti-inflammatory cytokines (IL-4, IL-10, TGFβ), tumour-promoting factors (IGF-1, EGF, PDGF), promote angiogenesis (VEGF, IL-8), disrupt metabolism (via ARG1- and IDO-mediated amino acid depletion), and express immune checkpoints as PD-L1 and CD39 (Rodriguez et al. 2004; Schartner et al. 2005; Hussain et al. 2006; Sharma et al. 2023). Similar to other cancers, myeloid-derived suppressor cells (MDSCs), which originate from monocytic or granulocytic cells, are also present in the glioma tumour microenvironment. These cells suppress tumour-specific effector T cells in a manner similar to GAMs (Raychaudhuri et al. 2015; Gabrusiewicz et al. 2016). Additionally, single-cell RNA sequencing (RNA-seq) studies have identified a significant presence of neutrophils in the glioma tumour microenvironment (Friebel et al. 2020; Klemm et al. 2020). While neutrophils generally play an immunosuppressive role in glioblastoma, their specific functions are not yet fully understood.

Natural killer cells

Natural killer (NK) cells are CD56⁺CD3⁻ lymphoid cells that conduct antigen-independent immune surveillance against pathogens and stressed cells through "missing-self" recognition, contributing to innate antitumor immunity. In glioblastoma, however, NK cells are scarce among the tumour-infiltrating immune cells, primarily consisting of the CD56dimCD16⁻ subtype (Kmieciak et al. 2013). Single-cell studies confirm the rarity of NK cells in primary gliomas, with CD16⁻ immature NK cells prevalent in IDH wild-type tumours and CD16⁺ cytotoxic NK cells found in IDH1 mutant tumours and brain metastases (Friebel et al. 2020). NK cells are inhibited by HLA-I molecules, which bind to inhibitory killer immunoglobulin-like receptors. Although HLA-I genes in gliomas often undergo loss of heterozygosity, complete deletion is rare. Additionally, the glioma tumour microenvironment suppresses NK cell function - TGFβ, secreted by both cancerous and noncancerous cells, downregulates the NKG2D-activating receptor on NK cells isolated from glioblastoma patients (Crane et al. 2010). Thus, the role of NK cells in anti-glioma surveillance has yet to be fully established. However, in preclinical settings, adoptive NK cells have shown cytotoxic activity against glioma cells, including glioma stem cells, and recent studies have

demonstrated that targeting certain inhibitory mechanisms can enhance NK cell function (Castriconi et al. 2009; Daher and Rezvani 2018; Shaim et al. 2021).

T lymphocytes

T lymphocytes can migrate into brain tumours, but glioblastomas typically show poor infiltration by lymphocytes, including NK and T cells, which comprise only a small fraction of the immune cells (Klemm et al. 2020). Despite the presence of CD8+ cytotoxic T cells and CD4+ helper T cells in glioblastoma, these T cells often become dysfunctional due to senescence, tolerance, anergy, or exhaustion (Woroniecka et al. 2018). There are conflicting reports on the clinical significance of T-cell infiltration in glioblastomas. Kmiecik et al. demonstrated that increased CD3+ T-cell infiltration correlates with prolonged survival in glioblastoma patients, independent of age, Methylguanine-DNA methyltransferase (MGMT) promoter methylation, or post-operative treatment (Kmiecik et al. 2013). On the contrary, another group found that PD-L1 is frequently expressed in T cells infiltrating glioblastomas, and the expression of both PD-L1 and PD-1 serves as negative prognostic indicators for clinical outcomes (Nduom et al. 2016). A meta-analysis by Song et al. concluded that high CD3+ and CD4+ T-cell infiltration in gliomas correlated with poor overall survival, while high FOXP3+ T-cell infiltration predicted poor progression-free survival (Song et al. 2020). These conflicting findings highlight the complex role of T-cell infiltration in gliomas. The extent to which glioma-infiltrating lymphocytes can mount an effective antitumor immune response remains unclear. Additionally, lymphocyte sequestration in the bone marrow, induced by tumour-imposed internalisation of the S1P receptor (S1P1) on T cells, impairs tumour infiltration in glioblastomas (Chongsathidkiet et al. 2018).

Neurons

Interactions between glioma cells and neurons are an emerging area of interest. Glioma cells often exhibit perineuronal satellitosis, a phenomenon first described in classical pathology studies (Scherer 1940). Neurons can interact with glioma cells through paracrine stimulation or synaptic transmission. Studies have shown that brain-derived neurotrophic factor (BDNF) and neuroligin 3 (NLGN3) play significant roles in these interactions. BDNF, which signals through the tropomyosin receptor kinase B (TrkB) receptor, is involved in neuronal growth and differentiation and is implicated in glioma progression (Miranda et al. 2019). NLGN3, a synaptic protein, has been shown to promote glioma cell proliferation through the phosphatidylinositol 3-kinase - mammalian target of rapamycin (PI3K–mTOR) pathway (Venkatesh et al. 2015). Blocking NLGN3 cleavage with A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) inhibitors inhibits glioma growth in xenografts (Venkatesh et al. 2017). Neuronal activity also affects immune function. However, the

impact on the glioma immune microenvironment is not fully understood (Monje et al. 2020).

1.2.1.2 Cellular interactions and metabolic dynamics in the tumour microenvironment of glioblastoma

Gap Junctions

Gap junctions, formed by connexin proteins (Cx), play a complex role in glioblastoma progression. Cx43 is expressed in most glioblastoma samples, affecting intercellular communication (Cottin et al. 2011). Inhibiting gap junctions by small molecular inhibitors sensitises glioblastoma cells to temozolomide treatment (Potthoff et al. 2019). Interestingly, glioblastoma cancer stem cells express Cx46, which enhances self-renewal, while Cx43 is predominantly found in non-stem cells (Hitomi et al. 2015). Cx43 can act as a tumour suppressor in some glioma cell lines (Huang et al. 1998), but it also promotes tumour invasion through glioma-astrocyte interactions (Zhang et al. 1999; Mccutcheon and Spray 2022). Gap junctions facilitate the transfer of functional microRNAs between GBM and endothelial cells, influencing angiogenesis (Thuringer et al. 2016).

Glutamate-Mediated Signalling

Glutamate signalling plays a crucial role in glioblastoma progression and invasion. Glioblastoma cells release glutamate, which acts as an autocrine/paracrine signal promoting cell invasion and migration (Lyons et al. 2007; So et al. 2021). This glutamate release occurs primarily via the system xC transporter and activates Ca^{2+} -permeable α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, inducing intracellular Ca^{2+} oscillations essential for cell migration (Lyons et al. 2007). Glutamate signalling also contributes to metabolic reprogramming and the formation of synaptic-like connections with surrounding cells (Pei et al. 2020). Metabotropic glutamate receptors (mGluRs) are involved in glioblastoma progression, with mGluR1 and mGluR3 antagonists showing anti-proliferative and anti-migratory effects (Pereira et al. 2017). Inhibition of glutamate signalling has been shown to reduce tumour growth and invasiveness in preclinical models (Lyons et al. 2007; Pereira et al. 2017). These findings suggest that targeting glutamate signalling pathways may offer new therapeutic strategies for glioblastoma treatment (Corsi et al. 2019; Mollazadeh et al. 2020; Lange et al. 2021). However, clinical trials targeting glutamatergic neurotransmission have not shown significant benefits (Shi et al. 2022).

Metabolic Factors

Metabolic reprogramming plays a crucial role in interactions between stromal and tumour cells. Glioma cells increase glucose and nutrient uptake to support rapid proliferation and variations in nutrient supply due to heterogeneous neo-angiogenesis affect their metabolic properties (Vaupel and Mayer 2014; Ibrahim-Hashim and Estrella 2019). Tumour cells within the same mass can exhibit different metabolic signatures influenced by extracellular pH and oxygen concentration.

Acidosis and hypoxia

Tumour acidosis, a characteristic of malignancies, promotes an immunosuppressive environment and treatment resistance (Corbet and Feron 2017). Acidosis supports tumour cell survival, invasion, and resistance to chemotherapy and radiotherapy by activating protective mechanisms and metabolic adaptations (Vaupel and Mayer 2014). Acidosis also contributes to immunosuppression and promotes the survival of dormant tumour cells (Peppicelli et al. 2017). Tumour cells' metabolic plasticity allows them to switch between glycolysis and oxidative phosphorylation, which could be therapeutically targeted with metabolic inhibitors (Duraj et al. 2021).

Acidosis is crucial factor affecting tumour progression and therapy resistance in glioblastoma (Reichert et al. 2002; Duan et al. 2018). Acidosis promotes hypoxia-inducible factors (HIF) function and cancer stem cell maintenance through heat shock protein 90 (HSP90), independent of the prolyl hydroxylase domain/ Von Hippel–Lindau (PHD/VHL) pathway (Filatova et al. 2016). Lactic acid induces metabolic reprogramming and facilitates tumour growth by modulating the epigenome in glioblastoma (Seyfried et al. 2022). One case study reported elevated lactate levels in a glioblastoma patient (Kahlon et al. 2016). Lactate transporters - monocarboxylate transporters (MCTs) and pH regulators are frequently overexpressed in glioblastomas, contributing to tumour aggressiveness (Miranda-Gonçalves et al. 2016). Targeting these proteins and metabolic pathways may offer new therapeutic strategies for glioblastoma treatment (Miranda-Gonçalves et al. 2016; Seyfried et al. 2022).

Hypoxia in tumours promotes therapy resistance and is associated with poor prognosis (Casazza et al. 2014; Hayashi et al. 2019). HIFs activate genes essential for cell survival under low oxygen conditions (Semenza 2014). Prolyl hydroxylase 2 (PHD2), which regulates HIF expression, exhibits pro- and antitumor properties depending on the context (Pistollato et al. 2009; Sun et al. 2014). PHD2's role in glioblastoma progression and its potential as a therapeutic target warrant further investigation. Hypoxia induces a glycolytic switch, upregulates proangiogenic genes, and enhances invasion-related proteins in glioblastoma cells (Chédeville et al. 2020). Hypoxia also triggers autophagy, a cellular recycling process contributing to glioblastoma survival and therapy resistance (Jawhari et al. 2016). The hypoxic microenvironment modulates angiogenic and inflammatory responses, involving the nuclear factor kappa-light-chain-enhancer of

activated B cells (NF- κ B) signalling pathway genes associated with chemotherapy resistance (Murat et al. 2009). Targeting hypoxia and HIF (e.g. via small molecular inhibitors) represents a promising therapeutic approach for glioblastoma, potentially improving treatment outcomes and patient survival (Kalkan 2015; Wang et al. 2017b).

1.2.2 Tumour microenvironment of brain metastases

The complex interplay between metastatic cells and the CNS microenvironment begins even before the establishment of brain metastases and continues to evolve throughout the disease's progression. As metastatic cells invade the CNS, they assimilate into and alter their surroundings via complex pathways, influencing tumour establishment, aggressiveness, and treatment response (Fig. 2).

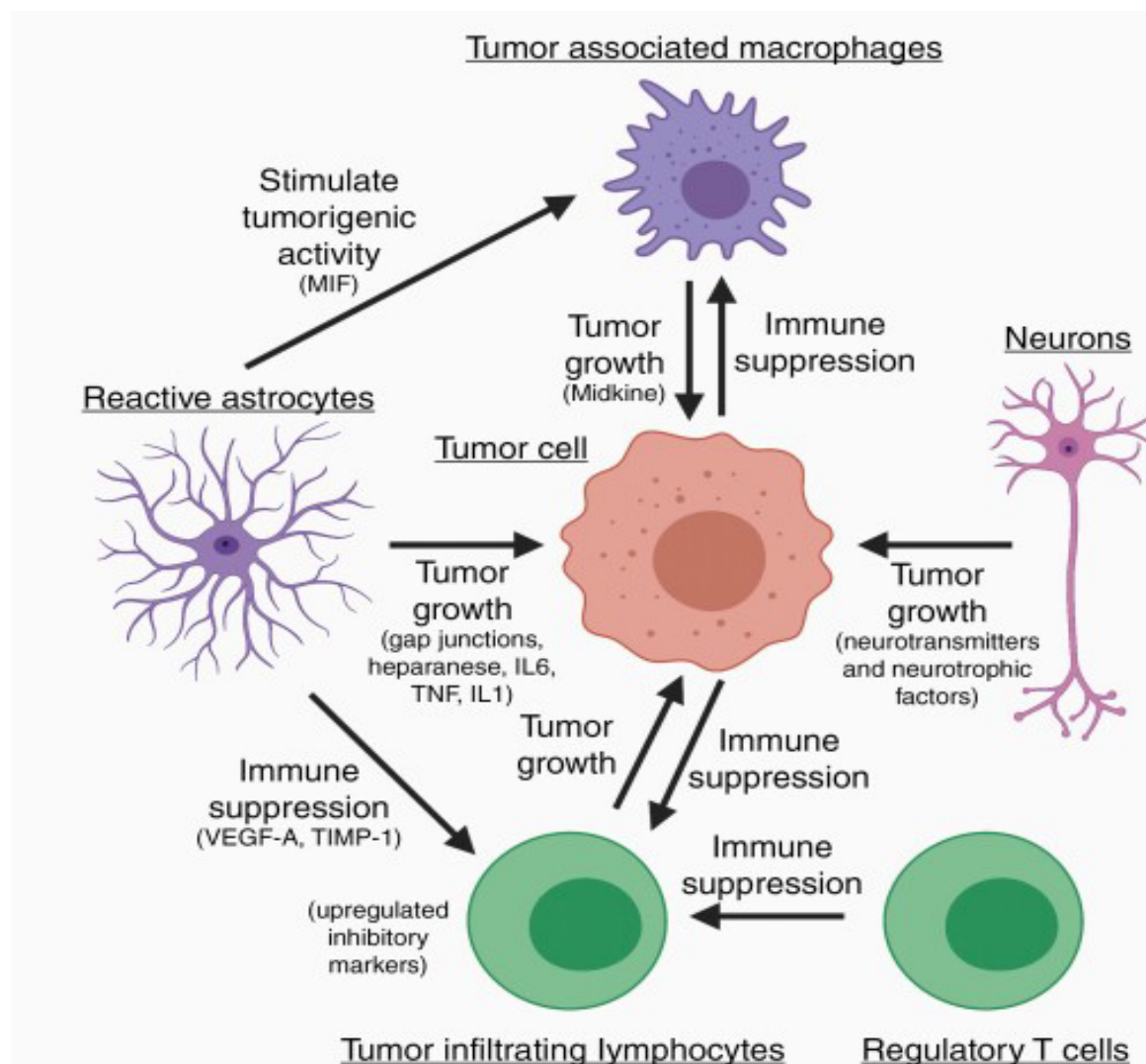


Figure 2. Overview of key interactions within the tumour microenvironment of brain metastases. Processes involving tumour cells, tumour-associated macrophages, reactive astrocytes, neurons, and tumour-infiltrating lymphocytes (Srinivasan et al. 2021).

1.2.2.1 Mechanisms underlying the establishment of brain metastases

Premetastatic niche and BBB disruption

Before metastatic cells breach the blood-brain barrier (BBB) and enter the CNS, a permissive "premetastatic niche" is often established in the brain. The formation of the premetastatic niche is induced by cytokines, chemokines, exosomes, and angiogenic factors secreted by the primary tumour and circulating tumour cells. Although less characterized in solid tumour brain metastases, similar factors involved in the formation of the premetastatic niche include TNF- α , TGF β , VEGF, and CXCL1 (Liu and Cao 2016; Wang et al. 2017a; Medeiros and Allan 2019).

A critical aspect of CNS metastasis is the initial disruption of the BBB. A study using a murine model of acute monocytic leukaemia showed that leukemic cells increase BBB permeability by secreting matrix metalloproteinases 2 and 9, which disrupt tight junctions between endothelial cells by degrading ZO-1, claudin-5 and occludin (Feng et al. 2011). VEGF similarly compromises the BBB, and VEGF inhibition via monoclonal antibodies has shown protective effects (Zhang et al. 2017). Higher brain metastasis incidence has been observed in small-cell lung cancer patients with elevated placental growth factor (PLGF), which breaks down tight junctions via a VEGFR-mediated pathway, facilitating CNS invasion by circulating tumour cells (Li et al. 2013). Changes in pericyte populations, such as an increase in desmin-expressing pericytes, have also been noted in high-permeability BBB metastases (Lyle et al. 2016).

Cellular adhesion and CNS invasion

Tumour-specific cellular adhesion molecules are upregulated in endothelial cells prior to the development of metastases. In a murine breast cancer model, brain endothelial vascular cells increase cell adhesion molecules (CAMs) like E-selectin, VCAM-1, ALCAM, ICAM-1, VLA-4, and β 4-integrin in response to circulating tumour cells, enhancing susceptibility to CNS infiltration (Soto et al. 2014).

The CNS microenvironment is modulated by circulating factors before metastases are established. Tumour-derived exosomes can enter the CNS parenchyma by transcytosis, as demonstrated in a breast cancer model (Morad et al. 2019). Exosomes containing miR-122 suppress glucose uptake in astrocytes, creating a favourable environment for metastatic cells, and inhibiting miR-122 restores normal glucose uptake, reducing metastasis incidence (Fong et al. 2015). Similarly, exosome-delivered miR-181c disrupts the BBB and increases brain metastasis incidence by affecting actin localisation via downregulating 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Tominaga et al. 2015). Cell migration-inducing and hyaluronan-binding protein (CEMIP) in exosomes promotes CNS metastasis by inducing pro-metastatic changes in brain endothelial cells and microglia, supporting a pro-inflammatory environment. High

CEMIP expression in primary tumours correlates with a shorter time to brain metastasis and poorer overall survival (Rodrigues et al. 2019).

Extravasation and colonisation

The process of metastatic cells entering the CNS involves several critical steps, including intravascular arrest, extravasation, and colonisation. Intravascular arrest leads to microthrombosis and reduced microvascular perfusion, impacting the brain microenvironment. Platelet aggregation and fibrin formation at the site of arrested tumour cells are essential for their persistence and extravasation (Feinauer et al. 2021).

Extravasation involves dynamic interactions between tumour cells and the blood vessel wall (Kienast et al. 2010). Most extravasated cancer cells do not survive in the tissue. Those that do, persist within the perivascular niche - a critical site for cancer stem cells that drive tumour growth and angiogenesis. In glioblastomas, these cancer stem cells may even differentiate into endothelial cells and/or pericytes (Eyler et al. 2008; Ricci-Vitiani et al. 2010; Wang et al. 2018). Thus, the perivascular niche in brain metastases is vital for tumour progression and possibly treatment resistance, similar to glioblastomas (Calabrese et al. 2007; Charles and Holland 2010; Infanger et al. 2013; Wang et al. 2021b).

Vascular co-option and angiogenesis

Reorganisation of the cerebral microvasculature is crucial for metastasis progression. Vascular co-option involves tumour cells utilising existing vasculature for nutrients and oxygen, while angiogenesis consists of the formation of new blood vessels. Tumour cells can utilise adhesion molecules like L1CAM and $\beta 1$ -integrin for vascular co-option (Carbonell et al. 2009; Valiente et al. 2014). In non-small cell lung cancer, cancer cells often rely more on co-option than angiogenesis, highlighting the differences between metastatic and primary sites (Jubb et al. 2011). Angiogenesis modifies the niche for perivascular cancer cells and plays a crucial role in tumour progression through the VEGF pathway. Breast cancer tumour cells have been shown to secrete VEGF to promote the development of brain metastases in preclinical murine xenograft models (Kim et al. 2004). Also, VEGF secretion by tumour cells has been linked to the activation of mediators such as integrin- $\alpha \nu \beta 3$, further promoting the progression of brain metastases and the formation of blood vessels (Lorger et al. 2009). Anti-VEGF therapy has shown promise in reducing angiogenesis, preventing an early angiogenic switch, and decreasing metastatic growth in preclinical models (Ilhan-Mutlu et al. 2016; Bohn et al. 2017).

1.2.2.2 Cells and metabolic dynamics in the tumour microenvironment of brain metastases

T lymphocytes

The presence of infiltrating immune cells in the tumour microenvironment of brain metastases presents both challenges and opportunities for therapy. Brain metastases have a higher proportion of invading CD8⁺ T cells than primary brain tumours. Regulatory T cells (Tregs) accumulate in brain metastases, promoting a permissive environment for tumour progression (Chaudhary and Elkord 2016; Friebel et al. 2020). Prognostic implications of T cell infiltration vary, with some studies showing no correlation and others linking it to oedema and overall survival (Harter et al. 2015; Berghoff et al. 2016). An analysis of brain metastatic samples revealed that melanomas and renal cell carcinomas are the most immunologically active, with diffuse infiltration of CD3⁺ and CD8⁺ lymphocytes, although with high expression of inhibitory markers such as PD-1 (Harter et al. 2015). Some studies show lower immune cell infiltration in brain metastases compared to primary breast tumours, indicating the need for further research across different tumour types (Ogiya et al. 2017). Brain tumours are also associated with systemic lymphopenia and lymphocytic exhaustion (Chongsathidkiet et al. 2018). Identifying therapeutics to stimulate immune cells in the brain tumour environment is crucial for future studies.

Microglia and Macrophages

The CNS contains parenchyma-associated microglia and nonparenchymal macrophages. CD68⁺ or Iba1⁺ macrophages are found in brain metastatic lesions from melanoma, breast and lung cancer. Although these tumour-associated macrophages display phagocytic markers, they do not appear to initiate inflammation or adaptive immune responses, suggesting that they have adopted a pro-tumorigenic role (You et al. 2020). This is supported by animal studies in a murine breast cancer brain metastasis model, where brain-resident microglia and infiltrating macrophages with a pro-tumorigenic phenotype have been detected (Rippaus et al. 2016).

Neutrophils

Neutrophils play a complex role in brain metastases, exhibiting both pro-tumorigenic and anti-tumorigenic functions. Neutrophils in brain metastases have been shown to upregulate anti-inflammatory markers like the adenosine A2A receptor (ADORA2A), inhibiting local T-cell proliferation and creating a pro-tumorigenic environment (Yang et al. 2018a; Klemm et al. 2020). They are recruited to brain metastases through mechanisms involving signalling through the interleukin 8 receptor beta, a chemokine receptor also known as CXCR2 (Safarulla et al. 2022), and hepatocyte growth factor (HGF) receptor (c-Met) mediated cytokine networks (Liu et al. 2023a). Tumour-

associated neutrophils (TANs) contribute to metastasis by promoting angiogenesis, immunosuppression, and cancer cell invasion (Zhang et al. 2020b; Wu et al. 2020). The neutrophil-to-lymphocyte ratio (NLR) in peripheral blood has emerged as a potential prognostic marker for brain metastasis patients, where higher NLR is associated with worse survival outcomes (Mitsuya et al. 2017). Recent studies have explored targeting neutrophil recruitment and function as potential therapeutic strategies for brain metastases, including CXCR2 inhibition and granulocyte colony stimulating factor (G-CSF) blockade (Zhang et al. 2020b; Safarulla et al. 2022).

Astrocytes and Neurons

Astrocytes, crucial for tissue homeostasis and BBB maintenance, play significant roles in CNS malignancies, including brain metastases. They can exhibit pro- and antitumour functions. Initially, astrocytes contribute to neuroprotection by inducing tumour cell death via a nitric oxide-dependent pathway (Samdani et al. 2004). At later stages however, astrocytes also facilitate brain metastatic formation and colonisation by protecting tumour cells from chemotherapy and promoting tumour growth (Lin et al. 2010; Chen et al. 2016).

Neurons mediate electrochemical signals crucial for CNS function, yet their role in brain metastases is not fully understood. Brain metastatic lesions cause stress on brain tissue, leading to neuronal loss and neurological dysfunction (Seano et al. 2019). Neurons can interact with tumour cells through paracrine stimulation and synaptic transmission, supporting tumour growth. Studies show brain-seeking breast cancer and melanoma cells acquire neuro-adaptive attributes and enhance expression of neurotrophic factor receptors, aiding their adaptation to the neural niche (Neman et al. 2014; Termini et al. 2014; Venkataramani et al. 2024).

Metabolic dynamics

Several studies have shown that metastatic cells undergo metabolic reprogramming to adapt to the brain's unique microenvironment (Chen et al. 2007; Liu and Zhang 2022). Key metabolic changes include increased glycolysis, enhanced oxidative phosphorylation, and upregulation of the pentose phosphate pathway (Chen et al. 2007; Fukumura et al. 2021). Brain metastatic cells also show increased glutamine utilisation and activation of the gamma-aminobutyric acid (GABA) metabolic pathway (Chen et al. 2015; Schnepf et al. 2017). Notably, these cells can survive without relying on glucose by utilizing gluconeogenesis and amino acid oxidation (Chen et al. 2015). Brain metastases also show metabolic adaptations to the nutrient-limited brain environment, particularly in serine and glycine metabolism. The enzyme 3-phosphoglycerate dehydrogenase (PHGDH) plays a crucial role in serine synthesis and brain metastasis growth (Ngo et al. 2020). The metabolic shift of cancer cells is regulated by oncogenic

pathways and hypoxia-inducible factors (Marie and Shinjo 2011). Understanding these metabolic adaptations may reveal new therapeutic targets for brain metastases, such as inhibiting key metabolic enzymes or targeting the GABA pathway (Schnepp et al. 2017; Tyagi et al. 2022).

1.2.3 Cancer-associated fibroblasts

Initially, fibroblasts were defined as collagen-producing cells present in connective tissue. They are now recognised as interstitial mesenchymal lineage cells that are neither epithelial, endothelial, nor immune cells (Chen et al. 2021c). These cells' exact origins and roles are difficult to determine due to their significant phenotypic and functional diversity and the lack of definitive markers (Kalluri 2016). These versatile cells support tissue structure and contribute to wound healing, becoming activated by tissue damage or neoplasia. Fibroblast activation involves ECM synthesis, the development of a secretory phenotype, and the expression of markers like alpha-smooth muscle actin (α SMA) (Ronnov-Jessen and Petersen 1993; Orimo et al. 2005; Driskell et al. 2013; Arina et al. 2016)

In the context of cancer, cancer-associated fibroblasts (CAFs) are fibroblasts located within or near tumours. CAFs present in primary and metastatic tumours play significant roles in tumour initiation, progression, and metastasis (Öhlund et al. 2014; Kalluri 2016; Sahai et al. 2020). Recent single-cell RNA sequencing (scRNA-seq) studies across various cancers have demonstrated that CAFs are highly heterogeneous mesenchymal lineage cells with diverse functions (Chen et al. 2021c). They can originate from various sources, including quiescent tissue-resident fibroblasts, pancreatic or hepatic stellate cells (Yin et al. 2013), bone marrow-derived mesenchymal stem cells (Mishra et al. 2008), endothelial cells (Zeisberg et al. 2007), and adipocytes among others (Bochet et al. 2013) (**Fig. 3**). However, the precise origins of CAFs remain uncertain, highlighting the need for further research in this area due to their plasticity and the lack of clear lineage markers.

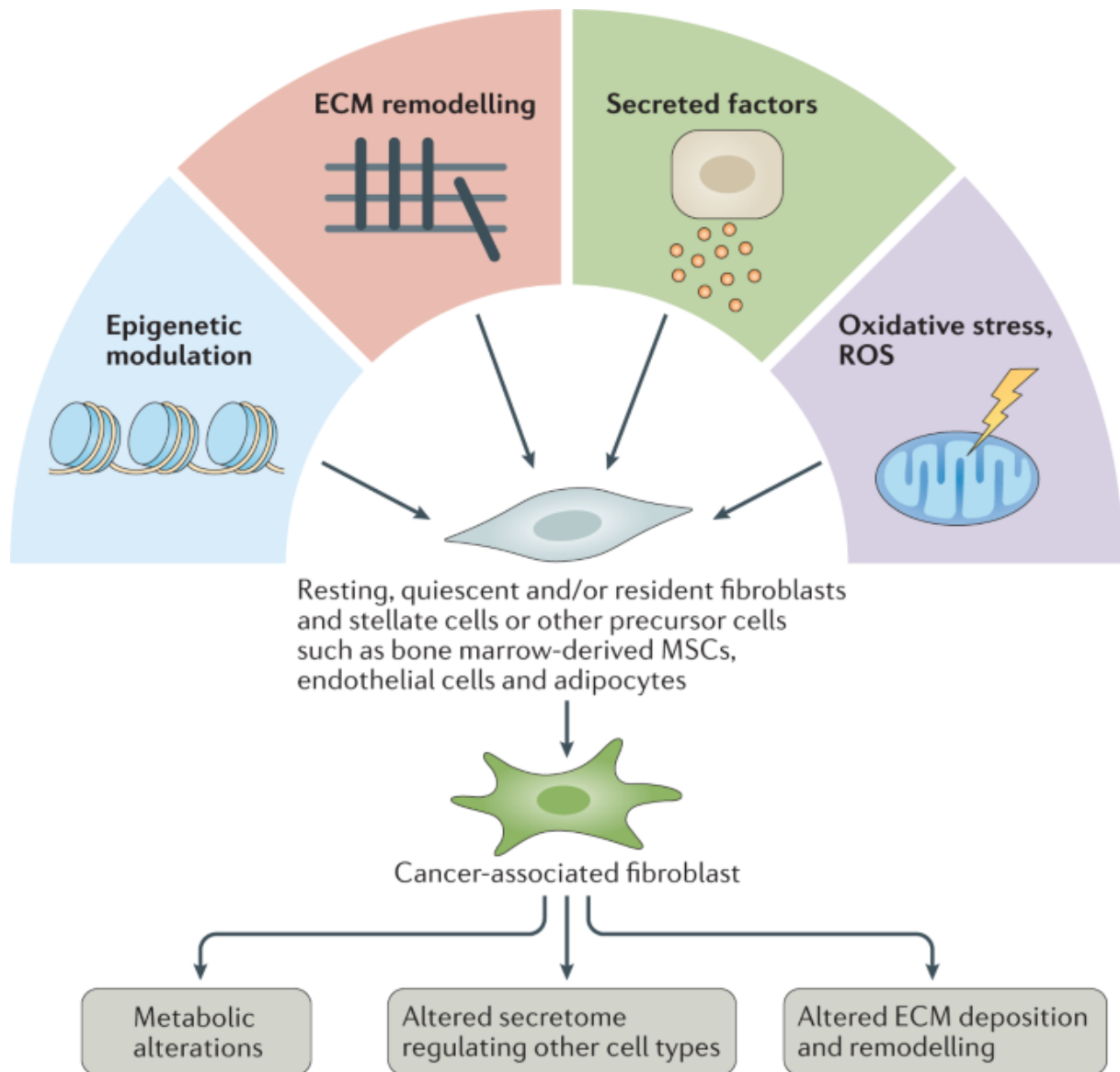


Figure 3. Mechanisms leading to the activation of cancer-associated fibroblasts (CAFs). Potential sources of CAFs include quiescent or resting fibroblasts, specific tissue-resident fibroblasts such as stellate cells, bone marrow-derived mesenchymal stem cells, endothelial cells, and other cell types. ECM stands for extracellular matrix, and ROS refers to reactive oxygen species (Chen et al. 2021c).

CAF subpopulations

CAFs are identified using various mesenchymal cell markers, including α SMA, fibroblast-specific protein 1 (FSP1 or S100A4), fibroblast activation protein (FAP), platelet-derived growth factor receptors (PDGFR) - PDGFR α , PDGFR β , desmin, discoidin domain receptor 2 (DDR2), and vimentin. Single-cell RNA sequencing has identified distinct CAF subpopulations with diverse origins, markers, and functions (Sebastian et al. 2020; Lavie et al. 2022). Common subpopulations include myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs), and antigen-presenting CAFs, each with unique gene expression profiles and metabolic characteristics (Sebastian et al. 2020; Hu et al. 2022). These subpopulations can either promote or

suppress tumour progression through various mechanisms, including ECM remodelling, cytokine production, and immune modulation (Simon and Salhia 2022; Yamamoto et al. 2023). Understanding CAF heterogeneity and its impact on tumour biology is crucial for developing more effective cancer treatments, potentially by targeting specific CAF subpopulations (Mhaidly and Mechta-Grigoriou 2021). Among the molecules expressed by CAFs, FAP has emerged as a potential target for imaging and therapy, particularly in metastatic and immunosuppressive contexts (Peltier et al. 2022).

Tumour-promoting functions of CAFs

CAFs produce ECM, ECM-degrading proteases (e.g., matrix metalloproteinases - MMPs) and ECM-crosslinking enzymes, which are crucial for ECM remodelling in the tumour microenvironment (Sternlicht et al. 1999; Boire et al. 2005). CAF-secreted cytokines, chemokines, and remodelled ECM regulate immune responses and angiogenesis, contributing to immunosuppressive TME and therapy resistance (Costa et al. 2018; Givel et al. 2018; Zhang et al. 2020a). CAF-derived TGF β has an immunosuppressive role, impacting T cells, macrophages, and neutrophils (Thomas and Massagué 2005; Fridlender et al. 2009; Mariathasan et al. 2018; Tauriello et al. 2018). CAFs also promote tumour progression through the secretion of CXCL12 and VEGFA, which enhance angiogenesis (Orimo et al. 2005; O'Connell et al. 2011). Other CAF-secreted factors like LIF, IGF1, HGF, IL-6, WNT5 α , and BMP4 induce signalling interactions that support tumour growth (Quante et al. 2011; Tape et al. 2016; Shi et al. 2019).

Depletion of α SMA⁺ or *Lrat* lineage-traced CAFs in cholangiocarcinoma mouse models reduces tumour burden, and deletion of *Has2* or *Hgf* in CAFs decreases tumour growth (Affo et al. 2021). Preclinical models and data from patient samples suggest a connection between specific CAF subsets, anti-tumour immune responses, and immunotherapy efficacy (Chauhan et al. 2019; Kieffer et al. 2020; Dominguez et al. 2020). FAP-expressing CAFs may have an immunosuppressive function, presenting opportunities to improve immunotherapies by targeting CAFs or CAF-derived factors like CXCL12 or CCL2 (Kraman et al. 2010; Feig et al. 2013; Yang et al. 2016).

CAFs have also been shown to supply pancreatic cancer cells with various metabolites including alanine (Sousa et al. 2016; Parker et al. 2020), proline (Olivares et al. 2017), deoxycytidine (Dalin et al. 2019), and lipids (Auciello et al. 2019). Exosomes derived from CAFs enhance cancer cell stemness, epithelial-mesenchymal transition (EMT), and aggressive phenotypes in various cancers, including breast, colorectal, and pancreatic cancer (Donnarumma et al. 2017; Yang et al. 2019). Specific microRNAs, such as miR-500a-5p and miR-92a-3p, have been identified as key players in this process (Hu et al. 2019; Chen et al. 2021a). CAF-derived exosomes also modulate cancer cell metabolism, inhibiting oxidative phosphorylation and promoting glycolysis (Zhao et al. 2016). Furthermore, these exosomes contribute to chemoresistance by

increasing the expression of resistance-inducing factors in cancer cells (Richards et al. 2017). Understanding the role of CAF-derived exosomes in cancer progression may lead to novel diagnostic and therapeutic approaches (Li et al. 2017; Peng et al. 2022).

CAFs in the tumour microenvironment can develop a senescence-associated secretory phenotype (SASP), which contributes to cancer progression (Zhang et al. 2024a). The SASP in CAFs is regulated by various mechanisms, including the miR-335/COX-2/PTEN axis (Kabir et al. 2016), enhancer of zeste homolog 2 (EZH2) downregulation (Yasuda et al. 2021), and p38MAPK signalling (Alspach et al. 2014). Therapy-induced senescence in CAFs can enhance tumour growth and invasiveness (Toste et al. 2016; Higashiguchi et al. 2023). The SASP includes pro-inflammatory cytokines like IL-6 and IL-8, which promote malignancy (Coppé et al. 2008). While senolytic drugs can eliminate senescent cells, some may have unintended effects on SASP cytokine secretion and tumour progression (Bogdanova et al. 2024). Understanding the mechanisms of SASP development in CAFs and its impact on cancer progression is crucial for developing targeted therapies that address the tumour microenvironment (Zhang et al. 2024a).

Tumour-suppressing functions of CAFs

The tumour-suppressing functions of CAFs include promoting anti-cancer immunity, secreting pro-inflammatory factors, activating tumour-inhibitory signalling, and producing ECM components that act as physical barriers to tumour invasion. Specific CAF subpopulations can inhibit tumour growth, particularly in early-stage cancers (Wang et al. 2021a). For example, type I collagen produced by myofibroblastic CAFs mechanically restrains tumour spread (Bhattacharjee et al. 2021). In mouse models of pancreatic cancer, depleting α SMA⁺ CAFs accelerates tumour progression, reduces fibrosis, and decreases survival. Higher levels of α SMA and stromal density correlate with better survival in pancreatic cancer patients (Özdemir et al. 2014; Torphy et al. 2018). Targeting the Sonic hedgehog (SHH)–Smoothed (SMO) pathway has been shown to inhibit stromal desmoplasia and diminish myofibroblasts, leading to tumour progression in models of pancreatic cancer (Rhim et al. 2014; Lee et al. 2014). SHH signalling and fibrotic stroma also have tumour-suppressing roles in bladder cancer (Shin et al. 2014), suggesting that α SMA⁺ CAFs, or myofibroblasts, restrain tumours partly through SHH–SMO signalling.

In pancreatic cancer models, Janus kinase (JAK) inhibitors suppress IL-1-induced LIF signalling in inflammatory iCAFs, shifting them to ECM-producing myCAFs, increasing the myCAF to iCAF ratio, and reducing tumour growth (Biffi et al. 2019). Deleting collagen I (Col1a1) in α SMA⁺ myofibroblasts decreases collagen deposition. It accelerates pancreatic tumour progression, creating a more immunosuppressive stroma and increasing myeloid-derived suppressor cells, which suppress CD8⁺ T cells (Chen et al. 2021b). Deleting Col1a1 in CAFs promotes liver colonisation of pancreatic

and colorectal cancers (Bhattacharjee et al. 2021), but does not affect cholangiocarcinoma growth (Affo et al. 2021). These findings highlight the specific tumour-suppressing functions of myofibroblasts and their stroma.

The discovery of CAFs' tumour-suppressing functions may explain why several clinical trials targeting CAFs or stroma have failed to demonstrate a clinical benefit (Catenacci et al. 2015; Ko et al. 2016; Benson et al. 2017; Hecht et al. 2017; Ramanathan et al. 2019; Van Cutsem et al. 2020). Future therapies could focus on reprogramming and normalising tumour-promoting CAF subsets, ideally converting them to tumour-suppressing CAFs.

The functional heterogeneity of CAFs (**Fig. 4**) across cancer types highlights the challenge of identifying universal markers and the need for biomarker-guided strategies for targeted therapeutics.

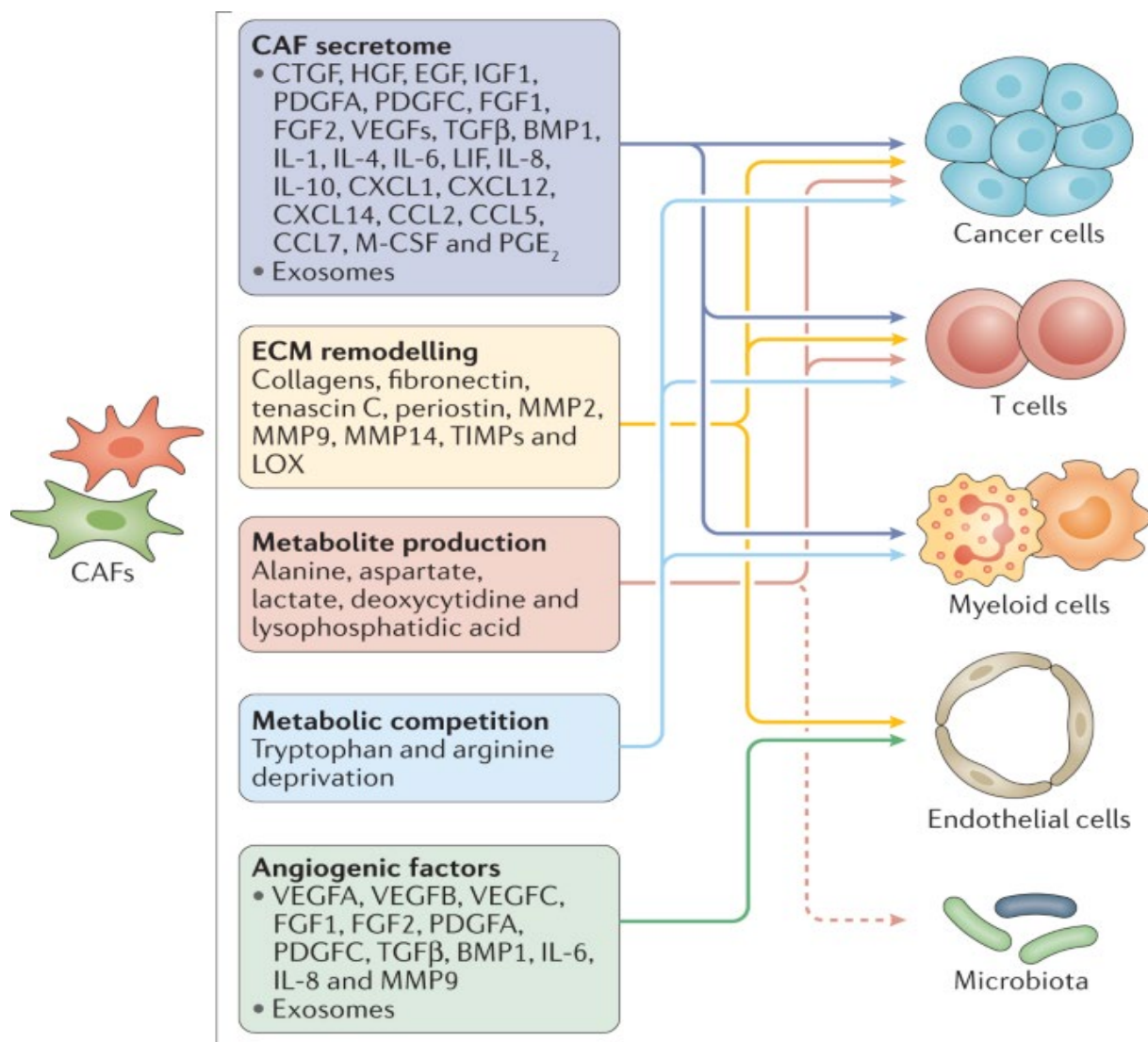


Figure 4. Interactions of cancer-associated fibroblasts (CAFs). CAFs affect various cell populations within the tumour microenvironment, including cancer cells, lymphocytes, myeloid cells, endothelial cells, and the microbiota. Used from (Chen et al. 2021c)

1.2.4 Fibroblast activation protein

FAP is a non-classical serine protease from the "Dipeptidyl peptidase (DPP)-IV activity and/or structure homologues" (DASH) family (Sedo and Malik 2001; Bušek et al. 2004). FAP was first identified as a membrane protein predominantly found in stromal fibroblasts across various solid tumours (Garinchesa et al. 1990). The dipeptidyl peptidase and endopeptidase enzymatic activity of FAP has been shown to cleave substrates such as fibroblast growth factor 21 (FGF21), neuropeptide Y, substance P, denatured type I collagen, colony-stimulating factor-1 (CSF-1), and C-X-C motif chemokine 5 (CXCL5) besides other factors (Busek et al. 2018). Therefore, FAP enzymatic activity may regulate metabolism, ECM remodelling and immune responses. Some studies have also demonstrated, that FAP promotes tumour growth and invasion of breast cancer cells through non-enzymatic functions (Huang et al. 2011). Recent research highlights FAP's unique expression in CAFs or tumour cells across various tumour types (Liu et al. 2015), indicating its potential for improving diagnostic precision and targeted therapy.

FAP expression in malignancy

Attachment 4 provides a comprehensive review of the role of FAP in malignant tumours (Busek et al. 2018). FAP in normal tissues typically remains at a low level or is not present at all. However, it is detected in over 80% of patients with solid tumours (Liu et al. 2015). FAP expression tends to vary notably in different tumour types: in distal cholangiocarcinoma (Byrling et al. 2020), hepatocellular carcinoma, oesophageal squamous cell carcinoma (Li et al. 2020), pancreatic adenocarcinoma, osteosarcoma, oesophageal adenocarcinoma, and epithelial ovarian carcinoma, FAP can be detected either within tumour stroma or in tumour cells. In differentiated carcinomas, such as endometrial carcinoma, colon adenocarcinomas, colorectal cancer, non-small cell lung cancer, gastric cancer, medullary thyroid carcinoma, urothelial carcinoma of the bladder, clear cell renal cell carcinoma, prostate cancer, and breast cancer, FAP is expressed dominantly within stromal cells and rarely within the tumour cells. Conversely, in the case of osteosarcoma and other sarcomas, FAP is dominantly present in tumour cells (Pure and Blomberg 2018; Busek et al. 2018; Zhang et al. 2024b). Tumours, where FAP expression is present in tumour cells and/or the stromal cells, tend to be associated with an increased probability of distant metastases, local tumour invasion, and lymph node metastases (Liu et al. 2015).

Regulation of FAP expression

Research over the past few decades has explored the regulation of FAP expression at both the mRNA and protein levels, revealing several mechanisms that modulate FAP functions. TGFbeta can increase FAP mRNA levels by facilitating the binding of smad

3/4 to the FAP gene promoter. However, this effect can be inhibited by c-ski, which suppresses the FAP promoter, particularly in invasive tumour cells (Tulley and Chen 2014). In human oral keratinocytes, TERT has been shown to increase FAP production through the upregulation of early growth response (EGR)-1 (Park et al. 2016). Additionally, the receptor for anticancer cytokine interferon 1 (IFN1), IFNAR1, has been found to inhibit FAP expression and subsequent stromagenesis (Cho et al. 2020). Specific miRNAs also regulate FAP expression; miR-30a-5p can block FAP expression, inhibiting proliferation, invasiveness and migration of oral cavity cancer cells (Ruan et al. 2017). Also, miRNA-204 has been hypothesised to inhibit FAP expression in glioma cells, with an added effect of reversing temozolomide resistance (Yang et al. 2018b). Hypoxia has also been suggested as a possible regulator of FAP. In one study, a hypoxic environment led to the upregulation of both hypoxia-inducible factor-1 α (HIF-1 α) and FAP in hepatocellular carcinoma cells (Zou et al. 2018). Another study has shown, that HIF-1 α and FAP are overexpressed in lung cancer CAFs. Also, hypoxia and HIF-1 α expression led to the upregulation of FAP and conversion of normal fibroblasts to CAFs in a murine model of lung cancer (Zhang et al. 2021).

1.2.4.1. Fibroblast activation protein in cancer

Extracellular matrix remodelling

FAP functions as a serine protease targeting ECM and ECM-associated proteins, cleaving substrates after proline residues, such as collagen types 1, 3, and 5, extracellular matrix protein 1 (ECM-1), and fibrillin-2. FAP expression also upregulates other proteases as MMPs (Zhang et al. 2019). Interestingly, both enzymatically active and genetically engineered inactive forms of FAP led to elevated MMP-9 levels suggesting that this effect is independent of FAP's enzymatic activity (Huang et al. 2011). FAP+ fibroblasts can produce ECM in the form of fibrillar networks with parallel alignment, which promotes tumour invasion in mouse xenograft pancreatic tumours (Lee et al. 2011). When high FAP-expressing mesenchymal cells are co-cultured with tumour cells, they create a complex network of collagen and fibronectin that attracts macrophages and neutrophils, enhancing tumour cell invasion (Samaniego et al. 2013). FAP-mediated cleavage of collagen I promotes adhesion of tumour-associated macrophages (TAMs) through the scavenger receptor-A (SR-A), which could enhance local immunosuppression (Mazur et al. 2016).

Invasiveness and shape of cancer-associated fibroblasts

By poorly defined mechanisms, FAP plays a role in maintaining CAF morphology by promoting the release of tissue plasminogen activator, pigment epithelium-derived factor, VEGF-C, and TGFbeta, which help sustain the fibroblastic shape of CAFs. Reduced FAP expression leads to an epithelial transformation characterised by fewer

spindle-shaped cells (Koczorowska et al. 2016). Cell membrane-bound FAP accumulates with its binding partners in lipid rafts in CAF pseudopodia and invadopodia during ECM remodelling (Knopf et al. 2015).

Angiogenesis

Angiogenesis is crucial for cancer progression due to increased nutrient supply to tumour cells. Overexpression of FAP in colorectal cancer cells increases VEGF-A production, activating human umbilical vein endothelial cells (HUVECs) through the Akt and ERK pathways (Cao et al. 2017). FAP expressing hepatic stellate cells induce recruitment of myeloid-derived suppressor cells (MDSCs) and epithelial-mesenchymal transition in cancer cells. These factors enhance vessel co-option, where tumours hijack normal tissue vessels, leading to resistance to antiangiogenic therapy in a liver metastasis model of colorectal cancer (Qi et al. 2022).

Malignant phenotype of cancer cells

FAP overexpression in breast cancer cells reduces the phosphorylation of the focal adhesion kinase (FAK), which enhances tumour migration, invasion, and metastasis (Jia et al. 2014). Similarly, in ovarian cancer, FAP in invasive tumour cells recruits the protein kinase, DNA-activated, catalytic subunit (PRKDC) to lipid rafts, activating the NF- κ B pathway and promoting cancer cell survival (Li et al. 2023). FAP also affects pathways like PTEN/PI3K/Akt and Ras-ERK, with FAP knockdown reducing phosphorylation in these pathways, leading to decreased EMT and MMP levels, thus suppressing cancer proliferation and metastasis (Wang et al. 2014). FAP is recruited by integrins like β 1 and α v β 3 in fibrosarcoma, activating signalling molecules such as FAK and Rac, facilitating tumour cell adhesion and invasion (Baird et al. 2015). Co-culture of fibroblasts expressing FAP with gastric cancer cells induced EMT, advancing tumour progression through mechanisms like upregulation of DKK-1 and LEF-1 linked to the Wnt/ β -catenin pathway and changes in N-cadherin, vimentin, E-cadherin, and ZO-1 levels (Liu et al. 2018).

Local immunosuppression

Several studies imply, that FAP may contribute to immune evasion in tumours by promoting the recruitment of immunosuppressive cells. In CAFs, FAP expression stimulates Akt phosphorylation (Wu et al. 2017), upregulates MAP2K1/2, and dephosphorylates PTEN (Wang et al. 2014; Higashino et al. 2019), leading to increased GSK-3 β , β -catenin, NF- κ B, and chemokines like CCL2, IL-6, and CXCL8, which attract M2 macrophages, MDSCs, and Tregs (Higashino et al. 2019). FAP in CAFs also activates STAT3 through the FAK-c-Src-JAK2 pathway, leading to secretion of CCL2, which recruits MDSCs (Yang et al. 2016). FAP in tumour-associated mesenchymal stem

cells (MSCs) inhibits CD4⁺ T cell proliferation and senescence through the Akt pathway (Wu et al. 2017). FAP's enzymatic activity cleaves CCL2 and CXCL-5 without altering their chemotactic properties (Zhang et al. 2019), and remodelled collagen I attract TAMs by exposing a macrophage-binding site (Mazur et al. 2016).

Taken together, as summarized in Attachment 4 and Figure 5, FAP expression in both stromal cells and cancer cells contributes to tumour progression through various mechanisms.

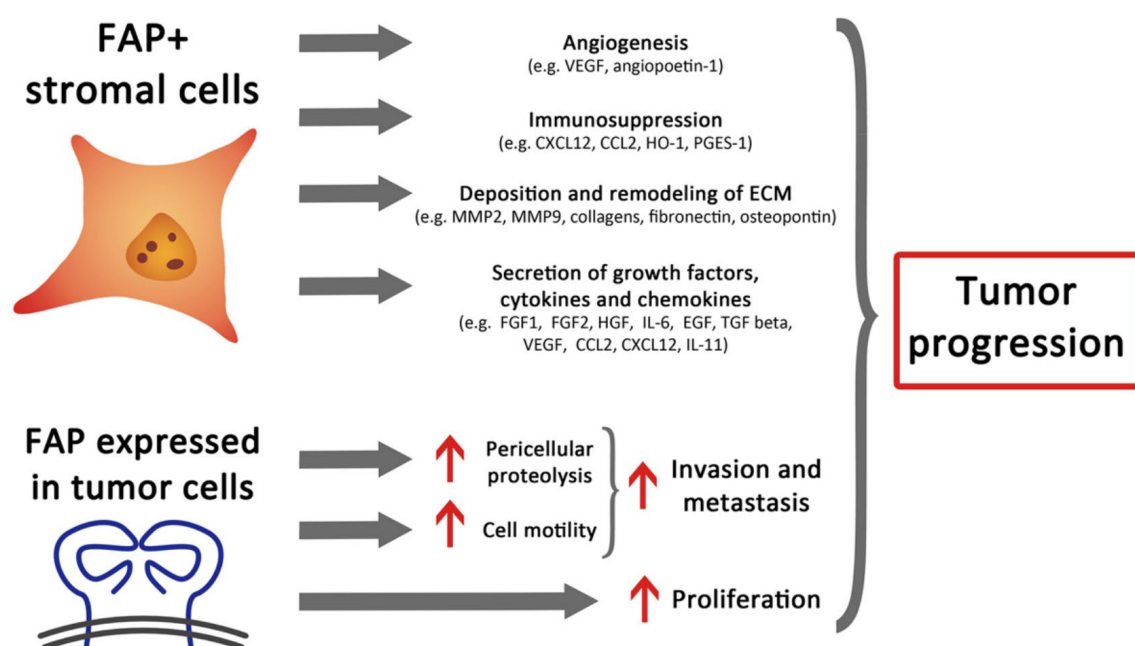


Figure 5. FAP in tumour progression. FAP⁺ stromal cells contribute to a pro-tumorigenic microenvironment by producing various structural and regulatory molecules that promote local immunosuppression, extracellular matrix remodelling, and angiogenesis. Additionally, FAP expressed in transformed cells may directly enhance tumour cell invasiveness and proliferation. VEGF = Vascular endothelial growth factor; HO-1 = Heme oxygenase 1; PGES-1 = Prostaglandin E synthase-1; MMP2, 9 = Matrix metalloproteinase 2, 9; FGF1, 2 = Fibroblast growth factor 1, 2; HGF = Hepatocyte growth factor; IL-6, 11 = Interleukin 6, 11; TGFbeta = Transforming growth factor beta (Attachment 4 (Busek et al. 2018)).

1.2.4.2 FAP-targeting in cancer therapy

As summarized in Attachment 4, FAP represents an emerging target in cancer treatment. Despite its role in tumorigenesis, direct inhibition using small molecules has shown minimal impact on tumour growth in preclinical mouse studies (Gunderson et al. 2019) and clinical trials (Narra et al. 2007). This limited efficacy is likely due to complex interactions between tumour cells and their microenvironment. Therefore, in cancer therapy, FAP is utilized in two primary ways: it can serve as a target for the selective delivery of cytotoxic drugs and immune cells or its enzymatic activity can be exploited to activate FAP-cleavable prodrugs specifically targeting FAP-expressing cells. These strategies include the use of chimeric antigen receptor T cells (CAR-T cells), tumour

vaccines, antibody-drug conjugates, bispecific antibodies, and drug delivery systems, which leverage FAP's exclusive expression in the tumour microenvironment to enhance specific delivery of cytotoxic drugs or radioligands.

CAR-T cells

CAR-T cell therapy has shown success in hematologic malignancies but faces challenges in solid tumours due to the dense, immunosuppressive stroma formed by CAFs, to some extent partly driven by FAP expression (Gulati et al. 2018). FAP-targeted CAR-T cells selectively deplete stromal cells, enhancing T cell infiltration and tumour inhibition in mice (Wang 2014). Recently, FAP-directed CAR-T cells have been combined with tumour-directed CAR-T cells to achieve synergistic effects (Li et al. 2022). Pre-treatment with FAP-targeted CAR-T cells depletes CAFs, degrades ECM (Das et al. 2023) and prevents immunosuppressive MDSC recruitment (Liu et al. 2023b), enhancing the efficacy of CAR-T cells targeting cancer proteins. A phase I clinical trial evaluated the safety of FAP-directed CAR-T cells administered via intrapleural infusion in advanced/inoperable pleural mesothelioma patients. This trial has demonstrated the safety and subsequent whole-body distribution of the FAP-directed CAR-T cells (Hiltbrunner et al. 2021).

Tumour Vaccines

Incorporating FAP into tumour vaccines targets both CAF and tumour cells, inducing CD8⁺ T cell infiltration, preventing desmoplasia, and counteracting immunosuppression. Exosome-like vaccines containing multiple tumour antigens can be enhanced by adding the FAP gene, promoting tumour ferroptosis and depleting CAFs (Hu et al. 2021). FAP-directed DNA vaccines combined with other tumour vaccines or anti-cancer agents weaken the tumour immunosuppressive environment and facilitate T-cell infiltration (Duperret et al. 2018; Geng et al. 2022; Zhang et al. 2022).

Antibodies

FAP antibodies, fused with drugs to create antibody-drug conjugates (ADCs) or with signalling molecules or immunomodulators, enhance specificity and concentrate in tumours while minimising normal tissue distribution (Fang et al. 2016). Bispecific T-cell engagers (BiTEs) and bispecific antibodies targeting FAP and other co-stimulatory molecules enhance T-cell contact and tumour cell killing (Trüb et al. 2020).

Prodrugs

FAP's unique enzymatic activity is utilised to activate prodrugs with inhibitory moieties containing a Gly-Pro linker. This allows prodrugs derived from doxorubicin (Huang et al. 2018; Casazza et al. 2022) or bufalin (Kim et al. 2017) to inhibit tumour growth effectively with reduced toxicity. Drug delivery systems also incorporate FAP-cleavable peptides into liposomes, allowing stepwise inhibition and selective killing of FAP-expressing stromal and cancer cells by locally releasing nanoparticles or cytostatic drugs (Hou et al. 2019; You et al. 2020).

Inhibitor conjugates

A novel approach in targeted therapy exploits low-molecular-weight FAP inhibitors as targeting ligands. Suitable chemical modifications of the inhibitor allow for the attachment of other compounds, and the resulting conjugates can be used for cancer therapy or diagnosis. iBodies, designed as antibody substitutes, consist of an N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer decorated with compounds that may act as targeting ligands, affinity anchors or imaging probes (Šácha et al. 2016). These versatile polymer conjugates have been tailored to target various proteins, including FAP. By incorporating a FAP-specific inhibitor, iBodies achieve low nanomolar inhibition of FAP. These iBodies have been shown to detect FAP from cell lysates and serum, showing potential for specific anticancer drug delivery and broad biomedical applications (Dvorakova et al. 2017).

2. Hypothesis, aims and objectives

Fibroblast Activation Protein (FAP) is overexpressed in a wide array of solid tumours, with emerging evidence suggesting a crucial role for FAP and FAP-expressing cells in fostering pro-tumorigenic features within the tumour microenvironment. Previous results of our research team have shown significant overexpression of FAP in the microenvironment of glioblastomas, particularly on cells exhibiting mesenchymal characteristics. This observation has led us to hypothesise that FAP expression could be notably increased in recurrent glioblastomas, where the mesenchymal subtype typically prevails. Therefore, a comparative analysis of FAP expression in primary and recurrent glioblastomas was conducted. Additionally, due to the limited data on FAP expression in brain metastases from solid tumours compared to primary tumours, this research investigated FAP expression in brain metastases and aimed to identify FAP-expressing cells within the tumour microenvironment of brain metastases. Given the underexplored functional role of FAP in both glioblastomas and brain metastases, this study further examined the biological properties of FAP-expressing cells in the brain tumour microenvironment. The selective expression of FAP in the tumour microenvironment of solid tumours suggests potential for targeted anticancer therapies. Therefore, this work also evaluated the feasibility of exploiting FAP enzymatic activity for targeted anti-cancer treatment. This research aimed to deepen the understanding of the role of FAP in tumour biology and apply this knowledge towards innovative treatment strategies for glioblastomas and brain metastases.

AIM I: To determine FAP expression in the tumour microenvironment of glioblastomas, recurrent glioblastomas and brain metastases

Objectives:

- Quantification of FAP expression in primary glioblastomas and paired samples of recurrent glioblastomas
- Quantification of FAP expression in brain metastases

AIM II: Identification of FAP-expressing cells in the tumour microenvironment of brain metastases

Objectives:

- Assessment of cell markers expressed on FAP+ cells
- Derivation of FAP+ stromal cells and evaluation of possible functions of FAP+ stromal cells in the tumour microenvironment

AIM III: Targeting FAP-expressing cells in the tumour microenvironment

Objectives

- *In vivo* toxicity assessment of a FAP targeting iBody conjugate in mice
- *In vivo* assessment of antitumor activity of a FAP targeting iBody conjugate in FAP expressing tumours

3. Materials and methods (see also corresponding sections in Attachments 1-3)

3.1 Patient samples

Tissue samples were collected from patients with glioblastomas, brain metastases and pharmacoresistant epilepsy who underwent neurosurgical resection at the Department of Neurosurgery, Hospital Na Homolce, Prague, and the Department of Neurosurgery and Neurooncology, First Faculty of Medicine, Charles University, and Military University Hospital, Prague and The Clinic of Neurosurgery of J. E. Purkyně University and Masaryk Hospital in Ústí nad Labem. Full informed consent was obtained from all donors prior to neurosurgical resection. Patient cohort characteristics are described in Attachments 1,2,3 (Zubal' et al. 2023; Krepela et al. 2021; Balaziová et al. 2021). The tumours were diagnosed and characterised according to the current World Health Organization classification (Louis et al. 2021). Formalin-fixed and paraffin-embedded (FFPE) blocks were used for the immunodetection of FAP and Masson's trichrome staining. Snap frozen glioblastomas, brain metastases and pharmacoresistant epilepsy tissues were stored at -78°C and used to prepare tissue lysates and frozen sections.

3.2 Preparation of tissue lysates, determination of total protein concentration, determination of FAP enzyme activity, FAP ELISA, TGFbeta ELISA, Masson's trichrome staining, immunohistochemical staining and fluorescent double labelling, immunocytochemistry

The details of these procedures are described in the corresponding publications. Refer to Attachments 1, 2, and 3 for further information (Zubal' et al. 2023; Krepela et al. 2021; Balaziová et al. 2021).

3.3 Cell culture

3.3.1 Derivation of primary cell cultures

For the derivation of primary cells cultures, fresh samples of glioblastomas, brain metastases, pharmacoresistant epilepsy and subgaleal connective tissue of the skull were transferred into MACS® Tissue Storage Solution (Miltenyi Biotec), transported to the laboratory at 4°C and processed either immediately or within 24 hours after resection. A standardized combined mechanic and enzymatic dissociation protocol was followed using the Tumor Dissociation Kit, human (Miltenyi Biotec, #130-095-929), utilizing the gentleMACS Dissociator system (Miltenyi Biotec) according to manufacturer instructions.

Necrotic regions, fibrous tissue and fat were removed from the tissues. The tissue was then cut into small fragments, approximately 2–4 mm in size, using sterile scalpels.

Afterwards, the tissue fragments were transferred into a gentleMACS C Tube, pre-loaded with an enzyme mix suitable for tumour tissue dissociation. The C Tube was tightly sealed and attached in an inverted position onto the gentleMACS Dissociator. Initial mechanical dissociation was performed using the pre-programmed h_tumor_01 protocol. Upon completion of the first dissociation cycle, the C Tube was incubated at 37°C for 30 minutes with continuous rotation. Afterwards, the gentleMACS Dissociator was used for a second mechanical dissociation cycle using the h_brain_02 protocol. After this cycle, the sample was once again incubated for 30 minutes at 37°C with continuous rotation. A final dissociation cycle was performed using the h_tumor_03 program to ensure thorough mechanical disaggregation of the tumour tissue. The resulting cell suspension was passed through a cell strainer (40 µm or 70 µm) placed over a sterile 50 mL conical tube to remove large tissue fragments and ensure a single-cell suspension. The filter was washed with 12 mL Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific). The filtered cell suspension was centrifuged at 300×g for 7 minutes at 4°C to pellet the cells. Following centrifugation, the supernatant was carefully aspirated without disturbing the pellet.

In cases where the sample contained excessive erythrocytes or debris, additional steps were performed. For erythrocyte removal, the cell pellet was resuspended in Red Blood Cell Lysis Solution (Miltenyi Biotec, #130-094-183) and treated according to the manufacturer's instructions. Eventually, the Debris Removal Solution (Miltenyi Biotec, #130-109-398) was used following the manufacturer's protocol to remove cell debris.

The final cell pellet (after erythrocyte lysis and debris removal if needed) was resuspended in culture medium – DMEM 10% of fetal calf serum (FCS) and antibiotic solution of penicillin/streptomycin (P/S, final concentration 1%). The cells were afterwards seeded and left to attach in cultivation flasks, typically T75 flasks (Thermo Fisher Scientific), and cultured in an incubator at 37°C and a humidified atmosphere containing 5% CO₂.

3.3.1.1 Anti-fibroblast magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) with Anti-Fibroblast MicroBeads, human (Miltenyi Biotec, # 130-050-601) was used for the positive selection of fibroblasts. The procedure was performed according to manufacturer's protocol.

Briefly, the cultured cells were trypsinised to prepare cell suspensions. The total cell number was determined using an automated cell counter. After cell counting, the suspension was centrifuged at 300×g for 10 minutes at 4°C to pellet the cells. Following centrifugation, the supernatant was carefully aspirated. The resulting cell pellet was resuspended in a buffer: phosphate-buffered saline (PBS), pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA); 80 µL of the buffer were used for 10⁷ cells. Anti-Fibroblast MicroBeads (20 µL per 10⁷ cells) were then added to the suspension, ensuring thorough mixing of the beads with

the cell suspension. This mixture was incubated for 30 minutes at room temperature (19–25 °C) to allow binding of the MicroBeads. After incubation, the cells were washed by adding 1–2 mL of buffer for 10^7 cells, followed by another round of centrifugation at $300\times g$ for 10 minutes at 4°C. The supernatant was aspirated, and the cell pellet was resuspended in 500 μ L of buffer for magnetic separation. The magnetic separation was carried out using MS MACS Columns (Miltenyi Biotec, #130-042-201) and the MiniMACS Separator (Miltenyi Biotec, #130-042-102) and MACS MultiStand (Miltenyi Biotec, #130-042-303). First, the MACS Column was placed in the magnetic field of the MACS Separator, and the column was rinsed with 500 μ L of the buffer. Next, the cell suspension was applied to the column. The flow-through, containing unlabelled cells was collected. The column was then washed three times with 500 μ L buffer, and the flow-through was combined with the effluent from the previous step. Once the washing steps were complete, the column was removed from the magnetic separator and placed onto a 15ml sterile plastic collection tube. The magnetically labelled cells were flushed out of the column by adding 1 mL of buffer to the MS Column, and the cells were collected. The cells were centrifuged (300g/7min/4°C). The supernatants were discarded, cell pellets were resuspended in cultivation medium – DMEM+10%FCS+1%P/S, seeded in T25-T75 flasks, and cultured in an incubator at 37°C and humidified atmosphere containing 5% CO₂. The positively selected cells were labelled as positive fraction (PF), while the remaining cells were labelled as the negative fraction (NF).

3.3.2 Cell lines

The lung adenocarcinoma cell line A549 was obtained from the European Collection of Cell Cultures (Salisbury, UK).

The human glioma cell line U-251MG was obtained from the American Type Culture Collection (ATCC).

U-251MG cells expressing FAP were prepared in previous projects. The cells were stably transfected using Lipofectamine 2000 (Invitrogen) either with the

1. pTRE-Tight_hFAP vector. U251 cells were first transfected with the pTet-On-Advanced plasmid (Clontech). Stable clones were selected and then transfected with the pTRE-Tight_hFAP plasmid, followed by hygromycin selection (Clontech). FAP expression was induced by adding 1 μ g/ml of doxycycline (Clontech) to the culture medium (Dvorakova et al. 2017).
2. pcDNA4_hFAP vector to stably express FAP.

All cell lines were cultured in DMEM+10%FCS in an incubator at 37°C and a humidified atmosphere containing 5% CO₂.

3.4 Preparation of conditioned media

For the preparation of conditioned media, we utilised a slight modification of a protocol as described in Attachment 3 (Balaziova et al. 2021) Briefly, cells were seeded at a density of 8×10^3 cells per cm^2 in 100 mm dishes and cultured in DMEM containing 10% FCS and 1% P/S for 24 hours. Then, the medium was removed, cells washed twice with PBS, and 10 mL of serum-free DMEM was added. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 . After 72 h, the medium was collected, centrifuged ($618 \times g$ at 4 °C for 10 min), and the supernatant filtered through a 0.22 μm pore filter (Merck) and stored in aliquots at -75°C until use. Media not exposed to cells were used as controls. Cells were counted after harvesting of the media. To standardise the number of cells used to prepare the conditioned media, the conditioned media were diluted with the control medium to achieve a final concentration of 5×10^4 cells conditioning 1 ml of medium before the biological assays.

3.5 Beta-galactosidase assay

Cells were seeded at a density of 7×10^3 cells per cm^2 in 24-well plates Corning™ Costar™, Fisher scientific) and cultured in DMEM containing 10% FCS and 1% P/S for 96 hours. Then, the cells were stained for beta-galactosidase activity using the Senescence Cells Histochemical Staining Kit (CS0030, Sigma-Aldrich) according to the manufacturer's instructions. The growth medium was aspirated, followed by two washes with 1 mL of PBS per well. Subsequently, 1.5 ml of 1x Fixation Buffer was added to each well, and the plate was incubated at room temperature for 7 minutes. The Staining Mixture was prepared during this fixation period according to the provided instructions. Following fixation, the cells were rinsed three times with 1 ml of PBS per well and 1 ml of the Staining Mixture was added to each well. The plate was then incubated at 37°C without CO_2 overnight, until the cells stained blue. To prevent the plate from drying out during the incubation, it was sealed with Parafilm. After staining, the cells were observed under a microscope. Four microscopic fields per well (10× objective) were photographed and blue-stained cells (indicative of senescence) and the total number of cells were manually counted. The percentage of β -galactosidase-positive cells (senescent cells) was calculated. Each cell culture was seeded in quadruplicates.

3.6 Transwell migration assay

A transwell migration assay was conducted as previously described with slight modifications (Busek et al. 2012; Balaziova et al. 2021). A549 cells ($4,5 \times 10^4$) were seeded in cell culture inserts with 8 μm pores suitable for 24-well plates (Corning, Glendale, AZ, USA) in serum-free medium and allowed to migrate towards either control or conditioned medium for 18 hours. Four inserts for each conditioned medium

were used. Non-migrated cells were removed with a cotton swab, and the cells on the lower surface of the insert were fixed with buffered 10% formalin and stained with Crystal Violet fixative solution (0.5 g Crystal Violet, C0775 Sigma-Aldrich; 270 ml buffered 10% formalin, 10ml methanol, 720ml PBS) for 30 min at room temperature. The inserts were afterwards washed twice in distilled water. Four microscopic fields per insert (10× objective) were photographed and the cells were manually counted.

3.7 Cell proliferation assay

A549 cells were plated at a density of 1×10^3 cells per well in a 96-well plate (Corning™ Costar™, Fisher Scientific) and cultured in DMEM containing 10% FCS for 24 hours at 37°C and humidified atmosphere with 5% CO₂. Then the culture medium was carefully removed and replaced with conditioned/control medium. For this experiment, the media were diluted at a 1:1 ratio with either serum-free DMEM (serum-free conditions) or DMEM containing 4% FCS (resulting in a final concentration of 2% FCS in the tested medium). A volume of 100 µl of each respective medium was added per well. The cells were cultured for 72 hours at 37°C and humidified atmosphere with 5% CO₂. All conditions were tested in hexaplicates.

Afterwards, the CellTiter-Glo® Luminescent Cell Viability Assay was performed according to the manufacturer's protocol. The CellTiter-Glo® Reagent (100 µl) was added to each well containing the cell culture medium. After the addition of the reagent, the contents of each well were thoroughly mixed on an orbital shaker for two minutes to ensure complete cell lysis. Following the mixing step, the lysates were transferred into a white opaque 96-well polystyrene plate (Pierce™, ThermoFisher Scientific). The plate was incubated at room temperature for ten minutes to allow the luminescence signal to stabilise. Afterwards, the luminescence was measured and recorded using a luminometer (Tecan).

3.8 Orthotopic implantation of CAFs in mice

The tumorigenic potential of CAFs derived from glioblastomas and brain metastases was assessed using an orthotopic xenotransplantation model. The procedure was performed as described previously (Hilšer et al. 2012). Briefly, the cells were collected and resuspended in serum-free DMEM at a concentration of 100 million cells/ml. A total of 5×10^5 cells in 5 µl were implanted intracranially into immunodeficient NOD-RAG mice. The cells were injected into the brains of the mice, 1.2 mm anterior to the bregma, 2.5 mm lateral from the midline, and at a depth of 3 mm, using a Hamilton syringe. Each cell culture was implanted in three mice. Mice were monitored daily for neurological symptoms or macroscopic signs of tumour growth. In the absence of neurological symptoms or visible tumour bulking, the brains were collected after 107 days, frozen in Tissue Freezing Medium (Leica, DE), and stored at -20°C.

3.9 Cytotoxic FAP-targeting compounds

Control and FAP-targeting iBodies were prepared at the Institute of Macromolecular Chemistry of the Czech Academy of Sciences (Dr. Etrych, Dr. Kostka and Dr. Šubr) and at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences (Dr. Šácha and prof. Konvalinka).

For detailed information on the structures of the iBodies, please refer to the diploma thesis of Petra Dvořáková (Dvorakova et al. 2017). Briefly, a FAP inhibitor with a (4-quinolinoyl)glycyl-2-cyanopyrrolidine scaffold (Jansen et al. 2013; Jansen et al. 2014) was attached to a HPMA copolymer using a polyethylene glycol (PEG) linker (Dvorakova et al. 2017). The tubulin inhibitor mertansine (emtansine, DM1) was attached via a disulfide bridge, forming the FAP-targeting iBody labelled as U156. The iBody without the FAP inhibitor was labelled as U157. The iBodies were dissolved in sterile H₂O at a concentration of 500 µM.

3.10 *In vitro* cytotoxicity assessment of iBodies

The U251 cells with doxycycline-inducible FAP expression were diluted in DMEM medium with 10% FBS and 100 nM doxycycline (for the FAP-expressing cells). The cells were seeded into a 96-well microplate, with 5000 cells in each well. The cells were cultured at 37°C and humidified atmosphere containing 5% CO₂ for 24 hours. Then a concentration series of free DM1, the anti-FAP polymer containing a FAP inhibitor and DM1 (U156) and a polymer with DM1 without the FAP inhibitor (U157) was added to the cells respectively. The cells were incubated for 6 hours at room temperature. Afterwards, the medium was removed and 100 µl of fresh medium was added. The cells were left for 3 days at 37°C. Afterwards, the medium was removed, the cells were washed once with PBS and stained with methylene blue solution (5g/l in 50% ethanol) for 20 minutes at 37°C followed by two washes with deionised water. After removing the deionised water, 100 µl of sodium dodecyl sulfate (1% solution in water) was added to the wells and left on an orbital shaker for two hours. Then, the absorbance was measured at 630nm using a microplate reader (Tecan). Each concentration of the conjugates was measured in quadruplicate. The experiment was repeated independently four times.

3.11 *In vivo* toxicity and effect on tumour growth of iBodies

For the assessment of the maximum tolerated dose, several rounds of dose escalation were conducted. The FAP-targeting iBody conjugate with DM1 (U156) was compared to free DM1 in C57Bl mice. The control mice received PBS. U156 and free DM1 were diluted from stock solutions to the final concentrations with PBS, delivering doses corresponding to 1200, 2000, 2800, and 3600 µg of DM1 per m² of body surface area. The compounds were administered intravenously via tail vein injection. Mice were

monitored and weighed daily. If the mice lost more than 20% of their initial body weight, they were euthanized. In cases of acceptable tolerance, repeated administrations were allowed in weekly cycles. The mice received up to three administrations of the respective compounds. The experiment was terminated and mice were euthanised one week after the last application. Each concentration was tested in five mice.

For the analysis of antitumor efficacy, subcutaneous tumour xenografts were established using U251 cells transfected to constitutively express FAP. The FAP expressing U251 cells were harvested and resuspended in DMEM to achieve a concentration of 50 million cells per milliliter. A 50 µl microliter aliquot of this suspension (containing 2,5 million cells) was then mixed in a 1:1 ratio with Corning® Matrigel® Basement Membrane Matrix Growth Factor Reduced. 100 µl of this cell-matrigel mixture was injected subcutaneously into the right or left flank of immunodeficient NOD-RAG mice. Tumours were allowed to grow for seven days before administration of the compounds (U156, U157, or PBS for control mice). U156 and U157 were applied intravenously via tail vein injection at a dose equivalent to 2800 µg of DM1 per m² of body surface area. In total three doses were applied in weekly intervals. After administration, the mice were monitored daily, including weight measurements. Tumour size was assessed using callipers and the approximate tumour volume was calculated ($\text{Volume} = \frac{1}{2} (\text{Length} \times \text{Width}^2)$). The experiment was terminated and mice were euthanised one week after the last application. Subcutaneous tumours were weighted at the end of the experiment, frozen in Tissue Freezing Medium (Leica, DE) and stored at -20°C.

3.12 Statistical analysis

Statistical analyses were performed with STATISTICA 12 (Tibco Software, USA) and GraphPad Prism 8.0.2 (GraphPad Software, USA). A p value <0.05 was considered statistically significant.

4. Results

4.1. FAP expression in primary, recurrent glioblastomas and brain metastases

FAP expression in primary glioblastomas

Previous and current work of our and other research groups has shown that FAP is significantly overexpressed in glioblastomas in comparison to non-tumorous brain tissue. In our more recent study (see Attachment 2, (Krepela et al. 2021)), we aimed to assess FAP expression in a larger cohort of glioblastomas and investigate whether TGFbeta could regulate FAP expression in the tumour microenvironment. We determined FAP expression in glioblastomas (n=76) and non-tumorous brain tissue from tissues of pharmacoresistant epilepsy specimen (PRE, n=10). FAP was significantly upregulated on the protein level, as determined by ELISA, and also its enzymatic activity assessed using a FAP specific substrate was higher in glioblastomas (Fig. 6A). There was a significant positive correlation between FAP protein concentration and FAP enzymatic activity (Fig. 6D).

ELISA results showed that TGFbeta 1 was the most prevalent isoform of TGF detected in glioblastomas (Fig. 6B). Further analysis showed that the levels of TGFbeta 1 protein were significantly elevated in glioblastomas compared to non-tumorous PRE brain tissues (Fig 6C). Additionally, a statistically significant positive correlation was observed between the concentrations of FAP and TGFbeta 1 proteins in glioblastomas (Fig. 6E).

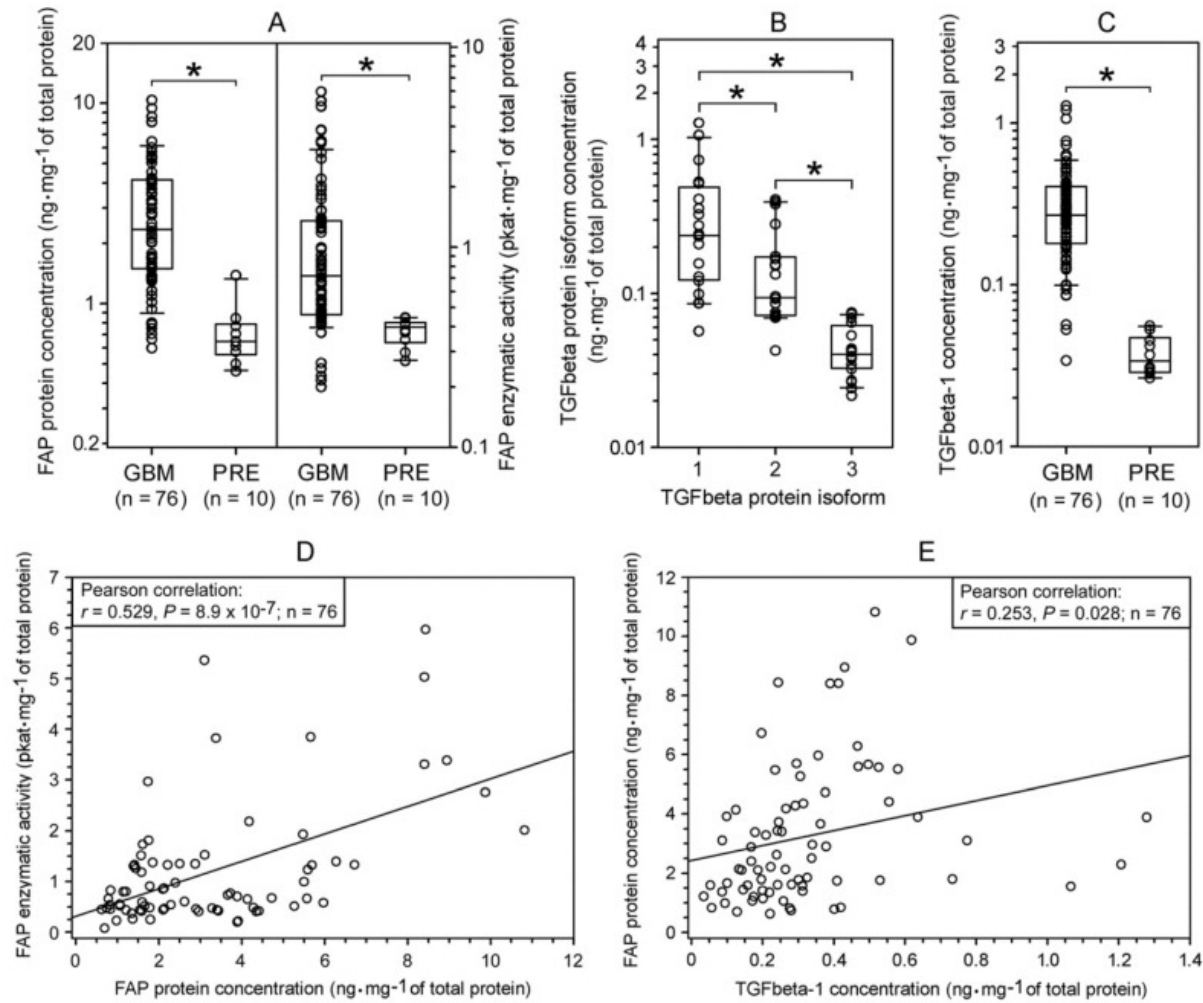


Figure 6. Expression of FAP and TGFbeta in human glioblastomas. (A) Upregulation of FAP protein (left panel) and FAP enzymatic activity (right panel) levels in glioblastomas in comparison to pharmacoresistant epilepsy (PRE) brain tissues, (B) Expression of TGFbeta protein isoforms in glioblastomas (n = 20), (C) Upregulation of TGFbeta 1 protein level in glioblastomas compared to PRE brain tissues. (D) Correlation between FAP enzymatic activity and FAP protein concentration in glioblastomas, (E) Correlation between FAP protein and TGFbeta 1 protein levels in glioblastomas. In (A–C), the sets of individual data points (each representing the mean of measurements in triplicate) are presented as a median with the box showing the 25th–75th percentile and whiskers indicating the 10th and 90th percentile. Note the logarithmic y-axis. In (A,C), * p < 0.01, Mann-Whitney rank sum test. In (B), * p < 0.05, Kruskal-Wallis one way ANOVA on ranks (Attachment 2, (Krepela et al. 2021))

Using chromogenic and fluorescence immunohistochemistry, we observed FAP immunopositivity in various cell subpopulations in glioblastomas, including perivascular stromal cells, some intraparenchymal cancer cells in approximately a quarter of the glioblastomas, and occasionally in the endothelial cells of certain blood vessels. In contrast, PRE brain tissues showed no FAP immunopositivity (Fig. 7A-E). Additionally, using fluorescence immunohistochemistry and confocal microscopy, we noted widespread TGFbeta 1 immunopositivity in the glioblastoma microenvironment.

TGFbeta 1 immunopositivity was observed in glioma cells and stromal areas, particularly near FAP-positive perivascular stromal cells (Fig. 8).

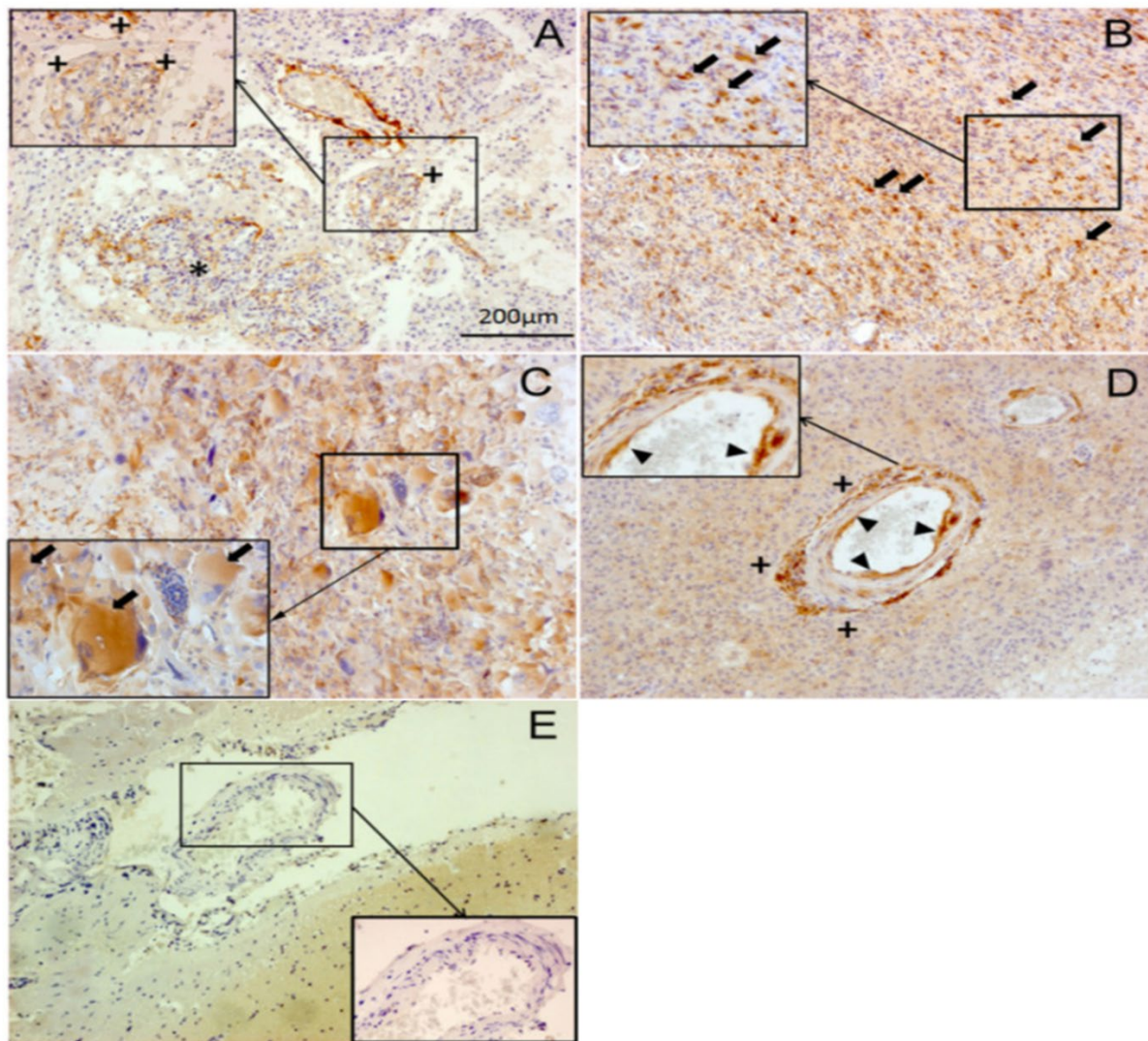


Figure 7. Immunohistochemical analysis of FAP in the tumour microenvironment of human glioblastomas. (A-D) FAP expression (brown staining) present in glioblastomas, but not in non-tumorous PRE brain tissue (E). In glioblastomas, FAP immunopositivity is found in perivascular stromal cells (+), including microvascular proliferations (*), and occasionally in some cancer cells (arrows). In rare cases, FAP expression is also detected in the endothelial cells of blood vessels (arrowheads indicating endothelial FAP in an arteriole (Attachment 2, (Krepela et al. 2021))).

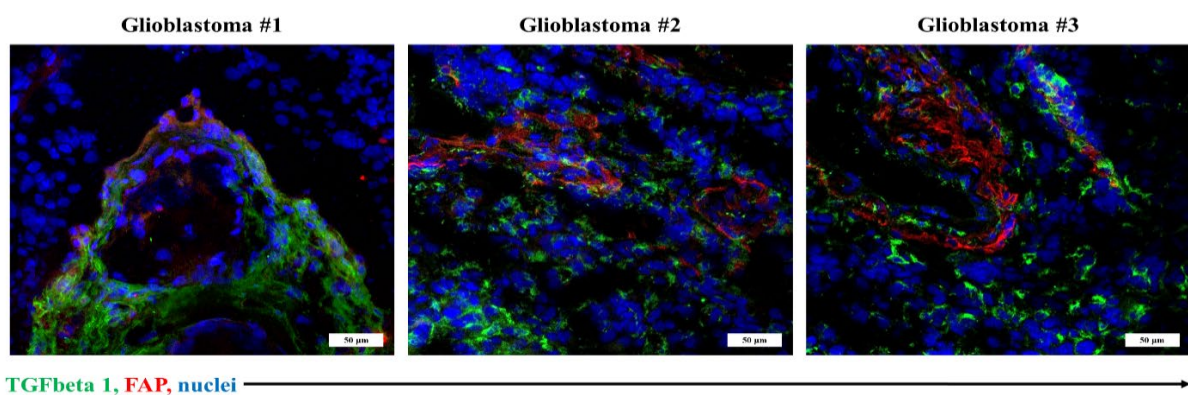


Figure 8. Immunopositivity of TGFb in human glioblastomas. Representative confocal fluorescence microscopy images of glioblastoma tissue sections showing TGFbeta 1 immunopositivity (green) in the tumour parenchyma and stromal regions in the vicinity of FAP expressing perivascular stromal cells (red). Nuclei (blue) were counterstained with the Hoechst 33258 dye (unpublished data and Attachment 2, (Krepela et al. 2021)). Glioblastoma#1,2,3 correspond to patients AZVU11, AZVU004, and H050, respectively.

Functional studies performed by my colleagues demonstrated that TGFbeta 1 induces the expression of FAP in non-stem glioma cells, pericytes, and glioblastoma-derived endothelial and FAP+ mesenchymal cells, but not in glioma stem-like cells. In glioma cells, this effect was mediated by the TGFbeta type I receptor and canonical Smad signalling, involving the activation of FAP gene transcription. This study demonstrated that TGFbeta plays a crucial role in regulating FAP expression within the microenvironment of glioblastoma (Attachment 2, (Krepela et al. 2021)).

FAP expression in recurrent glioblastomas

Recurrent glioblastomas often show a shift towards a mesenchymal phenotype (Wood et al. 2016). Since previous findings of our research group linked FAP expression in glioblastomas to the mesenchymal subtype (Busek et al. 2016), we investigated whether FAP is upregulated in recurrent glioblastomas.

FAP protein concentration and FAP enzymatic activity were measured in 22 paired samples of primary and recurrent glioblastomas. We did not observe a significant difference in FAP expression between primary and recurrent glioblastomas. There was a positive correlation between FAP enzymatic activity and protein concentration in primary glioblastomas. In recurrent glioblastomas, we also noted a positive correlation between FAP protein levels and enzymatic activity; however, the statistical significance in these cases was primarily influenced by a single sample with high FAP expression (Fig 9A).

Treatment information was available for ten patients. Of these, eight patients underwent standard concomitant chemoradiotherapy followed by adjuvant temozolomide. One patient received only radiotherapy following the resection and one patient refused adjuvant therapy. The analysis of the group that received concomitant chemoradiotherapy showed no significant difference or impact of the treatment on FAP expression in glioblastomas (data not shown).

In summary, we did not observe any significant trend in terms of up- or down-regulation of FAP in recurrent glioblastomas compared to the paired primary glioblastomas (Fig. 9B).

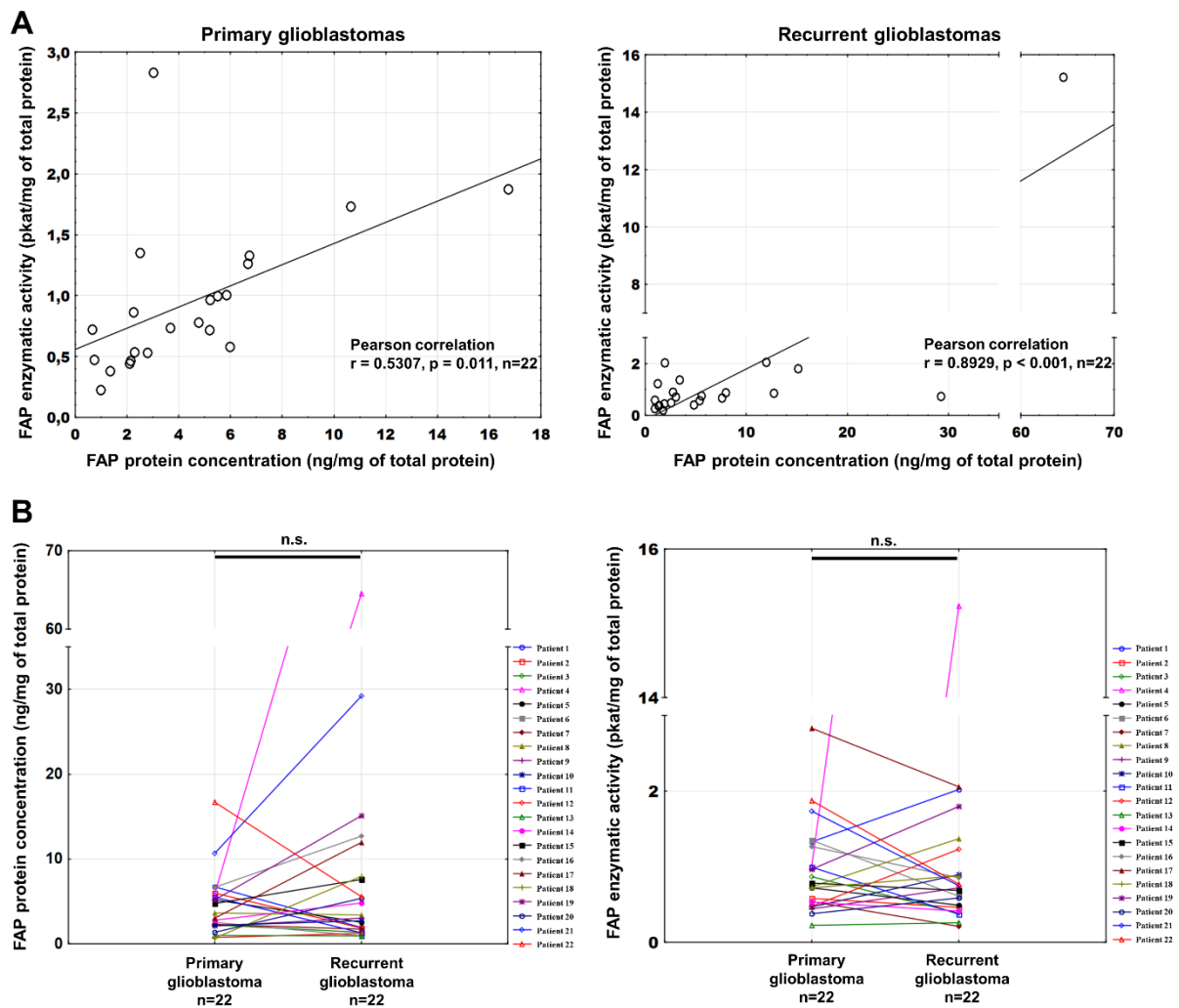


Figure 9. Expression of FAP in primary and recurrent glioblastomas. (A) Correlation between FAP enzymatic activity and FAP protein concentration in primary glioblastomas (left panel) and recurrent glioblastomas (right panel), circles = source data. (B) Comparison of FAP protein concentration (left panel) and FAP enzymatic activity (right panel) in paired samples of primary and recurrent glioblastomas. Wilcoxon matched pairs test, n.s.= non-significant, $p > 0.05$.

FAP expression in brain metastases of solid tumours

We assessed the expression of FAP in tissue lysates of brain metastases ($n = 58$) originating from various primary sites. PRE tissue samples ($n = 12$) were used as non-tumorous brain tissue controls. The concentration of FAP protein, determined by ELISA, was found to be more than 20 times higher in the tumour tissues compared to the control samples. To confirm the presence of enzymatically active FAP molecules in brain metastases, we performed a kinetic enzymatic assay using a FAP-specific substrate. The results showed that FAP enzymatic activity, in concordance with FAP protein concentration, was significantly higher in brain metastases compared to non-tumorous brain tissue (Fig. 10A). FAP enzymatic activity positively correlated with the levels of FAP protein (Fig. 10B). Interestingly, we observed that the expression of FAP in brain metastases was also significantly higher compared to glioblastomas (Fig. 10C).

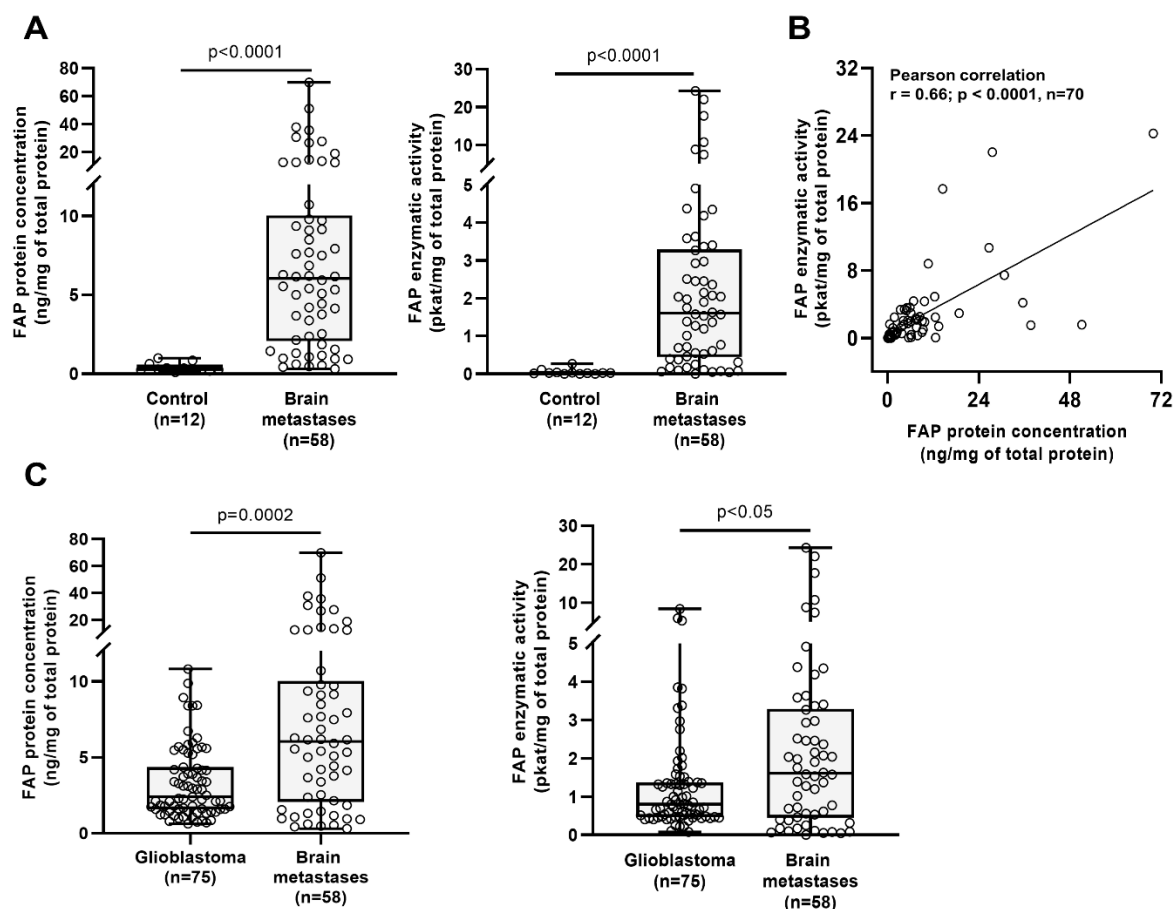


Figure 10. FAP expression in brain metastases, non-tumorous brain tissue (control=PRE) and primary glioblastomas. (A) Upregulation of FAP protein concentration (left panel) and FAP enzymatic activity (right panel) in brain metastases (n=58) as compared to control non-tumorous (PRE) brain tissues (n=12), Mann-Whitney U test. (B) Correlation between FAP protein levels and FAP enzymatic activity (n=70), circles – source data. (C) Upregulation of FAP protein concentration (left panel) and FAP enzymatic activity (right panel) in brain metastases (n=58) compared to glioblastomas (n=75, (Krepela et al. 2021)), Mann-Whitney U test; In (A) and (C) horizontal line – median, boxes – 25–75 %, whiskers – range values, circles – source data (Attachment 1 (Zubal’ et al. 2023)).

The presence of FAP in brain metastases was further validated by my colleague Ivana Matrasova using western blotting. Under denaturing and reducing conditions, FAP immunopositivity corresponding to a FAP monomer was observed at 90 kDa in the majority of brain metastases, while no immunopositivity was detected in non-tumorous brain tissue. Densitometric analysis confirmed a significantly higher expression of FAP in comparison to non-tumorous brain tissue. In native western blotting, FAP immunopositivity with electrophoretic mobility ranging from 100 to 140 kDa was observed in a substantial proportion of brain metastases. Additionally, high molecular weight forms of FAP were present in several tissues, like what has been previously observed in glioblastomas (Attachment 1, (Zubal’ et al. 2023), doctoral thesis (Matrasová 2023), Laboratory of Cancer Cell Biology).

Given our previous findings linking TGFbeta 1 to FAP expression in glioblastomas, we sought to investigate whether a similar relationship exists in brain metastases. TGFbeta 1 protein concentration was measured by ELISA in brain metastases (n=15) and non-tumorous PRE brain tissue (n=10), revealing significantly higher levels in brain metastases compared to non-tumorous PRE tissues. Additionally, there was a positive correlation between TGFbeta 1 and FAP protein concentration in brain metastases (Fig. 11). These findings suggest that TGFbeta 1 may play a similar role in regulating FAP expression in brain metastases as in glioblastomas, though the exact mechanism requires further investigation.

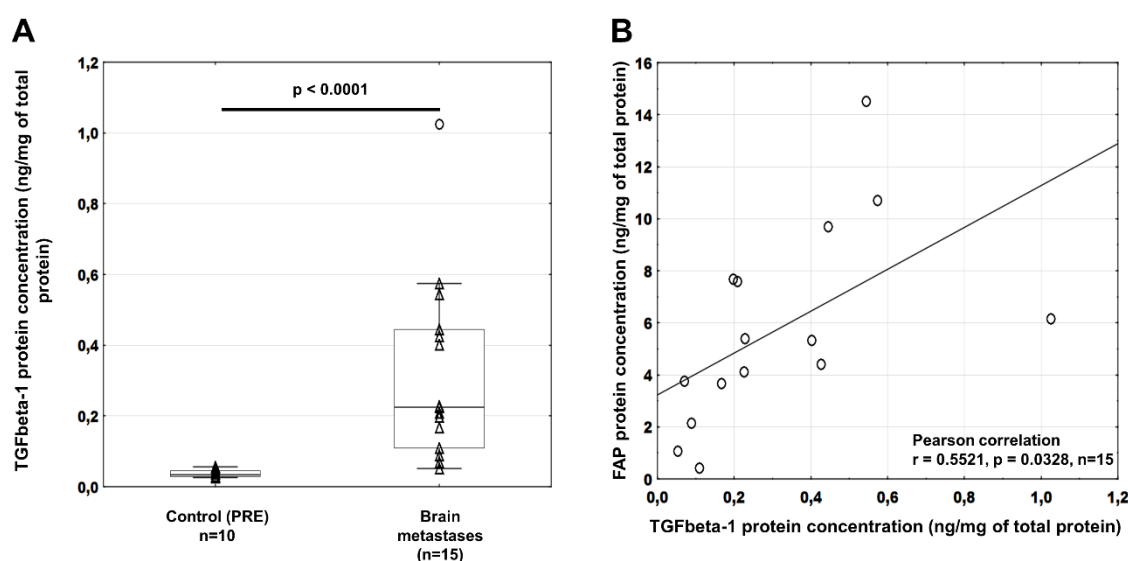


Figure 11. Expression of TGFbeta 1 and FAP in brain metastases and non-tumorous brain tissue (PRE). (A) Higher TGFbeta 1 protein concentration in brain metastases as compared to control PRE tissue, horizontal line - median, boxes - interquartile range 25–75%, whiskers - range of non-remote values, triangles—source data, circle - remote value; Mann-Whitney U test. (B) Correlation between TGFbeta 1 and FAP protein levels, circles – source data.

FAP protein levels and enzymatic activities were further evaluated with an aim to identify a relationship between FAP expression and the histological type of brain metastasis. Our cohort comprised various brain metastases originating from several types of cancer, including lung (n=16), breast (n=12), gastrointestinal (n=10), renal (n=5), ovarian (n=3) and primary peritoneal serous cancer (n=1). The cohort also included brain metastases from cancers of unknown primary origin (n=5), melanoma (n=4) and sarcoma (n=1). Relatively high FAP expression was found in brain metastases originating from lung, breast, gastrointestinal cancer, melanoma, and in one sample of brain metastasis from bladder cancer. As expected, a brain metastasis from sarcoma exhibited one of the highest levels of FAP expression. Low FAP expression, comparable to that in PRE tissues, was detected in approximately 25% of breast cancer samples (3 out of 12 tissues), 25% of melanomas (1 out of 4), 30% of gastrointestinal tract cancers (GIT) (3 out of 10), 19% of lung cancers (3 out of 16), 20% of cancers of unknown primary origin (1 out of 5), and 20% of renal cancer brain metastases (1 out of 5).

This analysis showed no significant association between FAP expression levels and the histological subtype of brain metastases (Fig. 12).

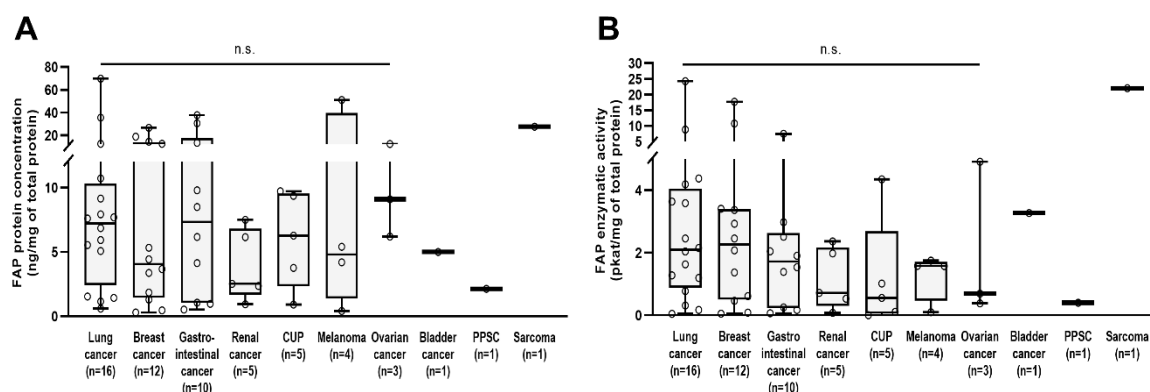


Figure 12. Expression of FAP in brain metastases categorized by their primary cancer origin. Neither FAP protein concentration (A) nor FAP enzymatic activity (B) showed a significant association with the histological type of brain metastasis. CUP, carcinoma of unknown primary; PPSC, primary peritoneal serous carcinoma; ns, non-significant; $p > 0.05$, Kruskal–Wallis test; horizontal line = median; boxes = 25–75%; whiskers = range of values; circles = source data (Attachment 1, (Zubal’ et al. 2023)).

4.2 Characterization of FAP-expressing cells in the tumour microenvironment of brain metastases

In collaboration with The Department of Pathology of Na Homolce Hospital, immunohistochemical staining for FAP was performed on formalin-fixed paraffin-embedded (FFPE) sections of brain metastases ($n=112$) originating from various primary sites to identify FAP expressing cells in the tumour microenvironment of brain metastases. FAP immunopositivity was detected predominantly in perivascular regions and stromal septa surrounding areas with cancer cells, with no immunopositivity detected in non-tumorous PRE brain tissue. FAP-positive stromal cells were identified in 100 (89%) of the examined brain metastases samples (Fig. 13A).

Given the substantial presence of FAP-positive stromal cells, we examined their distribution across different histological subtypes of brain metastases. No significant difference was observed in the prevalence of FAP-positive stromal cells across various histological subtypes of brain metastases (Fig. 13D, left panel). This finding aligns with the results for FAP enzymatic activity and protein levels. Therefore, it appears that FAP expression and the quantity of FAP-positive stromal cells do not significantly vary among brain metastases from different primary origins.

Fluorescent double labelling immunohistochemistry was performed on twelve tissues of brain metastases originating from breast ($n=4$), lung ($n=3$), gastrointestinal ($n=2$), ovarian ($n=1$) cancer, and carcinomas of unknown origin ($n=2$). To confirm the perivascular localization of FAP, we performed fluorescent immunohistochemical double-labelling of FAP and CD31 (platelet endothelial cell adhesion molecule;

PECAM-1), a canonical marker of endothelial cells. FAP immunopositivity was observed near CD31-positive endothelial cells. The double-labelling results provided further evidence of the distribution of FAP in brain metastases, showing a relationship to the tumour vasculature (Fig. 13B, lower panel).

In extracranial solid tumours, FAP expression is commonly associated with the remodelling and organization of extracellular matrix proteins, particularly collagens. To assess the presence of collagens in tissue sections of brain metastases, we conducted Masson's trichrome staining. The results confirmed the presence of collagen deposits in several brain metastases, and the corresponding regions also exhibited FAP immunopositivity (Fig. 13A). This observation was further validated through fluorescent immunohistochemical double labelling of FAP and collagen I, where FAP-positive cells were typically surrounded by collagen I immunopositivity (Fig. 13B, upper panel). Additionally, Masson's trichrome staining was scored in FFPE sections of brain metastases (n=106) and demonstrated a positive correlation with the immunopositivity of FAP in stromal cells (Fig. 13D, right panel).

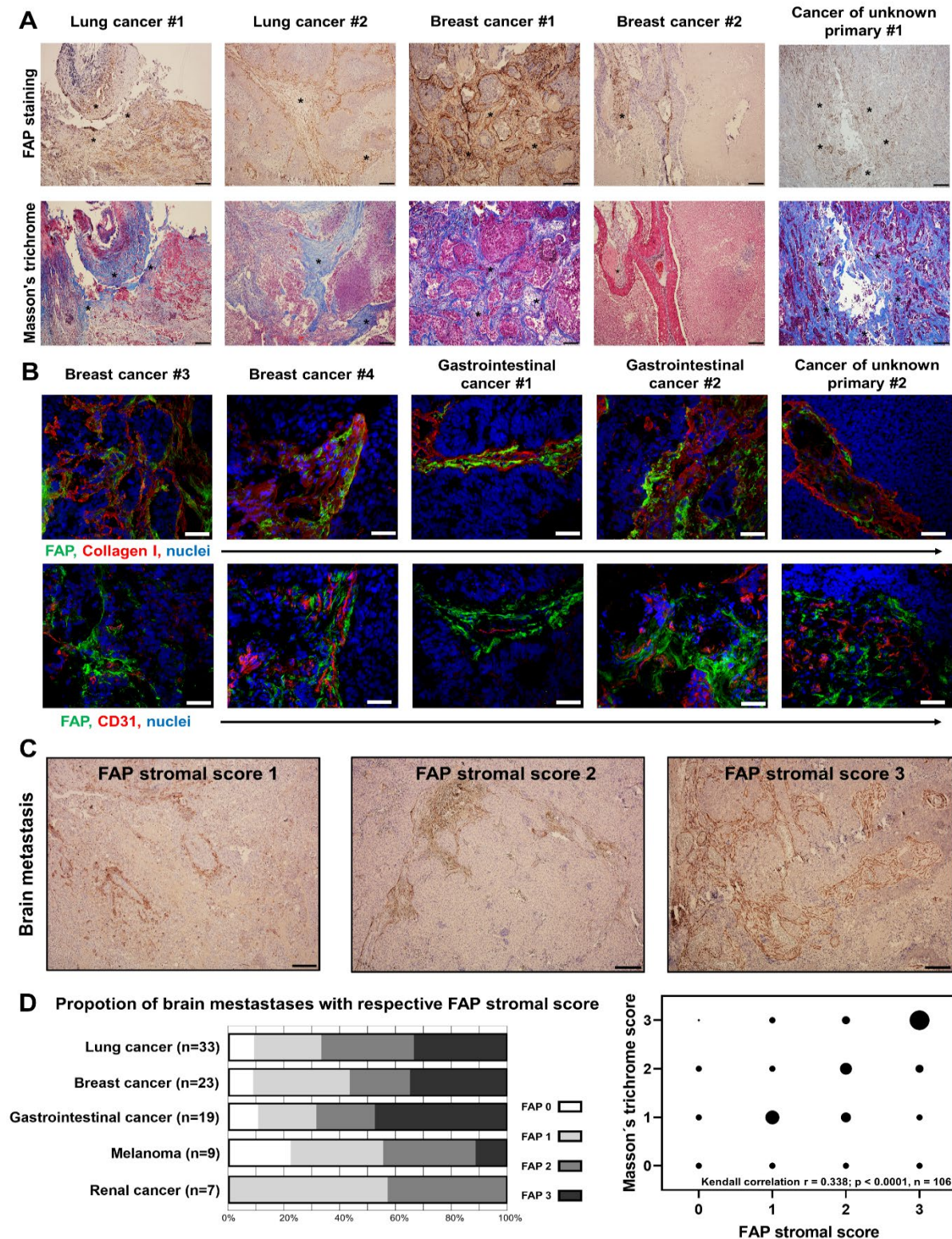


Figure 13. Immunohistochemical detection of FAP in stromal regions of brain metastases. (A) FAP immunopositivity in septal and perivascular regions (arrows) and Masson's trichrome staining of the corresponding areas (asterisks); scale bars = 200µm. Lung cancer #1,2 correspond to patients 1713/2019, 11120/19; Breast cancer #1,2,3,4 correspond to patients 11234/19, 5202/2019, H117, NCH106; Gastrointestinal cancer #1,2 correspond to patients H114, H093, Cancer of unknown primary #1,2 correspond to patients 10050, 8/5 (B) Immunohistochemical double labelling of FAP and collagen I or CD31, scale bars = 50µm. (C) Representative images of stromal FAP immunopositivity (score 1–3) in FFPE tissues; scale bars = 200µm. (D, left panel). Evaluation of stromal FAP immunopositivity in brain metastases of different origin. (D, right panel) Correlation between Masson's trichrome score and FAP stromal score in tissue sections of brain metastases (n=106). Frequencies of overlapping points between two variables are illustrated by the size of the point markers (Attachment 1, (Zubal' et al. 2023)).

In addition to the prevalent stromal positivity of FAP, FAP expression was also detected in cancer cells of brain metastases from various primary cancers (Fig. 14A), including melanoma (7 out of 9 cases), lung (16 out of 33), breast (6 out of 23), gastrointestinal (2 out of 19), renal (4 out of 7), ovarian (1 out of 3), carcinoma of unknown primary (3 out of 11), salivary gland cancer (1 out of 1), and sarcoma (2 out of 2). However, cells co-expressing FAP and the epithelial cell marker pancytokeratin were rarely observed in brain metastases from carcinomas (Fig. 14B). The highest numbers of FAP-positive cancer cells, with positivity in more than 30% of cancer cells, were observed in metastases from melanoma, lung, and breast cancer (Fig. 14D). As could be expected due to their mesenchymal origin, brain metastases from the two analysed sarcomas showed strong FAP expression in most cancer cells (Fig. 14C).

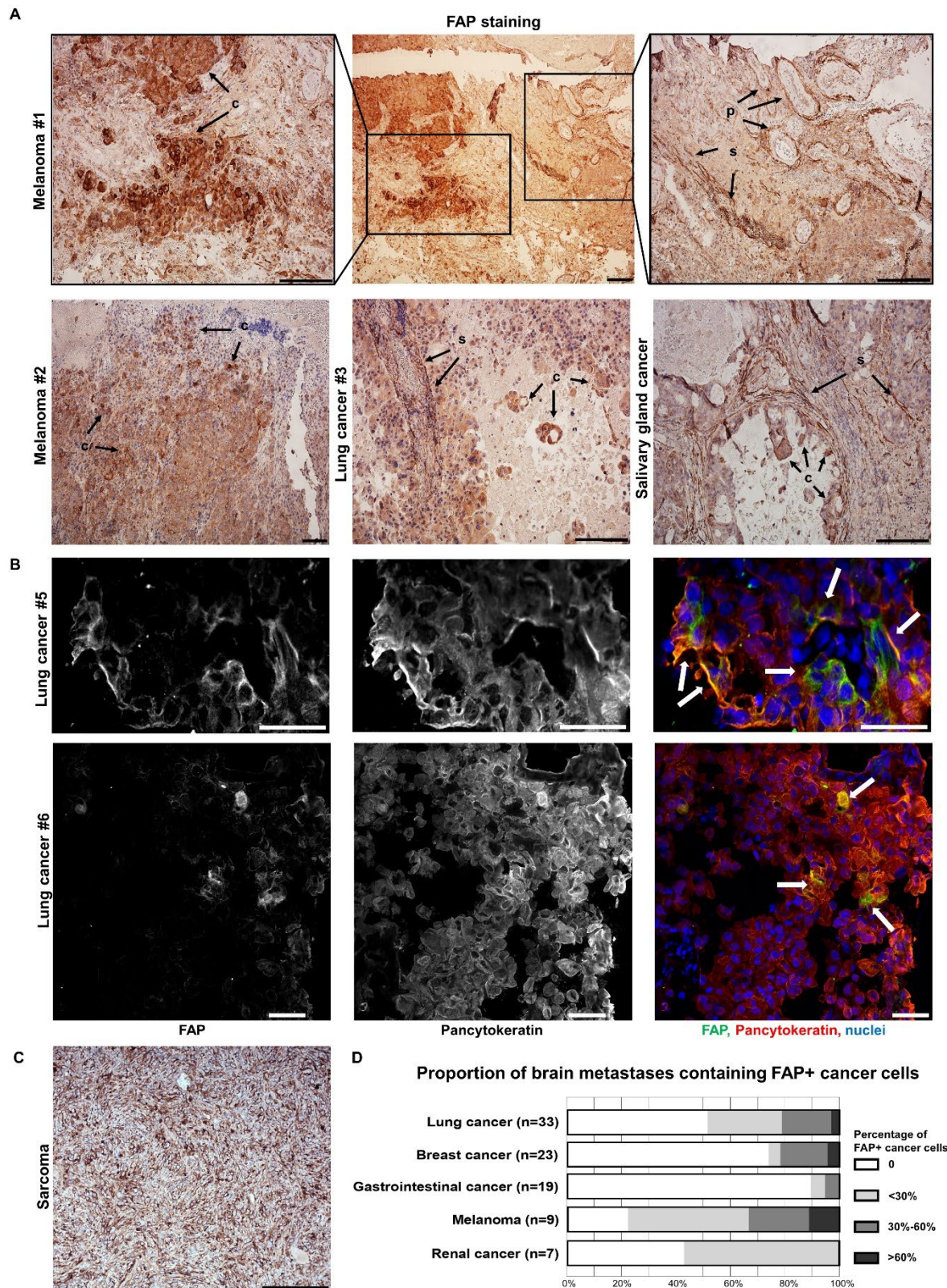


Figure 14. Immunohistochemical detection of FAP in cancer cells of brain metastases. (A) FAP immunopositivity in tissues containing FAP expressing cancer cells. c, cancer cells; p, perivascular regions; s, stromal/septal regions; scale bars = 200µm. Melanoma #1,2 correspond to patients UVN5706, UVN3963; Lung cancer #3 corresponds to patient 11231/19; Salivary gland cancer corresponds to patient 19046/19 (B) Immunohistochemical double labelling of FAP and pancytokeratin; arrows = rare occurrence of double positive cells, scale bar = 50µm. (C) FAP immunopositivity in brain metastasis of a sarcoma, scale bar = 200µm. (D) FAP expression in cancer cells in most frequent histological types of brain metastases - summary of immunohistochemical evaluation (Attachment 1, (Zubal' et al. 2023)).

Only a few brain metastases originating from lung (2 out of 33), breast (2 out of 23), gastrointestinal cancer (1 out of 19), carcinoma of unknown primary (1 out of 11), and one case of primary peritoneal serous carcinoma showed no FAP immunopositivity in either stromal or cancer cells. We assessed the FAP protein concentration in six of these tumours; among them, only one tumour exhibited low FAP expression similar to that observed in non-tumorous brain tissue, while the other five had substantially higher FAP levels.

Taken together, these data suggest that FAP in brain metastases is typically localized in perivascular regions and areas rich in collagen deposits. FAP is dominantly expressed on stromal cells, although some brain metastases, originating from melanoma, lung and breast cancer, may also contain FAP-positive cancer cells. Overall, FAP expression in the tumour microenvironment appears to be a common characteristic across most brain metastases originating from solid tumours, with a caveat that a minority of brain metastases may be FAP-negative.

FAP is considered a canonical marker of CAFs. Therefore, we wanted to explore if additional CAF markers are expressed in FAP-positive stromal cells. Immunohistochemical double labelling revealed that the microenvironment of brain metastases contained cells that were positive for both FAP and other CAF markers, including TE7, PDGFR β , and α SMA. FAP immunopositivity was localised in TE7-positive cells, while only a portion of the FAP immunopositivity overlapped with the signals of PDGFR β or α SMA (Fig. 15A).

Double labelling immunohistochemistry for FAP was also performed with other cell markers - pancytokeratin, an epithelial carcinoma marker, and GFAP, a marker of astroglia. We observed extensive pancytokeratin positivity in brain metastases originating from epithelial cancers/carcinomas. There was almost no expression of FAP in pancytokeratin-positive cells. This contrasts with our findings showing FAP immunopositivity in morphologically cancerous cells in FFPE sections. The lack of pancytokeratin in FAP-positive cells may be attributed the dedifferentiation/epithelial-mesenchymal transition of metastatic tumour cells (Ribatti et al. 2020). GFAP positivity indicates the presence of astroglia in the tumour microenvironment of brain metastases. Nevertheless, these astroglia cells are FAP-negative (Fig. 15B).

Overall, these findings suggest that FAP-expressing mesenchymal stromal cells in brain metastases may represent CAFs. Considering our previously published data in glioblastomas (Attachment 3 (Balaziová et al. 2021)), these CAFs may contribute to tumour vascularization and the remodelling of the extracellular matrix, potentially playing a significant role in the tumorigenesis of brain metastases.

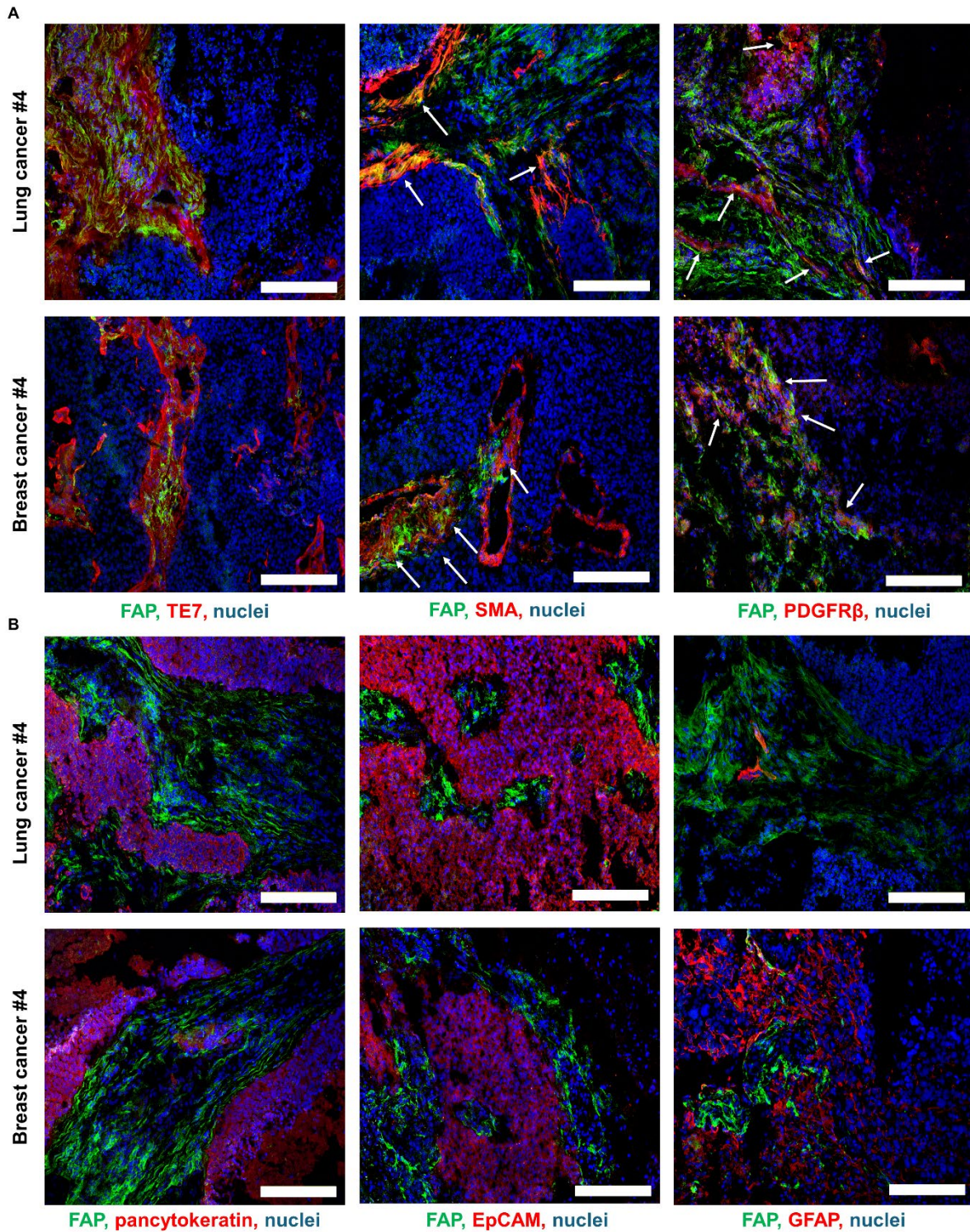


Figure 15. Immunohistochemical detection of FAP and markers of CAFs, epithelial cells and astroglia in brain metastases. (A) Immunohistochemical double labelling of FAP and other markers of cancer-associated fibroblasts – TE7, α SMA, PDGFR β ; arrows indicate double positive areas. (B) Immunohistochemical double labelling of FAP and markers of epithelial cells – pancytokeratin, and astroglia - GFAP, scale bars = 200 μ m. Lung cancer #4 = H034; Breast cancer #4 = NCH106 (Attachment 1, (Zubal' et al. 2023)).

4.3 Derivation and biological features of FAP+ stromal cells from brain tumours

Our previous data from glioblastomas and brain metastases have shown that FAP expressing stromal cells resemble cancer associated fibroblasts. To further assess their biological characteristics, we established primary cell cultures using anti-Fibroblast Magnetic-activated cell sorting (MACS). The cells were cultivated in DMEM with 10% FCS and 1% solution of antibiotics (penicillin+streptomycin). We derived cultures from glioblastomas (n=5), brain metastases of lung (n=7), breast (n=2), gastrointestinal (n=2) cancer, primary peritoneal serous carcinoma (n=1) and melanoma (n=1). Positively selected cells were labelled as positive fractions (PF), the rest was labelled as negative fraction (NF, supposedly comprised of other stromal cells and/or cancer cells, negative for the epitope targeted by the anti-fibroblast MACS antibody).

PFs from glioblastomas and brain metastases demonstrated a stronger capacity to grow attached to cultivating plastic. Also, PFs displayed limited *in vitro* propagability, slower growth rates, lower acidification of the cell culture medium and exhibited different morphology in comparison to NFs (Fig. 16). In the case of brain metastases originating from gastrointestinal cancer, we were only able to propagate the NFs.

Using the same procedure, we derived mesenchymal cells from pharmacoresistant epilepsy tissues (non-tumorous cerebral mesenchymal cells, “PRE control cells”) and subgaleal connective tissue (non-tumorous extracerebral mesenchymal cells, “subgaleal control cells”). These cells were used as controls for subsequent functional studies. Interestingly, PRE control cells had similar morphology in cell culture as PFs derived from glioblastomas and brain metastases (Fig. 17).

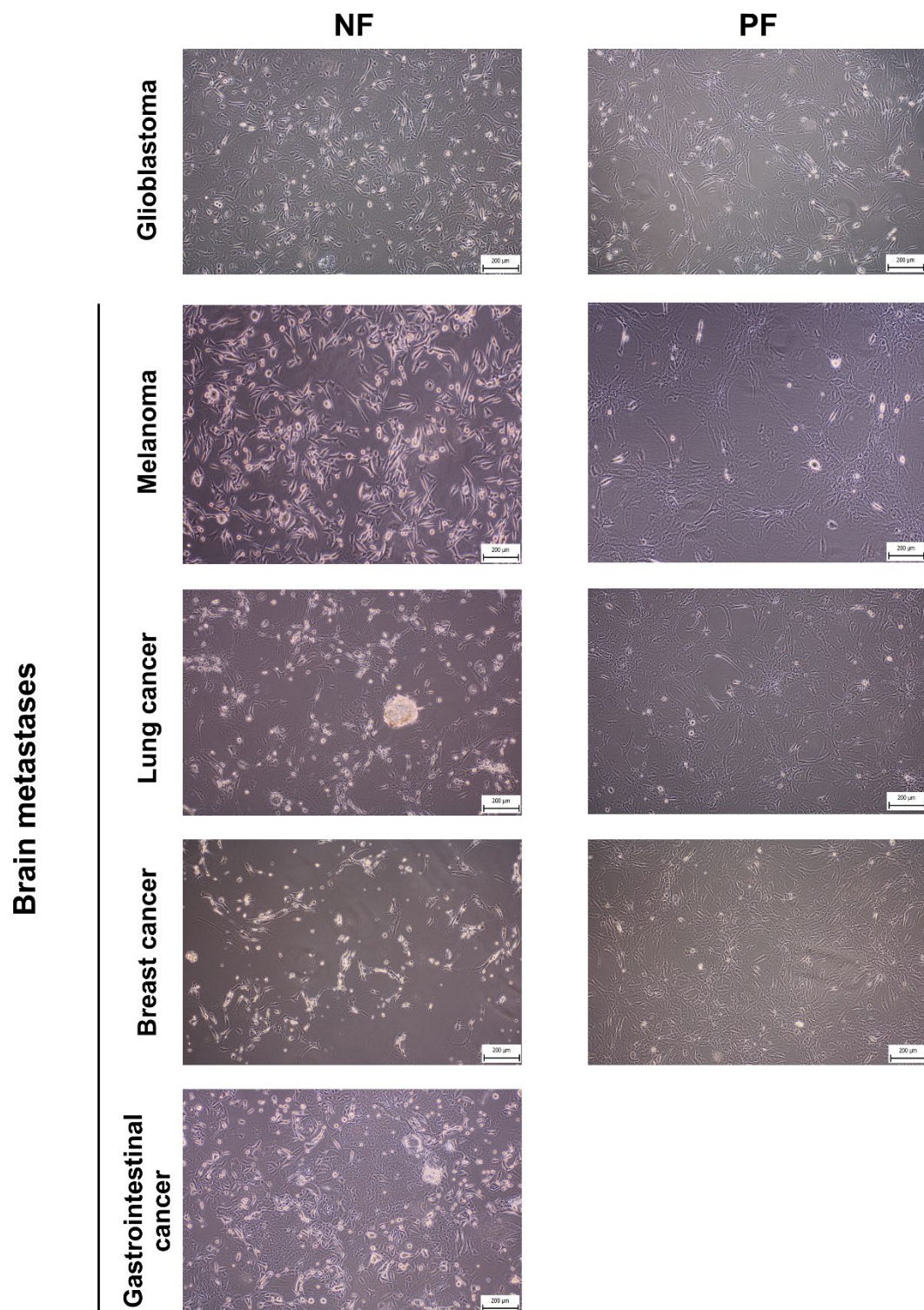


Figure 16. Morphology of cell cultures derived from glioblastomas and brain metastases using anti-Fibroblast MACS. NFs (left panels) exhibit asymmetrical shapes – spindles, “oat-like”, occasionally forming spheroids (lung, breast cancer metastases), frequently detaching from the cultivating plastic. PFs (right panels) show a more uniform growth pattern, forming trabecular structures. Interestingly, PFs share similar morphology across different histological origins of primary tumour, including PFs from glioblastoma. Respective cell cultures: Glioblastoma = NCH353, Melanoma = NCH 229, Lung cancer = NCH402, Breast cancer = NCH233, Gastrointestinal cancer = NCH381

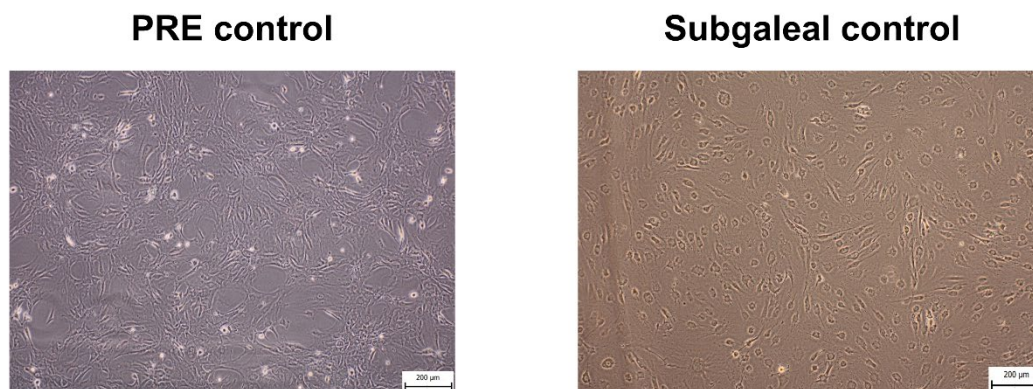


Figure 17. Representative images of cell cultures from control PRE and subgaleal tissues after anti-Fibroblast MACS.
Respective cultures: PRE control = NCH236, Subgaleal control = H131

To verify the mesenchymal/fibroblast phenotype of our PFs, we performed immunocytochemistry with CAF markers as used above – FAP, TE7, SMA, PDGF β . All PFs were positive for FAP (ranging from weak to intense staining), as could be expected in cultured fibroblasts (Rettig et al. 1988), TE7 (20-100% of cells) and PDGFR β (70-100% of cells). PFs derived from glioblastomas and brain metastases were SMA positive (50-95% of cells). All PFs were negative for pancytokeratin (in 5 cases of brain metastatic CAFs positivity under 5% of cells) or GFAP, ruling out malignant or astrocytic cells (data not shown). Therefore, PFs derived from glioblastomas and brain metastases were further labelled as CAFs (Fig. 18).

PFs from subgaleal connective tissue were SMA negative. Interestingly, PFs from pharmacoresistant epilepsy tissues also exhibited SMA positivity (2 of 3 cultures, 10 and 50% of cells), which could hint that mesenchymal cells in pharmacoresistant epilepsies could also contain a fraction of activated fibroblasts/myofibroblasts or could be activated in cell culture (Rettig et al. 1988).

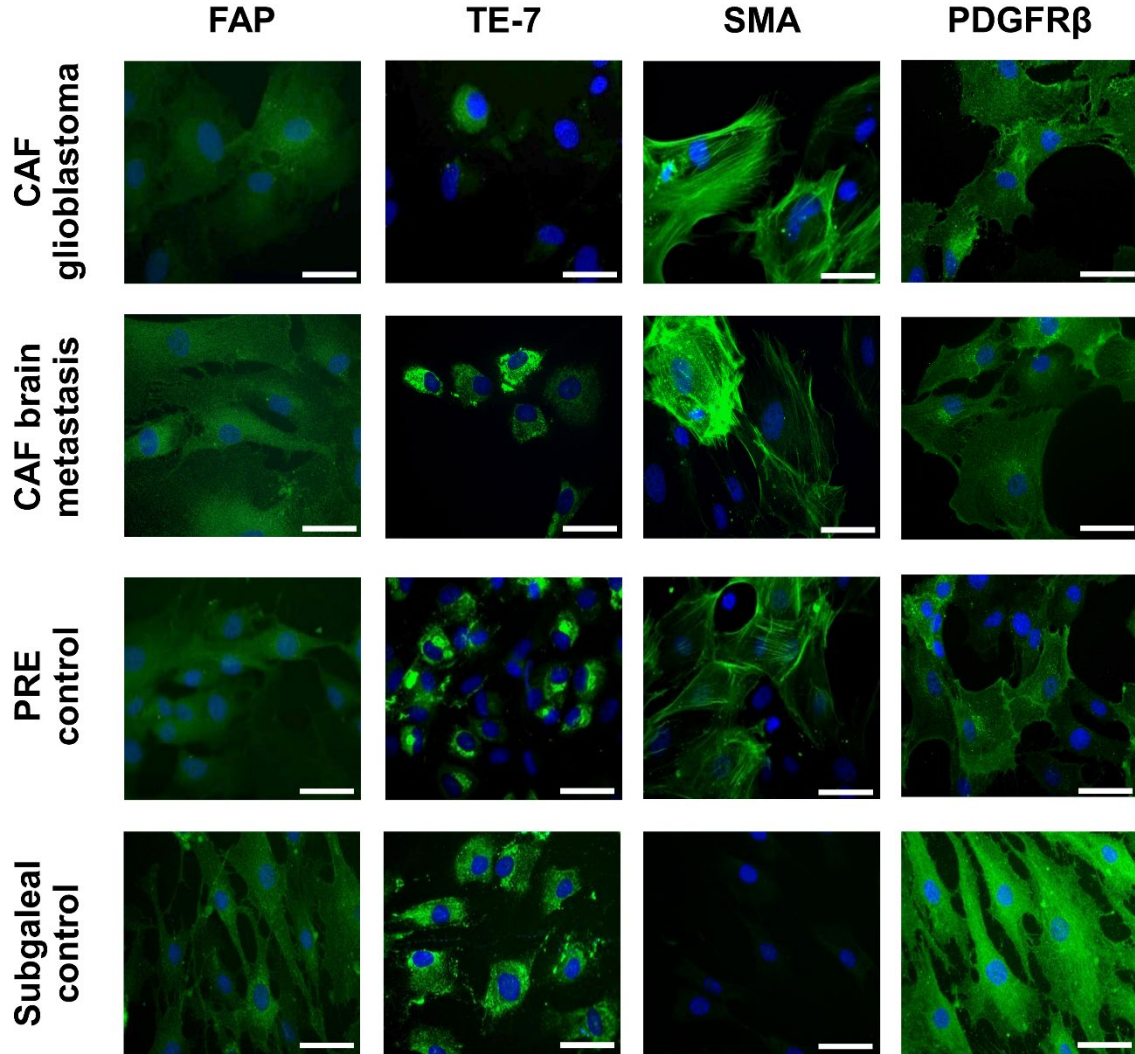


Figure 18. Immunocytochemical labelling of mesenchymal/CAF markers in PFs derived from glioblastomas, brain metastases, PRE and subgaleal control tissues. Mesenchymal cell markers (green) – FAP, TE-7, SMA, PDGFRβ. Nuclei (blue) were counterstained with the Hoechst 33258 dye. Scale bars = 50 μm. Respective cultures: CAF glioblastoma = NCH197, CAF brain metastasis = NCH233 (breast cancer metastasis), PRE control = NCH236, Subgaleal control = H131.

We noted that CAFs from brain metastases and glioblastomas exhibited a slower growth rate in cell culture compared to PFs derived from subgaleal tissue and PFs obtained from patients with pharmacoresistant epilepsy. Therefore, we hypothesised, that the reduced proliferation rate of CAFs might be attributed to senescence, a state known in some cases to confer pro-tumorigenic properties through a senescence-associated secretory profile (SASP) (Zhang et al. 2024a). To detect senescent cells, we performed a histochemical assay based on the detection of beta-galactosidase activity (Fig. 19A). We determined beta-galactosidase activity in CAFs from brain metastases (n=8; lung cancer n=4, breast cancer n=2, primary peritoneal serous carcinoma n=1) and glioblastomas (n=4), subgaleal (n=6) and pharmacoresistant epilepsy controls (n=3).

Brain metastatic CAFs had a significantly higher percentage of senescent cells compared to subgaleal control PFs. Also, CAFs from brain metastases also displayed lower cell densities (number of cells per field of view) in comparison to subgaleal control PFs. CAFs from glioblastomas exhibited a trend similar to brain metastatic CAFs, but the comparison with subgaleal control PFs did not reach statistical significance, possibly due to the low number of GBM CAF samples (Fig. 19B). The percentage of senescent cells and cell density in pharmacoresistant epilepsy control PFs fell between those of brain metastatic CAFs and subgaleal control PFs (data not shown). Given the relatively small number of pharmacoresistant epilepsy controls (n=3), they were excluded from the statistical analysis. Overall, our data suggests that the microenvironment of brain metastases, and potentially that of glioblastomas, may contain senescent CAFs.

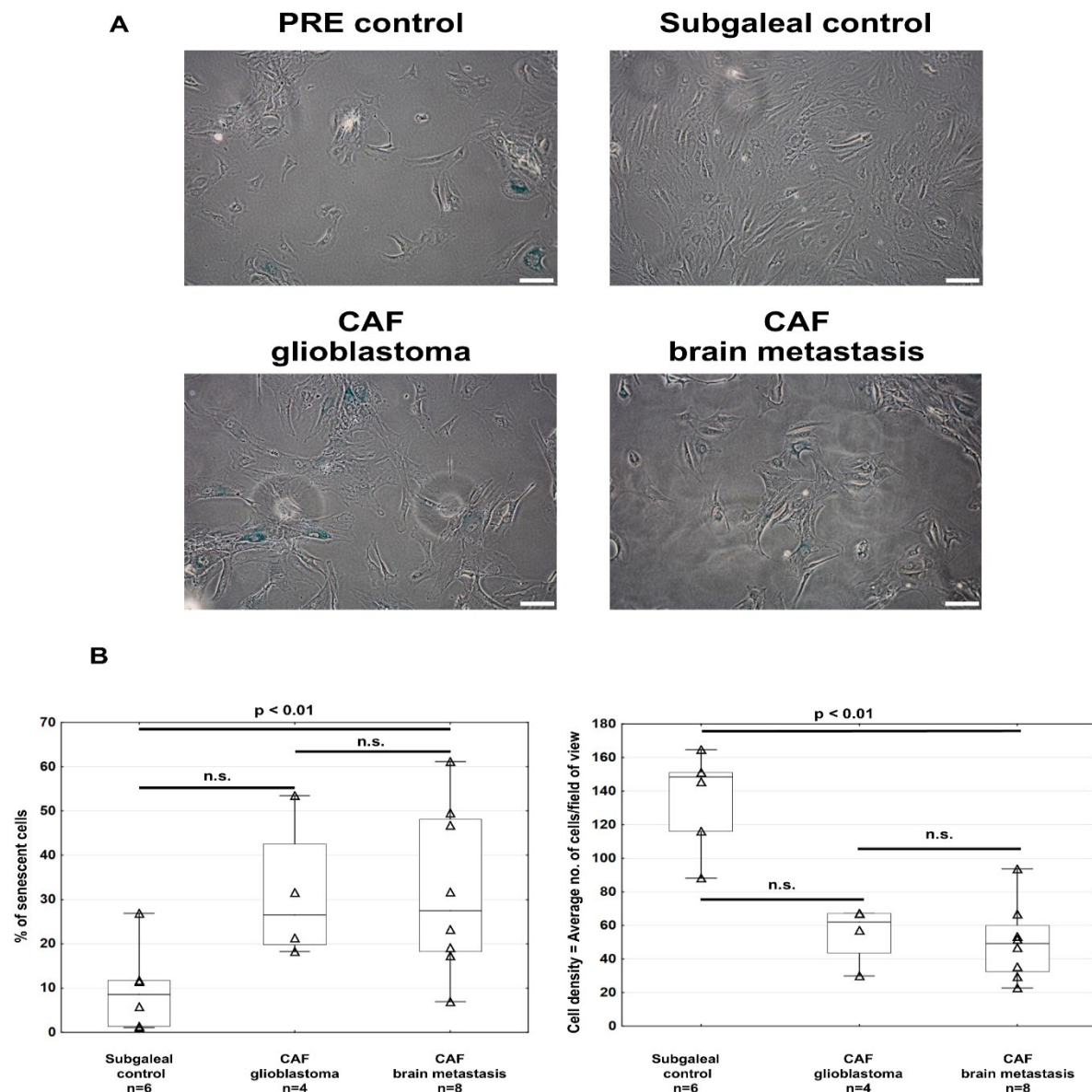


Figure 19. Quantification of senescent cells in CAFs from glioblastomas, brain metastases and subgaleal controls. (A) Representative images from wells at the end of the experiment. Blue cells are positive for beta-galactosidase activity. Scale bars = 100 μ m (B) Comparison of average percentage of senescent cells in all wells per culture (left panel), Comparison of cell density (right panel), horizontal line - median, boxes - interquartile range 25–75%, whiskers - range of non-remote values, triangles—individual patient derived cell cultures; Kruskal–Wallis one way ANOVA on ranks. n.s. – non significant, $p > 0.05$

To investigate, whether the isolated CAFs were indeed nonmalignant cells, we tested their tumorigenicity. Glioblastoma CAFs (n=3) and CAFs derived from brain metastases of lung cancer (n=2) were orthotopically implanted into the brains of immunodeficient NODRAG mice. No visible signs of macroscopic tumours were observed even after 107 days post-implantation. The brains were harvested and analysed immunohistochemically. The presence of human cells was evaluated using an antibody staining human nuclei (HuNu), human mesenchymal cells were detected by staining for the marker TE-7. We detected faint traces of human cells, some of which were positive for TE7. Xenotransplants of glioblastoma CAFs displayed a more pronounced positivity of TE-7, where TE-7 positive cells exhibited fibrillary or vessel-like morphology (Fig. 20). These findings corroborate the conclusion that the cell cultures isolated from brain metastases and glioblastomas are nonmalignant.

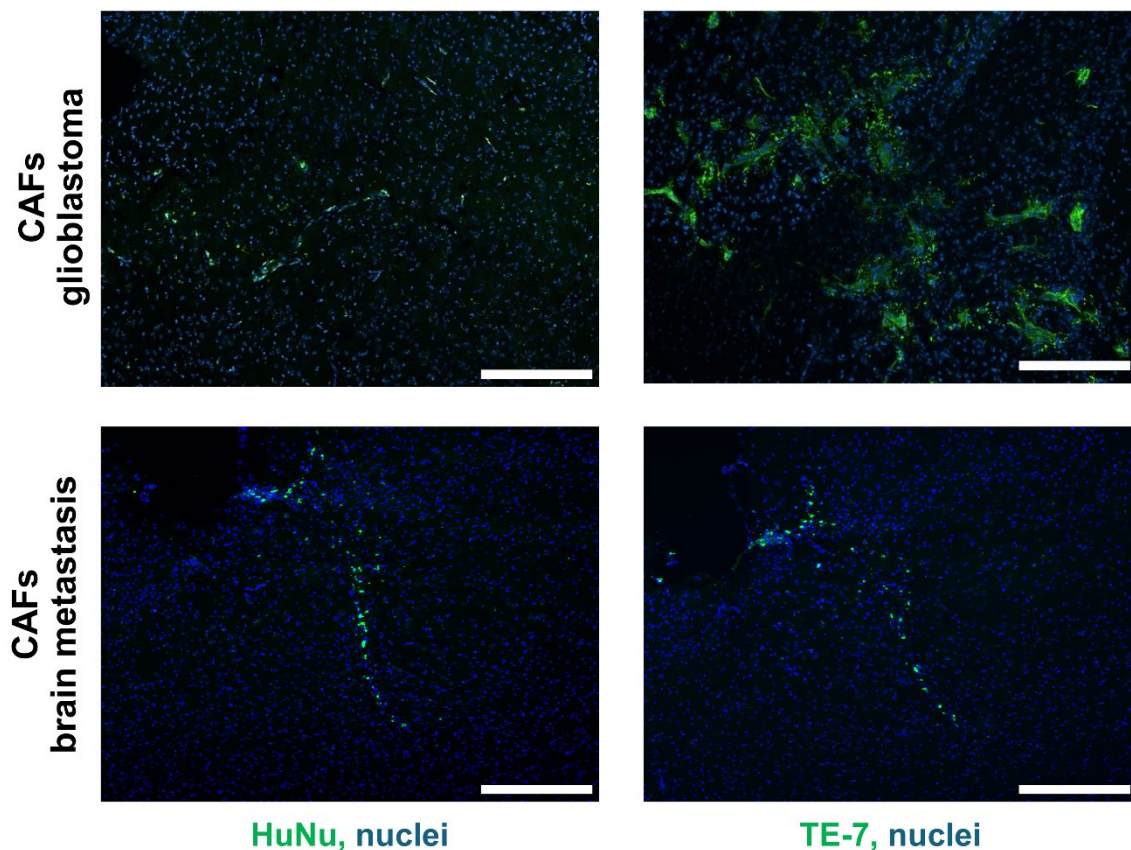


Figure 20. Representative images of intracerebral xenotransplants of CAFs from glioblastomas and brain metastases of lung cancer. Implanted murine brains without sign of bulky tumour - CAFs from glioblastoma (upper panel), CAFs from brain metastasis of lung cancer (lower panel). Cell markers (green) – HuNu (human nuclei) and TE-7. Nuclei (blue) were counterstained with the Hoechst 33258 dye. Scale bars = 200 μ m. Respective cultures: CAF glioblastoma = NCH353, CAF brain metastasis (lung) = NCH402

Based on our previous findings indicating that brain metastatic CAFs may exhibit some level of senescence, we aimed to explore whether these cells could display characteristics of a pro-tumorigenic secretory profile. Therefore, we sought to determine the impact of soluble factors secreted by CAFs from brain metastases on the migration

of cancer cells. Conditioned media from CAF cultures derived from lung cancer brain metastases (n=2) were used in a transwell migration assay with the lung adenocarcinoma cell line A549. Conditioned media from subgaleal controls (n=3), PRE controls, and conditioned media from cancer cells themselves were used as biological controls and non-conditioned medium served as a negative control. We observed a pro-migratory effect of the conditioned media from CAFs in comparison to the non-conditioned medium, subgaleal controls and cancer cells. PRE control conditioned media displayed a similar chemoattractive potential comparable to that of brain metastatic CAFs (Fig. 21).

These preliminary results suggest that brain metastatic CAFs secrete soluble factors that are chemoattractive to cancer cells. However, this conclusion is based on a small experiment with a limited number of cultures. Further validation with more cultures/ biological replicates is necessary.

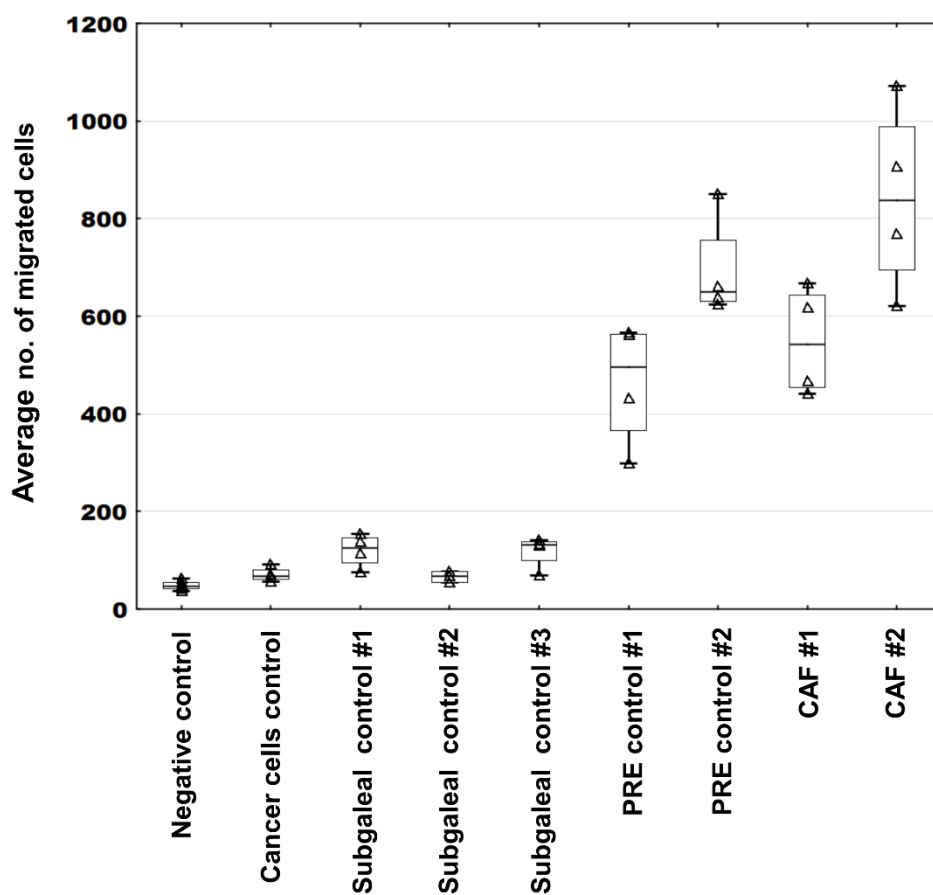


Figure 21. Effect of conditioned media from CAF cultures on cancer cell migration. A transwell migration assay was performed using lung adenocarcinoma cancer cells (A549) and conditioned media from cancer cells (A549), subgaleal controls, PRE controls and CAFs from lung cancer brain metastases. There is a pro-migratory effect of conditioned media from CAFs in comparison to all controls, except for conditioned media from PRE. Horizontal line - median, boxes - interquartile range 25–75%, whiskers - range of non-remote values, triangles – average no. of migrated cells per insert (performed in quadruplicates per culture). Subgaleal control #1,2,3 correspond to cultures: H131GB, NCH233GB, NCH379GB; PRE control #1,2 correspond to NCH236GF, NCH421GF; CAF #1,2 correspond to cultures NCH402GM, NCH131GM.

We further investigated whether the conditioned media that demonstrated a pro-migratory influence on the A549 lung adenocarcinoma cell line might also exert a pro-proliferative effect in comparison to conditioned media of control cells. Therefore, we conducted a proliferation assay using CellTiter-Glo®, where the A549 cells were incubated with the conditioned media from subgaleal controls (n=3), PRE controls (n=2) and CAFs derived from lung cancer brain metastases (n=2). The cells were seeded and left to attach for 24 hours. Afterwards the cultivation medium was switched for the respective conditioned media. The cells were cultivated in conditioned media for the next 72 hours. The incubations with conditioned medium were performed under serum-free conditions or with a final concentration of 2% FCS. There was no difference between conditioned media from CAFs and PFs from pharmacoresistant epilepsy. We observed a trend towards a pro-proliferative effect in the presence of 2% FCS with conditioned media from CAFs when compared to subgaleal PFs (Fig. 22).

This experiment did not show any significant pro-proliferative effect of conditioned media in lung adenocarcinoma cells. However, given the trend observed in 2% FCS, it's difficult to determine whether using more cultures might reveal a pro-proliferative effect of conditioned media from brain metastatic CAFs.

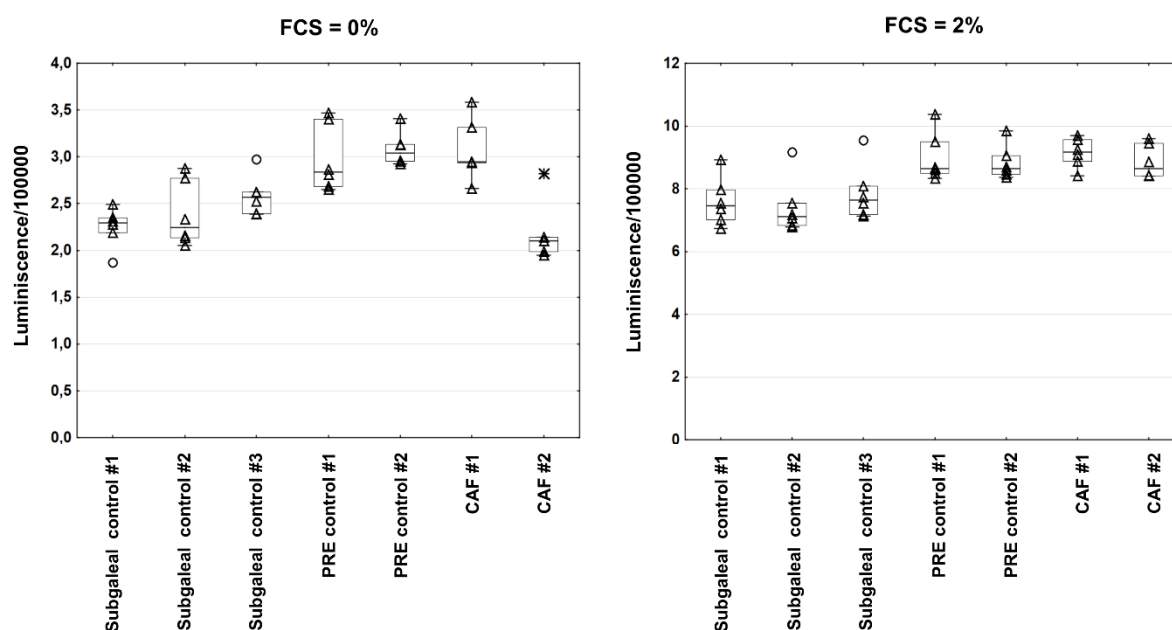


Figure 22. Growth of cancer cells in conditioned media prepared from CAFs and control fibroblasts. A CellTiter-Glo luminescent cell viability assay was performed after cultivation of A549 lung adenocarcinoma cells in conditioned media from subgaleal controls, PRE controls and lung cancer brain metastatic CAFs. Data are luminescence at the end of the experiment after cancer cell cultivation in conditioned media in serum free conditions (left panel) and in 2% FCS (right panel). Horizontal line - median, boxes - interquartile range 25–75%, whiskers - range of non-remote values, circles - remote values, asterisks - extremes, triangles – source data (hexaplicates for each culture). Subgaleal control #1,2,3 correspond to cultures: H131GB, NCH233GB, NCH379GB; PRE control #1,2 correspond to NCH236GF, NCH421GF; CAF #1,2 correspond to cultures NCH131GM, NCH402GM

4.4 Targeting FAP expressing cells in the tumour microenvironment

To explore FAP as a potential target for anticancer therapy, we investigated the efficacy of an iBody consisting of a HPMa copolymer conjugates linked to the cytostatic agent mertansine (DM1), with and without a FAP-specific inhibitor. The conjugates were synthesized at the Institute of Organic Chemistry and Biochemistry and Institute of Macromolecular Chemistry, Czech Academy of Sciences. The conjugates were labeled U156 (iBody with FAP-inhibitor) and U157 (iBody without FAP-inhibitor).

Initial *in vitro* analyses were conducted using the FAP-expressing glioma cell line U251, where FAP expression was induced using a doxycycline (dox)-inducible Tet-On system. *In vitro* analyses demonstrated the selective toxicity of the FAP targeting iBody in FAP-expressing glioma cells, performing comparably to free DM1. The FAP non-targeting iBody was less toxic in comparison to both FAP targeting iBody and free DM1 in FAP-expressing cells. Both the FAP-targeting and non-targeting iBodies exhibited lower toxicity in FAP-negative cells in comparison to free DM1 (Fig. 23). These experiments provided the rationale for conducting *in vivo* testing of the conjugates.

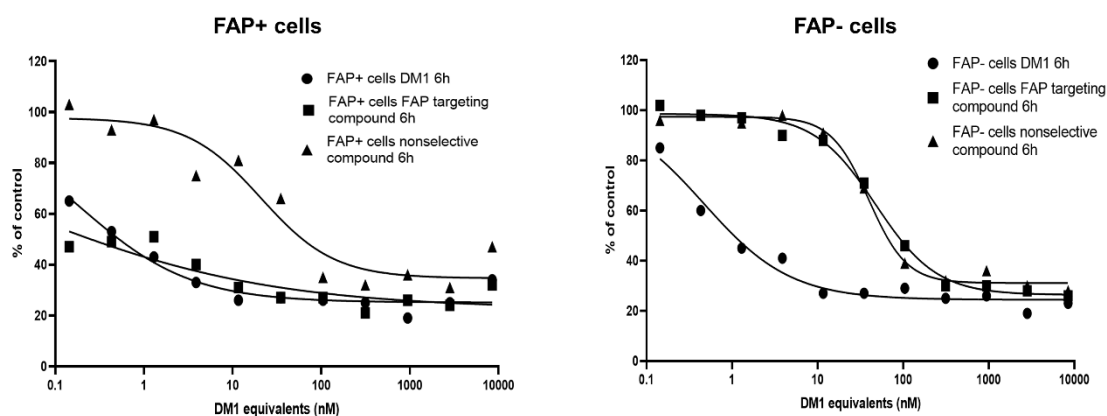


Figure 23. Cytotoxicity of FAP targeting iBodies *in vitro*. Percentage of surviving cells grown for 72 hours after a 6 hour treatment with a FAP-targeting iBody, a non-targeting iBody and free DM1 in FAP-expressing (left panel) and non-expressing cells (right panel). The experiments were performed by Rosana Mateu Sanz.

In the initial *in vivo* experiments, we determined the maximal tolerated dose of the FAP-targeting iBody (U156) in mice. Free DM1 was used as a comparator in the first rounds of assessment. The toxicity was primarily assessed by a decrease in body weight following the administration of the compounds (Fig. 24B). We noted a more significant decrease in body weight in mice injected with free DM1 at doses of 2000 and 2800 micrograms after one application, compared to mice receiving an equivalent dose of U156 (data not shown). Consequently, only the U156 group underwent repeated applications. The maximum tolerated dose of U156 was established at the equivalent of 2800 micrograms of DM1 applied per m² of body surface area for repeated intravenous applications. U156 was administered on a weekly basis up to 3 administrations in total

with an acceptable tolerance. In contrast, a dose equivalent to 3600 micrograms of DM1 per m² of body surface area proved lethal after the first application (Fig. 24A).

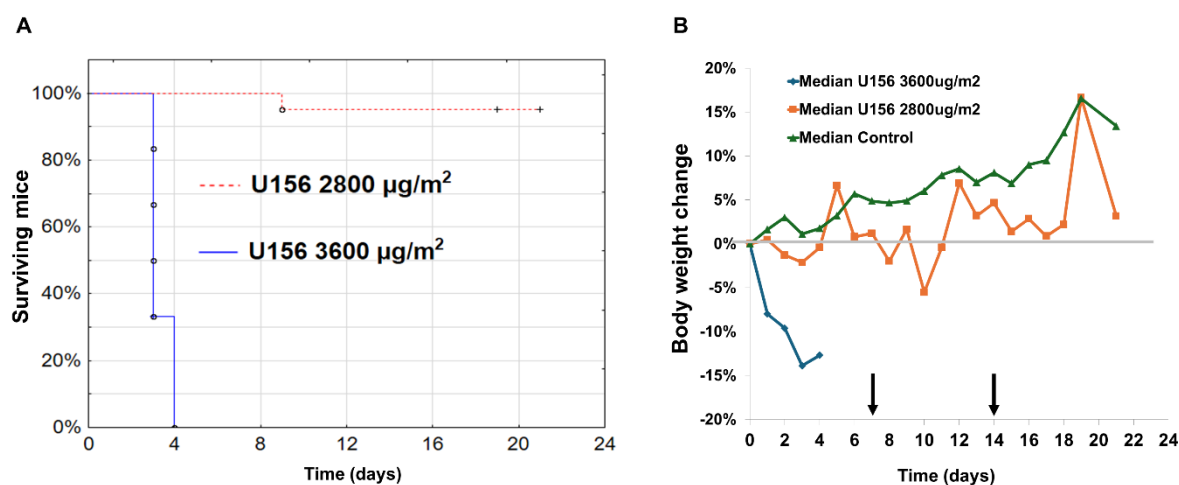


Figure 24. Toxicity of FAP targeting iBody *in vivo*. (A) Survival curves of mice after one intravenous administration of the FAP targeting iBody (U156) corresponding to a DM1 dose equivalent to 3600 µg/m² (n=6) and 2800 µg/m² (n=21), circles – death events, plus - censored data. (B) Gain/loss of body weight in mice after intravenous administration of the compound U156 at a dose equivalent to 3600 µg/m² (n=6), 2800 µg/m² (n=17) of DM1, or saline (control group, n=18). Mice receiving 3600 µg/m² died at day 3 and 4. Mice receiving 2800 µg/m² were given a second and third dose of U156 at days 7 and 14 – days of administration marked with arrows.

For the evaluation of the antitumor efficacy of U156 *in vivo* a dose equivalent to 2800 micrograms of DM1 per m² of body surface area was selected. U251 glioma cells with constitutive FAP expression were injected subcutaneously into flanks of immunodeficient NODRAG mice to establish FAP expressing tumours. The treatment group received three doses of U156 in weekly intervals, while the control group was administered only PBS. The tumour volume was measured using callipers. During the experiment, the tumour growth was significantly greater in the control group. One exceptionally large tumour from the control group was excluded from the volume analysis due to cystic formations that ruptured after harvesting (calculated volume approximately 3060 mm³, over 10x larger than the median of the control group). The median size of the control group tumours was approximately 6.6 times larger in comparison to the treatment group (Fig. 25A). At the end of the experiment, the weight of the control tumours was significantly bigger in comparison with the group treated with U156. The median tumour weight in the control group was approximately 7 times larger than that in the treatment group (Fig. 25B). Thus, in this experiment three doses of U156 exhibited significant anti-tumour activity in FAP-expressing tumours.

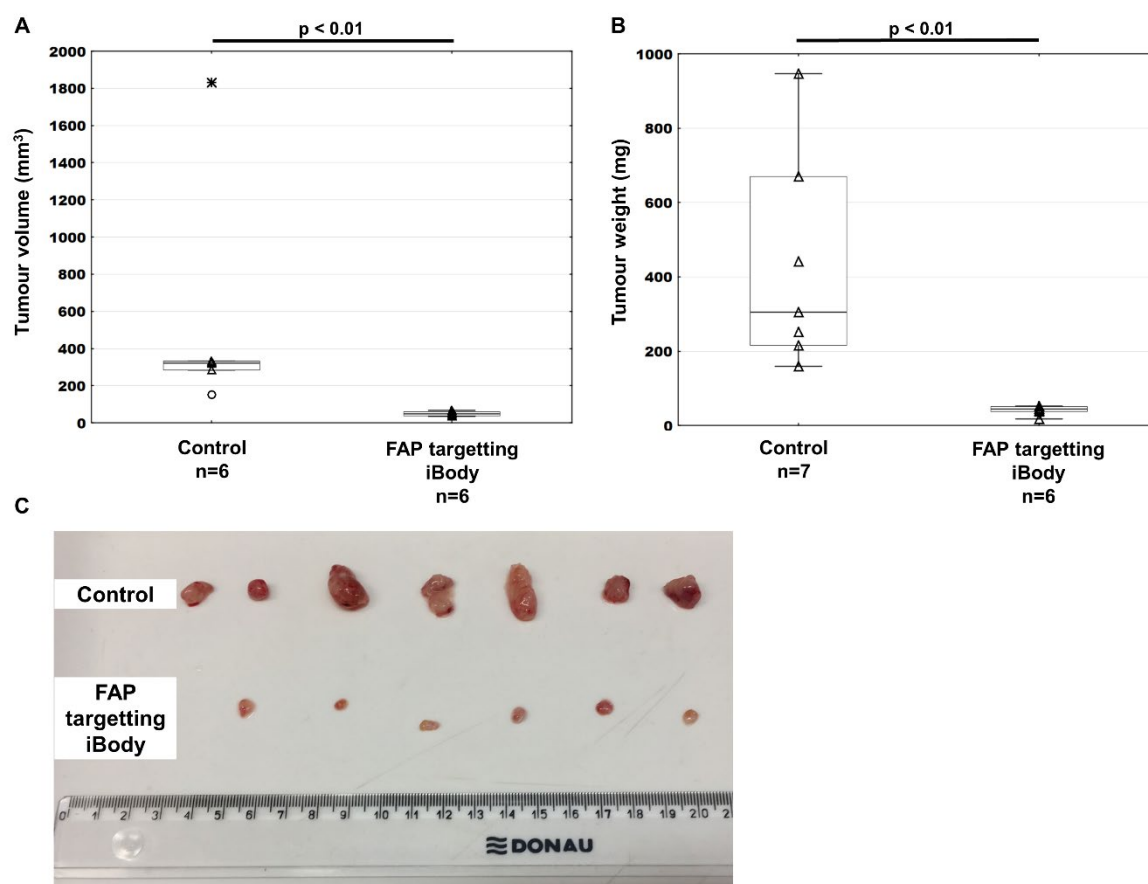


Figure 25. Anti-tumour activity of FAP targeting iBody (U156) in FAP expressing subcutaneous tumours from U251 cells. Comparison of estimated tumour volume before harvesting (A) and tumour weight after harvesting of tumours (B) showing significantly smaller tumours in the group treated with the FAP targeting iBody. (C) Photography of the harvested tumours. Tumours were harvested at day 21 (7 days after the 3rd weekly administration of FAP targeting iBody). Mann-Whitney U test, horizontal line—median, boxes—25–75 %, whiskers—range of non-remote values, white triangles—source data, white circles—remote values, asterisks—extremes

Subsequently, to determine whether the antitumour effect was due to the targeting of FAP, we conducted another experiment with a similar setup, including an additional control group that received the iBody without the targeting FAP inhibitor (U157). Thus, the setup consisted of three groups which received three treatment administrations of either the FAP targeting iBody (U156), the iBody without the FAP inhibitor (U157), or PBS as a control. During the tumour measurement, the tumours in the PBS control group were relatively larger than those observed in the previous experiment. However, we noted an antitumour effect of U157, which was further confirmed by measuring the weight of the tumours after harvesting. There was no significant difference in tumour growth inhibition between the FAP targeting and FAP non-targeting iBody groups (Fig. 26 A-B). The expression of FAP in the subcutaneous tumours was verified by immunohistochemical staining for FAP (Fig 26 D). This experiment suggests that the antitumor effect was primarily due to the nonspecific accumulation of the DM1-containing polymers, rather than the selective targeting and delivery of the cytostatic agent to FAP-expressing cells.

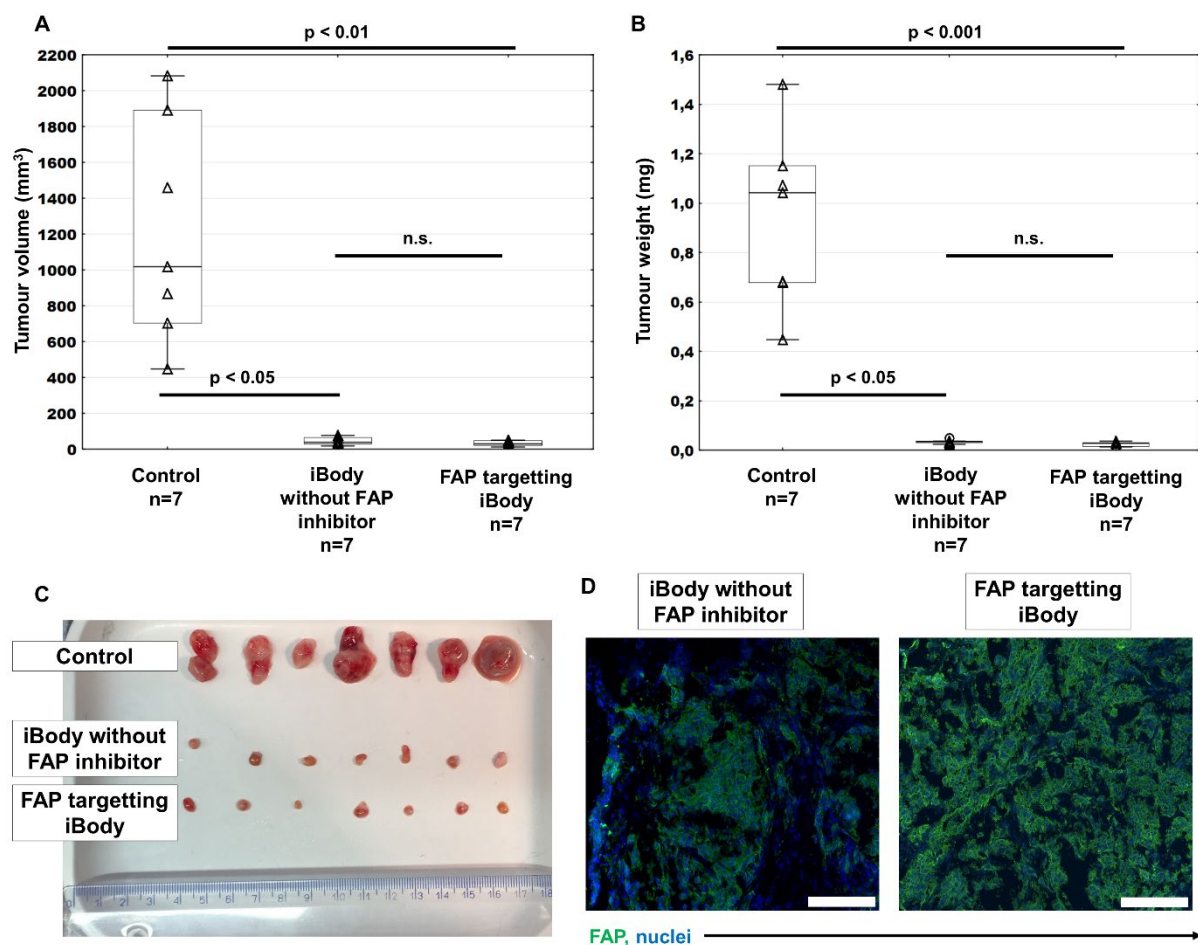


Figure 26. Anti-tumour activity of FAP targeting iBody (U156) and iBody without FAP inhibitor in FAP expressing subcutaneous tumours from U251 cells. Comparison of estimated tumour volume before harvesting (A) and tumour weight after harvesting of tumours (B) showing significantly smaller tumours in mice treated with the FAP targeting iBody and iBody without FAP inhibitor in comparison to controls. No significant difference in tumour growth observed between mice treated with either iBody (C) Harvested tumours. Tumours were harvested at day 21 (7 days after the 3rd weekly administration of iBody). Kruskal–Wallis one way ANOVA on ranks, n.s. = not significant, $p > 0.05$; horizontal line—median, boxes—25–75 %,whiskers—range of non-remote values, white triangles—source data, white circles—remote values, asterisks—extremes. (D) Immunohistochemical confirmation of FAP expression in treated tumours.

5. My contribution to the presented data

1. Preparation of frozen tissue sections and tissue lysates, biobanking/human tissue logistics.
2. Immunohistochemistry in frozen sections – sectioning, staining, photographing, evaluation.
3. Analysis of immunohistochemical data obtained from FAP-stained FFPE sections.
4. Derivation of primary cell cultures.
5. *In vitro* assays
 - a. Immunocytochemistry
 - b. Beta-galactosidase assay
 - c. Transwell migration assay
 - d. Proliferation assay
6. Preparation of subcutaneous and orthotopic xenotransplanted murine tumourigenicity models – cell culture, implantation, harvesting, sectioning, staining, photographing, data evaluation.
7. *In vivo* cytotoxicity assays using FAP-targeting compounds– dose titration, implantation of tumour cells, monitoring of weight, measurement of tumour size, harvesting of animals, weighing of tumours, staining of tumours.
8. Statistical analysis.
9. Attachment 1 (Zubal' et al. 2023) – Immunohistochemical staining, data analysis, figure preparation, manuscript assembly, proofreading.
10. Attachment 4 (Busek et al. 2018) – Writing of the subchapter 3.3. FAP in the tumor microenvironment.

6. Discussion

In this work, we have extended previous findings that FAP is overexpressed in primary glioblastomas compared to non-tumorous brain tissue (Busek et al. 2016; Ebert et al. 2020; Krepela et al. 2021). Recurrent glioblastomas often exhibit a shift towards the mesenchymal phenotype (Kim et al. 2021), which is associated with a worse prognosis and increased therapy resistance (Matsumoto et al. 2022). Prior results from our research group linked FAP expression with the mesenchymal subtype of glioblastoma (Busek et al. 2016). Based on this, it would be expected that FAP expression may be upregulated in recurrent glioblastoma, potentially contributing to the aggressive phenotype (Phillips et al. 2006; Wang et al. 2017c). However, in our patient cohort, we did not observe a difference in FAP expression between paired samples of primary and recurrent glioblastomas. This may be attributed to the relatively small size of the patient cohort, which consisted of 22 patients. Nevertheless, our observation is in concordance with data from other research groups. Although Ebert and colleagues have observed higher FAP levels in both primary and recurrent glioblastomas compared to low-grade gliomas, there was no significant overexpression of FAP in recurrent glioblastomas in comparison to primary glioblastomas (Ebert et al. 2020). Therefore, we conclude that, while FAP is upregulated in recurrent glioblastomas compared to non-tumorous brain tissue, there is no further increase or shift in FAP expression when the disease recurs.

Our results have shown that FAP is upregulated in brain metastases of solid tumours to an even greater extent than in glioblastomas. Interestingly, FAP expression does not vary significantly between brain metastases of different histological origin. This suggests a universal presence of FAP-expressing cells in the brain metastatic microenvironment, regardless of the primary tumour type (Zubal' et al. 2023).

Regulation of FAP expression in glioblastomas appears to be linked to TGFbeta signalling. TGFbeta plays a crucial role in glioblastoma progression, promoting cell migration, invasion, and immunosuppression (Wick et al. 2006; Birch et al. 2020). Of the TGFbeta isoforms, TGFbeta 1 is the most abundant in glioblastomas and correlates with poor patient survival (Frei et al. 2015; Roy et al. 2018). Our data confirm that TGFbeta 1 is the most abundantly expressed isoform in primary glioblastomas and positively correlates with FAP expression. The functional studies presented in Attachment 2 also indicate that FAP expression in glioblastomas is regulated by the canonical TGFbeta type I receptor/Smad pathway. TGFbeta 1 stimulates FAP expression in glioma cells, pericytes, glioblastoma-derived endothelial cells and mesenchymal cells, but not in glioma stem-like cells. Selective synthetic small-molecule inhibitors of the TGFbeta type I receptor, A8301 and A7701, led to a significant decrease in both FAP protein levels and activity in treated glioma cells. This finding, along with immunohistochemical data showing TGFbeta expression in stromal regions and parenchyma in glioblastomas, supports the hypothesis that autocrine/paracrine TGFbeta 1 signalling is involved in influencing the tumour microenvironment of glioblastoma (Jennings et al. 1991; Krepela et al. 2021).

In this thesis, we also present data indicating that TGFbeta 1 is upregulated in brain metastases. We found a positive correlation between TGFbeta 1 protein concentration and FAP protein concentration in our samples, suggesting a potential link between TGFbeta 1 signalling and FAP expression in brain metastases. Other studies have also suggested a connection between TGFbeta signalling and brain metastasis. Zhang et al. have shown that autocrine TGFbeta 2 expression is necessary for melanoma metastasis to the brain parenchyma (Zhang et al. 2009). In breast cancer brain metastasis, astrocyte-derived TGFbeta 2 upregulates angiopoietin-like 4 (ANGPTL4) expression in tumour cells, facilitating metastatic growth in the brain parenchyma. TGFbeta induces epithelial-mesenchymal transition, facilitating metastatic spread particularly in lung adenocarcinoma and brain metastases (Tsubakihara and Moustakas 2018; Piña Batista et al. 2019). However, further studies are needed to confirm the potential regulatory role of TGFbeta in FAP expression and to determine its impact on the tumour microenvironment of brain metastases.

Previous and current data of our research group reveal that FAP expression in glioblastomas is present in both transformed and stromal cells (Busek et al. 2016). In Attachment 3 (Balaziová et al. 2021) we show that FAP-positive mesenchymal cells are associated with increased tumour vascularization in glioblastoma. FAP-expressing mesenchymal cells derived from glioblastomas exhibit upregulation of angiogenesis-promoting factors such as angiopoietin-2, endothelin-1, IGFBP-2, and VEGF. This is supported by the functional experiments demonstrating that FAP-expressing mesenchymal cells derived from glioblastomas secrete chemoattractive factors, inducing endothelial cell migration and promoting vascular sprouting *in vitro*. Also, the addition of FAP-positive mesenchymal cells from glioblastomas to glioma cells led to a significant increase in haemorrhages observed in a chorioallantoic membrane glioblastoma model. In summary, this work shows that FAP-expressing mesenchymal cells in glioblastoma may contribute to glioblastoma progression by enhancing neovascularisation and stimulating glioma cell growth and migration via paracrine signalling (Balaziová et al. 2021). In a recent study, my colleagues also identified FAP-expressing mesenchymal cells as a crucial cell population responsible for the production of ECM proteins like collagen I and fibronectin in the microenvironment of glioblastoma. The ECM produced by the FAP-expressing mesenchymal cells enhances glioma cell migration, adhesion, and FAK signalling, possibly contributing to glioma progression (Vymola et al. 2024). Given the expression of FAP and the mesenchymal characteristics of these cells, they could potentially be identified as cancer-associated pericytes or cancer-associated fibroblasts (CAFs). Recent studies have identified cancer-associated fibroblasts (CAFs) in glioblastoma, challenging the previous assumption that these cells are absent in brain tumours (Jain et al. 2021). CAFs have been shown to exhibit pro-tumoral effects by enhancing glioblastoma stem cell (GSC) enrichment, promoting tumour growth, and influencing the tumour microenvironment also possibly through TGFbeta (Jain et al. 2023). Single-cell RNA sequencing and spatial transcriptomics have revealed CAF subtypes and their proximity to mesenchymal GSCs,

endothelial cells, and M2 macrophages (Jain et al. 2023). CAFs secrete factors like fibronectin, osteopontin, and HGF, which facilitate glioblastoma cell migration and invasion (Trylcova et al. 2015; Galbo et al. 2024). While they represent a rather minor subpopulation, CAFs may significantly impact glioblastoma progression, making them an intriguing target for future therapeutic interventions.

In brain metastases, we have shown that FAP is expressed in areas containing blood vessels and regions rich in collagen I. FAP-positive stromal cells in the tumour microenvironment expressed TE7, α SMA, and PDGFR β , markers of CAFs. Literature on CAFs in brain metastases is accumulating in recent years. The presence of CAFs expressing markers like PDGFR- β and α -SMA in brain metastases originating from various primary cancers was also shown by another group (Akanda et al. 2023). However, evidence on the influence of CAFs in the tumorigenesis of brain metastases is contradictory. Duda et al. demonstrated that human brain metastases originating from lung cancer contain fibroblast-like cells. Furthermore, they presented evidence that CAFs from the primary tumour site metastasise together with cancer cells, aiding in the establishment of brain metastases (Duda et al. 2010). Another group has shown, that CAFs from breast cancer brain metastases chemoattract cancer cells *in vitro*, likely by the chemokines CXCL16 and CXCL12 (Chung et al. 2017). A recent work by Song et al. using scRNA-seq identified CAFs in tissues of lung and breast cancer brain metastases. These CAFs showed high expression of type I collagen. The authors hypothesise that CAFs in brain metastases play a dominant role in cell-cell interactions within the tumour microenvironment. This is mediated through producing type I collagen, contributing to the remodelling of the tumour microenvironment into a collagen-I-rich matrix similar to the primary tumour site. Additionally, this group observed M1 activation in native microglial cells and infiltrating macrophages, which may support a proinflammatory tumour microenvironment (Song et al. 2023). On the other hand, Tew et al. derived CNS metastasis-associated stromal cells (cMASCs), with an expression profile similar to CAFs. Functionally, these CAFs restricted tumour growth and induced desmoplasia *in vivo*, indicating that CAFs may contribute to a protective host response aimed at limiting tumour progression in the brain metastatic microenvironment (Tew et al. 2019). These conflicting results may reflect the heterogeneity of CAFs within brain metastases. Our detection of α SMA and PDGFR β positivity in only a subset of FAP-expressing cells supports the notion of diverse CAF subpopulations within the tumour microenvironment of brain metastases.

We have successfully derived CAFs from brain metastases (and glioblastomas) that exhibited a higher degree of senescence compared to control cranial fibroblasts. Senescent stromal cells in the glioblastoma tumour microenvironment, particularly senescent astrocytes, have been shown to play a crucial role in tumorigenesis. Studies in glioblastoma have demonstrated, that these cells develop a senescence-associated secretory phenotype (SASP), secreting factors like HGF that activate receptor tyrosine kinases in cancer cells, enhancing cancer cell invasion and tumour growth (Fletcher-Sananikone et al. 2021). Senescent fibroblasts in solid tumours can induce epithelial-

mesenchymal transition in nearby epithelial cells, facilitating tumour progression (Coppé et al. 2008; Laberge et al. 2012). Our preliminary experiments suggested that CAFs from lung cancer brain metastases may secrete chemoattractive factors that promote lung cancer cell migration *in vitro*. However, these findings are based on a limited number of experiments and cell cultures, and further research is required to confirm whether the brain metastatic CAFs exhibit pro-tumorigenic properties. Additionally, it remains to be determined if the potential pro-tumorigenic effects of CAFs in brain metastases could be attributed to the senescence-associated secretory phenotype.

We observed FAP immunopositivity in cancer cells in a subset of brain metastases from various primary tumours, including melanoma, lung, breast, renal, and gastrointestinal cancers, as well as sarcomas and carcinomas of unknown primary origin. Our immunohistochemistry analysis showed FAP+ cancer cells in approximately 38% (42/112 tissues) of brain metastases samples. However, FAP immunopositivity was rare in cells expressing pan-cytokeratin or EpCAM (Zubal' et al. 2023). The absence of epithelial markers in brain metastatic carcinoma cells could be possibly due to epithelial-mesenchymal transition, in which cancer cells have been observed to lose their original epithelial characteristics in favour of a fibroblast-like phenotype, potentially enhancing their invasive and metastatic capabilities (Natarajan et al. 2014; Zeisberg and Neilson 2009; Ribatti et al. 2020). Previous studies have linked FAP expression in cancer cells to increased proliferation, invasiveness, and tumorigenic potential. However, some research suggests FAP expression in cancer cells may also promote apoptosis and cell cycle arrest under certain conditions (Wesley et al. 1999; Wesley et al. 2004). Therefore, further studies are needed to elucidate whether FAP acts as a promoter or suppressor of tumorigenicity in brain metastases.

FAP has been shown to be expressed in most solid tumours (Busek et al. 2018). Our data further demonstrate that FAP is also expressed in malignant brain tumours - glioblastomas and brain metastases, in both stromal and cancer cells (Busek et al. 2016; Balaziová et al. 2021; Zubal' et al. 2023). Given the selective expression of FAP in the tumour microenvironment, FAP represents a highly promising target for theranostic (therapeutic and diagnostic) applications in oncology. Various FAP-targeted approaches have been developed, including activatable immunoliposomes for fluorescence imaging (Ruger et al. 2014), radiolabelled FAP inhibitors for PET imaging (Peltier et al. 2022) and inhibitor-decorated polymer conjugates – “iBodies” (Dvorakova et al. 2017). In this work, we evaluated the anti-tumour efficacy of a FAP-targeting iBody conjugate with the cytostatic agent mertansine (DM1). Our initial *in vitro* experiments showed promising results, with the FAP-targeting iBody exhibiting significant selective toxicity towards FAP-expressing cells compared to FAP non-expressing cells. The iBody conjugate exhibited lower toxicity both *in vitro* and *in vivo* compared to free DM-1. *In vivo*, both iBody conjugates (with and without the FAP inhibitor) have shown significant inhibition of tumour growth in FAP-expressing tumours. Therefore, the FAP-targeting iBody did not demonstrate selective toxicity in FAP-expressing tumours *in vivo*. We

hypothesize that the properties of the iBody backbone had a greater influence *in vivo*, outweighing the effect of the FAP-targeting moiety. The iBody is based on an HPMA copolymer as its core structure (Dvorakova et al. 2017). HPMA copolymers have shown promise as drug carriers for cancer treatment due to their ability to accumulate in tumours through the enhanced permeability and retention (EPR) effect (Ulbrich et al. 2016; Dozono et al. 2016; Nakamura et al. 2016). The EPR effect is a phenomenon in solid tumours that allows macromolecules and nanoparticles beyond certain sizes (typically above 40 kDa) to concentrate in tumour tissues due to leaky vasculature and impaired lymphatic drainage (Wu 2021). A better understanding and optimization of the EPR effect could be crucial for improving targeting conjugates and/or nanoparticle-based cancer treatments (Shinde et al. 2022).

Although our *in vivo* experiments did not show additional benefits of the FAP-targeted polymers over the untargeted ones, several ongoing studies using low molecular FAP inhibitor conjugates are demonstrating promising outcomes in cancer. Fibroblast Activation Protein Inhibitor (FAPI) theranostics is an emerging field in cancer research by targeting FAP-expressing cells in the tumour microenvironment (Zhao et al. 2022; Sidrak et al. 2023). FAPI-PET imaging has been shown to complement or even outperform the standardly used ^{18}F -fluorodeoxyglucose (FDG)-PET in certain diagnoses (Sharma et al. 2021; Fu et al. 2023). The most widely used compounds, FAPI-04 and FAPI-46, contain dodecane tetraacetic acid (DOTA)-chelators enabling the use of various radionuclides (^{68}Ga , ^{90}Y , and ^{177}Lu) allowing for both diagnostic and therapeutic applications (Mori et al. 2023). Studies have shown the potential of FAPI PET/CT in detecting primary tumours, metastases, and lymph node involvement, particularly in head and neck, lung, breast, prostate and gynaecological malignancies (Hathi and Jones 2019; Syed et al. 2020; Li et al. 2024). FAPI PET/CT offers high tumour-to-background contrast, facilitating improved tumour delineation and staging, even in tumours in FDG avid organs such as glioblastomas and brain metastases (Fu et al. 2021; Caresia et al. 2024; Yao et al. 2022). Also, FAPI PET/CT has demonstrated improved tumour uptake and retention in a murine model of glioblastoma (Lai et al. 2023). However, in a pilot study in humans, FAPI PET/CT has been shown to detect fewer lesions in comparison with MRI (Yao et al. 2022). Therapeutic use of ^{177}Lu radiolabelled FAPI has shown promising outcomes in patients with advanced-stage breast cancer (Yadav et al. 2024). Notably, in a case involving a breast cancer patient with brain metastases treated with ^{177}Lu -FAPI, the therapy was well tolerated, and the patient experienced clinical improvement with a reduction in headache severity (Ballal et al. 2021). Another case study involved a patient with high-grade medullary thyroid cancer, which progressed after conventional therapies. A theranostic approach using ^{68}Ga -DOTA-FAPI-guided ^{177}Lu -DOTA-FAPI radionuclide therapy was administered, which led to a sustained reduction in the neck tumour mass and significant improvement in quality of life (Ballal et al. 2022). While FAPI theranostics shows promise, this approach is still in early clinical stages, and further research is needed to establish its full potential in cancer diagnosis and treatment (Sidrak et al. 2023).

In summary, this dissertation presents evidence that FAP is significantly overexpressed in glioblastomas and brain metastases compared to non-tumorous brain tissue. FAP is predominantly expressed in mesenchymal stromal cells resembling CAFs, and also to a lesser extent in cancer cells within the brain tumour microenvironment. The selective presence of FAP in both stromal and cancer cells lays basis and strengthens the rationale for targeting FAP in the treatment of glioblastomas and brain metastases especially.

7. Conclusions

AIM I: We have demonstrated, that FAP is upregulated in glioblastomas and brain metastases compared to non-tumorous brain tissue. In brain metastases, FAP is also significantly overexpressed in comparison to glioblastomas (Zubal' et al. 2023). FAP expression does not differ between primary and recurrent glioblastomas. In both glioblastomas (Krepela et al. 2021) and brain metastases, TGFbeta 1 is overexpressed and shows a positive correlation with FAP expression.

AIM II: In brain metastases, FAP is predominantly expressed in mesenchymal stromal cells resembling CAFs. To a lesser extent, FAP expression is also present in cancer cells. CAFs in brain metastases are linked to ECM deposition. *In vitro*, CAFs have a higher proportion of senescent cells in comparison to control cranial fibroblasts. Preliminary *in vitro* experiments suggest that these CAFs may have a chemoattractive effect on cancer cells.

AIM III: FAP targeting represents an attractive approach in cancer treatment (Busek et al. 2018). The FAP-targeting iBody conjugate U156 demonstrates promising selective toxicity towards FAP-expressing cells *in vitro* and exhibits acceptable tolerability *in vivo*. This FAP-targeting iBody conjugate inhibits the growth of FAP-expressing tumours *in vivo*. However, this tumour inhibition is not mediated through selective targeting of FAP possibly due to the properties of the iBody polymer backbone outweighing the selective FAP targeting moiety.

8. References

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9. Attachments

Attachment 1: Zubal M, Výmolová B, Matrasová I, Výmola P, Vepřková J, Syrůček M, Tomáš R, Vaníčková Z, Křepela E, Konečná D, Bušek P, Šedo A. Fibroblast activation protein as a potential theranostic target in brain metastases of diverse solid tumours. *Pathology*. 2023 Oct;55(6):806-817. doi: 10.1016/j.pathol.2023.05.003. Epub 2023 Jun 16. PMID: 37419841. **IF₂₀₂₃: 3,6**; Journal Citation Reports category and quartile (2023): **PATHOLOGY, Q1**; Times cited according to Web of Science: **7**

Attachment 2: Krepela E, Vanickova Z, Hrabal P, **Zubal M**, Chmielova B, Balaziova E, Vymola P, Matrasova I, Busek P, Sedo A. Regulation of Fibroblast Activation Protein by Transforming Growth Factor Beta-1 in Glioblastoma Microenvironment. *Int J Mol Sci*. 2021 Jan 21;22(3):1046. doi: 10.3390/ijms22031046. PMID: 33494271; PMCID: PMC7864518. **IF₂₀₂₁: 6,208**; Journal Citation Reports category and quartile (2023): **BIOCHEMISTRY & MOLECULAR BIOLOGY, Q1**; Times cited according to Web of Science: **18**

Attachment 3: Balaziova E, Vymola P, Hrabal P, Mateu R, **Zubal M**, Tomas R, Netuka D, Kramar F, Zemanova Z, Svobodova K, Brabec M, Sedo A, Busek P. Fibroblast Activation Protein Expressing Mesenchymal Cells Promote Glioblastoma Angiogenesis. *Cancers (Basel)*. 2021 Jul 1;13(13):3304. doi: 10.3390/cancers13133304. PMID: 34282761; PMCID: PMC8267680. **IF₂₀₂₁: 6,575**; Journal Citation Reports category and quartile (2023): **ONCOLOGY, Q1**; Times cited according to Web of Science: **20**

Attachment 4: Busek P, Mateu R, **Zubal M**, Kotackova L, Sedo A. Targeting fibroblast activation protein in cancer - Prospects and caveats. *Front Biosci (Landmark Ed)*. 2018 Jun 1;23(10):1933-1968. doi: 10.2741/4682. PMID: 29772538. **IF₂₀₁₈: 2,214**; Journal Citation Reports category and quartile (2020): **BIOCHEMISTRY & MOLECULAR BIOLOGY, Q2**; Times cited according to Web of Science: **116**