Studies on Glioblastoma Associated Thrombosis:

Impact of Tumor Cell Heterogeneity and Procoagulant

Extracellular Vesicles

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Abstract

Anomalies within the vasculature, including local and peripheral thrombosis, are a hallmark of glioblastoma (GBM) and an aftermath of deregulation of the cancer cell genome and epigenome. In this thesis project we examined the hitherto unstudied relationships between the complexities of single cell architecture of human GBM and the distribution and impact of vascular effector genes and the resulting pro-thrombotic consequences. We interrogated how the interplay between driver mutations and epigenetic programs control the emergence of coagulant and non-coagulant cellular GBM subpopulations, which impact both local and systemic vasculature through release of extracellular vesicles (EVs) carrying hemostatic effectors. The focal point of these efforts was the role of podoplanin (PDPN), a platelet activating protein, expressed by GBM cells and recently linked to an increased risk for venous thromboembolism (VTE) in patients.

We used single-cell and bulk transcriptome data mining, as well as cellular and xenograft models in mice, to analyze the nature of cells expressing PDPN, as well as their impact on the activation of the coagulation system and platelets. We report that PDPN is expressed by distinct (mesenchymal) GBM cell subpopulations and downregulated by oncogenic mutations of EGFR and IDH1 genes, along with changes in chromatin modifications (enhancer of zeste homolog 2) and DNA methylation.

We found that Glioma cells exteriorize their aberrantly expressed effectors, including PDPN and/or tissue factor (TF) as cargo of exosome-like extracellular vesicles (EVs). We observed that the injection of PDPN-carrying EVs activates platelets, whereas TF carrying EVs activate the clotting cascade. At variance with these systemic events, the extent of intratumoral thrombosis depended on co-expression of PDPN and TF by GBM cells.

Finally, we explored biological effects of PDPN beyond thrombosis. These studies suggested thus far that the PDPN status may influence experimental GBM progression in a context dependent fashion, both without and with intervention involving temozolomide (TMZ) chemotherapy, including in settings where natural killer (NK) cells play a meaningful role in outcomes. Thus, unlike some other hemostatic effectors, PDPN may principally affect the vascular aspect of GBM progression.

Overall, our study suggests that in GBM, distinct cellular subsets have the potential to engage specific facets of cancer-associated thrombosis (CAT) mechanism and may thereby represent targets for phenotype- and cell type–based diagnosis and antithrombotic intervention.

Résumé

Les anomalies du système vasculaire y compris les thromboses locales et périphériques, sont des caractéristiques importantes des glioblastomes (GBM) et le résultat de la dérégulation des programmes génétiques et épigénétiques des cellules cancéreuses.

Dans ce projet de thèse nous avons étudié la relation, encore inconnue, entre la complexité de l'architecture des populations de cellules individuelles constituantes des GBM avec la distribution des gènes reliés au système vasculaire et ainsi leur impact sur le développement des thromboses. Nous avons examiné la relation entre les mutations conductrices et les programmes épigénétiques dans l'émergence des sous populations de cellules cancéreuses liées ou non au processus de coagulation. Ces sous populations affectent le système vasculaire local et systémique par le biais de la sécrétion de vésicules extracellulaires (EVs) qui contiennent des effecteurs hémostatiques. En particulier, nous étudions le rôle de la podoplanine (PDPN), une protéine activatrice des plaquettes exprimée par les GBM et qui a été récemment liée avec l'augmentation du risque de thromboembolie veineuse (VTE).

L'analyse des cellules uniques et du transcriptome de masse ainsi que les modèles cellulaires de xénogreffe de souris nous ont permis de déterminer la nature des cellules exprimant le PDPN ainsi que leur rôle dans l'activation des programmes de coagulation et des plaquettes. Nous démontrons ainsi que le PDPN est exprimé par une sous population cellulaire spécifique des GBM, en particulier les cellules mésenchymateuses. Nous montrons aussi que le PDPN est sous régulé par les oncogènes EGFR et IDH1. Cette dérégulation est accompagnée par des modifications au niveau de la chromatine (enhancer of zeste homolog 2) et de la méthylation de l'ADN. De plus notre travail indique que les EVs des cellules cancéreuses contiennent du PDPN et/ou le facteur tissulaire (TF). L'injection des EVs comportant le PDPN active les plaquettes, alors que celles comportant le TF activent la cascade de coagulation. L'étendue de la thrombose intratumorale dépend largement de la co-expression du PDPN et du TF dans les cellules cancéreuses.

Finalement, nous avons exploré le rôle du PDPN dans les GBM outre que son implication dans la thrombose. Nos études suggèrent que l'effet du PDPN sur la progression des GBM dépend du contexte, et que le traitement avec la temozolomide (TMZ) n'affecte pas ce rôle. De même la

présence des cellules naturelles tueuses n'influent pas l'effet du PDPN sur la progression des GBM. Ainsi, et contrairement aux autres effecteurs hémostatiques, le PDPN permet la progression des GBM en influant le système vasculaire.

En résumé, notre étude suggère qu'il existe dans les GBM des sous populations cellulaires distinctes qui jouent des rôles différents dans le développement des thromboses, et ainsi ces populations constituent des cibles phénotypiques et cellulaires pour le diagnostic et les traitements antithrombiques.

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First and foremost, I want to thank my supervisor Dr. Janusz Rak for his tremendous help and support throughout this journey. His involvement in my project was instrumental to my success and to my growth as a scientist. I especially want to thank him for being very understanding and supportive at times when I needed support the most.

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Dr. Rak's lab has been the perfect incubator for personal and professional growth. The members in this lab are not just colleagues but rather friends, always there to support and provide any help I needed Brian Meehan and Laura Montermini, our Lab's senior members have been instrumental in providing the crucial support in terms of both experimental logistics and execution as well as design and thought process. One way or another, Cristiana Spinelli, Lata Adnani, Dongsic Choi, Shilpa Chennakrishnaiah, Thupten Tsering, Saro Aprikian, Mia Araujo, and Esterina D'Asti have all played a role in propping me throughout this path and considering the time spent in this lab with these people it is easy to say this lab has been a second home, and the people have been a second family.

Funding from CIHR, MICRTP, McGill's faculty of medicine and FRQS has been the driving financial force for this project and without it many of the experiments we did could not have happened. In that same spirit, I want to thank several key collaborators of ours including but not restricted to Dr. Hamed Najafabadi, Dr. Ali Nehme, Dr. Kevin Petrecca, and Dr. Charles Couturier, for their invaluable help and support. They were the driving force behind all the complex bioinformatical analyses and as such they helped pursue avenues in my research that would have been impossible otherwise. I would also like to mention our lab is blessed with wonderful neighboring labs, the labs of Dr. Nada Jabado, and Dr. Kolja Eppert. Over the years, good friendships were formed with members of both groups and their kindness and neighborly support will always be remembered. I would be amiss if I fail to mention, that I was truly lucky to have

the chance to mentor one of the brightest summer students I encountered throughout my academic journey, Rayhaan Bassawon. Rayhaan showed full dedication, amazing commitment and in no time became a partner on my project, one I was grateful to have.

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The help and dedication my friends showed me was unparalleled, especially when time seemed tight, and deadlines creeped in. It'll be hard to mention everyone, but I would like to mention Martin Karam who is always there at critical moments, ready to give his all to help. Samer Monzer, Harout Akilian, Jalal Kazan, Ali Darwiche, Azzam Faour, Leeanna El- Houjeiry, Rola Hammoud, Bettina Habib, and others, their friendship made life brighter, especially at first when I was a stranger in a new country that now I am happy to call home.

Finally, while there are certainly many names I could not list, I want to thank every person, past and present, for they shaped who I am today. We, as humans, are the products of our experiences, and my past and present encounters allowed me to be here today. I am truly thankful for every single one, the good and the bad.

I want to dedicate this thesis to my mother and my grandma

I love you and you will forever be a blessing!

Contributions to original knowledge

The following PhD thesis includes material from my three first-authored and published review papers as well as my first-authored and published original research manuscript. This work contains the following contributions to original knowledge:

1) The coagulome of GBM tumors is as heterogeneous as the tumors themselves whereby the net balance of differentially expressed procoagulant effectors across the tumor dictates the overall resultant coagulant phenotype (2018)

   Tawil, N., Chennakrishnaiah, S., Bassawon, R., Johnson, R., D'Asti, E., & Rak, J. (2018). Single cell coagulomes as constituents of the oncogene-driven coagulant phenotype in brain tumors. Thrombosis research, 164, S136-S142.

2) Biological mechanisms driving human cancers are highly dynamic and heterogeneous, at both the inter-individual and intra-tumoral levels. It follows that, mechanisms triggering or modulating cancer associated thrombosis (CAT) could likely be diverse and mutable over time. Additionally, driver mutations and epigenetic modifications have a documented role in the dysregulation of genes that control tumor angiogenesis or coagulant phenotype, both potentially involved in CAT (2019).

   Tawil, N., Bassawon, R., & Rak, J. (2019, June). Oncogenes and clotting factors: the emerging role of tumor cell genome and epigenome in cancer-associated thrombosis. In Seminars in thrombosis and hemostasis (Vol. 45, No. 04, pp. 373-384). Thieme Medical Publishers.

3) It is increasingly appreciated that cancer-specific drivers of CAT include a constellation of oncogenic mutations and their superimposed epigenetic states that shape the transcriptome, phenotype and secretome of cancer cell populations, including the repertoire of genes impacting the vascular and coagulation systems (2020).

   Tawil, N., Spinelli, C., Bassawon, R., & Rak, J. (2020). Genetic and epigenetic regulation of cancer coagulome–lessons from heterogeneity of cancer cell populations. Thrombosis research, 191, S99-S105.

4) a) We identified that PDPN is expressed by distinct (mesenchymal) GBM cell subpopulations and downregulated by oncogenic mutations of EGFR and IDH1 genes, along with changes in chromatin modifications (enhancer of zeste homolog 2) and DNA methylation.
   b) We discovered that glioma cells exteriorize their aberrantly expressed PDPN (and/or tissue factor (TF)) as cargo of exosome-like extracellular vesicles (EVs) shed from cells *in vitro* and *in vivo*.
   c) We observed that the injection of glioma-derived podoplanin carrying extracellular vesicles (PDPN-EVs) activates platelets, whereas tissue factor carrying extracellular vesicles (TF-EVs) activate the clotting cascade. Similarly, an increase in platelet activation (platelet factor 4) or coagulation (D-dimer) markers occurs in mice harboring the corresponding glioma xenografts expressing PDPN or TF, respectively.

d) We uncovered differential mechanisms driving CAT at systemic versus intratumoral levels. While systemic CAT appears to be largely dependent of PDPN in GBM, the co-expression of PDPN and TF by GBM cells seems to exhibit a cooperative impact on tumor microthrombosis exacerbating intratumoral vascular occlusion (2021).

Tawil, N., Bassawon, R., Meehan, B., Nehme, A., Montermini, L., Gayden, T., De Jay, N., Spinelli, C., Chennakrishnaiah, S., Choi, D., Adnani, L., Zeinieh, M., Jabado, N., Kleinman, C., Michael Witcher, M., Yasser Riazalhosseini, Y., Key, N., Schiff, D., Grover, S., Mackman, N, Couturier, C., Kevin Petrecca, K., Mario L. Suvà, M., Anoop Patel, A., Tirosh, I., Najafabadi, H., & Rak, J. (2021). Glioblastoma cell populations with distinct oncogenic programs release podoplanin as procoagulant extracellular vesicles. Blood Advances, 5(6), 1682-1694.

This work was conducted entirely by me. Nonetheless, several experimental elements of this investigation and the corresponding published original research manuscript were accomplished with the assistance of co-authors. Their contributions are broadly outlined in the "Contributions of Authors" section below.

Contributions of Authors

The chapters (4 to 6) below constitute the bulk of this PhD thesis and are part of our published original research. Chapter 7 delineates a preliminary study investigating biological effects of PDPN in glioblastoma progression.

Accordingly, contributions of authors are as follows:

- I (Nadim Tawil) performed the bulk of experimentation.
- Concept and design were achieved with Dr. Janusz Rak.
- Laura Montermini and Dr. Dongsic Choi provided invaluable assistance and guidance with several techniques pertaining to extracellular vesicles.
- Brian Meehan provided crucial assistance and guidance with experimental procedures pertaining to the *in vivo* segment of our work.
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- General data analysis and interpretation was performed by Nadim Tawil, Rayhaan Bassawon, Dr. Cristiana Spinelli, Laura Montermini, Brian Meehan, Dr. Dongsic Choi, Dr. Lata Adnani, Dr. Michele Zeinieh and Dr. Janusz Rak.
- Drs. Claudia L. Kleinman, Nada Jabado, Nigel S. Key, David Schiff, Michael Witcher, Yasser Riazalhosseini, and Steven P. Grover contributed to manuscript review and edits.
- Dr. Janusz Rak, Dr. Hamed Najafabadi, Dr. Nada Jabado, Dr. Kevin Petrecca, and provided project oversight and guidance.
- Dr. Janusz Rak (mainly) and I contributed to funding acquisition.
- I (Nadim Tawil) and Dr. Janusz Rak led the initial critical writing and bulk intellectual content.
- All authors contributed to the final approval of the version published.

Other Contributions

El-Saghir, J., Nassar, F., **Tawil, N**. and El-Sabban, M., 2016. ATL-derived exosomes modulate mesenchymal stem cells: potential role in leukemia progression. Retrovirology, 13(1), pp.1-13.

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List of abbreviations

2-D-HG	D-2-hydroxyglutarate
5 Aza	5-Azacytidine
ABs	Apoptotic Bodies
ACC	Animal Care Committee
ACVR1	Activin A Receptor Type 1
adMYF	Alveolar Duct Myofibroblasts
ADP	Adenosine Diphosphate
ALDH1	Aldehyde dehydrogenase 1
Alix	Programmed Cell Death 6-Interacting Protein (PDCD6IP)
AP1	Activator Protein-1
APC	Activated Protein C
AS	Astrocytic
ASMase	Sphingomyelinase
AT	Antithrombin
ATE	Arterial Thrombosis
ATP	Adenosine Triphosphate
ATRX	ATP-dependent helicase ATRX
AUP	Animal Utilization Protocol
BCA	Bicinchoninic Acid
BEC	Blood Endothelial Cell
BFP	Blue Fluorescent Protein
BLI	Bioluminescent Imaging

BMP-9	Bone Morphogenic Protein 9
BTSCs	Brain Tumor Stem-like Cells
C19MC	Chromosome 19 microRNA Cluster
C1R	Complement component 1R
C1S	Complement Component 1s
CAFs	Cancer Associated Fibroblasts
cAMP	Cyclic Adenosine Monophosphate
Cas	Crk-Associated Substrate
Cas9	CRISPR-associated Protein 9
CAT	Cancer Associated Thrombosis
CBC	Complete Blood Count
CBTRUS	Central Brain Tumor Registry of the United States
CCAC	Canadian Council of Animal Care
CD133	Prominin-1
CD15	Alpha-(1,3)-fucosyltransferase 4
CD44	CD44 Molecule (Chondroitin Sulfate Proteoglycan 8)
CD58	Lymphocyte Function-associated Antigen 3
CD61	Integrin Beta-3
CD81	CD81 Molecule (TAPA1)
CD9	CD9 Molecule (Tetraspanin 27)
CDK4	Cyclin Dependent Kinase-4
CDK5	Cyclin Dependent Kinase 5
CDKN2B	Cyclin Dependent Kinase Inhibitor 2B

CDKN2	Cyclin-dependent Kinase Inhibitor
CHMP4	Charged Multivesicular Body Protein
CL	Classical
CLEC-2	C-type Lectin-like Type II Transmembrane Receptor
CM	Conditioned Medium
CNS	Central Nervous System
CNV	Copy Number Variation
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
CTNNB1	β-catenin
D-dimer	Fibrin Degradation Product (or FDP)
DAXX	Death-associated Protein 6
DCs	Dendritic Cells
DEX	Dexamethasone
DIC	Disseminated Intravascular Coagulation
DLL1	Delta-Like Protein 1
DRSS	Diagnostic and Research Support Service
dsDNA	Double Stranded DNA
DVT	Deep Vein Thrombosis
EANO	European Association for Neuro-Oncology
EC	Endothelial Cell
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor

EGFRvIII	Epidermal Growth Factor Receptor variant III
EMT	Epithelial to Mesenchymal Transition
EPCR	Endothelial Protein C Receptor
ephrinA3	EPH-related receptor tyrosine kinase ligand 3
ERM	Ezrin-Radixin-Moesin
ESCRT	Endosomal Sorting Complexes Required for Transport
ETMR	Embryonal Tumors with Multilayered Rosettes
EXs	Exosomes
EZH2	Enhancer of Zeste Homologue 2
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FOS	Proto-oncogene c-Fos
FRC	Fibroblastic Reticular Cell
G-CIMP	CpG island methylator phenotype
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GBM	Glioblastoma Multiforme
GMP	Guanylate Monophosphate
GPCR	G-coupled Protein Receptors
GSC	Glioma Stem Cells
GSEA	Gene Set Enrichment Analysis
H&E	Hematoxylin and Eosin
H3F3A	Histone H3.3
HEVs	High Endothelial Venules

HIF-1	Hypoxia-inducible factor 1
hPC	Human Podocyte
IDH1	Isocitrate Dehydrogenase
IFN-γ	Interferon-γ
IGRT	Image-Guided Radiotherapy
IL	Interleukin
ILV	Intraluminal Vesicle
IVC	Inferior Vena Cava
Jak	Janus Kinases
KEAP1	Kelch-like ECH-associated protein 1
KO	Knock Out
LEC	Lymphatic Endothelial Cell
LECT1	Chondromodulin-1
LMWH	Low Molecular Weight Heparin
LOs	Large Oncosomes
LPS	Lipopolysaccharide
Luc	Luciferase
LYVE-1	Lymphatic Vessel Endothelial Receptor 1
MB	Medullablastoma
MDCK	Madin Darby Canine Kidney
MES	Mesenchymal
MET	Mesenchymal to Epithelial Transition
MET	Tyrosine-protein Kinase Met

Met-VEL	Metastatic Variant Enhancer Locus
MGMT	Methylation of the O-6-Methyl Guanine Methyl-DNA-Transferase
miRNA	Micro RNA
MMP	Matrix Metalloproteinase
MPs	Microparticles
MRI	Magnetic Resonance Imaging
MSB	Martius Scarlet Blue
MSs	Migrasomes
MUHC RI	McGill University Health Center Research Institute
MVB	Multivesicular Body
MVs	Microvesicles
MVT	Microvascular Thrombosis
NCCN	National Comprehensive Cancer Network
NEAT1	Nuclear Enriched Abundant Transcript 1
NETs	Neutrophil Extracellular Traps
NEU	Neural
NF1	Neurofibromatosis type
NK	Natural Killer
NKG2D	Killer Cell Lectin Like Receptor K1
NKG2DL	Killer Cell Lectin Like Receptor K1 Ligand
NMWL	Nominal Molecular Weight Limit
NO	Nitric Oxide
NPC	Neural Progenitor Cell

NSCs	Neural Stem Cells
NSG	NOD-Scid IL2Rgammanull, NOD-Scid IL2Rgnull, NOD Scid Gamma
NTA	Nanoparticle Tracking Analysis
OD	Optical Density
OLIG1	Oligodendrocyte Transcription Factor 1
OPC	Oligodendrocyte Progenitor Cell
OS	Overall Survival
OSs	Oncosomes
p21Cip1	Cyclin-Dependent Kinase Inhibitor 1
PAI-1	Plasminogen Activator Inhibitor
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PC	Protein C
PCA	Principal Component Analysis
PCA	Procoagulant Activity
PD1	Programmed Cell Death Protein 1
PDGFRα	Platelet Growth Factor alpha
PDPN	Podoplanin (OTS8, gp38, T1α, PA2.26)
PE	Pulmonary Embolism
PF4	Platelet Factor 4
PFA	Paraformaldehyde
pGBM	Pediatric Glioblastoma
PGI2	Prostacyclin

PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PLAG Domain	Platelet Aggregating Domain
PN	Proneural
PPP	Platelet Poor Plasma
PRC2	Polycomb Repressive Complex
PS	Protein S
Prox1	Prospero homeobox 1
PSGL-1	P-selectin Glycoprotein Ligant-1
PTEN	Phosphatase and tensin homolog
QoL	Quality of Life
Rab27a/b	Ras-associated Binding Protein a/b
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat Sarcoma
RB1	Retinoblastoma 1
RBC	Red Blood Cell
REB	Research Ethics Board
RHOA	Ras homolog family member A
ROCK	Rho-associated Protein kinase
RRE	Rev Response Element
RT	Radiation Therapy
RTEL1	Regulator of Telomere Elongation Helicase 1
RTK	Receptor Tyrosine Kinase

SCC	Squamous Cell Carcinoma
SCID	Severe combined immunodeficiency
SCID	Severe combined immunodeficiency
scRNA-seq	Single Cell RNA Sequencing
SERPINE1	Plasminogen Activator Inhibitor-1
SHH	Sonic Hedgehog
SLP-76	Lymphocyte cytosolic protein 2
SMAD	Homologues of the Drosophila Protein, Mothers Against Decapentaplegic (Mad) and the Caenorhabditis Elegans Protein Sma
SMPD3	neutral sphingomyelinase
SNARE	Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor
SNO	Society of NeuroOncology
SNPs	Single Nucleotide Polymorphisms
SOX-2	SRY-box 2
SP1/3	specificity protein1/3
Src	Proto-oncogene tyrosine-protein kinase Src
STAT	Signal Transducer And Activator Of Transcription
STK11	Serine/Threonine Kinase 11
SVZ	Subventricular Zone
Syk	Spleen tyrosine kinase
TCGA	The Cancer Genome Atlas
TEM	Transmission Electron Microscopy
TET	Ten-Eleven Translocation

TERT	Telomerase Reverse Transcriptase
TF	Tissue Factor (CD142)
TFPI	Tissue Factor Pathway Inhibitor
TGF β	Transforming Growth Factor-B
TIC	Tumor Initiating Cell
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TM	Thrombomodulin
TMZ	Temozolomide
TNF	Tumor Necrosis Factor
TP53	Tumor protein P53
tPA	tissue-type Plasminogen Activator
TPM	Transcript per Million
TSG101	Tumor susceptibility gene 101
TSP-1	Thrombospondin
TTF	Tumor Treating Fields
uPA	Urokinase-type Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
VSVG	Vesicular Stomatitis Virus G
VTE	Venous Thromboembolism
vWF	Von Willebrand Factor
WNT	Wingless
WT	Wild Type
YFP	Yellow Fluorescent Protein

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1 Introduction

Tawil et al. 2018 Thrombosis Research; Tawil et al. 2019 Seminars in Thrombosis and Hemostasis; Tawil et al. 2020 Thrombosis Research

1.1 Glioblastoma

Glioblastoma multiforme (GBM) is a class of primary, high grade astrocytic brain tumors that affect both adults and children and constitutes over 57% of gliomas as per Central Brain Tumor Registry of the United States (CBTRUS) statistical report (2007-2011 and 2013-2017) and Canadian Cancer Registry (2009-2013) (Ostrom et al., 2014; Ostrom et al., 2020; Walker et al., 2019). Despite the progress in understanding the underlying tumor biology, GBM still presents a major therapeutic challenge as one of the leading causes of brain cancer related deaths, and a generally incurable disease regardless of age (Pine et al., 2020; Siegel et al., 2015). This is compounded by heterogeneity among GBM cases resulting in distinct disease subtypes associated with diverse repertoires of transcriptomic profiles and oncogenic drivers (Patel et al., 2014; Sturm et al., 2012; Tirosh and Suvà, 2020), targeting of which has thus far been unfortunately ineffective (Jalali et al., 2016; Simon et al., 2020; Weenink et al., 2020) with 5-year survival rates as low as 5% to 15% (Borgo et al., 2010; Marenco-Hillembrand et al., 2020; Olar and Aldape, 2014) and 10-year survival rate of 0.71% (Tykocki and Eltayeb, 2018).

1.1.1 *Epidemiology*

As per the 2020 consensus review on management of glioblastoma and future directions from the Society for Neuro-Oncology (SNO) and European Association for Neuro-Oncology (EANO), the overall incidence of glioblastoma in the United States following age adjustment is 3.22/100,000 persons (Wen et al., 2020). This incidence rate is said to increase with advanced age

and male sex (Ostrom et al., 2019a). Global worldwide incidence is reported as variable (Leece et al., 2017), with inconsistencies regarding the existence of an upward trend: reports from UK indicate an increase in incidence (Philips et al., 2018), while data from Canada or US indicate a very minor upwards trend (Davis et al., 2020). Several explanations were proposed to justify the difference in trends and those include variations in monitoring approaches, coding and differences in adoption of amendments in classification (Louis et al., 2016).

Clinically, GBM (grade IV glioma) may arise through progression of lower grade lesions, especially grade II and III gliomas (secondary GBM) (Ohgaki and Kleihues, 2005), while grade I gliomas represent a different class of diseases (Wen and Kesari, 2008). In some instances, this progression can be influenced by mutational load acquired during chemotherapy of lower grade tumors, especially using temozolomide (TMZ) (Johnson et al., 2014). However, much more frequently (95% of cases) GBMs arise *de novo*, especially in older patients, without any evidence of prior lesions and within staggeringly short periods of time (Chittiboina et al., 2012) (primary GBM).

There are several measures by which a disproportional contribution of GBM to cancer related morbidity, mortality and disease burden can be more fully evaluated. For example, as per the National Program of Cancer Registries (2012-2016), the 5-year overall relative survival in GBM is as low as 6.8%, with variations by age at diagnosis and sex (Ostrom et al., 2019b). As for risk factors, a clear consensus is still missing with only an exposure to ionizing radiation to the head and neck validated by several independent studies (Ostrom *et al.*, 2019b). However, latency period for disease development following exposure is still unknown and hence SNO recommends

close surveillance and investigation of any changes in incidence trend (Wen *et al.*, 2020). There has been no implication of family history of cancer in around 95% of glioblastoma patients, leaving only 5% of gliomas that fall into the familial category (Ranger et al., 2014).

However, studies aiming to elucidate genetic predispositions utilizing genome-wide approaches have identified 25 single nucleotide polymorphisms (SNPs) as genetic risk factors for glioma, with 11 of them specifically implicated in predisposition to glioblastoma (Melin et al., 2017). Those include loci harboring genes critical for glioma such as telomerase reverse transcriptase (TERT), RTEL1, epidermal growth factor receptor (EGFR), cyclin-dependent kinase inhibitor 2B (CDKN2B) (Melin *et al.*, 2017) and TP53, including syndromes such as Li-Fraumeni whereby glioblastomas and astrocytomas were reported as the most common CNS tumor types in affected individuals (Amadou et al., 2018; Valdez et al., 2017). Certainly, continuous improvements in the fields of risk factor assessment and genetic risk factor identification should ultimately shed light on the etiological and biological basis of different histologies manifested by gliomas and consequently better assist and guide gene discovery initiatives that in turn can have profound impacts on the development of new therapeutic approaches.

1.1.2 *GBM Biology, Molecular Pathogenesis and Classification*

Increasing evidence suggests that brain tumors recapitulate, albeit in a distorted form, many physiological elements of the central nervous system (CNS) development. However, while such processes are highly fine-tuned during normal development, they may be inadequately reactivated at later timepoints of life leading to development of brain tumors (Jung et al., 2019). Despite the sharp decline of neurogenesis post infancy (Paredes et al., 2016; Sanai et al., 2011), in humans,

the subventricular zone (SVZ) of the brain contains neural stem cells (NSCs) that persist throughout adulthood (Sanai et al., 2004). Those NSCs are surrounded by a complex microenvironment that promotes the sustainability of stem cell characteristics (Alvarez-Buylla et al., 2008; Bjornsson et al., 2015). Whether and to what extent these cells contribute to restorative processes in the adult brain is controversial, but they may become targets of transforming mutations setting off growth processes under permissive conditions. Elements of such micro-ecology become subsequently incorporated into the tumorigenic process, including deregulated cell proliferation and self-renewal, among other hallmarks of cancerous cells (Hanahan and Weinberg, 2011; Riquelme et al., 2008).

Particularly, a cell subpopulation endowed with stem cell properties and initially identified as expressing NSC markers, such as nestin and CD133 has been uncovered in brain tumors and designated as 'brain tumor stem-like cells' (BTSCs), brain tumor initiating cells (BTICs) or glioma stem cells (GSCs) (Galli et al., 2004; Singh et al., 2003). Whether these cells represent transformed NSCs or result from aborted differentiation programs of other cells is under consideration (Friedmann-Morvinski et al., 2012). Nonetheless GSCs/BITCs resemble NSCs in terms of molecular traits maintained by both intrinsic programs and a specific microenvironment, and are closely associated with perivascular niches in the SVZ (Calabrese et al., 2007; Charles et al., 2010). Interestingly, BTSCs were found to further echo NSC behavior in terms of their response to tissue injury. In the context of GBM this program can be triggered by the therapeutic tumor bulk reduction, which promotes the repopulation of the tumor mass due to increased recruitment and proliferation of otherwise quiescent BTSCs (Gao et al., 2013b; Shankar et al., 2014). This suggests the existence of feed-back loops analogous to those present in neurogenesis

(Daynac et al., 2013; Doetsch et al., 1999) and multiple layers of cell-cell communication that sustain multicellular equilibria in brain pathology and cancer (Tirosh and Suvà, 2018). These observations further substantiate the rationale that throughout disease progression, brain tumors, in part, recapitulate elements of the physiological developmental programs and cellular hierarchies (Couturier et al., 2020).

Adult GBMs are often observed to develop in close proximity to, or possibly within the SVZ (Barami et al., 2009) containing neurogenic niches of immature cells that have been demonstrated by several groups to be much more prone to oncogenic transformation in comparison to their more differentiated counterparts in the rest of the brain, in part due to preferential reliance on the error-prone non-homologous end joining DNA repair pathway (Siebzehnrubl et al., 2011; Stiles and Rowitch, 2008). Moreover, neural stem cells affected by oncogenic mutations have a greater potential to proliferate and propagate these errors relative to their terminally differentiated counterparts. Recent work by Lee et al. relying on driver mutation and clonal tracing in human specimens of glioblastomas suggested that the SVZ NSCs are indeed the precursor cells from which these tumors originate (Lee et al., 2018; Llaguno et al., 2019).

GBMs have been reported to double in size within the span of 10 to 20 days (Yin et al., 2005). Cellular growth advantage in glioblastomas, and gliomas in general, has been found to be attributable to several regulatory levels. Multiple growth stimulatory cues can be generated through overexpression, activating and inactivating mutations within core cellular signalling pathways encompassing receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR) and its mutants (EGFRvIII), as well as RTK downstream effectors (RAF,

RAS/PI3K), and regulators, such as inactivating mutations of phosphatase and tensin homolog (PTEN). This is compounded with alterations that confer resistance to growth suppression mechanisms (loss of CDKN2A, RB1, and TP53 tumor suppressor genes), those enabling replicative immortality (mutations in TERT and ATRX), induction of angiogenesis (HIF-1 and VEGF overexpression), resistance to cell death (TP53 or RB pathway mutations), evasion of immune destruction and reprogramming of cellular energetics (Nørøxe et al., 2016) . Additionally, interaction with the glioma microenvironment including reactive astrocyte proliferation, microglia, vascular alterations (below) and infiltration with bone marrow derived cells, all of which account for 30-40% of the GBM tumor mass exert a powerful regulatory influence on malignant progression (Broekman et al., 2018). Furthermore, neurotransmitter signaling, and overall neuronal activity have been implicated in the promotion of cell growth in gliomas (Barres and Raff, 1993; De Groot et al., 2008; Duman et al., 2019; Ishiuchi et al., 2002; Rzeski et al., 2001; Smits et al., 2012; Van Vuurden et al., 2009; Venkatesh et al., 2015; Venkatesh et al., 2017). It should also be mentioned that while the local network of GBM driver events in the brain is increasingly well understood, the disease also triggers systemic consequences spectacularly illustrated by the states of immune suppression and peripheral thrombosis, all of which require mechanisms of long-range communication, which are presently poorly understood (Grabowski et al., 2021; Tawil et al., 2019). This emerging complexity represents both a daunting conceptual and analytical challenge and also a possible area of opportunity to dissect and rationally oppose the critical molecular mechanisms of gliomagenesis.

One remarkable development in the analysis of GBM complexity has been the identification of several molecular subtypes of this disease. From the genomic standpoint, The

Cancer Genome Atlas (TCGA) classification distinguishes four molecular subtypes of GBM: proneural (PN), neural (NEU), classical (CL), and mesenchymal (MES), each associated with a distinct transcriptional signature and network of oncogenic drivers (Verhaak et al., 2010). While the recent World Health Organization 2016 classification revised these designations, placing emphasis on the oncogenic mutation of the isocitrate dehydrogenase 1 gene (IDH1/R132H; IDH mutant subgroup) or its absence (receptor tyrosine kinase [RTK] I, RTK II, CL and MES GBM subgroups) (Reifenberger et al., 2017), the TCGA nomenclature is still in use due to well annotated linkages to gene expression profiles and the underlying recurrent oncogenic mutations (Verhaak *et al.*, 2010). Further molecular profiling dispensed of the NEU subtype initially proposed by TCGA and classified glioblastoma into 3 main subgroups identified on the basis of enrichment for specific somatic alterations (Wen *et al.*).

Thus, the PN GBM subtype, occurring mostly in relatively younger adults, is characterized by amplifications of cyclin-dependent kinase 4 (CDK4) and platelet derived growth factor alpha (PDGFRα). PN lesions often present with overexpression of SOX-2 and high frequency of IDH1 mutations (Neftel et al., 2019; Verhaak *et al.*, 2010). The MES subtype is dominated by lesions with pronounced macrophage infiltration and the presence of neurofibromatosis type 1 (NF1) loss. The CL subtype exhibits homozygous loss of CDKN2A/B, PTEN loss and a high frequency of EGFR amplifications along with the occurrence of a unique constitutively active mutant form of EGFR referred to as EGFR variant III (EGFRvIII). It is important to note that glioblastomas normally exist as mixed entities and are frequently associated with TERT promoter mutations (Brennan et al., 2013; Ceccarelli et al., 2016; Sturm *et al.*, 2012; Wang et al., 2017).

Such molecular classification certainly provides a framework for research efforts, but so far resulted in no immediate actionable information that could engender major changes, or improvements, in the clinical care of patients with GBM. (Wen *et al.*, 2020). This is important as it signals gaps in our understanding as to how genetic progression and emerging cellular and molecular patters in GBM impinge upon mechanisms driving the local and systemic aspects of the disease.

There is no doubt that genomic profiling and classification contributed significantly to the advancement of our understanding of GBM, however unfortunately this has not yielded any major impact on the ability to manage the disease progression, in part, due to relevant information not being captured. In this regard 'en masse' processing of bulk tumor samples fails to overcome two major obstacles. First, random sampling of GBM is highly prone to overlooking regional differences within the tumor mass that could hold key information about the rapid molecular progression of these lesions (Eskilsson et al., 2016; Wen *et al.*, 2020). Second, global profiling strategies may fail to tease out regulatory genomic and epigenetic events occurring at the level of single cells or distinct subpopulations within the tumor mass.

Over the recent years, the development of single cell sequencing technology brough about another major shift into the realm of molecular subtypes in GBM. Work by led by Suva, Berstein and their colleagues demonstrated that a given GBM lesion is composed of a mosaic of malignant cells existing in a quantitative equilibrium where cells exhibit signatures of not only a particular subtype but also intermediate signatures reflecting more than one subtype at once. Thereby a lesion classified as PN would merely contain a preponderance of PN-like cells and a MES tumor would

be numerically dominated by cells exhibiting MES-like characteristics, but in each case other cells would coexist with the dominant population (Patel *et al.*, 2014).

More recently, several groups have made additional strides in characterizing GBMs making use of single cell sequencing. Their efforts revealed that the molecular phenotypes of cells within the tumor bulk is flexible and under epigenetic control oscillates between MES-like, oligodendrocyte progenitor cell (OPC)-like, astrocytic (AS)-like and neural progenitor cell (NPC)-like states, whereby the oncogenic driving forces (NF1, PDGFRA, EGFR, and CDK4) govern the directional bias of this phenotypic flux more towards one or another phenotype (Neftel *et al.*, 2019; Suvà and Tirosh, 2020). This paradigm was further reinforced by studies utilizing RNA-velocity analysis suggested that the phenotypic flux is indeed non-chaotic and rather dictated by differentiation pathways (referred to as roadmaps) characteristic of central nervous system (CNS) development. Accordingly, such analysis postulates the existence of progenitor cells that travel phenotypically along alternative quasi-differentiation paths ultimately oriented towards mesenchymal, astrocytic, oligodendroglial or neuronal phenotypes (Couturier *et al.*, 2020).

1.1.3 *The Interplay Between GBM Progression and the Epigenome*

Efforts aiming to refine GBM subtype definition focused on several aspects of malignant progression including analysis of driver mutations, copy number variations (CNVs), characterization of micro RNA (miRNA) (Cho and Przytycka, 2013; Clarke et al., 2013), as well as epigenetic changes, particularly DNA and histone methylation profiles (Brennan *et al.*, 2013; Noushmehr et al., 2010; Sturm et al., 2014). In reference to the latter, through methylation profiling, Sturm et al. identified six epigenetic subgroups of GBM with distinct methylation

patterns, and methylation clustering allowed Noushmehr et al to identify three subgroups within the TCGA dataset (Brennan *et al.*, 2013; Noushmehr *et al.*, 2010). Despite significant progress, epigenetic profiling in GBM remains under development and has not reached diagnostic significance similar to the recommendations outlined by the SNO, for example, to exclude IDH1 mutant lesions from GBM classification (Wen *et al.*, 2020).

GBM genome and epigenome are interlinked in a number of ways. It is worth highlighting that prominent GBM associated oncogenic drivers like EGFR amplification/mutation and IDH1 mutations, particularly EGFRvIII and IDH1R132H respectively, could have an impact on the epigenetic landscape. EGFR alterations (amplifications, deletions and point mutations) are a hallmark of specific GBM subsets. Deletion alterations result in a number of EGFR variants whereby N-terminal deletion gives rise to EGFRvI, EGFRvII is the result of deletion of exons 14-15, EGFRvIII is the product of exons 2-7 deletion, and EGFRvIV and vV are a consequence of deletions of exons 25-27 and 25-28 respectively; of those variants, vII and vIII have been documented as oncogenic drivers (Furnari et al., 2015). EGFRvIII is the most common EGFR modification (Brennan *et al.*, 2013) and often occurs on a background of EGFR amplification (Eskilsson *et al.*, 2016) often occurring along with polysomy of chromosome 7, which in gliomas, is the most frequent EGFR CNV (Maire and Ligon, 2014). As a matter of fact, it has been found that 50% of EGFR amplified gliomas had EGFRvIII expression (Nishikawa et al., 1994). The resultant oncoprotein lacks the ligand binding domain and hence conformational regulation of the extracellular domain and, as alluded to earlier, exhibits low level but constitutive phosphorylation and activation (Sugawa et al., 1990). Hence, in line with the above-mentioned findings, the presence of EGFRvIII adds further ligand independent constitutive EGFR pathway activation on

top of a preexisting elevated expression of wild type (WT) EGFR. Moreover, GBM cells expressing EGFRvIII and those with wild type EGFR co-exist and symbiotically interact driving disease progression (Inda et al., 2010).

The IDH1R132H mutation on the other hand, was identified as somatic point mutation in isocitrate dehydrogenase 1 (IDH1) gene, targeting codon 132 and leading to the substitution of the arginine residue by histidine (Noushmehr *et al.*, 2010; Parsons et al., 2008; Yan et al., 2009). This change results in a gain of function enabling directly catalysis of α-ketoglutarate conversion to D-2-hydroxyglutarate (2-D-HG) (Dang et al., 2009). As a consequence, 2HG-mediated inhibition of DNA and histone demethylases, namely the ten-eleven translocation enzymes and lysine demethylases, respectively, results in hypermethylation of DNA and histones (Figueroa et al., 2010). This ultimately contributes to epigenetic reprograming of affected glioma cells' transcriptome (Figueroa *et al.*, 2010; Núñez et al., 2019).

At a global DNA methylation level, a hypermethylator phenotype has been tightly linked to the presence of the IDH1R132H mutation. The study by Turcan et al. showed that glioma CpG island methylator phenotype (G-CIMP) which is a powerful determinant of tumor pathogenicity, is the result of methylome remodeling which is in turn highly dependent on the presence of IDH1 mutation (Turcan et al., 2012).

Oncogenic drivers can operate at the level of DNA or histone modifications. Thus, integrative analysis of chromatin landscape induced by EGFRvIII identified 2,245 putative enhancers that were specifically activated in EGFRvIII-expressing GBM cells. These EGFRvIII

activated enhancers were identified by H3K4me1 and H3K27ac activating histone modifications, which were significantly enriched near EGFRvIII upregulated transcripts (Liu et al., 2015; Liu et al., 2016). In contrast to the enhancer activating effect documented by Liu et al., in another study by Chen et al., EGFR pathway activity was shown to upregulate the levels of a long non-coding RNA, Nuclear Enriched Abundant Transcript 1 (NEAT1). NEAT1 was further shown to have a significant impact in promoting the activity of the enhancer of zeste homologue 2 (EZH2), the catalytic subunit of the polycomb repressive complex (PRC2), in catalyzing the placement of the repressive H3K27me3 (Chen et al., 2018). In a separate work by Zhang et al. alterations in EZH2, mainly amplification, have been also documented to exhibit a tendency to co-occur with EGFR alterations (Zhang et al., 2017).

These observations strongly reinforce a notion that the epigenetic landscape dictated by DNA and histone modifications is profoundly altered in transformed cells and suggests a link between upstream oncogenic signalling pathways and chromatin modifications that could be highly consequential for malignant progression (Liu *et al.*, 2016). As this work is progressing medical applications are still to be realized.

1.1.4 *Medical Management and Current Treatment Modalities in Glioblastoma*

The prognosis of GBM patients remains grim despite all recent advances in understanding of glioma biology and molecular pathology. The standard of care remained unchanged for decades and consists of surgery, radiation, and temozolomide chemotherapy amounting to a median overall survival (OS) of 15-18 months and 5-year survival remaining below 10% (Gilbert et al., 2013; Stupp et al., 2009; Stupp et al., 2005). Although a slight improvement of survival by ~3 months

has been achieved with surgery followed by image-guided radiotherapy (IGRT), recurrence driven by invasive cells disseminated throughout the brain and inaccessible to surgical resection, remains inevitable. Tumors recur at locations nearby the primary lesion in about 80% of the cases, but evidence of wide-spread dissemination at distant sites exists whereby distant recurrence is plausible and occurs in about 18% of the cases and in approximately 4% of the cases recurrence could be observed in the contralateral hemisphere (Agnihotri et al., 2013; Berens and Giese, 1999; Sherriff et al., 2013).

Clinical trials and preclinical explorations are conducted at feverish pace in hopes to achieve a regimen that would allow for better management and control over GBM. These efforts include new combinations of elements of the traditional standard of care (surgery, radiation and chemotherapy) and novel agents that aim to target oncogenic and angiogenic pathways (Furnari et al., 2007; Mellinghoff et al., 2005; Vredenburgh et al., 2009) or immune responses (Lim et al., 2018), as well as physical methods like tumor treating fields (TTF) (Fabian et al., 2019; Wen *et al.*, 2020). However, despite being promising, none of the approaches has been able to provide curative results.

Efforts have been directed towards enhancing the efficiency of surgical debulking by maximizing the margin of surgical resection (Wen and Kesari, 2008), but despite all advances including surgical resection with magnetic resonance imaging (MRI) guided neuro navigation, intraoperative MRI, functional MRI, intraoperative mapping and fluorescence-guided surgery, GBMs remain unamenable to complete surgical removal (Sanai and Berger, 2008; Stummer, 2009; Stummer et al., 2011a). It is worth mentioning that aggressive surgical resection can have

detrimental impacts on the quality of life (QoL) sometimes due to the unavoidable neurological impairments, but studies have shown that more aggressive surgical resection could enhance the efficacy of subsequent radio and chemo therapies and is associated with better overall outcomes (Moliterno et al., 2012; Stummer, 2009; Stummer et al., 2011b; Wang and Jiang, 2013).

In the context of GBM, systemic chemotherapy with Temozolomide (TMZ) was introduced in 2005 in an effort to reduce the chances of relapse and malignant cell dispersion following surgery. This became known as the Stupp protocol and has been a standard of care since. Mechanistically, TMZ is an alkylating agent that impairs DNA replication and cell division and selectively induces GBM cell death (Lashkari et al., 2011; Stupp *et al.*, 2009; Stupp *et al.*, 2005). The pivotal work by Stupp and colleagues has shown the survival benefit of ~2.5 months and an improvement of two-year survival up to 26.5% with the use of RT plus concomitant daily oral TMZ followed by adjuvant TMZ dose of 75mg/m^2/day compared to RT alone resulting in median survival of 12.1 months and two year survival of 10.4% (Stupp *et al.*, 2009). It is worthy, however, to note that the efficacy of TMZ relies on the existence of drug sensitivity differential between a cell subset of the GBM lesion and normal tissues. In turn, the sensitivity of GBM cells to TMZ is predicated on the methylation of the O-6-methyl guanine methyl-DNA-transferase (MGMT) promoter locus resulting in silenced expression of the enzyme which otherwise confers protection from drug cytotoxicity. Indeed, MGMT-expressing subsets of GBM cells are resistant to RT and TMZ double treatment (Chahal et al., 2012) and comparative studies showed that RT/TMZ treatment increased the two-year survival to 46% in patients with MGMT methylation, while in those with active MGMT such survival was only 14% (Hegi et al., 2005).

Despite the progress in treating MGMT methylated GBMs, TMZ is ineffective in over 50% of GBM patients as MGMT methylated tumors represent approximately 45% of the cases and hence there is dire need to develop alternative treatment strategies. Additionally, the inevitability of tumor relapse continues to plague even the most successfully controlled cases with TMZ and relapsed tumors exhibiting treatment dependent molecular alterations including drug-induced genetic instability and various mechanisms of resistance to TMZ (Garnier et al., 2018; Johnson *et al.*, 2014; Wang et al., 2016).

In this light, several forms of salvage therapy have been considered and explored including secondary surgery and chemoradiation as well as antiangiogenic treatment modalities such as, bevacizumab which is currently FDA approved for recurrent GBMs. To date, however, none of these approaches, including bevacizumab, has yielded anything beyond palliative effect in recurrent GBMs (Wen *et al.*, 2020). Despite promising initial responses documented by decreases in MRI enhancement (Vredenburgh et al., 2007), subsequent phase III trials revealed it does not confer any overall survival benefit when used against treatment-naïve GBMs (Chinot et al., 2014; Gilbert et al., 2014).

Current efforts are being directed towards immunotherapeutic strategies, targeted agents, and novel radiotherapy approaches. In terms of targeted therapy, multiple therapeutic targets expressed by GBM cells and/or GSCs have been explored and some evaluated, but sadly thus far these attempts have produced little to none in terms of improved patients' survival (Pearson and Regad, 2017). The case in point are trials with inhibitors of oncogenic EGFR which was

convincingly rationalized as a suitable therapeutic target in GBM. In this regard several agents have been tested in clinical trials and their effects have been disappointing and baffling.

Among the reasons for lack of efficacy of EGFR inhibitors are the heterogeneity of cancer cells for expression of EGFR and EGFRvIII (Inda *et al.*, 2010), loss of episomal genetic material containing EGFR sequences during therapy (Nathanson et al., 2014), existence of redundant signaling pathways that can circumvent EGFR inhibition, including PDGFR, MET and others (Hegi et al., 2011), concomitant loss of PTEN and sustained AKT signalling (Mellinghoff *et al.*, 2005) and possibly several others.

As far as immunotherapy is concerned, in GBM several approaches have been attempted including vaccines against EGFRvIII and other antigens and immune checkpoint blockade (Preusser et al., 2015; Reardon et al., 2014). These efforts were met with limited and transient successes thus far. It is increasingly clear that GBMs are immunologically 'cold' tumors – a simple term used to describe tumors in which poor recruitment of immune and accessory cells results in lack of efficacy associated with the checkpoint blockade (Lim *et al.*, 2018). Similarly to other rational approaches, initial studies were promising, yet the preclinical results did not translate into meaningful benefit for patients with GBM (McGranahan et al., 2019). This challenge is best exemplified by the phase III trial of nivolumab (monoclonal antibody against PD-1) which despite the promise demonstrated by the drug in multiple preclinical contexts (Zeng et al., 2013), had to be terminated prematurely after failure to meet its primary endpoint of overall survival (Jackson et al., 2019). Some studies have documented an upregulation of alternative immune checkpoints in association with development of adaptive resistance to therapeutic PD-1 blockage (Koyama et

al., 2016) and accordingly, concomitant targeting of multiple immune checkpoints is being evaluated with once again promising preclinical results emerging from the concurrent targeting of PD-1 and TIM-3 (Kim et al., 2017). GBM exhibits resistance mechanisms to all phases of antitumor immune response with intrinsic resistance preventing the initiation of an immune response, the tumor infiltrating immune cells are 'deactivated' via adaptive resistance, and acquired resistance helps protect the tumor from complete elimination when immune activation is transiently achieved. As such, in order to successfully achieve tumor regression in an immunotherapy-resistant malignancy like GBM, all the arms of immunoresistance will have to be targeted synergistically and this will require a deeper understanding of the complex interactions between GBM and the host immune system (Jackson *et al.*, 2019)

Unfortunately, as mentioned earlier, despite various iterations of the same treatment regimen, as well as more novel modalities, none of the treatments are curative and the National Comprehensive Cancer Network (NCCN) recommends that eligible patients are enrolled in clinical trials in hopes that new approaches, could ultimately result in better outcome or cure (Weller et al., 2017).

1.1.5 *Accompanying Complications and Thrombosis in GBM patients*

Common GBM-accompanying complications include cognitive deficits, changes in personality, and mood disturbances (Pace et al., 2017) in conjuncture with peritumoral vasogenic edema and seizures, which are often managed with corticosteroids and anti-epileptic drugs (Wen *et al.*, 2020). In addition, GBM induces systemic perturbations including immunosuppression and thrombosis. The latter is both life threatening and concerning among GBM-associated

complications as the high risk for venous thromboembolism (VTE) presents during the perioperative period and extends well beyond, as indicated by the one-year incidence of 20% (Czap et al., 2019).

The elevated VTE risk in GBM patients represents a startling reminder that this disease occurring almost exclusively within the CNS exerts potent and puzzling perturbations across the vascular system, both locally and systemically. Indeed, vascular abnormalities represent a pathognomonic hallmark of GBM progression. Important local aspects of GBM-associated vascular pathology include several events such as endothelial cell proliferation and increased vascular permeability, along with perivascular invasion and vessel cooption by subsets of cancer cells (Cheng et al., 2013; Kuczynski and Reynolds, 2020). Moreover, intratumoral activation of the coagulation pathway (microstroke-like lesions) (Brat et al., 2004; Perry, 2012) formation of platelet-rich microthrombi, inflammatory stroma and angiogenic blood vessels are interspersed with areas of hypoxia and pseudopalisading necrosis, which are signature features of GBM, thought to represent responses to microvascular thrombo-occlusion of pre-existing or newly formed blood vessels (Brat *et al.*, 2004).

Intratumoral vaso-occlusion occurs with staggering frequency of 92% in GBM, but not in grade III, II or I astrocytomas or brain tumors with other histology (Tehrani et al., 2008). In addition, in spite of exclusively intracranial location, and as mentioned earlier, GBM elicits extraordinarily high risk of systemic venous thromboembolism (VTE, 2% per month of survival) comprising deep vein thrombosis (DVT) and pulmonary embolism (PE). In a study by Simanek et al. pulmonary embolism was diagnosed in 9 out of the 15 patients (60%) who developed VTE

within the study cohort; 2 out of the 9 cases were fatal (Simanek et al., 2007). This is alarming as in one study VTE was found to be the second leading cause of cancer-related deaths (Khorana et al., 2007). Yet another study on 450 patients with GBM, reported that 145 (32.2%) developed VTE and of these, 11 (7.6%) experienced PE, 117 (80.7%) had DVT and 16 (11%) had DVT, as well as PE (Edwin et al., 2016). It should be mentioned that VTE worsens patient prognosis and outcomes across a wide spectrum of cancers (Timp et al., 2013). The frequency and severity of these comorbidities in GBM is second only to pancreatic cancer and mechanistically poorly understood, which is an area of concern and one of the driving forces of this thesis project.

1.2 Hemostasis – Physiology and Pathology

Thrombosis and hemorrhage associated with cancer and other disease states exemplify pathological dysregulation in the regulatory circuitry that maintains the integrity of blood circulation within the vascular system and is referred to as the hemostatic system. Hemostasis is a tightly controlled and well conserved machinery (from zebrafish to humans) at the center of which is the process of blood clotting, also known as coagulation. Hemostasis allows for three major and vital processes to take place: the re-sealing of a damaged blood vessel, maintaining the blood's fluidity, and the eventual resolution of clots following the restoration of vascular integrity (Versteeg et al., 2013). These processes involve at least three different hemostatic compartments including the activities associated with the vascular wall (endothelium and subendothelium), circulating platelets and the network of clotting proteins in plasma, the functions of which are integrated and molecularly interlinked (Adams and Bird, 2009).

1.2.1 *Discovery and Components of the Hemostatic System*

The hemostatic system is complex, and its discovery occurred gradually over centuries. Plato described the fact that blood forms fibers outside the human body approximately two millennia ago and proposed the term fibrin used until today to refer to the key protein essential for the formation of fiber-like structures, the scaffold on which blood clotting occurs (Becker, 2000). Plato's views, shared by others like Aristotle and Galen, did not change fundamentally until the 19th century, during which ground-breaking work was conducted to further understand the biological mechanisms of blood coagulation. Platelets were discovered around 1865 and their critical implication in hemostasis was established (Bizzozero, 1881). Later, around 1872 Alexander Schmidt proposed the existence of a protein, "thrombin", that could induce the formation of fibrin and in 1894 prothrombin was discovered by Pekelharing (Shen, 2006)

In 1905, the first coagulation model was proposed in which thromboplastin, now more commonly referred to as tissue factor (TF), was released upon vessel damage resulting in the conversion of prothrombin to thrombin in the presence of calcium (an interpretation significantly revised in recent decades). Nonetheless, this process subsequently allows the formation of insoluble fibrin from the circulating soluble precursor, fibrinogen (Riddel Jr et al., 2007) resulting in generation of a clot. The exact chain of events required for orderly clot formation involves several additional proteins, which were revealed by deficiencies occurring in bleeding disorders, such as hemophilia, von Willebrand disease and other alterations in normal hemostasis (Adams and Bird, 2009). Many of these remaining coagulation factors known today, such as von Willebrand factor (vWF) and coagulation factors V, VII, VIII, IX, and XI (FV, FVII, FVIII, FIX, FXI) were characterized around the 1950s, at which point the initial links to pathological states

were drawn (FVIII deficiency in hemophilia A and FIX deficiency in hemophilia B) (Dent et al., 1990).

The early model incorporating these factors appeared around the 1960s and resembled a waterfall or cascade, hence the name "coagulation cascade" used until today. According to the cascade model, which includes two cascades termed as the intrinsic and extrinsic pathways, each clotting factor comprises a proenzyme that is converted into the active form by the clotting factor that is upstream of it. The intrinsic pathway got its name by virtue of the presence of all of its components within the blood, while the extrinsic pathway is initiated by an external factor – TF from the extravascular space that must come into contact with blood coagulation factors for the clotting process to occur (Davie and Ratnoff, 1964; Macfarlane, 1964).

1.2.2 *Coagulation Pathways*

Activation of the intrinsic coagulation pathway is triggered when blood comes in contact with a hydrophilic surface, such as exposed collagen of an injured blood vessel, resulting in autoactivation of FXII and interaction with functional kallikrein, ultimately leading to the activation of factors FXI, FIX, FX, and prothrombin cascade. The extrinsic pathway, on the other hand, is initiated by the interaction of TF with activated FVII (FVIIa), which is translated into direct and sequential activation of FX and prothrombin. The events involving formation of the active FXa, FIXa and FIIa (thrombin) are referred to as the common pathway, the effects of which are modulated by FVa and FVIIIa (Adams and Bird, 2009). The common pathway is presently the main target of anticoagulants used in clinical practice (DeWald et al., 2018; Weitz and Harenberg, 2017). These agents either interfere with the synthesis of clotting factors in the liver (Vitamin K

inhibitors), activate endogenous anticoagulants (low molecular weight heparin; LMWH), or directly inhibit FXa (Rivaroxaban, Apixaban, Edoxaban), or thrombin (Dabigatran) (Wolberg et al., 2015).

Under normal physiological conditions, following an insult to the blood vessel wall and endothelial damage, platelets adhere to subendothelium at the site of damage via the interaction of their integrin receptors with extracellular ligands and soluble proteins. Next, the exposure of blood to subendothelial mural cells expressing TF triggers the activation of the extrinsic cascade which results in formation of a small burst of thrombin activity that triggers platelet activation and feeds into the multiple reinforcement loops of the intrinsic cascade. The resulting generation of a strong thrombin burst culminates in generation of fibrin at the site of vessel damage and stabilizing the earlier formed platelet thrombi (Adams and Bird, 2009). It should be mentioned that hemostasis represents a solid state biochemistry where coagulation factors are assembled on lipid membranes or platelets, perivascular cells, parenchyma, or their derived extracellular vesicles, or occur on contact with the ECM (Versteeg *et al.*, 2013).

Three main phases comprise the process of coagulation in accordance with the current model (Monroe and Hoffman, 2006). First an initiation phase, whereby relatively low amounts of active thrombin are generated, followed by an amplification phase that boosts the amounts of active coagulation factors and finally a propagation phase during which binding of coagulation factors to highly procoagulant membranes of activated platelets and fibrin clot formation occurs (Adams and Bird, 2009; Mackman, 2009). In further detail, and as alluded to earlier, at the vertex of the initiation phase is the exposure of TF to blood and thus elements of the coagulation cascade,

resulting in the formation of the catalytic complex with FVIIa and culminating in the production of small amounts of thrombin. The essential role played by TF is highlighted by the fact that no known human cases of TF deficiency exist, and all murine TF knockout models exhibit embryonic lethality strongly suggesting that lack of TF is incompatible with viability (Carmeliet et al., 1996; Grover and Mackman, 2018; Mackman et al., 1993). In the amplification phase the 'tenase' complex is formed through the interaction of FIXa and FVIIIa and the resulting complex is crucial for sustaining hemostasis via enhanced FXa formation and thus accelerated thrombin production downstream (Mann et al., 2006). Finally, the propagation phase relies on the continued recruitment of activated platelets in order to enhance thrombin generation and create what is referred to as the 'thrombin burst', which in turn through the activation of FXIIIa will lead to generation of fibrin and stabilize the progressing clot (Adams and Bird, 2009).

To ensure the tight regulation of hemostasis and to prevent a widespread uncontrolled clot formation, an extensive negative control of coagulation exists. This negative control is accomplished mainly at two levels. First, the endogenous protease inhibitors (antithrombin, heparin cofactor II, TFPI, and C1 inhibitor) inactivate coagulation factors through targeting of their active sites. A second level of control is established via the enzyme-based protein C/protein S pathway (Versteeg *et al.*, 2013). The protein C (PC) pathway is a key anticoagulant mechanism the main function of which is to downregulate thrombin formation. Mechanistically, the PC system regulates and attenuates the activation of FX and prothrombin (FII) which promote fibrin formation. The key elements that make up the PC pathway are thrombin (IIa), thrombomodulin (TM), the endothelial PC receptor (EPCR), PC and protein S (PS). Thrombin bound to thrombomodulin, expressed on endothelial cells, activates PC. In turn activated PC (APC) cleaves

peptide bonds in activated coagulation factors FVIIIa and FVa and acts as a promoter of fibrinolysis via the inhibition of plasminogen activator inhibitor-1 (PAI-1) (Anastasiou et al., 2012). The latter is the main inhibitor of the fibrinolytic system that represents an additional layer of negative regulation that achieves its function through clot dissolution (fibrinolysis) driven by plasminogen following its activation to plasmin by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Rijken and Lijnen, 2009). The pathological inhibition or overactivation of the fibrinolytic system results in a distortion in the balance between the coagulation system and its major negative feedback regulators and may lead to either thrombosis or bleeding. (Chapin and Hajjar, 2015; Lin et al., 2020). In sepsis, trauma, and certain cancers (e.g., acute promyelocytic leukemia) coagulation may assume catastrophic and widespread characteristics often referred to as disseminated intravascular coagulation (DIC) where exhaustion of clotting factors may lead to bleeding (Levi and Sivapalaratnam, 2018).

1.2.3 *Vascular Hemostasis*

At the level of vasculature, the endothelium constitutes a dynamic interface separating the blood and deeper layers of the vascular wall. This unique position enables endothelium to participate in regulating the pro- and anti-coagulant forces (Furie and Furie, 2008; Hajjar and Muller, 2021). Endothelial cells exhibit region-specific phenotypes that result from either genetic wiring or external biochemical and biomechanical stimuli that trigger posttranscriptional and/or post translational changes. Such heterogeneity allows them to thrive in different microenvironments (e.g., the relatively hypoxic and hyperosmolar microenvironment of the kidney medulla versus the oxygen rich capillary bed in the lung) and help meet the specific metabolic needs of the surrounding tissue (e.g., tight junctions in the blood-brain barrier versus

the discontinuous endothelium in hepatic sinusoids) (Aird, 2007b; c). Importantly, endothelial cells produce thromboregulatory molecules. On the procoagulant end, endothelial cells in certain situations like sepsis could become triggered to express vWF which extends the stability of FVIII in circulation and mediates platelet adhesion to damaged vascular subendothelium (Peyvandi et al., 2011).

Pro-inflammatory conditions result in induced expression of TF and PAI-1 by endothelial cells and promote a procoagulant state (Sturtzel, 2017). Endothelial cells also produce anticoagulant mediators such as eicosanoids, nitric oxide (NO), CD39 (an ecto-adenosine phosphatase), tissue factor pathway inhibitor (TFPI), heparan, TM, EPCR and tPA which allow them to modulate the vascular and platelet reactivity throughout the early stages of thrombus formation process (Aird, 2007a; Hajjar and Muller, 2021). Prostacyclin (PGI2), which is the most important endothelial eicosanoid, blocks platelet reactivity, locally promotes the relaxation of the blood vessel and induces the production of cytokines (Marcus et al., 2005). NO is similarly an inhibitor of platelet activity and a short spanned vasodilator which exerts its function through cyclic guanylate monophosphate (GMP) (Moncada and Higgs, 1995). CD39 is membrane-associated apyrase that functions as an inhibitor of platelet activation by converting adenosine diphosphate (ADP) in releasate of activated platelets, and thus contributes to blunting of the propagation of further platelet activation and recruitment via the modulation of ambient ADP (Marcus *et al.*, 2005). TFPI, a proteinase inhibitor exerts its anticoagulant effect by precluding the formation of the TF/FVIIa complex while heparan potentiates the function of antithrombin (AT). Additionally, endothelial cells participate in regulation of coagulation at late stages of thrombus formation including thrombus resolution. In part, this is achieved by preventing excessive

thrombin generation by providing heparan proteoglycans that act as cofactors for antithrombin (AT) which functions as an inhibitor of activated coagulation factors X and II (FXa and FIIa i.e. thrombin) (Hajjar and Muller, 2021). However more importantly, endothelial cells provide the framework for the activity of the fibrinolytic system, the key anticoagulant thrombomodulatory pathway described earlier in section 1.2.2.

1.2.4 *Regional Regulation of Hemostatic Processes*

It is worth noting that endothelial-derived pro-and anti-coagulant factors are not expressed evenly throughout the vasculature. It was noted early on that bleeding events associated with hemophilia occur in specific vascular beds (joints) and not (rarely) elsewhere, which suggests that underlying coagulation mechanisms possess regional significance, rather than being uniformly utilized to maintain vascular integrity (Mackman, 2005). This is of interest as cancer-related thrombotic perturbations may bear some site-specific features based on these inherent features of the hemostatic system.

Several hemostatic effectors have been implicated in specific regions of the vasculature. For instance, while TM is expressed in all blood vessels, vWF acts mostly in veins, endothelial-derived NO is a predominant attribute of the arterial side of circulation, EPCR is expressed mostly in large veins and arteries and TFPI is a predominant feature of the capillary endothelium (Aird, 2007a). While reflective of endothelial cell heterogeneity, this also suggests that endothelial cells in different sites modulate the hemostatic balance using different combinations of pro and anti-coagulants in response to perturbations in hemostasis. Coupled with endothelial cell heterogeneity resulting in the deployment of varying repertoires of thromboregulatory molecules, there are other site specific properties that can play a role in the predisposition for and progression of a thrombotic

event (Aird, 2007a). For instance, certain features of the venous vasculature confer a layer of predisposition for the development of thrombosis (VTE/DVT) in the presence of additional triggers. Particularly in the lower extremities, venous valves are numerous and interestingly venous thrombosis in the lower limbs often occurs particularly in the venous pockets (Mackman, 2012). Blood flow in venous valve pockets is irregular and is often characterised by low oxygen tension, especially in conditions of reduced mobility (Bovill and van der Vliet, 2011). Thus, the pronounced presence of elements of the Virchow's triad (conditions required for thrombosis such as stasis, endothelial damage and blood hypercoagulability), like blood stasis, facilitated by the nature of the venous valve anatomy in combination with prothrombotic triggers renders venous valve pockets particularly susceptible to thrombosis (Brooks et al., 2009). Prothrombotic triggers setting off VTE are still subject of debate, however several studies proposed the involvement of TF in certain pathological conditions including cancer where TF is expressed by tumor cells, activated monocytes, circulating microvesicles (MVs) and possibly by activated ECs (Pawlinski and Mackman, 2010).

Activated endothelium contributes to setting the stage for the development of VTE. Indeed, a hypoxic or inflammatory insult to the endothelial lining promotes the expression of adhesion receptors P-selectin and E-selectin as well as vWF, rendering the endothelium prone to capture circulating leukocytes, platelets and TF+ MVs. Bound TF+ MVs, in combination with leukocytes (triggered to express TF) promote procoagulant conditions which could be very potent with pre-existence of blood stasis and could overwhelm the protective anticoagulant pathways (Mackman, 2012; von Brühl et al., 2012).

Arterial thrombosis on the other hand most commonly occurs in the context of atherosclerosis. Arteries have their own anatomical aspects that render them prone to atherosclerosis and subsequently a thrombotic event. Arterial branching points and extensive curvatures in arterial circulation are regularly exposed to blood flow disturbances under normal conditions and the corresponding proximal ECs are permanently sensitized for activation (Aird, 2007a). Pathological conditions, like atherosclerosis have been shown to render the primed endothelial lining overlaying an atherosclerotic plaque dysfunctional: exhibiting increased expression of adhesion molecules, TF and TFPI and/or reduced expression of TM and EPCR. However, the most dramatic occurrence in the arterial circulation is the atherosclerotic plaque rupture with fulminant thrombosis upon exposure of the pathological content, leading to acute regional ischemic events such as myocardial infarction or stroke (Aird, 2006; Crawley et al., 2000; Cybulsky and Gimbrone, 1991; Laszik et al., 2001; Walski et al., 2002).

1.2.5 *Hemostatic Role of Platelets*

Platelets are the second most prevalent cellular component of the circulating blood. They range in size from 2 to 5 μm (Bussel et al., 2000). The average life span of platelets once formed from their precursor megakaryocytes and released into blood stream, is between 7 and 10 days (Ghoshal and Bhattacharyya, 2014). Platelets lack nuclei, but they contain proteins and various forms of RNA prepacked into them during the process of their biogenesis which entails formation of long processes (proplatelets) by megakaryocytes. Being scattered throughout the entire vasculature, platelets can respond to various signals from the endothelium, circulating cells and other blood components (Koupenova et al., 2018).

A particular feature of platelets is their content of three distinct types of granules referred to as α-granules, dense or δ-granules, and lysosomes assembled by megakaryocytes during platelet formation (Morrell et al., 2014; Semple et al., 2011). In this regard, α-granules contain an array of proteins including chemokines, cytokines and growth factors essential for normal functionality. Dense or δ-granules, on the other hand, harbor small molecules like ADP, serotonin, glutamate, polyphosphates, histamine and calcium that play crucial roles in hemostasis (Koupenova et al., 2017).

Upon vessel wall injury, platelets adhere to the site of vessel damage through a process coordinated by multiple signaling cascades that are ultimately dependent on the expression of glycoproteins on the platelets surface (Jurk and Kehrel, 2005). This process is further facilitated by the decrease in the inhibitory functions exhibited by the otherwise intact endothelium including production of prostacyclin, NO and CD39 expression, and the exposure of the extracellular matrix proteins to circulation (Koupenova *et al.*, 2018). The mechanism underlying initial platelet binding is influenced by shear stress in the circulation, whereby under high shear stress the rapid unfolding and deposition of vWF occurs upon contact with subendothelial collagen at injury site. The unfolding process allows for the exposure of binding sites to platelets' CD42 receptor. The binding of CD42 to collagen bound vWF slows down platelet velocity and creates low shear stress conditions allowing direct adhesion between platelets and collagen fibers, predominantly mediated through their GPIa/IIa integrins. The result is platelet activation which triggers multiple physiological and cytoskeletal changes that include a major shape shift from the discoid (inactive) form to the highly irregular form possessing numerous pseudopodia (activated) (Ghoshal and Bhattacharyya, 2014; Jurk and Kehrel, 2005).

When injury is sufficient to expose blood coagulation machinery components to subendothelial TF, the extrinsic cascade is triggered, culminating in thrombin generation which mediates robust platelet activation and aggregation and firm anchorage of the formed hemostatic plug (Koupenova *et al.*, 2018). Thrombin-mediated platelet activation occurs through protease-activated receptors PAR1 (PAR-3 in mice) and PAR4, whereby PAR1 is favored under low concentrations of thrombin and PAR4 triggers platelet activation only at high thrombin concentrations (Coughlin, 2000; Offermanns, 2006) . Binding of thrombin to PAR1/4 activates downstream PLC signalling resulting in a series of platelet activation events including granule release, integrin activation and thromboxane A2 synthesis (Estevez and Du, 2017; Li et al., 2010). In depth, the formation of the hemostatic plug is a process resulting in a stringently governed architecture and starts with a core of fibrin and tightly packed activated platelets deposited at the site of exposure to extravascular space (Stalker et al., 2013). Activated platelets in turn, undergo internal trafficking of their granules to the surface membrane where the calcium mediated conformational change promotes membrane fusion to occur and granule cargo to be released (Golebiewska and Poole, 2014).

Platelet degranulation is highly complex and regulated, progress has been made towards deconvoluting the process and it has been established that Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor (SNARE) proteins are involved but the exact protein-protein interactions have not yet been teased out (Heijnen and Van der Sluijs, 2015). Detailed mechanistic data regarding how exactly internal calcium is implicated is also missing and given the ‘core and shell architecture’ of a growing thrombus (Crittenden et al., 2004), one could ask whether the

degranulation process is polarized and oriented in a way that facilitates such an organization (Joshi and Whiteheart, 2017). The released granule cargo, particularly ADP and thrombospondin, cooperates with secreted thromboxane and thrombin generated on the surface of activated platelets. Platelets expose phosphatidylserine providing binding sites for procoagulant zymogens and enzymes (Wolberg and Campbell, 2008), to allow for the creation of a platelet activation gradient. This gradient extending outwards from the inner core of the thrombus allows for further platelet activation, recruitment and thrombus growth (Koupenova *et al.*, 2018).

ADP secreted from δ-granules upon initial platelet-activating trigger along with the release of thromboxane are major contributors to further propagation of platelet activation which ensures rapid progression of hemostasis. Two receptors for ADP exist on platelet surface, P2Y1 and P2Y12, and both are G-coupled protein receptors (GPCRs) (Dorsam and Kunapuli, 2004) (Woulfe et al., 2001). P2Y1 is involved in platelet shape change and calcium mobilization. P2Y12 on the other hand, upon binding to ADP, functions to inhibit the activity of adenylyl cyclase which is responsible for cAMP generation and subsequent inhibition of platelet activation (Smolenski, 2012) (Dorsam and Kunapuli, 2004).

Thrombospondin (TSP-1), secreted from α-granules upon platelet activation, has been shown to be involved in modulation of the inhibitory cAMP signalling (Roberts et al., 2010) along with other functions such as inhibition of angiogenesis. Thromboxane A2 is a soluble potent agonist that binds to its GPCR on platelets which ultimately activate PKC via PLC and promote cytosolic calcium accumulation (Rucker and Dhamoon, 2020).

A number of pathological conditions can overwhelm the normal hemostasis resulting in an uncontrolled clot formation that could ultimately lead to vessel occlusion. One particularly relevant state involving platelets is a process often termed as immunothrombosis, whereby in response to a tissue damage (e.g. induced by a blood-borne pathogen like COVID-19 virus), platelets contribute to the immune reaction by acting in concert with immune cells, namely neutrophils (Bonaventura et al., 2021; Yang et al., 2020; Zhou et al., 2020). This results in the formation of a physical barrier in an attempt to confine the spread of infection (Engelmann and Massberg, 2013). It should be mentioned that different populations of leukocytes play a role in immunothrombosis, including monocytes that can be induced to express TF and neutrophils, which trigger clotting events through expulsion of genomic DNA and chromatin as neutrophil extracellular traps (NETs) (Hisada and Mackman, 2017; Kapoor et al., 2018; Martinod and Deppermann, 2021). Accordingly, several viral or bacterial infections have been shown to increase the risk for thrombosis and while immunothrombosis could contribute to infection clearance the interplay between neutrophils and platelets can form a vicious cycle that sets the stage for high risk of thrombosis. It should be noted that infection may not be the only factor activating the mechanism of immunothrombosis, through interaction of immune cells and the hemostatic system, and similar processes (e.g. NETosis) occur in cancer (Bunce et al., 2011; Tichelaar et al., 2012; Yang *et al.*, 2020).

1.2.6 *Non-canonical Roles of the Hemostatic System*

A growing body of evidence implicates various elements of the hemostatic system in cellular responses that extend beyond the regulation of hemostasis. These processes frequently involve activation of intracellular signalling and changes in gene expression induced by elements of the hemostatic system, which is a part of the larger wound healing program operative in normal tissues (Zelaya et al., 2018). Various cell populations have been shown to possess the required

molecular machinery to respond to signals from members of the hemostatic system (Magnus et al., 2014a). Hemostatic system proteins implicated in non-hemostatic roles include TF, PAR-1, uPAR, TM, EPCR, as well as integrins and growth factor receptors that become activated in response to interaction with elements of the coagulation system. As a consequence of such interactions, intracellular signalling pathways are triggered, and the expression of several genes is altered, including genes involved in angiogenesis and inflammation (Abe et al., 1999; Albrektsen et al., 2007; Bromberg et al., 1999; Camerer et al., 2000; Mackman, 2008; Ruf et al., 2011). For instance, TF expression by fibrosarcoma cells was linked to an enhanced pro metastatic phenotype (Morrow et al., 2018). In adipocytes, TF expression caused alterations in their metabolic circuitry in diabetic setting (Schwalie et al., 2018). Under proinflammatory conditions, TF expressed on the surface of monocytes has been shown to modulate complement-coagulation crosstalk (Zelaya *et al.*, 2018). Elements of cancer progression such as cell migration, invasion, proliferation, and cell death evasion, have been linked to TF-FVIIa complex signaling through PAR2 (van den Berg et al., 2012; Versteeg and Ruf, 2006), including evidence for cooperation between EGFR and TF-PAR2 signaling pathways in promoting drug resistance and hence poor survival in cervical cancer (de Almeida et al., 2018). Our own group and others have shown that TF expression in a cell line model of glioblastoma that otherwise produces indolent xenografts *in vivo*, is capable of promoting the cessation of tumor dormancy and initiating full progression of tumor growth (Dillekås and Straume, 2019; Magnus et al., 2014b).

Additionally, activated platelets release a broad spectrum of active molecules, one of which is TGF-β, and platelets contain the largest amount of TGF-β in the body (Semple *et al.*, 2011). Numerous works have suggested that platelet-derived TGF-β is implicated in numerous

physiological and pathological scenarios that range from normal lung development (Suzuki-Inoue and Tsukiji, 2020), to direct suppression of natural killer cell activity (Kopp et al., 2009) as well as influencing the function of particular subsets of regulatory T cells (Yu et al., 2008a). Interestingly, work on dormant lesions of lipo- and osteosarcomas by Cervi et al. suggested an antiangiogenic role for another platelet derived chemokine, platelet factor-4 (PF4), released from α-granules upon activation (Cervi et al., 2008). The authors speculated that PF4, through its capacity to bind VEGF (Rybak et al., 1989), could play a role in counterbalancing the tumor cell-released VEGF and thus contribute to maintaining the tumor in a nonangiogenic dormant state (Cervi *et al.*, 2008).

Taken together, the aforementioned observations support the existence of a complex crosstalk between effectors of the coagulation system and other non-hemostatic cellular systems, acting as components of the tissue injury response program and wound healing processes. At the same time these mechanisms possess a considerable disease-causing/exacerbating potential, and their aberrant or unscheduled triggering could have serious implications for the progression of a given pathology, including human malignancies.

1.2.7 *Extracellular Vesicles in Hemostasis and Thrombosis*

Remote transmission of coagulant and regulatory signals is often attributed to release and trafficking of cellular fragments known as extracellular vesicles (EVs). EVs constitute an increasingly recognized route of intercellular communication and regulation due to their ability to interact with recipient cells and homeostatic systems, including the coagulation cascade (Mathieu et al., 2019). Under both, physiological, as well as pathological conditions, cells release these

cargo-loaded and lipid-bilayer enclosed particles which are capable to exert local and systemic effects (Mause and Weber, 2010; Minciacchi et al., 2015; Pilzer et al., 2005; Ratajczak et al., 2006; Yuana et al., 2013). Extracellular vesicles is a term that covers a broad spectrum of heterogeneous vesicles that differ in size and content, as well as mode of biogenesis (Zijlstra and Di Vizio, 2018). EVs are thought to contribute to a wide range of biological processes mainly through constituting an alternative mode of secretion of cellular mediators and molecular transfer, as well as elimination of excess or unrequired material from cells of origin (Rak, 2013).

Many types of EVs have been distinguished on the basis of different criteria such as size, source, cargo and underlying mechanism of biogenesis. While the main EV subsets are often classified as small and large exosomes (EXs), microvesicles, (MVs), and apoptotic bodies (ABs) other terms have also been coined such as microparticles (MPs), oncosomes (OSs), large oncosomes (LOs), migrasomes (MSs) and several others to reflect the structural, functional or molecular features of particles in question (Al-Nedawi et al., 2008; Camussi et al., 2010; Ratajczak *et al.*, 2006; Zhang et al., 2018). In addition, cells produce non vesicular extracellular particles, of which exomeres represent one of the recently described examples (Zhang *et al.*, 2018). While EV classification has enabled more systematic studies of their distinct categories their true molecular diversity is far greater and can be inferred from proteomic datasets that predict the existence of dozens of EV subsets (Choi et al., 2019b). This diversity could be partially revealed by the emerging technologies such as single EV flow cytometry and molecular profiling using microfluidic devices (Choi et al., 2019a)

Broadly, the segmentation of EV subsets is largely predicated on their biogenesis. This approach differentiates two main processes: membrane budding and exocytosis of intracellular vesicles and both processes produce EVs of a wide range of size and physical properties (Van Niel et al., 2018). Thus membrane-derived MVs which are generated by membrane budding and often referred to as ectosomes exhibit a size range of ~160nm - 1μm, enrichment of integrins and resemblance to their cells of origin. EXs on the other hand, are vesicles of a smaller size range of ~30nm – 150nm and pronounced presence of tetraspanins (CD81, CD82, CD9, CD63, and TSPAN8), heat-shock proteins (HSP70, HSP90), ESCRT complex related proteins as well as endosomal proteins (TSG101, ALIX and LAMP2) and others (Kowal et al., 2016; Mathivanan et al., 2010; Simons and Raposo, 2009).

EVs could also be generated through the process of apoptosis, which results in vesicles that are referred to as ABs. Although ABs could exceed 2μm in size, small apoptotic vesicles (50nm to 150nm) have also been described (György et al., 2011; Montermini et al., 2015). Another category of mostly large EVs left behind on cellular migratory tracks have been recently identified and dubbed as migrasomes (Ma et al., 2015). In the context of cancer cells, another subset of large EVs associated with ameboid motility are referred to as LOs, their size ranges between 1μm and 10μm, and they are often enriched in cellular proteins including oncogenes (Di Vizio et al., 2009). However, it is worth noting that the term LOs should not be used synonymously with oncosomes, which are cancer cell derived particles conforming in size to EXs and MVs categories, and were the first to be identified to carry oncoproteins whence their original name (Al-Nedawi *et al.*, 2008; Meehan et al., 2016).

As mentioned earlier, the current classification of EVs into different categories is in part tied to their respective process of biogenesis. The blebbing of cellular membranes generated MVs or ectosomes, the inward budding of endosomal membrane leading to the formation of the multivesicular body (MVB) followed by the generation of intraluminal vesicles and their translocation to the plasma membrane results in EXs, an ABs, as alluded to earlier, result from the breakdown of dying cells. LOs are the product of 'pinching off' of cellular protrusions of migrating malignant cells, while particles referred to as exosome-like small EVs may detach from tips of cellular cilia (Al-Nedawi et al., 2009; Di Vizio *et al.*, 2009; Johnstone, 2006; Nager et al., 2017; Rak, 2010; Simpson et al., 2009; Théry et al., 2009; Tilley et al., 2008).

Significant efforts invested in understanding the differences between various subsets of EVs have shed light on many aspects of the vesiculation machinery and highlighted the highly complex nature of the process. In particular, enzymes that control the symmetry of the plasma membrane phospholipid bilayer, sphingomyelinase (ASMase), flippases and scramblase (TMEM16F) were implicated in the formation of MVs. Other small GTP-ases like ARF6 and Rho and kinases like LIMK and MKK4 were also found to contribute to the control of the process. The close association of MVs with plasma membranes enriched in lipid rafts, is often reflected by their cargo. Molecules associated with lipid rafts such as TF, flotillin-1, P-selectin glycoprotein ligand-1 (PSGL1) and integrins are often present on MVs (Al-Nedawi *et al.*, 2009; Bianco et al., 2009; Del Conde et al., 2005; Garnier et al., 2012; Heijnen et al., 1999; Muralidharan-Chari et al., 2009; Pilzer *et al.*, 2005; Thomas et al., 2009).

Exosome biogenesis is controlled by specific cellular pathways that either utilize the endosomal signalling complex required for export (ESCRT) or are ESCRT independent and involve neutral sphingomyelinase (SMPD3) and tetraspanins (Van Niel *et al.*, 2018). In some of these instances the activation of the cell surface receptor recycling pathway is a key initial step (Pilzer *et al.*, 2005; Simons and Raposo, 2009; Théry *et al.*, 2009; Van Niel et al., 2011). Initiation begins with an inward invagination of the plasma membrane microdomain coated with clathrin, a structure often referred to as clathrin-coated pit (Murphy et al., 2009). Upon detachment into the cytosol, these clathrin-coated invaginations fuse and give rise to early endosomes which mature into late endosomes/multivesicular bodies filled with small intraluminal vesicles under the control of the multimolecular assembly of proteins known as elements of ESCRT pathway (Van Niel *et al.*, 2018). Alternatively to ESCRT mediated formation, the biogenesis of ILVs can involve bioactive, curvature generating membrane lipids, such as ceramide, generated by neutral sphingomyelinase (NSMase2) from sphingomyelin (Trajkovic et al., 2008). Recent work by Baietti et al demonstrated a role for syndecan-syntenin-Alix driven regulation of exosome biogenesis where syndecan, a cell surface transmembrane glycoprotein interacts with Alix through syntenin, which functions as an adaptor between syndecan's cytoplasmic portion and Alix. This interaction combined with the association of Alix with TSG101 and charged multivesicular body protein (CHMP4) was implicated in driving ILV formation and cargo sorting (Baietti et al., 2012; Odorizzi, 2006). Other processes of vesicular biogenesis exist and rely on complex multimolecular interactions, some of those include RHOA/ROCK signalling and other regulators of actin cytoskeletal rearrangement (Di Vizio *et al.*, 2009; Montermini *et al.*, 2015; Morley et al., 2014).

It is important to note that just like EVs are highly heterogeneous, their molecular content could display an equally profound diversity. It has been estimated that an average of ~2500 proteins could be contained in the vesiculome of a given cell and given size limitations, an EV would permit the accommodation of up to ~200 proteins. This results in an almost endless range of protein combinations associated with EVs released from a population of cells (Choi et al., 2017; Garnier et al., 2013; Zhong et al., 2018). Consequently, as mentioned earlier, technologies have been developed to allow for single EV analysis and pattern analysis including complex molecular composition of individual EVs in general and cancer derived EVs especially. Two prominent examples of such emerging technologies are microfluidic devices and nano-flow cytometry (Choi et al., 2018; Shao et al., 2018).

A substantial body of literature provides support for a role of EVs in hemostasis and thrombosis while outlining their procoagulant characteristics and mechanisms by which they influence the thrombotic process (Geddings and Mackman, 2013; Hisada and Mackman, 2017; Zarà et al., 2019). Among the first observations to this effect, coagulant properties of the 'platelet dust', particles related to activated platelets (Hargett and Bauer, 2013), emerging role of microparticle-associated phospholipids (Dachary-Prigent et al., 1997) and works from Nemerson's group indicating that EVs (microparticles) carry bioactive TF (Giesen et al., 1999). Indeed, EVs are likely to have a role in physiological hemostasis as indicated by hemorrhagic disorders linked to genetic defects in platelet vesiculation (e.g. Scott syndrome) (Burnier et al., 2009) and bleeding phenotype of Rab27a/b deficient mice (Tolmachova et al., 2007). Since Rab27a/b genes have been shown as crucial effectors in exosome biogenesis (Van Niel *et al.*, 2018) it could be reasoned that their disruption reveals the role of exosomes in hemostatic functions.

In malignant setting various types of EVs have been implicated in thrombosis through their content of bioactive TF, mucins, phosphatidylserine (PS) and other components involved in clotting events (Geddings and Mackman, 2013). While in certain cancers TF-carrying EVs have been shown to possess procoagulant activity (Yu and Rak, 2004) and were implicated in triggering thrombosis (Wang et al., 2012), the analysis of blood samples from GBM patients failed to demonstrate a predictive value of TF-EVs (also referred to as microparticles) in VTE (Thaler et al., 2012). It is possible that while TF is readily released from cancer cells as cargo of EVs (Garnier *et al.*, 2012), in some instances, this process might involve TF encryption (steric modification) (Bach, 2006) resulting in the absence of the related procoagulant activity in the circulation (Thaler *et al.*, 2012).

To date, PDPN remains the strongest correlate of VTE in the context of GBM (Riedl and Ay, 2019; Riedl et al., 2017; Watanabe et al., 2019). In this thesis we document that PDPN carrying EVs act as potential effectors of peripheral thrombosis. PDPN is released by GBM cells *in vitro* and *in vivo* and retains platelet stimulating activity in experimental settings (Tawil et al., 2021). However, further research is required to determine whether PDPN-EVs also contain other accessory molecules involved in thrombosis and are true predictors of VTE. Moreover, such EVs may possess other biological activities which are presently largely unstudied.

1.2.8 *Pathomechanisms of Thrombosis*

Pathological states within the hemostatic system may either emerge due to its insufficiency and resulting bleeding disorders (e.g., in hemophilia) or may stem from overactivation of clotting processes in either arterial, venous, or capillary circulation, defined respectively, as arterial

thromboembolism (ATE), VTE or microvascular thrombosis (MVT). In all forms of thrombosis underlying pathophysiology is often explained by the components of the Virchow's triad which postulates that thrombosis may arise through perturbations in blood flow, vascular wall integrity and hypercoagulable state of the circulating plasma. For example, prothrombotic conditions may arise in the face of diminished blood flow (stasis), changes in the state of the vessel wall (damage), and alterations in the blood composition (hypercoagulability) one or more of which are at the core of thrombosis (Næss et al., 2007).

VTE is the most studied form of thrombosis and one with the best understood implications of the interplay between cancer and the hemostatic system. This link is attributable, in large part, to the high frequency of VTE in cancer patients, albeit with a degree of disease specificity (Falanga et al., 2017). Therefore, in the context of GBM, understanding the processes leading to thrombotic occlusion of peripheral veins is of paramount interest. Advancements in the understanding of VTE reveal a role of all components of the Virchow triad. Blood stasis and low oxygenation, the presence of venous valves and local blood flow perturbations, may lead to localized endothelial cell activation and recruitment of innate immune cells, all of which may breach the threshold for antithrombotic mechanisms and activate coagulation cascade and platelets. While platelets are central to ATE (Mackman, 2008) they also have a role in VTE, as do red blood cells, leukocytes, and procoagulant extracellular vesicles (EVs), also known as microparticles (Wolberg *et al.*, 2015).

Inflammation is closely intertwined with thrombosis. Inflammatory mediators may lead to the exposure of TF on the surface of activated endothelial cells and monocytes, or emission of

neutrophil extracellular traps (NETs) contributing to the VTE risk in various settings (Wolberg *et al.*, 2015). Also an imbalance between pro and anti-coagulant effectors in the blood, including levels of plasminogen activator inhibitor 1 (PAI-1), tissue factor pathway inhibitor (TFPI) and other regulators may also be involved in the development of VTE (Mackman, 2012; Reitsma et al., 2012). It should be mentioned that this complexity makes prediction of VTE in individual patients very challenging, and efforts are underway to develop the VTE risk scores linked to various clinical and pathobiological variables (Unlu and Versteeg, 2018). Work from our laboratory, including this thesis suggests that such scores should incorporate not only purely hematological considerations, but also unique molecular and cellular traits of underlying pathology, especially in cancer.

Changes in concentrations and functions of hemostatic proteins as a result of genetic, regulatory or pharmacological influences could drive the VTE risk. For example, common germline mutations affecting coagulation factor V (factor V Leiden) may cause increased VTE risk (thrombophilia) due to insensitivity of this mutant protein to inhibitory effects of activated protein C (APC). Several other germline mutations may also increase the risk of thrombosis (Dautaj et al., 2019).

The focus on the role of coagulation factors has shaped the basis underlying the currently adopted treatment of VTE relying on heparins and vitamin K antagonists as well as direct inhibitors of factor Xa and thrombin (DOACs) (Posch et al., 2015). However, more recent works suggest that inflammation-induced alterations in cells, cellular components and platelets can be equally pivotal in promoting thrombotic risk (von Brühl *et al.*, 2012). The interpretations of such studies

are in part echoed in the current consensus regarding venous thrombosis clinical risk factors like surgery, obesity, smoking as well as acute and chronic infection (Anderson Jr and Spencer, 2003; Goldhaber, 2010; Reitsma *et al.*, 2012), as well as in studies reporting a positive impact of aspirin on VTE prevention and prophylaxis (Nam et al., 2015; Simes et al., 2014), thus further implicating the platelet compartment in VTE. A perspective shift could become very important from the VTE management standpoint. For instance, if platelets and inflammation are equally critical determinants of thrombotic risk, treatment approaches that account for platelets involvement could present an alternative and possibly safer and more effective strategy in managing VTE in cancer (Versteeg *et al.*, 2013). Of course, such changes must be based on evidence and exercised with caution.

1.3 The Evolving Landscape of Cancer Associated Thrombosis

Progression of human cancers has long been known to increase the risk of thrombosis, a comorbidity that worsens the outcomes and affects disease biology (Falanga *et al.*, 2017; Hisada and Mackman, 2018; Rickles and Falanga, 2009; Trousseau, 1865). Indeed, CAT is relatively prevalent (in some cases more than 20%) (Wun and White, 2009), morbid, and sometimes life-threatening (Khorana *et al.*, 2007). However, a consensus as to its biological underpinnings, optimal medical interventions, and follow-up strategies is still to emerge due to baffling pathogenic complexities and diagnostic challenges (Falanga *et al.*, 2017; Hisada and Mackman, 2018). A striking disconnect seems to exist between the relatively limited spectrum of interventions used to manage CAT in cancer patients (Kahale et al., 2018) and the dizzying diversity of the underlying (CAT-inducing) neoplastic states (D'Asti and Rak, 2016).

Moreover, the increasingly personalized approaches to cancer treatment based on molecular distinctions between disease-causing biological mechanisms and tumor subtypes stand in contrast to the analysis and management of clinical manifestations of CAT often studied across wide boundaries of fundamentally different disease entities (Kahale *et al.*, 2018). The implicit assumption in this area is that there is certain basic similarity between the causes, consequences, mechanisms, and targeting points between thromboses occurring in different cancers and that their salient characteristics converge, at least to some extent, with coagulopathy associated with non-malignant conditions. Therefore, similar diagnostic approaches, clinical analyses, and prophylactic and therapeutic countermeasures could be applied to thrombosis in these diverse contexts. While this has been a useful paradigm it may require revision if further progress is to be achieved. It could be argued that CAT deserves a special consideration among different procoagulant states recognizing the complexity of the triggering disease (D'Asti and Rak, 2016; Falanga *et al.*, 2017; Geddings and Mackman, 2014; Kuderer and Lyman, 2014; Rickles and Falanga, 2009).

In cancer, the hemorrhage, thrombosis, and vaso-occlusion may occur both within the tumor capillary network (microthrombosis) and in peripheral blood vessels, and the exact linkage between these aspects of the disease is presently unclear. In clinical terms, CAT is manifested by the heightened risk of thromboembolism in either the venous (VTE) or arterial (ATE) macrocirculation (Falanga *et al.*, 2017; Navi et al., 2017), the former being more frequent, and these events are associated with considerable morbidity and mortality, at least in part, due to pulmonary embolism (PE) which is a frequent event in VTE along with accompanying deep vein thrombosis (DVT) (Khorana *et al.*, 2007).

The onset of CAT is generally associated with worsening of the overall survival and unfavorable disease outcomes (Blom et al., 2005; Ouaïssi et al., 2015; Timp *et al.*, 2013). This relationship is documented in several studies which point to VTE as the second leading cause of cancer-related deaths (Khorana *et al.*, 2007) and as a condition otherwise negatively affecting overall survival. For example, in one study, patients with cancer and VTE, cancer alone, or VTE alone were found to face the risk of death that is up to 30-, 7-, and 3-fold higher, respectively, relative to the control cancer-free population (Timp *et al.*, 2013). Several mechanistic triggers of such impact have been investigated, including the role of activated coagulation system in invasion and metastasis (D'Asti et al., 2016).

This emerging link is, at least in part, due to the fact that the coagulation system in cancer exerts multiple biological effects outside of its canonical hemostatic role. For instance, clotting proteins and platelets influence the phenotypes of cancer cells, as well as stromal, inflammatory, immune, vascular, and perivascular cell compartments (Haemmerle et al., 2018; Zelaya *et al.*, 2018). Among more striking examples in this regard are findings that suggest that pre-existing thrombophilia, such as homozygous Factor V Leiden mutation is associated with elevated risk for colorectal cancer development (Vossen et al., 2011). Experimentally, genetic modulation of thrombin activity drives colitis associated neoplasia in mice (Turpin et al., 2014), the coagulation system is crucial for cancer metastasis (Adams et al., 2015), while the expression of TF in glioma xenografts regulates the dormant state or tumor progression impacting the evolution of cancer cell genome and epigenome (Magnus *et al.*, 2014b). These interactions are often reciprocal, as indicated by the cross-talk between coagulation pathways and inflammatory circuitry

(Steinbrecher et al., 2010), innate immunity (Massberg et al., 2010; Palumbo and Degen, 2010), and connected with mechanisms that collectively impact cellular growth, angiogenesis, invasion, metastasis, and therapeutic responses (Degen and Palumbo, 2012; Morrow *et al.*, 2018; Schwalie *et al.*, 2018; van den Berg *et al.*, 2012). The respective signals elicited by cell contact with clotting proteins are transmitted into cells through membrane receptors that act as "sensors" of various facets of the coagulation system. Such sensors include protease activated receptors (PARs), tissue factor (TF), thrombomodulin (TM), endothelial protein C receptor (EPCR), urokinase receptor (uPAR), and other cellular proteins (Coughlin, 2005; Ruf, 2007).

In this regard it is of interest that the expression of the TF gene (*F3*) was identified in the epigenetic screen as a crucial event linked to the dissemination of fibrosarcoma cells driven by a metastatic variant enhancer locus (Met-VEL) (Morrow *et al.*, 2018). TF expression (CD142+ phenotype) of adipocytes was also linked to their metabolic functions in diabetes (Schwalie *et al.*, 2018), a finding that enforces cellular roles of this and other coagulation effectors. Several examples of cancer-related signaling events mediated by coagulation receptors have been characterized and implicated in various aspects of disease pathogenesis, ranging from metastasis and angiogenesis to drug resistance (de Almeida *et al.*, 2018; van den Berg *et al.*, 2012; Zelaya *et al.*, 2018). It is presently unclear whether, and to what extent, the clinically used pharmacological thromboprophylaxis and anticoagulation measures meaningfully impact cellular events associated with cancer and whether their modification may impact patient survival (Falanga *et al.*, 2017; Griffiths et al., 2009; Lee and Peterson, 2013). From a mechanistic standpoint, it is reasonable to consider CAT as an aftermath of dysregulated expression and/or function of canonical effector

mechanisms within the hemostatic system caused by cancer cell phenotype, either directly or indirectly.

Among hemostatic effectors deregulated in cancer, TF plays a central and initiating role, which has long been of interest in the context of CAT (Grover and Mackman, 2018). TF acts as a transmembrane receptor for the blood borne coagulation factor (F) VII/VIIa, with which it forms a complex (TF/FVIIa) that activates coagulation FIX and FX to FIXa and FXa, respectively. FXa generates thrombin (FIIa), which then activates platelets (through PARs), while also triggering intracellular signalling (Zelaya *et al.*, 2018). Extracellularly these events trigger the amplification phase of the coagulation cascade with the involvement of FVIIIa and FVa (Adams and Bird, 2009). The resulting burst of thrombin activity leads to the conversion of plasma fibrinogen to an insoluble fibrin clot accompanied by the additional activation of platelets (Adams and Bird, 2009). These processes are also influenced by the extrinsic coagulation pathway (FXII, FXI) and several regulatory feedbacks (Aharon and Brenner, 2009; Lacroix et al., 2013) that control the magnitude and duration of the hemostatic response. An excessive, prolonged, unscheduled, or displaced clotting process results in thrombosis, vascular occlusion, factor consumption, poor hemostasis, and other pathological manifestations frequently associated with CAT. Cancer cells enter these processes at multiple levels as expressors of TF, FVII (and other clotting factors, PAI-1, PDPN and other molecules (Tawil *et al.*, 2019).

Consequently, current interventions target key points in the aforementioned cascade focusing mostly on the common coagulation pathway (FIIa and FXa) (Geddings and Mackman, 2014; Kahale *et al.*, 2018). While clinically effective, this approach inherently leads to the

weakening of physiological hemostasis and to an increased and often severe risk of bleeding (Falanga *et al.*, 2017; Mackman, 2008). It is noteworthy that, for practical reasons, current medical approaches to targeting hemostatic pathology and thrombosis, such as CAT, usually do not take into consideration context specific peculiarities of the disease. For example, structural, mechanistic, and disease-specific properties of the clot formation process are generally not factored into prophylactic or therapeutic protocols and neither are the intricacies of regulatory balances in specific cases, spatial distribution of coagulation effectors such as TF, role of microparticles/extracellular vesicles (MPs/EVs), myeloid cells and platelets, heterogeneity of the vasculature, fine architecture of the fibrin polymer, or other biologically meaningful but difficult to measure individual variables (Davies et al., 2015; Grover and Mackman, 2018; Stark et al., 2018).

Nonetheless, efforts are underway to understand what is unique about pathological thrombosis versus physiological hemostasis and, more specifically, what are the upstream and more cancer specific causes (inducers and modulators) of CAT in comparison to cancer-unrelated mechanisms of thrombosis (Geddings and Mackman, 2014; Hisada and Mackman, 2018). The emerging picture suggests that some of the elements of the coagulation cascade may be nonessential for hemostasis (e.g., FXII) and thereby potentially targetable in the context of CAT without a cost of bleeding (Geddings and Mackman, 2014). Moreover, specific neoplastic disease states may exhibit intrinsically, quantitatively, and qualitatively different propensities to engage, directly or indirectly, the various compartments of the coagulation system and trigger thrombosis (D'Asti and Rak, 2016; Rak and Klement, 2000)

1.4 Coagulopathy in GBM: Incidence and Mechanistic Underpinnings

As mentioned earlier, GBM is not only the most aggressive and common form of primary astrocytic brain tumors (Wen and Kesari, 2008), but also the most procoagulant among them (Perry, 2012). This includes both highly prevalent microthrombosis within the tumor vasculature (90%) (Tehrani *et al.*, 2008) and a high risk of peripheral VTE (26–30%) (Perry, 2012). While high TF expression and circulating TF-exposing microvesicles (TF-MVs) have been reported in GBM patients (Sartori et al., 2013; Thaler *et al.*, 2012; Unruh et al., 2016), to date, one of the strongest molecular correlates of VTE in this setting appears to be the expression of podoplanin (PDPN) (Riedl *et al.*, 2017). PDPN is an activating ligand for platelet receptor CLEC2 and a crucial regulator of vascular development and platelet activity (Takemoto et al., 2017a). Notably, GBM patients with high levels of tumor associated PDPN exhibit the highest VTE risk and low levels of platelets, likely due to consumption (Riedl *et al.*, 2017). While this correlation points to the rate limiting role of PDPN it does not preclude contributions of other factors, such as TF, as will be documented in this thesis project. Notably, the nature of processes regulating PDPN, TF, and other aspects of GBM coagulome is not fully elucidated, but there are several emerging links with the oncogenic "wiring" of cancer cells, both at the level of genome and epigenome.

Genetic characteristics of cancer cells exert a profound impact on the regulators of vascular responses including CAT. This notion was originally proposed by our laboratory (Milsom et al., 2008; Rak and Klement, 2000; Yu et al., 2004; Yu et al., 2005) and later supported by findings from several groups (Boccaccio et al., 2005; Rong et al., 2009; Rong et al., 2005). Clinically, colorectal cancer patients with lesions positive for oncogenic KRAS mutations have been reported to suffer from an elevated frequency or VTE (Ades et al., 2015), while the opposite effect was

observed in the case of glioma harbouring mutations of the IDH1 gene (Unruh *et al.,* 2016). A recent dramatic validation of this linkage was reported by Dunbar and colleagues who identified a list of oncogenic mutations that significantly correlated with VTE in a large cohort of patients (11,695) with diverse cancers (Dunbar et al., 2020).

Coagulant effects of oncogenic mutations may be mediated through several mechanisms. First, oncogenes and tumor suppressors are known to regulate angiogenesis and vascular morphogenesis in various tumor settings (Rak et al., 1995) including GBM. These effects are often amplified by hypoxia (Mazure et al., 1996) and epigenetic influences leading to deregulation of the vascular endothelial growth factor (VEGF), including in glioma stem cells (Bao et al., 2006). VEGF expression may also be induced by inflammatory cytokines and the exposure to EGFRvIII-carrying EVs (oncosomes) (Al-Nedawi *et al.*, 2008). VEGF induces vascular permeability and formation of extravascular fibrin while facilitating contacts between circulating coagulation zymogens and procoagulant tissue microenvironment (Dvorak et al., 1992). While increase vascular permeability could logically enhance the accessibility of systemic circulation to cancer-derived extracellular vesicles, there is evidence that supports their successful bidirectional crossing of intact blood-brain barrier (Alvarez-Erviti et al., 2011). Nonetheless, abnormal vascular architecture leads to stasis while inflammation is among the likely triggers of TF expression and local GBM microthrombosis, all of which may potentially impact several aspects of CAT. Thus, oncogenic circuitry may induce thrombosis indirectly through aberrations in the vascular architecture and inflammatory microenvironment.

Oncogenic lesions may also directly influence GBM-associated repertoire of coagulation effectors (coagulome) expressed by cancer cells. Our earlier studies revealed that the profile of coagulation-related genes is strikingly different between TCGA-designated GBM subtypes in that TF upregulation strongly correlates with EGFR expression in CL GBM, whereas both TF and PDPN are downregulated in PN tumors, which parallels their enrichment for mutant IDH1 (Magnus et al., 2013; Tawil et al., 2018). We have also demonstrated a direct impact of oncogenic EGFRvIII on TF expression and activity in glioma cell lines (Magnus et al., 2010), along with its biological role in GBM progression (Magnus *et al.*, 2014b; Milsom *et al.*, 2008). EGFRvIII also influences the levels of PAR1, FVII, and other factors (Magnus *et al.*, 2013). The impact of EGFR on TF was widely corroborated in multiple model systems (Rong *et al.*, 2009) and extended to other genetic events, such as loss of PTEN, especially when the latter is combined with hypoxia (Rong *et al.*, 2005). These changes were shown to impact the coagulant phenotype of GBM cells and are likely relevant for tumor vascular interactions (Rong *et al.*, 2005). While these are intriguing possibilities, they are often inferred from model GBM cell lines, which may not recapitulate the genetic epigenetic and microenvironmental complexity of tumors in GBM patients. Therefore, the question as to whether EGFR/EGFRvIII signalling acts as a direct regulator of coagulome in GBM cells *in vivo* or facilitates respective epigenetic rearrangements has not been fully investigated and will be explored in this thesis project.

In line with these findings, Unruh et al. have recently described a remarkable effect of the IDH1 R132H mutation status on the extent of CAT in GBM patients. Thus, while patients with IDH1 wildtype lesions (mostly CL, MES, or NEU) manifested the expected high VTE rates (26–30%) and reduced number of platelets, their counterparts with mutant IDH1 tumors experienced

virtually no VTE and almost no intratumoral microthrombosis. IDH1 mutant tumors exhibited low level of TF immunostaining, and their corresponding levels of EV-associated TF activity (TF-MPs) in blood showed a tendency toward reduction, relative to the IDH wild-type disease (Unruh *et al.*, 2016). In another subsequent study, it was found that combined low PDPN and IDH1 mutation signify the lowest VTE risk among all GBM patients (Mir Seyed Nazari et al., 2018), even though PDPN is likely downregulated by mutant IDH1 at the level of the epigenome (Tawil *et al.*, 2018). It is of note that in the recent study Unruh and colleagues attributed the suppression of TF levels in IDH1 mutant GBM tumors to epigenetic effects associated with *TF/F3* gene methylation (Unruh et al., 2019).

The molecular landscapes of pediatric brain tumors are vastly different than those in adults and are increasingly well characterized (Gröbner et al., 2018; Northcott et al., 2012; Reifenberger *et al.*, 2017; Sturm *et al.*, 2014), but they are infrequently discussed in the context of CAT (Bajzar et al., 2006; Piovesan et al., 2014; Tabori et al., 2004). While this is understandable due to a lesser clinical burden of apparent thrombosis in children, the biological effects of hemostatic perturbations are likely relevant. For example, in pediatric medulloblastoma (MB), the expression of TF, PAR-1, FX, and other elements of the vascular coagulome and angiogenesis effectors (angiome) is highly divergent between the four main disease subtypes: sonic hedgehog (SHH), wingless (WNT), group 3, and group 4 (D'Asti et al., 2014a) . Not only are the various coagulation mediators (e.g., TF) dysregulated by MB-related oncogenic pathways, such as SHH, WNT/β-catenin (CTNNB1), or MET, but coagulation-related cellular signaling also contributes to further changes in the expression of genes involved in hemostasis (SERPINE1), inflammation (IL1B), and angiogenesis (LECT1) in the presence of their respective agonists (D'Asti et al., 2014). It is

of note that the WNT subtype of MB is associated with high vascular density and permeability, which is believed to contribute to beneficial therapeutic outcomes (Phoenix et al., 2016). Likewise, the rare embryonal tumor with multilayered rosettes (ETMR) driven by the oncogenic microRNA cluster (C19MC) exhibits highly hemorrhagic phenotype in association with miR520 g-mediated epigenetic downregulation of TF, which also impacts cellular stemness (D'Asti *et al.*, 2016). Indeed, the role of epigenome and microRNA networks in both coagulant phenotype of cancer cells and transduction of coagulation-related signals is emerging as a fascinating and understudied area of research on CAT (D'Asti et al., 2017; D'Asti *et al.*, 2016). Furthermore, the apparent histological similarity between adult and pediatric GBM (pGBM) is not paralleled by comparable manifestations of CAT, with VTE being rare in pGBM patients (Tabori *et al.*, 2004).

In line with this discordance, the genetic landscape of pGBM is completely different than that of adult GBM and dominated by mutations of genes directly regulating cellular epigenome, such as oncohistones (H3F3A), epigenetic modifiers (ATRX, DAXX) in addition to transforming growth factor receptors (PDGFRA, ACVR1) and other lesions (Reifenberger *et al.*, 2017). These comparisons enforce the notion that in brain tumors, oncogenic transformation mechanisms intersect with host factors (such as age) in regulating the interplay between cancer cells and the hemostatic system. However, clinical manifestations of CAT may not be the only sign of such interactions, which may occur asymptomatically and as a part of a broader and consequential oncogene-driven change in the tumor microenvironment (D'Asti and Rak, 2016; Hisada and Mackman, 2018).

1.5 Podoplanin

As mentioned in prior sections, the enigma of CAT occurrence in GBM patients has recently been alleviated by a discovery of a striking correlation between the levels of PDPN expression by cancer cells and the corresponding risk of VTE (Riedl *et al.*, 2017). As PDPN plays important roles in formation and homeostasis of the vascular system and platelets, this observation raised the possibility that, likewise, the mechanisms, biological implications and remedies for GBM-associated CAT may lie beyond the traditional realm of the common coagulation pathway. Indeed, these events may include the interactions of cancer cells with platelets and involve a wide spectrum of their biological roles (Haemmerle *et al.*, 2018). For this reason, the biology of PDPN deserves a greater scrutiny.

1.5.1 *Historical Overview*

Podoplanin (PDPN) is a 36-43 kDa mucin-type transmembrane protein that owes its current name to its function in governing the shape of kidney podocytes (Breiteneder-Geleff et al., 1997). Despite being most commonly known today as podoplanin, PDPN has been described in several biological contexts within a relatively narrow time frame and consequently several names have been attributed to it. It was first described in 1990 as a molecule upregulated in osteoblasts in response to treatment with phorbol ester and was given the name OTS8 (Nose et al., 1990). In 1992 it was described under the name gp38, a glycoprotein preferentially expressed by thymic epithelial cells and fibroblastic reticular cells (FRCs) of lymphoid organs (Farr et al., 1992). In 1995 and 1996, Rishi et al. and Williams et al. respectively, identified PDPN as T1α, a marker of type-1 alveolar epithelial cells (Rishi et al., 1995; Williams et al., 1996). Also in 1996, Wetterwald and colleagues dubbed it as the E11 antigen of lymphatic endothelial cells (LECs) (Wetterwald et

al., 1996) and in 1999 the name PA2.26 was attributed to PDPN when it was identified as a cell-surface protein, the expression of which was induced in skin keratinocytes as a result of epidermal carcinogenesis and skin remodeling processes upon injury (Scholl et al., 1999). Finally, the name Aggrus was bestowed upon PDPN in 2003 when Kato et al. described PDPN's capacity to induce platelet aggregation in absence of plasma components (Kato et al., 2003).

1.5.2 *Structure*

Human PDPN is a type-I transmembrane sialomucin comprised of 162 amino acid residues. Its larger extracellular domain, in its N-terminal portion, contains three tandem repeats (EDXXVTPG; where "X" could be any amino acid) and is richly O-glycosylated. Also, these extracellular sequences, often termed as platelet aggregation-stimulating sub-domain (PLAG1-3 sub-domain) are known to be capable of interacting with the C-type lectin-like receptor (CLEC-2) on platelets whereby they induce platelet aggregation (Swain and Routray, 2018). Among mammals these triplicated subdomains are highly conserved and act as carriers for the O-glycosidic chain attached at Thr^{52} of PLAG3 (Sekiguchi et al., 2016) and believed to be critical for podoplanin capacity to bind to CLEC-2. In fact, studies involving treatment with O-glycanase as well as glycosylation-deficient CHO cell mutants and genetically modified yeast showed that lack of O-glycosylation resulted in reduced potential of PDPN to activate and aggregate platelets (Kaneko et al., 2004; Kaneko et al., 2007; Kunita et al., 2007; Toyoshima et al., 1995). In 2016, Sekiguchi et al. reported on the existence of an additional, fourth, platelet aggregation-stimulating sub-domain (PLAG4) spanning amino acids 81-85 in human PDPN. This sub-domain consists of another highly conserved EDXXT sequence closely related to the consensus sequence of the PLAG domain (EDXXVTPG), and the deletion of which results in a drastically attenuated capacity

of PDPN to bind to CLEC-2 and induce platelet aggregation. Sekiguchi and colleagues also demonstrated that even a point mutation within PLAG4 could have deleterious consequences on platelet aggregation induction potential of PDPN and antibodies raised against PLAG4 of PDPN inhibited its CLEC-2 binding function along with reduced demonstrated platelet aggregation (Sekiguchi *et al.*, 2016).

The intracellular domain of PDPN is comprised of a rather short, 9 amino acid sequence, and to-date is known to function in binding to the ezrin-radixin-moesin (ERM) complex and impact the actin cytoskeleton rearrangement to mediate filopodia formation (Swain and Routray, 2018). The cytoplasmic tail of PDPN contains 2 serine residues (S167/S171 in murine PDPN and S157/S161 in human PDPN) which are highly conserved across mammals. Those are believed to be implicated in the protumorigenic influence of PDPN represented by enhanced cell motility that is reported to be modulated by the phosphorylation (or lack thereof) of those serines by protein kinase A (PKA) and cyclin-dependent kinase 5 (CDK5) (Krishnan et al., 2013; Krishnan et al., 2015).

Finally, the transmembrane domain of PDPN is comprised of 20 amino acids and in combination with the cytosolic membrane is reported to mediate localization of PDPN in lipid rafts of the plasma membrane (Astarita et al., 2012) (Fig 1.1). This property, may, however be cell type specific. For example, in alveolar type I epithelial cells, PDPN was found within detergent-insoluble fractions (Barth et al., 2010) and in Madin-Darby Canine Kidney (MDCK) type-II cells it was reported to be localized within lipid rafts (Fernández-Muñoz et al., 2011).

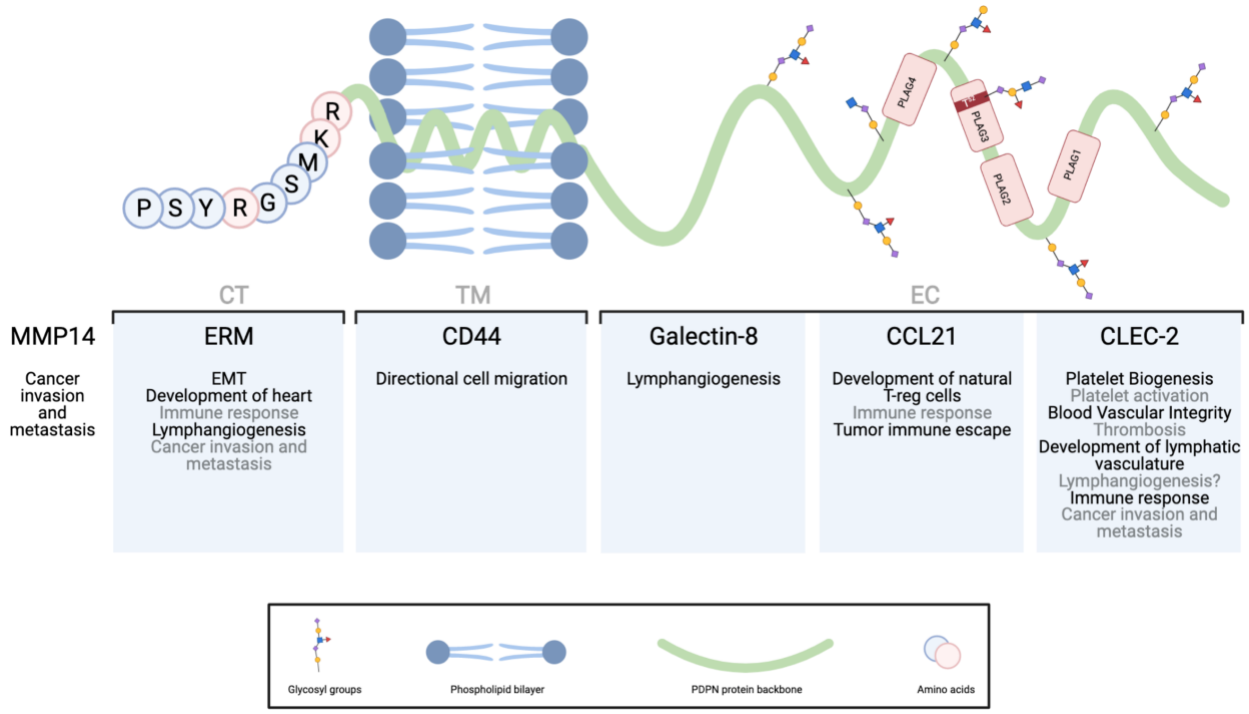

Figure 1.1 ***Schematic representation of the structure of human podoplanin (PDPN)***

The schema shows the amino-acid sequence of the short cytosolic (CT) domain. A number of interacting proteins and the biological processes in which the interaction with PDPN is involved are presented. EC (ectodomain), TM (transmembrane region) and CT (cytosolic domain) designate the main structural domains of PDPN involved in ligand binding are indicated. In the case of matrix metalloproteinase 14 (MMP14), the domain involved in this interaction is presently unknown.

1.5.3 *Expression Landscape*

Under normal circumstances, PDPN expression has been detected in alveolar epithelial type I cells in lung, kidney podocytes, lymphatic endothelial cells, fibroblastic reticular cells (FRCs) of lymph nodes, and in the central nervous system (Breiteneder-Geleff *et al.*, 1997; Breiteneder-Geleff et al., 1999; Farr *et al.*, 1992; Malhotra et al., 2012; Rishi *et al.*, 1995; Shibahara et al., 2006). Other reports have identified PDPN expression in osteocytes, basal keratinocytes, and mesothelial cells (Kimura and Kimura, 2005; Schacht et al., 2005; Wetterwald *et al.*, 1996). In pathological setting, overexpression of PDPN has been reported in various malignancies including brain tumors (Kolar et al., 2015; Mishima et al., 2006; Riedl *et al.*, 2017; Shibahara *et al.*, 2006), squamous cell carcinomas (oral cavity, tongue and pharynx) (Cueni et al., 2010b; Martín-Villar et al., 2005; Sikorska et al., 2019; Yuan et al., 2006), lung tumors (Kadota et al., 2010; Kato et al., 2005; Shimada et al., 2009), germ cell tumors (Idrees et al., 2010; Mishima *et al.*, 2006), mesotheliomas (Kato et al., 2004; Kimura and Kimura, 2005; Maruyama et al., 2018; Ordóñez, 2005), gastric cancers (Hu et al., 2020), mammary carcinomas (Grzegrzolka et al., 2017; Wicki et al., 2006), among others (Kan et al., 2014; Kato *et al.*, 2003; Monzani et al., 2007; Ochoa-Alvarez et al., 2012; Ogasawara et al., 2016; Watanabe et al., 1990). The expression of PDPN in GBM cells is therefore considered as an anomaly as the corresponding normal astrocytes are generally negative for this signal (Riedl *et al.*, 2017). It follows, then, that molecular mechanisms involved in GBM onset and progression are the likely regulators of PDPN expression acting at transcriptional or post-transcriptional levels.

1.5.4 *Transcriptional Regulation of PDPN Expression*

The human PDPN gene is located on Chromosome 1 (1p36.21) and has 6 exons, whereby exon 1 encodes the signal sequence (5'UTR) and exons 2 to 4 encode the extracellular domain. The transmembrane domain and the first 8 amino acids of the intracellular domain are encoded by exon 5 and the last amino acid of the intracellular domain is encoded by exon 6.

The wide distribution of PDPN expression across various heterogenous tissues suggests that multiple transcription factor binding sites are present within its promoter region and respond to tissue-specific cues (Swain and Routray, 2018). Indeed, PDPN promoter contains consensus sequences for SP1/3 (specificity protein1/3) transcription factors, members of a ubiquitous transcription factor family that bind to the GC box of many gene promoters and implicated in regulating metabolism, proliferation, differentiation, senescence, death and tumorigenesis (Beishline and Azizkhan-Clifford, 2015; Peng et al., 2020). In addition, Prox1 (Prospero homeobox 1; the major regulator of LEC differentiation) has been shown to regulate PDPN transcription levels and consequently the expression, through binding to the 5' regulatory sequence. In fact, it has been shown that enforced Prox1 expression in differentiated blood endothelial cells (BECs) induced a LEC-like phenotype along with PDPN (Hong et al., 2002). Additionally, during embryogenesis, IL-3 which is constitutively expressed by LECs is believed to be an essential factor for the upregulation of Prox1 and PDPN throughout the transdifferentiation process of blood endothelial cells into lymphatic endothelial cells (Gröger et al., 2004).

Several cytokine signalling pathways have also been implicated in PDPN regulation, including transforming growth factor-β. Additionally, interferon γ, which acts via the Jak-STAT pathway, as well as IL-6 and IL-22 via STAT3 phosphorylation, can impact PDPN gene transcription. Another observation implicated activated Src in the induction of PDPN expression through the induction of FOS, a component of the AP1 transcription factor complex, an effect involving phosphorylation of focal adhesion adaptor protein Cas (crk-associated substrate) (Inoue et al., 2012; Retzbach et al., 2018; Shen et al., 2010). AP1 involvement in PDPN transcription regulation was also supported by the work of Durchdewald et al. who identified PDPN among the genes the transcription of which is Fos-dependent (Fig 1.2) (Durchdewald et al., 2008; Peterziel et al., 2012).

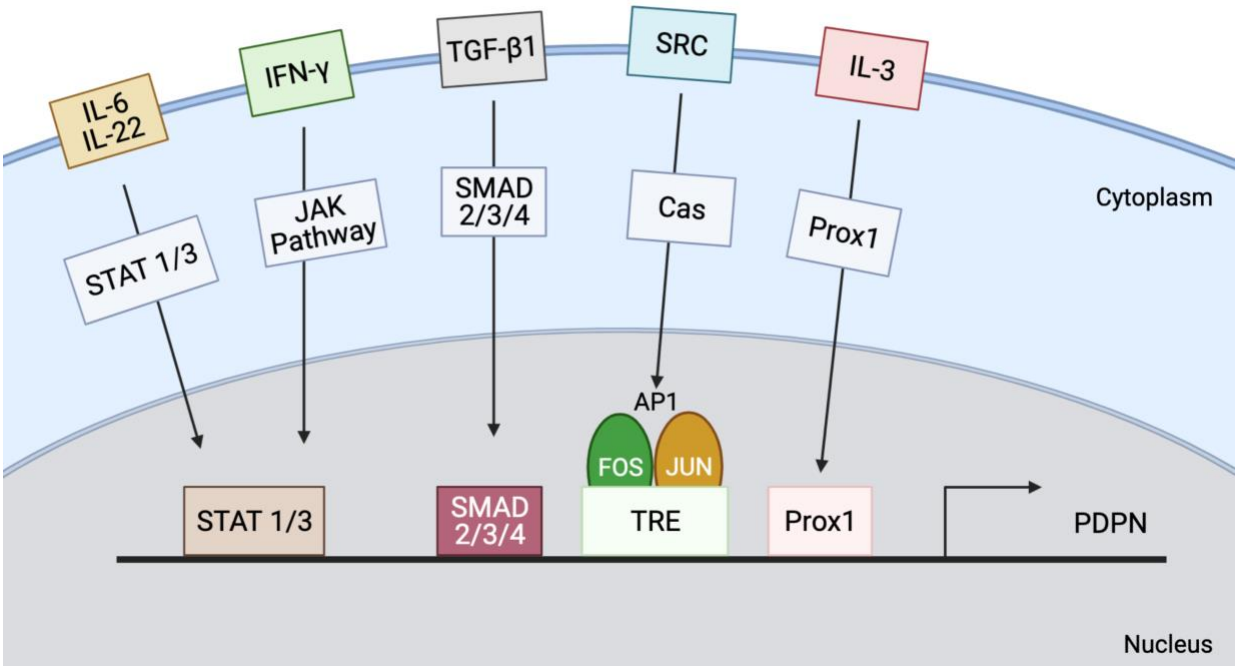

Figure 1.2 ***Transcriptional regulation of PDPN expression***

While the exact signaling pathways involved are mostly unknown, upregulation of PDPN expression can be achieved through a number of pro-inflammatory cytokines. These include IL-22, IL-6, IFN-γ, TGF-β, IL-1β, and TNF-α, PDPN upregulation induced by IL-6 and IL-22 depends on STAT3 while upregulation induced by IFN-ψ depends on STAT1 and STAT3. TGF-β requires Smad2/3 and 4 activity. According to Peterziel et al., the PI3K-AKT-AP-1 pathway can also induce PDPN expression in brain tumors that have lost the negative regulation normally provided by PTEN. AP-1, a transcription factor comprised of Fos and Jun proteins, binds to the tetradecanoyl phorbol acetate-responsive element (TRE) in the promoter of PDPN, which is heavily methylated.

1.5.3.2. Regulation of PDPN by epigenetic mechanisms.

While rapid responses to growth factors and signaling cues play a role in PDPN gene transcription, more lasting effects may be exerted by the epigenome including DNA methylation, chromatin architecture and microRNA networks. While conclusive evidence to this effect already exists, there is no consensus as to the exact or dominant mechanisms involved (Tawil *et al.*, 2019; Tawil et al., 2020).

One of the best studied aspects of epigenetic PDPN regulation is the methylation of CpG elements adjacent to the PDPN coding sequence. For instance, the earlier work of Hantusch et al. (2007) had already suggested the involvement of epigenetic control of PDPN and the level of DNA methylation, in that in MG63 cells treatment with 5-aza resulted in PDPN down regulation (Hantusch et al., 2007). Later in 2012, and in contrast to findings by Hantusch et al., Peterziel et al. made use of large set of human GBM samples in which Noushmehr et al identified the glioma-CpG island methylator phenotype (G-CIMP). These tumors were largely classified as proneural, and characterized by the early onset, improved outcomes, and characteristic presence of an IDH1 mutation. In this setting, PDPN was shown to be among the top-ranked hypermethylated genes, subsequently implicating PDPN promoter methylation in the expression regulation of this protein (Noushmehr *et al.*, 2010; Peterziel *et al.*, 2012). In the same context of gliomas, the most recent work by Sun et al. compared large cohorts of IDH1wt and IDH1mut gliomas and supported the findings reported by Peterziel and colleagues in that the observed downregulation of PDPN in the presence of mutant IDH1 is a function of pronounced PDPN promoter hypermethylation (Sun et al., 2020). In this thesis we further corroborate this result using an independent cohort of GBM patients and extend this analysis to other epigenetic mechanisms.

Additionally, the PDPN gene was also identified as target of several regulatory microRNA. Several miRNAs have been predicted, via various miRNA candidate prediction platforms in silico, to be capable of interacting with the 3' UTR region of PDPN, among those are miR-29b, miR-497, miR-125a, as well as miR-101 (Eisenreich et al., 2016) (Cortez et al., 2010). In silico data initially suggested that both miR-29b and miR-497 were capable of generating miRNA/target mRNA complexes with the PDPN 3'UTR, however work by Eisenreich et al conducted in human podocytes (hPC) validated an appreciable impact of only miR-29b which led to significant reductions of PDPN at both mRNA and protein levels (Eisenreich *et al.*, 2016). Impact of miR-29b on PDPN expression was further supported through the work of Cortez and colleagues, who followed a similar "in silico – to – bench" approach and pinpointed that in LN319 cells, PDPN is a direct target of both miR-29b and miR-125a (Cortez *et al.*, 2010).

Regulatory effects of the chromatin architecture on PDPN gene expression are relatively poorly studied. In simple terms, these mechanisms could entail various contributions of histone modifications such as activating histone acetylation (ac) marks or the activating/suppressive methylation (me) marks (An, 2007). Acetylation and methylation on specific lysine residues are highly consequential for epigenetic gene regulation. Particularly, lysine methylation can happen in three different forms: monomethylation (me1), dimethylation (me2) and trimethylation (me3) and the impact of methylation on transcription depends on its level and the residue. For instance, trimethylation of fourth lysine residue in histone 3 (H3K4) promotes transcription activation while a H3K9m3 or H3K27me3 mark is associated with transcriptional repression. Conversely, all acetylation marks, being localized to transcription start sites and/or enhancers of actively transcribed genes are generally correlated with transcriptional activation (Kimura, 2013). As such,

it is important to note, however, that histone modifications are not to be regarded as “on” and “off” switches of gene transcription, but nonetheless they have significant impact on chromatin architecture and serve as good epigenetic indicators of chromatin state which in turn reflects on the accessibility of particular regions to transcription machinery (Henikoff and Shilatifard, 2011) (Kimura, 2013).

In this thesis we document a role of the PRC2 complex in silencing PDPN expression in glioma cells harbouring specific oncogenic mutations. It is also puzzling that changes in stemness and differentiation status of glioma cells influence PDPN levels (Chapter 4), but the epigenetic mechanisms involved remain unknown.

These aforementioned complexities of PDPN regulation suggest that this protein possesses important functional properties that must be deployed in a specific temporal, special and cell-specific manner.

1.5.5 *Podoplanin Functions*

Podoplanin emerges as a functionally multifaceted protein that plays various roles in various settings that span the developmental stages, normal physiology as well as pathology (Ugorski et al., 2016).

1.5.5.1 Physiological roles of Podoplanin

PDPN is essential for normal development. PDPN deficient (KO) mice exhibit either embryonic lethality as a result of cardiovascular malformations, or die shortly after birth as a

consequence of alveolar malfunction resulting in respiratory failure (Quintanilla et al., 2019). Cardiac defects in PDPN-KO mice involve impairment of myocardial formation resulting from overexpression of E-cadherin and downregulation of RhoA GTPase disrupting normal EMT of the coelomic epithelium (Douglas et al., 2009; Mahtab et al., 2009; Mahtab et al., 2008). In the frame of respiratory defects, in PDPN KO mice, expanded alveolar sacs fail to form as a result of disrupted differentiation of type I alveolar cell. This results in narrow airspaces and upon birth, lungs fail to inflate (Ramirez et al., 2003). While uncertainty prevails in regard to the exact molecular mechanisms at play, one study reported perturbed expression of a number of genes in the lungs of term PDPN KO mice, notably *ephrinA3* and *p21Cip1* involved in cell signaling and cell cycle regulation (Millien et al., 2006). Another gene implicated in platelet-expressed CLEC-2/LEC PDPN interaction is TGF-β the release of which normally facilitates the differentiation of lung mesothelial cells into alveolar duct myofibroblasts (adMYFs) in the developing lung (Suzuki-Inoue and Tsukiji, 2020).

An important element in the phenotype of PDPN null mice is the disruption of lymphatic function. In the absence of PDPN-CLEC2 interaction, a proper separation of blood and lymphatic vessel fails to occur compounding circulatory pathology. In fact, PDPN null mice show defective lymphatic vasculature, which could further impede the successful clearance of fluid that fills up the lumen of the developing lung at birth and contribute to respiratory failure shortly after (Navarro-Núñez et al., 2013; Pan and Xia, 2015; Suzuki-Inoue et al., 2017). The development of murine lymphatic endothelial cell lineage occurs following the formation of the blood vascular system around day E9.5. In this regard the turning point is the activation of Prox1 expression, a master regulator of LEC identity, in a subpopulation of venous endothelial cells within the wall of

the cardinal vein. As a consequence, the expression of lymphatic endothelial markers is switched on (lymphatic vessel endothelial hyaluronan receptor LYVE-1, vascular endothelial growth factor VEGFR-3 and PDPN), and proliferation and migration commence initiating lymphangiogenesis. This process is supported by VEGF-C secreted by surrounding mesenchymal cells stimulating VEGFR-3/neuropilin-2 signaling. This program results in formation of embryonic lymph sacs, which will constitute the origin from which the entire lymphatic system will be ultimately derived (Quintanilla *et al.*, 2019).

Around E11.5, PDPN assumes one of its most studied functions. PDPN present in the emerging lymphatic vasculature is able to bind CLEC-2 on the surface of platelets leading to their activation and thrombotic occlusion of blood vessel – lymphatic connections. This process ensures the adequate separation between the blood and lymphatic vascular systems which is essential for their functionality. This notion is supported by the phenotype of PDPN-null mouse embryos and neonates which exhibit poor blood/lymph separation and open connections between them, resulting in blood-filled lymphatics and edema (Schacht et al., 2003; Uhrin et al., 2010).

Upon binding to CLEC-2 on the surface of platelets, PDPN induces CLEC-2 clustering which activates the downstream signalling (Src and Syk tyrosine kinases) resulting in the activation of PLCγ2 (Pollitt et al., 2014; Suzuki-Inoue et al., 2006; Suzuki-Inoue et al., 2007; Séverin et al., 2011). In turn, the Syk/SLP-76 signalling initiated within platelets triggers their degranulation (Fig 1.3) and aggregation which is responsible for sealing the separation zone of the cardinal vein and lymph sacs (Bertozzi et al., 2010; Uhrin *et al.*, 2010). The activation of platelets by LECs carries a further importance in regulating the separation of blood and lymphatic vessel

systems; upon their activation, platelets release a number of factors that inhibit LEC proliferation and migration. Among platelet derived factors implicated in regulation of LEC proliferation and/or migration BMP-9 is of interest as a member of the bone morphogenic protein (BMP) subfamily of TGF-β-related growth factors. In addition, platelets are a rich source of TGF-β, and platelet factor 4 (PF4) both of which were shown to modulate LEC proliferation (TGF-β) and migration (TGF-β and PF4) (Osada et al., 2012).

Several lines of evidence suggest the existence of additional mechanisms involved in lymphatic-blood vessel separation. Thus, PDPN-CLEC-2 interaction may contribute to prevention of backflow at the lympho-venous junction, while CLEC-2-induced clustering of PDPN drives downstream signaling through ERM proteins. Nonetheless, despite the absence of full consensus regarding the governing mechanism, these mechanisms are not necessarily mutually exclusive (Suzuki-Inoue *et al.*, 2017).

The very same PDPN-CLEC-2 interaction is similarly implicated in cerebrovascular patterning and integrity. PDPN expression is observed in the developing neural tube and both *PDPN* null and *CLEC-2* null mice exhibit an absence of neurovascular integrity within the embryonic brain. PDPN expressed on neuroepithelial cells of the developing neural tube induces the activation and aggregation of platelets that leak out of the neighboring developing blood vessels. This prevents hemorrhage and concomitantly, the platelet released-factors facilitate the maturation of the developing vasculature (Lowe et al., 2015). Apart from the involvement in lung development mentioned earlier, additional physiological processes in which the PDPN-CLEC-2 axis has been implicated are megakaryocyte growth and platelet production (Tamura et al., 2016),

entry of dendritic cells (DCs) into lymphatic vessels (Acton et al., 2012), and maintenance of high endothelial venules (HEVs) integrity (Fig 1.4) (Herzog et al., 2013; Quintanilla *et al.*, 2019).

PDPN has been shown to participate in important physiological processes independently of its interaction with CLEC-2. These effects include the maintenance of normal podocyte morphology and establishment of podocyte foot processes likely mediated by its anchorage to the actin cytoskeleton through ERM proteins and the control of cytoskeletal organization by regulating the activity of small Rho GTPases (IJpelaar et al., 2008; Koop et al., 2008). PDPN is also involved in development of natural regulatory T cells (T_{reg}) via the podoplanin–CCL21 interaction in thymic fibroblastic regulatory cells (FRCs) co-expressing both PDPN and CCL21, which appears to be critical for T_{reg} development (Fuertbauer et al., 2013). Another role of PDPN is the in proper function of stem cells in the context of mammary development through the modulation Wnt/β-catenin signaling activity and likely involving interaction with ERM proteins and/or CD44 (Bresson et al., 2018).

Overall, PDPN emerges as an important player in the development of multiple organs and those include the lungs, heart, and lymphatic system. Its impact seems to encompass a broad spectrum of functions and in certain instances it exerts its role in partnership with CLEC-2, however in others, PDPN seems to affect proliferation, migration or differentiation of a given cell type through alternative mechanisms. This suggests that the functionality of PDPN could be, to a certain extent, tissue specific and the range of downstream physiological effects might be a consequence of PDPN interactions with a range of proteins under diverse physiological conditions (Fig 1.1) (Astarita *et al.*, 2012).

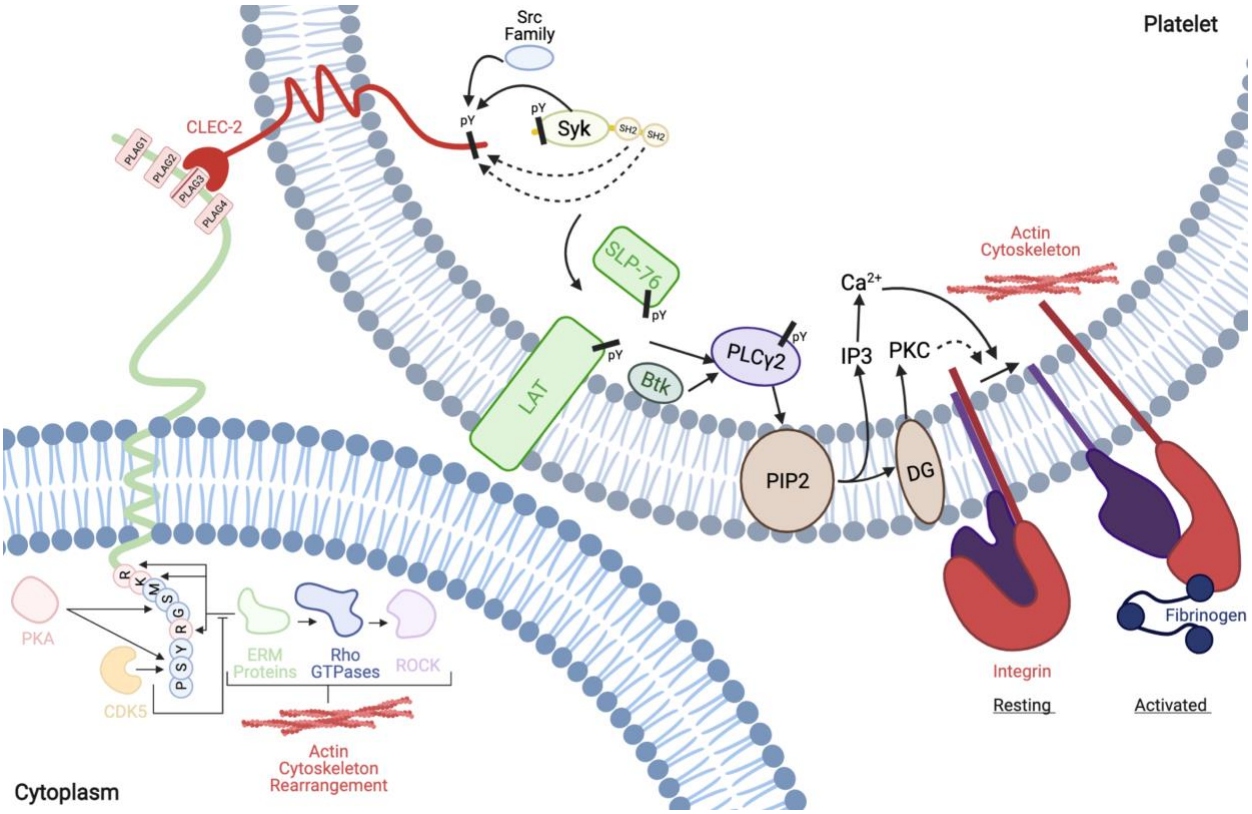

Figure 1.3 ***PDPN CLEC-2 interaction***

Upon binding to PDPN, CLEC-2 clustering is initiated. Initially, the phosphorylation of a tyrosine residue in hemi-ITAM (immunoreceptor tyrosine-based activation motif) by the Src family kinase occurs. This is followed by the binding of spleen tyrosine kinase (Syk) to the phosphorylated hemi-ITAM through its SH2 domain. As a result, Syk is activated, leading to phosphorylation of the downstream adaptor proteins, LAT and SLP76, and subsequently phosphorylation/activation of the downstream tyrosine kinase Btk and phospholipase Cγ2 (PLCγ2). In turn, PLCγ2 activation promotes the generation of inositol trisphosphate (IP3) and diacylglycerol, resulting in Ca2+ mobilization and protein kinase C (PKC) activation. Mobilization of intracellular Ca2+ and PKC activate integrin αIIbβ3, which allows for fibrinogen binding and platelet aggregation. (pY, phosphorylated tyrosine).

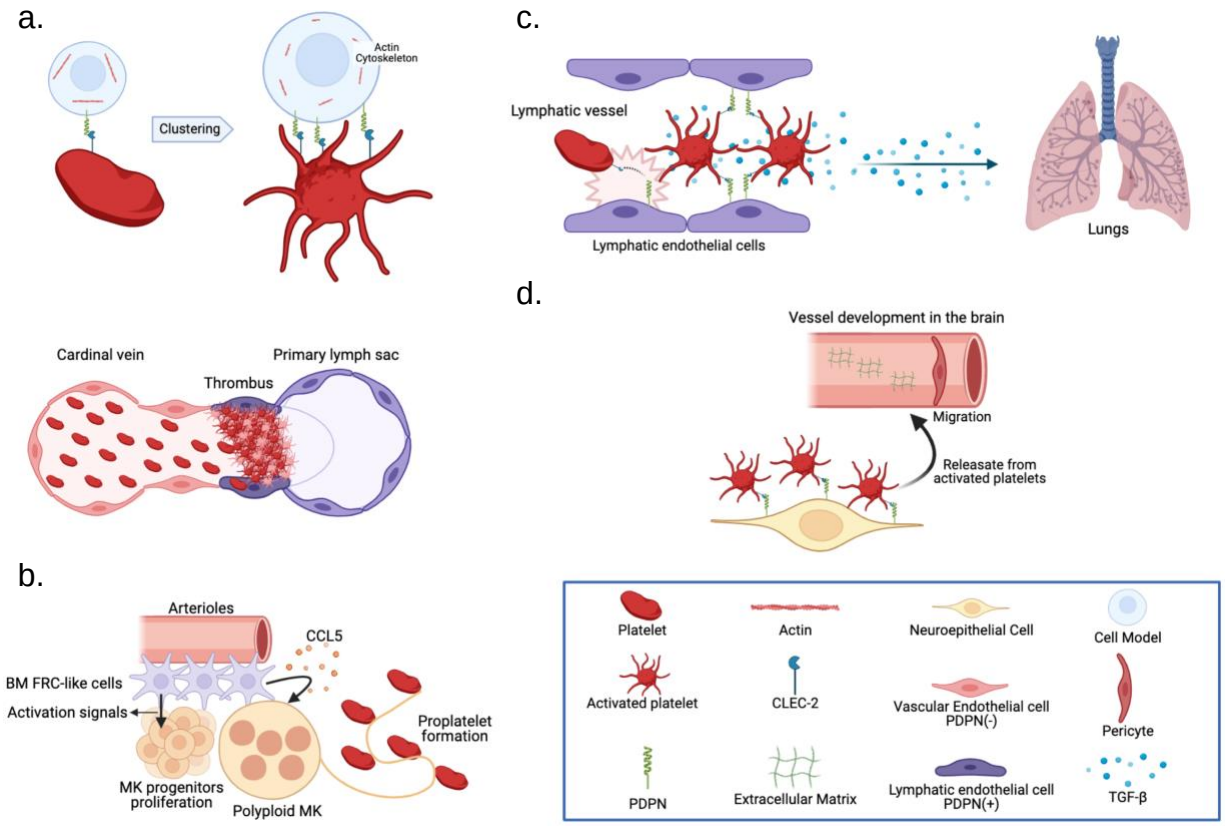

Figure 1.4. ***Physiologic processes modulated through the interaction between C-type lectin-like receptor 2 (CLEC-2) and podoplanin (PDPN).***

a. Blood–lymphatic vessel separation during developmental stages: CLEC-2 and PDPN interaction between platelet and LECs at lymphovenous (LV) junctions results in platelet activation and the formation of platelet thrombi. This prevents backflow of blood. **b**. Megakaryocyte (MK) proliferation and formation of proplatelet: bone marrow (BM) fibroblastic reticular cell (FRC)-like cells found in the BM form a novel niche, the peri-arteriolar megakaryocytic microenvironment. These cells express PDPN, and the interaction between CLEC-2 in MKs and PDPN of BM FRC-like cells results in an activation signal downstream of CLEC-2. In turn, this leads to proliferation of MKs and stimulates CCL5 release from BM FRC-like cells, facilitating proplatelet formation. **c.** A suggested contribution of platelet C-type lectin-like receptor 2 (CLEC-2) to lung development. During development, platelets are activated by binding between CLEC-2 and lymphatic endothelial cell podoplanin (PDPN). Activated platelets release transforming growth factor-β (TGF-β) which facilitates the differentiation process of lung mesothelial cells (luMCs) into alveolar duct myofibroblasts (adMYFs). AdMYFs are crucial for the capacity of lungs to inflate as they generate the necessary elastic fibers. **d.** Cerebrovascular development and integrity: neuroepithelium-expressed PDPN around cerebral vessels interacts with CLEC-2 on the surfaces of leaking platelets. This results in platelet aggregation, and degranulation. Contents of platelet granule facilitate pericytes recruitment. The recruited pericytes in turn produce the extracellular matrix that helps to guide the maturation and maintain the integrity of the developing vasculature.

1.5.5.2 Podoplanin in Pathology

Naturally, with such a broad involvement in normal physiology, PDPN is implicated in a number of pathological conditions including various malignancies. It comes as no surprise that PDPN has been implicated in thrombosis in general, and in inflammation-driven thrombosis (immunothrombosis) in particular. This is important because venous thromboembolism which, as described earlier, includes deep vein thrombosis (DVT), and of which pulmonary embolism (PE) is a major life threatening complication is a leading cause of cardiovascular deaths (Quintanilla *et al.*, 2019).

Studies have drawn a tight link between venous thrombosis and inflammation and it has been shown that depletion of platelets exerts a protective effect against DVT in mice (von Brühl *et al.*, 2012). The PDPN-CLEC-2 axis was demonstrated to play a critical role in the thrombotic process in mouse models utilizing stenosis of the inferior vena cava (IVC) whereby CLEC-2-deficient mice exhibited a complete protection against thrombotic vascular obstruction. Interestingly, in the IVC stenosis model, during thrombosis, PDPN was found to be upregulated in a subset of cells in the wall of the inferior vena cava, which allowed for the interaction with platelets and, expectedly, an exacerbated DVT. Despite the fact that the nature and identity of this subpopulation remains to be identified, PDPN's involvement was further confirmed by the fact that antibody-mediated PDPN neutralization resulted in formation of significantly smaller thrombi (Payne et al., 2017). Additionally, PDPN expression has been detected in a highly phagocytic ($F4/80^+$) subset of inflammatory macrophages within the peritoneal cavity and spleen. TNF-α as well as other pro-inflammatory stimuli, such as lipopolysaccharide were implicated as triggers of PDPN upregulation in macrophages (Hou et al., 2010; Kerrigan et al., 2012). Such PDPN-

expressing macrophages were shown to be capable of activating platelets and thus speculated to possess a potential to trigger extravascular platelet activation during wound healing and inflammatory processes, such as atherosclerosis. In this scope, Hatakeyama et al., in their post-mortem immunohistochemical analysis of abdominal aortas identified increased PDPN expression in smooth muscle cells and macrophages associated with advanced atherosclerotic lesions and necrosis (Hatakeyama et al., 2012). Three years later, and in line with the above findings, another group presented evidence for PDPN upregulation in subsets of macrophages in the vicinity of the blood vasculature during liver inflammation after systemic infection with *Salmonella typhimurium*. These authors observed an IFN-γ- mediated increase in numbers of PDPN-expressing monocytes in the hepatic parenchyma and perivascular sites, which ultimately resulted in the activation of platelets leaked out from damaged vessels via the PDPN-CLEC-2 interaction (Hitchcock et al., 2015). Notably, thromboinflammation may also lead to the expression of PDPN by vascular endothelial cells which may directly contribute to platelet activation and thrombosis (Suzuki-Inoue, 2019)

PDPN figures prominently in the context of wound healing, tissue injury and fibrosis. For example, PDPN upregulation by basal epidermal keratinocytes has been reported, under proinflammatory or hyperproliferative conditions, wound healing and in psoriasis. This effect occurred in response to proinflammatory cytokines such as TGF-β1, IFN-γ, interleukin 6 (IL-6), and IL-22 through their canonical signaling pathways, which shed some light on mechanisms of PDPN regulation. Thus, TGF-β1 triggers PDPN expression through the Smad pathway, whereas IFN-γ, IL-6, and IL-22 via signal transducer and activator of transcription (STAT)-1 and STAT-3 (Honma et al., 2012). Similarly, in the brain, two independent reports also showed that PDPN is

upregulated during inflammatory processes. In one study, two mouse models of brain injury, needle-induced injury and ischemic insult, demonstrated PDPN upregulation in reactive astrocytes, cells which have been also spotted in close proximity of malignant gliomas (Kolar *et al.*, 2015). On the other hand, during neuroinflammation induced by intraventricular injection of LPS in rats, PDPN expression was shown to be highly enhanced in neurons, but not in astrocytes, within the brain cortex (Song et al., 2014). Finally, a number of studies showed that PDPN contributes to lymphangiogenesis, in both inflammatory and malignant settings (Bieniasz-Krzywiec et al., 2019; Cueni et al., 2010a; Cueni *et al.*, 2010b; Hur et al., 2014; Maruyama et al., 2014; Sikorska *et al.*, 2019), as well as in chronic inflammatory autoimmune diseases (Chaitanya et al., 2013; Chihara et al., 2018; Del Rey et al., 2014; Gao et al., 2016; Nazari et al., 2016; Noack et al., 2016; Nylander et al., 2017; Peters et al., 2011). In the latter condition the contribution of PDPN to disease pathogenesis remains unclear (Quintanilla *et al.*, 2019).

1.5.5.3 *Podoplanin expression in cancer*

More pertinent to our scope of work is the emerging role of PDPN in the context of malignancy. Indeed, PDPN expression is pronounced in a broad spectrum of cancers, including but not limited to angiosarcomas, chondrosarcomas, osteosarcomas, malignant mesotheliomas, germ-cell tumors, squamous cell carcinomas (SCC), gliomas, and glioblastomas (Astarita *et al.*, 2012; Ordóñez, 2005; Quintanilla *et al.*, 2019; Renart et al., 2015; Ugorski *et al.*, 2016). PDPN expression in tumors is not always restricted to the cancer cells; stromal cells, particularly cancer associated fibroblasts (CAFs), are often $PDPN^{+}$. The overall PDPN overexpression in tumors is associated with poor prognosis and documented examples include SCCs of the skin, esophagus and head and neck, as well as glioblastomas (Acton *et al.*, 2012; Renart *et al.*, 2015; Ugorski *et*

al., 2016). In SCCs, it has been suggested that PDPN expression in the neoplastic epithelium is induced via cytokine stimulation from surrounding stroma, as PDPN-expressing cells are often seen to be semi-restricted to the outer edges of the tumor nests (Dumoff et al., 2005; Martín-Villar *et al.*, 2005; Rahadiani et al., 2010; Shimada *et al.*, 2009; Yuan *et al.*, 2006). Work by Kunita et al. subsequently showed that indeed, inflammatory cytokines secreted by $CD45^+$ (hematopoietic) stromal cells drive the induction of PDPN expression at the invasive edge in SCCs of the cervix (Kunita et al., 2018). However, in contrast to GBMs and SCCs of the skin, esophagus or head and neck, several reports associated PDPN expression with good prognosis in uterine cervical cancer (Dumoff *et al.*, 2005; Ito et al., 2009; Shimada *et al.*, 2009; Suzuki et al., 2011). Since prognosis may be defined by a range of factors including intrinsic disease aggressiveness, its biological determinants, stage at diagnosis, response to treatment and complications (e.g. thrombosis), a better understanding of the role PDPN plays in cancer would likely require more context-specific considerations. In the context of this thesis, it is noteworthy that while PDPN upregulation predicts VTE risk in GBM this protein is also upregulated in cancers with inherently low risk of thrombosis (SCC) pointing to the significance of a wider molecular context.

1.5.5.4 *Podoplanin expression and function across cancer stem cell hierarchies*

The prevailing dogma states that tumors have a well-established hierarchical organization, at the apex of which there is a subpopulation of cells capable of self-renewal and clonal initiation of tumor growth. These cells are often referred to as cancer stem cells (CSCs) or tumor-initiating cells (TICs). Inspired by the structure of the hematopoietic system CSCs or TICs in solid tumors are thought to remain largely quiescent, while they possess the capacity to give rise to more committed daughter cells that may exhibit proliferative capacity and populate the tumor bulk. In

contrast to CSCs/TICs the more committed cells (transitory amplifying or differentiated non-stem cells), individually are believed to be unable to re-establish or maintain the tumor (Kreso and Dick, 2014).

Recently, the hierarchical CSC model has been challenged by a growing body of evidence that suggests a more transitory and complex nature of tumor cell populations. It has also been suggested that in cancer the cellular 'stemness' could be seen as multicellular quality determined by a unique nexus of clonal growth capacity and niche effects afforded by daughter cells and stroma (Rak, 2006). The 'collective' nature of cancer cell aggressiveness is illustrated by single cell transcriptomes and methylation profiles in GBM. In this setting the analysis of complex cell populations suggests that cell subsets with progenitor-like phenotypes may retain considerable proliferative capacity (rather than being quiescent) and that stemness may represent a cellular state (rather than permanent attribute) adopted in response to microenvironmental cues (Meacham and Morrison, 2013). In fact, recent body of work by several groups presented compelling evidence in favor of cellular plasticity within GBM and implicated oncogenic circuitry, retained developmental programs and microenvironmental influences as major drivers that control the frequency of cells in each of several phenotypic states, including stemness and differentiation biases or roadmaps among stem cell progeny (Couturier *et al.*, 2020; Dirkse et al., 2019; Gupta et al., 2019; Neftel *et al.*, 2019).

Despite the ongoing debate, isolated GBM cell subpopulations consistently differ with respect to their engraftment and tumor initiation capacity, which reinforces the operational usefulness of the CSC concept (Li et al., 2009; Singh *et al.*, 2003; Stiles and Rowitch, 2008). In

this sense, CSCs are still believed to be at the root of the major challenges associated with cancer control and contributors to tumor recurrence, metastasis, and the development of resistance to therapy (Agliano et al., 2017; Chen et al., 2017; Cojoc et al., 2015; Gupta *et al.*, 2019; Li et al., 2007; Massagué and Obenauf, 2016; Peitzsch et al., 2017). These considerations drove a sustained effort to better define molecular indicators with which to identify and ultimately target CSCs. In GBM, some of the candidate CSC markers include CD133, CD44, SOX2, and ALDH1 (Bresson *et al.*, 2018; Miyashita et al., 2017), but their distribution may differ between proneural and mesenchymal glioma stem cell (GSC) subtypes (Garnier *et al.*, 2018; Mao et al., 2013) or between cells in tumor centre versus periphery (Nakano, 2015). In this increasingly complex landscape, it is reasonable to ask whether PDPN expression is an attribute of CSCs or their progeny, and how is it affected by the tumor type and molecular pathways of disease progression.

Interestingly, a report form Atsumi and colleagues (2008), suggested PDPN as a novel marker of CSCs/TICs in a cellular model of the human cervical SCC, as epitomized by the A431 cell line. The authors isolated A431 podoplanin-positive cells and demonstrated that they could differentiate into podoplanin-negative cells. The former had a higher colony formation efficiency, were more tumorigenic, and expressed increased CD44 levels, in line with several documented properties of CSCs/TICs (Atsumi et al., 2008; Quintanilla *et al.*, 2019). Incidentally, similar properties were found in A431 cells expressing high levels of TF, another effector of hemostasis and CAT (Milsom et al., 2007).

Since then, and with varying degrees of success, PDPN has been proposed to be a marker of CSCs in several cancers (Quintanilla *et al.*, 2019). Most of the related evidence, however, is of

indirect and correlative nature. In the context of esophageal SCCs, cases with podoplanin-high lesions were correlated with more advanced disease stage, lymphatic and vascular invasion, recurrence, and poor prognosis for patients. In contrast, podoplanin-low cases showed better overall and disease-free survival (Islam et al., 2015). In GBM Ernst et al. implicated PDPN as one of the molecular markers associated with clinical outcome and Kolenda and colleagues suggested an impact for hypoxia on the upregulation of CSC markers, one of which they consider to be PDPN (Ernst et al., 2009; Kolenda et al., 2011). Several investigational threads from the group led by Kenneth Aldape and Erik Sulman suggested a link between PDPN and GBM stem cells (GSCs), whereby they even suggested it to be a superior marker to CD133, which was often co-expressed with PDPN, but in their hands failed to identify tumor-initiating and treatment-resistant cells in the absence of PDPN (Sulman et al., 2008). Additionally, they speculated that PDPN expression is important for the stem-like phenotype and which is further pronounced in cell subpopulations co-expressing PDPN CD44, CD15, and/or CD58 (Goodman et al., 2010). While these are compelling observations genetic disruption of PDPN in GBM cells and GSCs produced no change in tumor cell engraftment in mice, suggesting that this trait is not obligatory for tumor initiation per se (Eisemann et al., 2019)

Lastly, PDPN expression was correlated with increased dsDNA repair activity in response to ionizing radiation, thus drawing a link to treatment resistance and tumor regrowth post therapy (Ezhilarasan et al., 2011). In light of varying models and culture conditions used to investigate PDPN implication in the context of CSCs and sometimes divergent conclusions drawn from *in vitro* versus *in vivo* studies the role of PDPN as a marker and/or effector of stemness requires further scrutiny. Indeed, our studies (below) document a disconnect between PDPN expression

and GBM stemness. These disparities are compounded by doubts regarding the true stem cell nature of many existing models of patient derived GSCs, a circumstance that fuels the related controversies.

1.5.5.5 *Podoplanin-related aspects of tumor aggressiveness*

GBM is a paradigm of co-existence and cooperation of cellular populations with distinct molecular and biological traits (Bonavia et al., 2010). It is therefore possible that while PDPN does not have to be present on CSCs/GSCs/BITCs it may still enhance their pathological effects through contributions of other cell populations. Indeed, there is an emerging clear evidence that PDPN expression does have important roles to play at various intersections of malignant progression and associated vascular complications, such as CAT.

Regardless of cellular stemness, PDPN was found to promote tumor-cell migration, invasion, extracellular matrix (ECM) remodeling, epithelial to mesenchymal transition (EMT), and metastasis. In regard to tumor cell migration and EMT, PDPN was found to be concentrated at cellular protrusions, such as ruffles, filopodia and microvilli implicated in migratory cellular phenotypes associated with aggressive cancer cells (Martín-Villar et al., 2006). Being dependent on, and shaped by, the actin cytoskeleton rearrangements, the formation of these structures can be induced by PDPN through the recruitment of ezrin and subsequent cytoskeletal rearrangements. Some studies reported an involvement of PDPN in E-cadherin downregulation and delocalization out of intercellular contact points resulting in reduction of calcium-dependent adhesion between cells (Inoue *et al.*, 2012; Martín-Villar *et al.*, 2005; Scholl *et al.*, 1999; Wicki *et al.*, 2006). In another study, overexpression of PDPN in MDCK epithelial cells resulted in full blown EMT

accompanied by the loss of epithelial markers and concomitant upregulation of mesenchymal ones (Martín-Villar *et al.*, 2006). EMT is often regarded as an essential and initial step in the metastatic cascade (Brabletz et al., 2018); interestingly cells expressing PDPN and EMT characteristics were found to exhibit enhanced migratory and invasive capacities and produced lymph node metastasis (Scholl et al., 2000).

Interestingly, while it is unclear if PDPN induced EMT is critical for cell dissemination, it has been documented that induction of EMT may, in some instances, lead to the acquisition of stem cell-like properties (Polyak and Weinberg, 2009). Along these lines, recent work focusing on triple negative breast carcinomas indicated that deletion of PDPN may result in diminished tumorigenesis and tumor sphere formation capacity, and that PDPN-deficient tumors exhibited a more differentiated phenotype and showed signs of mesenchymal-to-epithelial transition (MET), a reversal of EMT (Bresson *et al.*, 2018). Furthermore, gain/loss of function experiments suggested a potential cooperation between PDPN and CD44 in promoting directional migration in carcinoma cells (Martín-Villar et al., 2010) and other studies presented findings of CD44 contribution to invadopodia-like protrusion in GBM CSCs (Petropoulos et al., 2018). Some authors speculated on the possibility of CD44 cooperating with PDPN in promoting the assembly and maturation of invadopodia and the subsequent recruitment of MMP14 to these membrane structures resulting in enhanced aggressiveness (Quintanilla *et al.*, 2019).

PDPN was reported to play a role in ECM remodeling that facilitates cancer cell invasion. In this regard, PDPN has been implicated in orchestrating an upregulation of multiple matrix metalloproteinases (MMPs) in oral SCC, both directly in $PDPN^+$ cancer cells (MMP14) and

indirectly by activating and inducing surrounding fibroblasts. In the latter case TGF-β secretion was shown to upregulate stromal MMP2 and MMP14 expression. Activated fibroblasts (CAFs) within the tumor associated stroma, in turn, stimulate PDPN upregulation in tumor cells through the secretion of 'host' TGF-β, combined effects leading to increased invasion capacity (Li et al., 2018; Suzuki et al., 2008).

PDPN has been reported to promote hematogenous cancer metastasis (Takemoto *et al.*, 2017a). It has been established that platelets support tumor cell dissemination and metastasis particularly by coating tumor cells that have entered the blood stream and thus providing protection from circulation-related sheer stress, immune surveillance (e.g., NK cells) and through facilitating the subsequent adhesion to vascular endothelium (Haemmerle *et al.*, 2018; Palumbo and Degen, 2010). The latter step is critical for tumor cell extravasation and ultimately the establishment of metastatic deposits. It is important to note that platelets coating tumor cells are activated and they release bioactive factors, including TGF-β and PDGF, which additionally blunt the natural killer (NK) cell response (Kopp *et al.*, 2009; Palumbo and Degen, 2010). These factors also modify cancer cells by inducing EMT (Labelle et al., 2011) and directly facilitate their transendothelial migration and ultimately promote the growth of metastatic lesions (Fujita and Takagi, 2012; Lowe et al., 2012; Suzuki-Inoue, 2011). Notably, several of these processes are blocked by agents that inhibit coagulation cascade, platelet function and NK cell activity (Fig 1.5) (Palumbo and Degen, 2010). Moreover, the role of PDPN-CLEC-2 interactions in settings of experimental metastasis are documented by anti-metastatic effects of anti-PDPN or anti-CLEC-2 neutralizing antibodies that also diminish platelet aggregation (Kato et al., 2008; Kato et al., 2006). In addition, in CLEC-

2 deficient mice, pulmonary metastasis of PDPN+, but not PDPN- tumors was greatly reduced (Shirai et al., 2017).

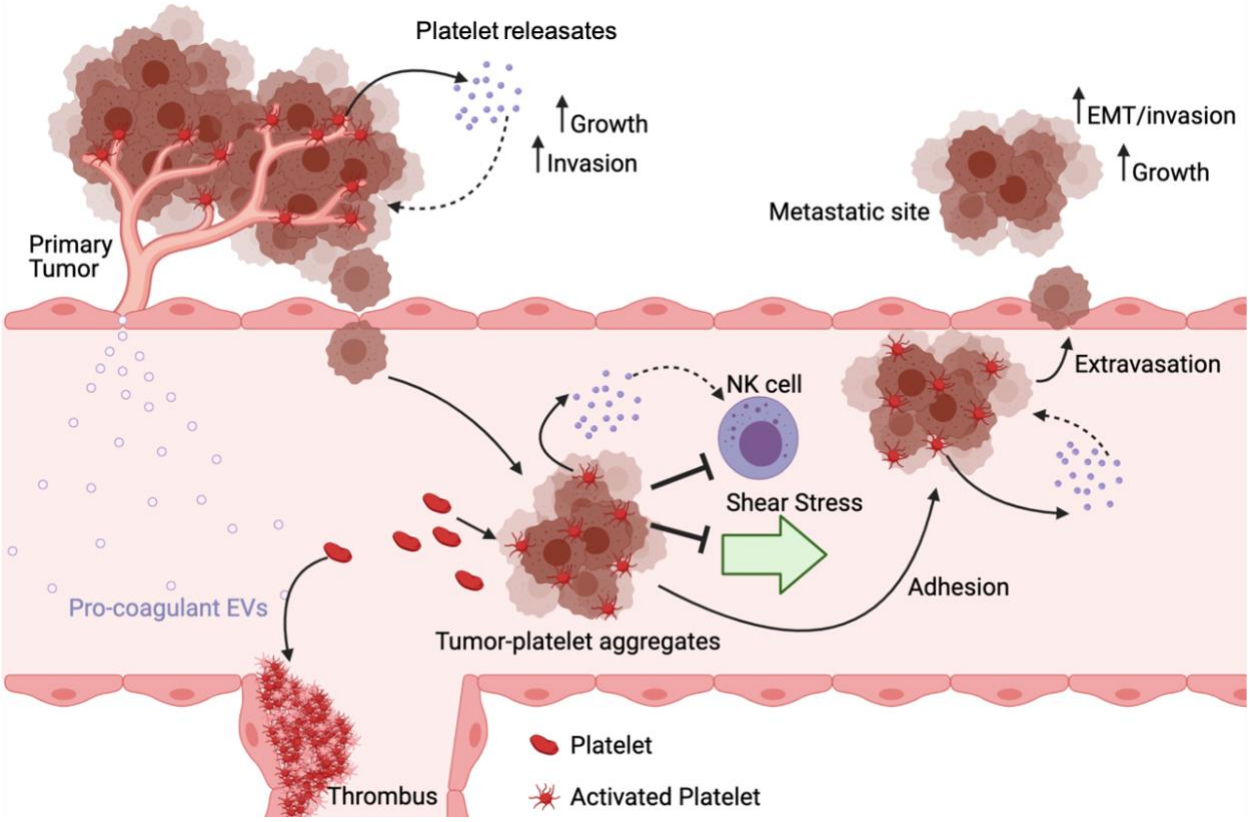

Figure 1.5 ***PDPN CLEC-2 axis in facilitating metastasis***

Throughout the course of malignancy, tumor-induced activation of platelets has the capacity to facilitate and promote tumor progression. The interaction of PDPN expressing tumor cells with platelets in the circulation produces tumor–platelet aggregates. Such aggregates shielded from sheer stress by activated platelets also exhibit resistance against immunological attacks by NK cells through the display MHC class I as well as platelet releasates. Platelet aggregates covering intravasated tumor cells further facilitate adherence to the vessel wall and the formation emboli in microvasculature. These in turn could facilitate extravasation and promote metastasis. Formation of the pre-metastatic niche is facilitated by platelet releasates which also contribute to tumor growth EMT/invasion which could propel extravasation. Similarly to the scenario in circulation, it is possible that tumor-expressed PDPN interaction with platelets leaked from vessels at the site of primary tumor contributes to tumor progression through factors released as a result of platelet activation.

1.5.5.6 Podoplanin-related paraneoplastic pathologies

As mentioned earlier, in section 1.4, the very same potential to drive platelet activation makes PDPN a prime risk factor for both inflammation and cancer driven thrombosis (CAT), and particularly in patients with leukemias and brain tumors (Lavallée et al., 2018; Riedl *et al.*, 2017; Zwicker, 2017). While mechanistics of PDPN involvement in CAT and contribution to promoting GBM associated VTE have been discussed earlier, it is interesting to note that in promyelocytic leukemia, Lavallée et al. showed that only PDPN-expressing cells were capable of inducing thrombocytopenia and prolonged bleeding times (Lavallée *et al.*, 2018).

In contrast to leukemias, when it comes to brain tumors, the way in which PDPN expression may impact systemic VTE is not obvious. This is because brain tumor cells are largely confined within the cranium or CNS, and thereby have limited access to circulating blood and no contact with sites of peripheral thrombosis, which in GBM usually occurs in extremities and visceral vessels (Tawil *et al.*, 2021) . This thesis project investigates the possible resolution of this paradox and suggests that GBM cells shed PDPN as coagulant extracellular vesicles that activate platelets in the peripheral circulation (Tawil *et al.*, 2021).

In more general terms, PDPN has been detected in biological fluids of patients with several advanced cancers. This includes larger and more aggressive multifocal tumors (bladder cancers), metastatic tumors, and those with generally poor prognosis (Zhao et al., 2018). The inference as to cancer cells being the major source of circulating PDPN comes from reports indicating a drop in blood levels of PDPN following cytoreductive treatment with chemotherapy, or surgery followed by chemotherapy (Sankiewicz et al., 2016; Zhao *et al.*, 2018).

1.5.5.7 The genesis and role of circulating podoplanin

As mentioned earlier PDPN is a transmembrane protein not amenable for conventional pathways of secretion. Therefore, the presence of PDPN in the circulation implies an unconventional form of release. In the case of other coagulant transmembrane proteins, such as TF, their extracellular release may entail packaging them as cargo of extracellular vesicles (Giesen *et al.*, 1999; Yu and Rak, 2004), cleavage of the ectodomains, or alternative splicing of the mRNA to produce a soluble protein isoform (Bogdanov et al., 2003). Importantly these release processes may profoundly alter the coagulant and biological activity of the protein in question (van den Berg et al., 2009). These are important consideration in cancers, such as a subset of GBM, where tumor associated PDPN is implicated in triggering peripheral thrombosis and platelet activation (Costa et al., 2019; Riedl *et al.*, 2017) without evidence of the physical presence of cancer cells at the sites of clot formation.

While systematic studies on extracellular release of PDPN are lacking, there is mounting evidence for circulating PDPN in various biological settings. For example, mice engineered to express chimeric PDPN in their epidermis exhibited signs of disseminated intravascular coagulation (DIC) and hemorrhages, occasionally with lethal outcome. This was seen as a consequence of the detected presence of PDPN in the circulation (Cueni *et al.*, 2010a). PDPN was also reported in the circulation of mice with metastatic cancer (Suzuki-Inoue, 2019). Likewise, PDPN was detected in an incompletely defined soluble form (sPDPN) in plasma of patients with breast cancer (Zhu et al., 2020), adenocarcinoma, SCC, lung cancer, gastric cancer and rectal cancer (Zhao *et al.*, 2018) and correlated with metastasis, albeit without clear implications for CAT (Zhao *et al.*, 2018). While the exact nature of sPDPN often remains undefined, the emerging

evidence suggests that MDCK cells (Carrasco-Ramirez et al., 2016) and glioblastoma cells (Tawil *et al.,* 2021) may release this protein in a bioactive form as exosome-like extracellular vesicles. However, in spite of these developments and threads of evidence from various cancer models, particularly in GBM, the mechanisms of PDPN release into the circulation and the vascular consequences of this process in cancer remain to be investigated (a subject of this thesis project).

In the remainder of this thesis, we explore the nexus between transformation pathways operative in GBM cells and the expression and function of genes with vascular activity and thereby likely responsible for the underlying vascular pathology, especially the occurrence of CAT. In this regard we focus on PDPN and the cells expressing this potent regulator across GBM cellular landscape. Finally, we investigate the interactions between GBM cells and the hemostatic system, including the role of PDPN release as coagulant EVs.

2 Project Rationale, Hypothesis and Research Questions

2.1 Unanswered Questions

GBM is the most malignant subset of brain tumors and exhibits high levels of biological complexity. This complexity is reflected at the intratumoral level in the interplay and functional integration of different cellular constituents (Bonavia *et al.*, 2010; Choi *et al.*, 2017; Couturier *et al.*, 2020; Jung *et al.*, 2019; Neftel *et al.*, 2019; Osswald et al., 2015; Patel *et al.*, 2014). GBM complexity is also apparent at the systemic level in how GBM affects the host. The latter is exemplified by the impact on the hemostatic system and manifestation of the high risk for CAT (Edwin et al., 2015; Jenkins et al., 2010; Marras et al., 2000; Muster and Gary, 2020; Streiff et al., 2015).

Targeting thrombosis in GBM is hampered by the lack of mechanistic understanding of processes involved. As mentioned earlier, several putative effectors of GBM-associated VTE have been studied, including the role of tissue factor (TF). As the main trigger of the coagulation cascade TF is understood to govern thrombin generation, platelets activation and clotting, and is overexpressed in GBM cells (Magnus *et al.*, 2014b). These circumstances led to a long-standing prediction that TF would likely act as the driver and biomarker of CAT in this setting. However, paradoxically, TF expression does not predict the magnitude of the VTE risk in patients with GBM (Thaler 2013). Indeed, more recent works suggest the involvement of PDPN, aberrantly expressed by GBM cells, capable of triggering direct platelet activation, and whose tumor expression levels have been found to be predictive of VTE risk in GBM patients (Riedl and Ay, 2019; Riedl *et al.*, 2017). In this thesis we sought to understand some of the reasons behind these discrepancies.

While local activation of the coagulation system in GBM is easy to conceptualize, the emergence of VTE and thus a systemic procoagulant impact is far less clear. Hence the importance of investigating how could the pro-coagulant GBM lesions induce high frequency of VTE (20-30%) in peripheral veins with which they do not have any direct contact and what are the mediators (Perry et al., 2012). Riedl et al. documented a startling correlation between PDPN and platelet activation and VTE in GBM patients. Platelet activation in patients with GBM has also been corroborated by the laboratory of Craig Horbinski at Northwestern University (Unruh *et al.*, 2016). The exact mechanisms of this process, however, its local and systemic impact, and the role of accessory molecules (e.g., TF) remain unexplained. In particular, it remains unclear whether PDPN in some form may act as the long sought circulating tumor coagulant in GBM, at least in some contexts.

The expression levels of PDPN in malignant brain tumors are variable (Shibahara *et al.*, 2006) but why this is so remains unclear. Generally, PDPN expression was correlated with poor prognosis in GBM patients (Birner et al., 2014; Ernst *et al.*, 2009), hence highlighting the link to mechanisms of disease aggressiveness and necessitating a better understanding of whether this correlation is spurious, microenvironmentally driven or interlocked with molecular drivers operating in cancer cells.

Furthermore, induction of thrombosis may lead to secondary vascular and inflammatory changes that permanently reprogram GBM genome, epigenome and biological aggressiveness and while PDPN expression correlates with VTE risk in GBM, it is unknown how perpetual activation

of these clotting processes alters the biology of GBM cells and what are the related therapeutic challenges as well as opportunities (Magnus *et al.*, 2014b).

2.2 Project Rationale

The overall aim of this doctoral research project is to contribute to a better understanding of the mechanistic underpinnings of GBM-associated thrombosis and attempt to shed light at potential reciprocal relationship suspected to be present between malignancy and the procoagulant state it induces. Particularly, we aimed to investigate the patterns of PDPN expression within GBM, to explore the modulatory forces impacting it and subsequently probe the involvement of extracellular vesicles (EVs) as carriers and potential route of PDPN systemic dissemination and possible contributor to GBM-induced VTE.

There are several documented observations that form the foundation upon which we built our reasoning that shaped the project rationale. As outlined earlier, there has been ample documentation of increased risk of VTE with high-grade glioma (Czap *et al.*, 2019), which was recently found to be particularly strongly correlated with pronounced PDPN expression (Riedl *et al.*, 2017; Watanabe *et al.*, 2019).

In our hypothesis building efforts we had to confront the prior body of literature, including contributions from our group (Magnus *et al.*, 2010) as well as others (Unruh *et al.*, 2016), which pointed to TF as a paradigm of cancer and GBM-driven thrombosis. While this contention was based on compelling preclinical evidence and well-defined model systems these biologically appealing findings did not withstand the scrutiny of clinical analysis and had to be reconsidered.

Indeed, clinical studies found no correlation between either TF levels or circulating TF-carrying microparticles (MPs)/EVs and VTE risk in GBM patients (Thaler *et al.*, 2012). Instead, such risk was found for PDPN but without an explanation of how PDPN confined to intracranial cancer cells may act systemically to induce clotting. We chose to embark on seeking the answer.

Additionally, work from our own lab and others had demonstrated that oncogenic driver mutations in GBM, particularly EGFR, are capable of modulating the expression of elements of GBM coagulome. The work by Magnus et. al had previously shown an upregulation of TF expression driven by the expression of oncogenic EGFRvIII (Magnus *et al.*, 2010; Magnus *et al.*, 2013). This led to the notion that GBM oncogenes may directly impact VTE. The general correctness of the link between VTE risk and cancer genetics has recently been confirmed in a large clinical study (Dunbar *et al.*, 2020). However, since PDPN emerged as the main candidate driver of VTE in GBM its plausible regulation by oncogenic pathways became an important research priority.

The role of cancer cell epigenome in malignant transformation represents an area of great interest. While transforming alterations in DNA methylation patterns and chromatin architecture have been implicated in cancer progression, including GBM (Sturm *et al.*, 2014), only a handful of studies explored the impact of these events on the coagulome. A stark reminder of the relative importance of epigenetic modulation in shaping the phenotype of cancer cells stems from single cell sequencing studies where individual cells, often with a similar genomic make up, may exhibit different phenotypic traits, including subtype signatures (Patel *et al.*, 2014) or differentiation

programs (Couturier *et al.*, 2020). Thus, we asked whether these processes influence the expression and function of PDPN in GBM.

It is becoming increasingly clear that EVs constitute an important emerging player in the realm of intercellular communication both in physiology and pathology. Several reports have documented mechanisms involving EV emission and their cargo being implicated in various aspects of different pathological states of the vasculature (Lo Cicero et al., 2015; Ruan et al., 2021; Spinelli et al., 2021; Van Niel *et al.*, 2018). This suggested a possibility that GBM-expressed PDPN might be emitted as cargo of tumor-derived EVs, could spill into systemic circulation and promote the propagation of procoagulant state via its capacity to induce platelet activation (Kato *et al.*, 2003; Riedl *et al.*, 2017; Sekiguchi *et al.*, 2016; Takemoto *et al.*, 2017a). This became a key question we wished to address.

Finally, Magnus et al. demonstrated the reciprocal nature of the relationship between malignancy and the procoagulant state. According to this model overexpression of procoagulant effectors, namely TF, was shown to be capable of changing the tumor microenvironment by recruitment of inflammatory cells and new blood vessels, which in the fulness of time would impact intrinsic properties of tumor cells contributing to their escape from dormancy. Interestingly, these secondary changes involved a massive loss of DNA methylation and dramatic changes in gene expression (Magnus *et al.*, 2014b; Magnus et al., 2014c). Whether these effects are restricted to TF, unspecific or may accompany PDPN expression remained unknown and open to question.

The plausible impact of PDPN expression on cancer cells (tumorigenicity) is of interest for several reasons. PDPN possesses the capability to activate platelets, which are known to exert several biological effects on cancer, ranging from increased metastasis to immunosuppression (Haemmerle *et al.*, 2018). Thus, we hypothesized that PDPN/platelet axis could impact other facets of oncogenic progression, including recurrence in GBM. We reasoned that even small recurrent lesions expressing PDPN could activate platelets, trigger clotting and exert biological effects on cancer cells to accelerate disease progression, especially given the occurrence of GBM in older patients prone to cerebrovascular events. One aspect of such influence could be that releasates from activated platelet may be able to suppress the activity of NK cells and reduce the innate control over the incipient brain malignancy (Kopp *et al.*, 2009). Thus, while we appreciated the earlier findings of PDPN being a 'marker' of aggressive cancer cells, we embarked on the analysis as to whether the reverse may also be true, i.e., whether PDPN expression itself could confer aggressiveness to GBM cells *in vivo*.

2.3 Hypothesis

This project aims to investigate the PDPN expression pattern and regulation by oncogenic drivers and the epigenome, as well as the link between PDPN, thrombosis and GBM progression. The key components of our working hypothesis are as follows:

1. PDPN is a regulatory target and effector of certain oncogenic driver mutations and epigenetic alterations in GBM (EGFRvIII, IDH1, PRC2, DNA methylation pathways) resulting in changes in PDPN expression and systemic release. Such a link would render PDPN expression and activity specific to molecular subtypes of GBM driven by distinctive oncogenic mechanisms.

2. PDPN acts systemically driving the risk of VTE as a result of its release as cargo of extracellular vesicles (EVs) which interact with platelets both locally and systemically.

3. PDPN may exhibit cooperative effects with the TF pathway and other elements of GBM 'coagulome' known to be affected by oncogenic drivers. This synergy may not only impact coagulopathy in GBM, but also elicit signals that could alter various aspects of the tumor and possibly alter tumor progression.

4. We postulate that coagulation events in GBM are targetable in a manner that would aid in more effective disease management.

2.4 Research Objectives

The research objectives driving this doctoral project are as follows:

1. To understand the regulation of PDPN across the landscape of grade IV glioma

   (a) Investigate the pattern of PDPN expression and characterize PDPN expressing cell populations among molecular subtypes of GBM and at the single cell level.

   (b) Evaluate the contribution of oncogenic driver pathways (EGFRvIII, IDH1) to PDPN expression using tumor datasets and cellular GBM models.

   (c) Explore the mechanisms of PDPN regulation by key oncogenic mutations in GBM, especially EGFRvIII and IDH1 R132H, respectively defining the classical GBM an a subset of grade IV gliomas with methylation phenotype (Chapter 4).

2. To understand the local and systemic role of PDPN in GBM-associated thrombosis.

   (a) Investigate the potential involvement of extracellular vesicles as route for systemic dissemination of GBM-related PDPN.

   (b) Examine the role of GBM-associated and systemically disseminated PDPN in peripheral activation of platelets.

   (c) Assess the contribution of PDPN to intratumoral thrombosis and explore the possible cooperation with TF. (Chapter 5 and 6).

3. To explore the possible contribution of PDPN expression on intrinsic aggressiveness of GBM cells

   (a) Evaluate the impact of PDPN expression in GSC models of tumorigenicity.

   (b) Investigate whether PDPN's contribution to tumorigenicity is an intrinsic or extrinsic phenomenon.

   (c) Investigate potential avenues to target the implicated mode of action. (Chapter 7).

3 Experimental Procedures

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3.1 Cell culture conditions, cell lines, reagents and treatments

Cell culture conditions have been previously described (Magnus *et al.*, 2014c; Spinelli et al., 2018). Briefly, U373P and U87P derived cells were maintained in Dulbecco's Modified Eagle's minimal essential medium (DMEM), supplemented with 10% Fetal Bovine Serum (Multicell FBS) and 1% Penicillin-Streptomycin (Gibco). GBM patient derived glioma stem cell lines (GSC), GSC157 and GSC1079 as well as GSC 528 (characterized as PN) and GSC 1123 (characterized as MES) were derived and characterized as proneural in the laboratory of Ichiro Nakano (Minata et al., 2019). GSC lines were maintained as sphere cultures, unless otherwise indicated, in DMEM-F12 media (GIBCO) supplemented with EGF (GIBCO), FGF (GIBCO), Heparin 0.2% (STEMCELL), B27 serum free supplement (GIBCO), Glutamax (GIBCO) and 1% penicillin-streptomycin (P/S) (GIBCO).

We used several isogenic variants derived from either U87P or U373P glioma cell lines (Magnus *et al.*, 2014b; Tawil *et al.*, 2021) . Notably, cells that were only maintained in culture and did not undergo passage in mice were designated as parental (U373P, U87P). The corresponding cell lines transfected with EGFRvIII oncogene were designated as U373vIII and U87vIII respectively and were generously supplied by late Dr. Abhijit Guha (University of Toronto). U373P cells were also transfected with TF, injected subcutaneously, and allowed to form primary tumors (PT) in immunodeficient mice after a long latency period, followed by their re-establishment in culture. The respective designation of such cell line used in this study is U373-

TF-G11-PT (Magnus *et al.*, 2014c; Tawil *et al.*, 2021) . Finally, while U373P cells are indolent and form dormant lesions in mice on rare occasions we were able to isolate tumors from mice injected with these cells and re-establish them in culture (U373-PT). To produce similar, mouse-derived cell lines expressing EGFRvIII, the U373vIII cell line was injected into mice and tumors isolated, dissociated and cultured (U373vIII-PT).

It should be mentioned that batches of U373P and U373vIII cells used in the present study were previously described by their originators (Micallef et al., 2009), extensively characterized (Magnus *et al.*, 2014b) and maintained in the laboratory for over 10 years, while exhibiting a remarkable phenotypic stability and data reproducibility. Using short tandem repeat (STR) assays the U373P cells were subsequently assigned genetic identity common with the commercially available U373MG cell line which was found to be identical with another commercially available U251MG cells, resulting in recent renaming of these cell lines to reflect their common origin (https://www.phe-culturecollections.org.uk/collections/ecacc.aspx). However, the biological properties observed in the case of our U373P cell line maintained from early passage in our laboratory, including their stable, astrocytic, and indolent phenotype as well as low TF expression (Magnus *et al.*, 2014b; Magnus *et al.*, 2010), were found to be different than those described in the literature for more aggressive U373MG cells available commercially (Albrektsen *et al.*, 2007) . Therefore, we believe our cells represent a variant, possibly less altered of the commercially available U373MG cells and we chose to adopt their unique designation, "U373P" to avoid possible confusion. For all cell lines their designation as "PT" (e.g., U373PT) indicates that the cells were isolated from the primary tumor initiated by the indicated cells (e.g., U373).

For GSCs their serum induced differentiation protocol involved maintenance in DMEM-F12 supplemented with 10% FBS, 1% P/S and 1% Glutamax. For Dacomitinib treatment, U373P and U373vIII cells were incubated with the drug (PF 00299804) (Selleckchem) at concentrations of 0.125 μM and 1 μM in DMEM supplemented with 10% FBS growth medium replaced every 24hr and extracts for western blot analysis were collected at 72h. For Pictilisib treatment, two batches of U373P and U373vIII cells were treated for 72h with the drug (GDC-0941 Catalog No. S1065) at the concentration of 5 μM in DMEM supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin, and treatment medium was replaced every 24hr. For 5-aza-2′-deoxycytidine (DAC) treatment. U373P, U373vIII and U87vIII cells were incubated with 5 μM of the drug (Sigma-Aldrich) 24h after plating in growth medium, and drug-containing medium was replaced every 24h. Western blot analysis was performed on extracts harvested 1, 2 and 3 days after the beginning of DAC treatment. For EZH2 inhibitor treatment, U373vIII cells were incubated with the drug (UNC1999; Selleckchem, Catalog No. S7165) at the concentration of 2.5 μM in complete growth medium for 25 days. Drug-supplemented growth medium was replaced every 48hrs. Extracts were collected on days 0, 5, 10, 15, 20 and 25 for western blotting. In combination treatment with Dacomitinib experiment, UNC1999 was used at concentrations of 1.25 μM or 2.5 μM and treatment duration was 7 days followed by western blotting. All drug concentrations were based on the prior data reported in the literature and in house testing across several concentrations.

3.2 RNA Analysis

Total cellular RNA was extracted using the RNeasy Mini RNA extraction kit (Qiagen Catalog No.74104) and RNA was reverse transcribed to single-stranded cDNA using QuantiTech Reverse Transcription kit (Qiagen Catalog No.205310) according to the manufacturer's protocol. Additionally, for mirR-520g, RNA was isolated from cells using the miRNeasy kit (Qiagen), and quantification was done using the Nanodrop (D'Asti *et al.*, 2016). Reverse transcription of miRNA was performed using the TaqMan miRNA reverse transcription kit (Thermofisher Cat# 4427975) according to the manufacturer's protocols.

3.2.1 *Quantitative Real-time q-PCR*

The relative mRNA expression levels of PDPN, miR-520g (along with control GAPDH mRNA and U6, respectively) were quantified using real-time PCR analysis (D'Asti *et al.*, 2016) . PDPN mRNA levels assessment was performed on the LightCycler480 (Roche) and amplification of specific PCR products was detected using the RT2 SYBR Green Fluor PCR Master Mix (Qiagen Catalog No.330510) according to the manufacturer's protocol. Amplification of miR-520g was performed using LightCycler96 (Roche) using the TaqMan Universal PCR Master Mix without amperase UNG and TaqMan miRNA primer assays (Life Technologies): miR-520g-3p (1121), and U6 snRNA. Forward and reverse primers for PDPN mRNA were used at a final concentration of 200 nM and all primers employed were cDNA specific and were synthesized by Integrated DNA Technologies – IDT. Primer combinations are shown in Table 1.

Gene	Primer forward (5′–3′)	Primer reverse (5′–3′)
PDPN	TCCTCGGGAGAGATAAATGCTGACT	CCGGAGAGGGAGGGTGCCCGAGCTT
GAPDH	GAAGGTGAAGGTCGGAGTCA	TTGAGGTCAATGAAGGGGTC
Beta Actin	CTCTTCCAGCCTTCCTTCCT	TGTTGGCGTACAGGTCTTTG

Table 3.1 ***List of primers***

Blank and standard controls were run in parallel to verify amplification efficiency within each experiment. Within each run, a melting curve analysis was performed to confirm the specificity of amplification and lack of primer dimers. The $2^{-\Delta\Delta Ct}$ equation was applied to calculate the relative expression of genes of interest in the corresponding cell lines and the equation E: 10−1/slope to calculate the efficiency of the RQ-PCR (values averaged around 2.1) was used to validate the efficiency of PDPN primers. The mean Ct value of the parental cell line (U373 and U87) was used as the calibrator point and reference. Average expression relative to reference was plotted with error bars representing SEM (Livak and Schmittgen, 2001).

3.3 Immunoblotting

Indicated cells and EVs were lysed and harvested using Laemmli Lysis-buffer with complete proteinase inhibitor cocktail (Roche Applied Science). Protein concentration was quantified using the micro-BCA protein assay kit (ThermoScientific), and samples containing 25-50 μg of total protein were resolved on 12% gradient SDS-PAGE. After blotting, PVDF transfer membranes (G&E Amersham) were blocked with 5% skimmed milk (5% BSA for phospho- antibodies) and probed with the indicated antibodies, including: anti-PDPN (Abcam Catalog No.128994; 1:1000

dilution), anti-EGFR (CellSignaling Catalog No. 4267L; 1:1000 dilution), anti-pEGFR Y1068 (CellSignaling Catalog No. 2234S; 1:1000 dilution), anti-pAKT S473 (CellSignaling Catalog No. 9271S/4060S; 1:1000 dilution), anti–H3K27me3 (CellSignaling Catalog No. 9733S; 1:1000 dilution), anti–CD63 (Abcam Catalog No. ab134045; 1:1000 dilution), anti-TF (American Diagnostica Sekisui Catalog No. 4509; 1:250 dilution), anti-Flotillin 1 (BD Transduction Laboratories Catalog No. 610821; 1:1000 dilution), anti–CD81 (Abcam Catalog No. ab79559; 1:1000 dilution), anti-Syntenin (Abcam Catalog No. ab133267; 1:1000 dilution), anti-CD9 (Abcam Catalog No. ab2215; 1:1000 dilution), anti–β-actin (Sigma Catalog No. A5441; 1:10000 dilution) and anti-GAPDH (Sigma Catalog No. G8795; 1:5000 dilution). Signal was developed using ECL detection reagents (Amersham RPN2106/RPN2232) (Choi *et al.*, 2018) .

3.4 Lentiviral production and transfection

Initially, 2.5 x10^6 293T cells were seeded onto 150 mm dish (15ml) and 24 hrs later the cells were transfected with the plasmid mixture: VSV-G (8454 Addgene), pRRE (12251 Addgene), REV (12253 Addgene) and transfer plasmids were added. The transfer plasmids used to deliver sgRNAs in pCLIP105 Dual-SFFV-ZsGreen for PDPN CRISPR targeting were TEDH-1058829, TEDH-1058830, TEDH-1058827, TEDH-1055978 (Transomic). Cas9 was delivered using the pCLIP-Cas9-Nuclease-hCMV-tRFP (SHB_2264 Transomic). All of which were generous gifts from Dr. Sidong Huang at McGill university. For generating luciferase positive cells, we utilized a Luc-BFP dual expression lentiviral vector kindly provided by the laboratory of Dr. Kolja Eppert at the Research Institute of the McGill University Health Centre. Transfection was carried out using the calcium phosphate transfection method (2X HBS mixed with a solution with 2M $CaCl_2$ in addition to the plasmid mixtures in accordance with what was mentioned above). Cells were

then incubated over-night (8- 12hr) at 37C, 5% CO2. On the next day the media was changed, cells were incubated and media containing viral particles was collected 24 and 48 hrs following media change. Collected media was spun down at 1,500 x g for 10min (4^{o}C) to eliminate large debris and filtered with 0.4 µm syringe filter. Finally, media was mixed with Lenti-X reagent, incubated for 1hr on ice and pellets of viral particles were obtained by spinning at 1500 rpm for 1.5 hrs. Obtained pellets were re-suspended in 50 uL of PBS. Luc-BFP lentiviral vectors were used to transduce GSC 1123 and GSC 528 lines. After 16 h post transduction, appropriate media were replenished, transduction efficiency assessed by florescent microscopy and using flow cytometry followed by sorting for BFP, to isolate successfully transduced cells. Stable expression of PDPN in GSC1123 was achieved via lipofectamine mediated transfection of PDPN plasmid (Origene catalog no. sc125342) followed by 4 rounds of sorting for PDPN-expressing cells over a period of 1.5 months in culture. Stable expression of PDPN was validated by immunoblotting performed on cell lysates taken at various time points over the period of 3 weeks succeeding the last round of FACS.

3.5 CRISPR-CAS9 gene knockout

Knock out of PDPN in PDPN-positive GSC 528 clones was achieved by lentiviral transduction employing the sgRNA and Cas-9 carrying vectors outlined above. First, Cas-9 was introduced and Cas-9 expressing cells were isolated by RFP expression using FACS. Next, Cas-9 positive cells of each clone were transduced with the previously validated sgRNA viral vector (TEDH-1058827) harboring a combination of 2 guide RNAs and GFP. Cells in which successful knock out of PDPN was achieved were identified and sorted out as the RFP-positive, GFP-positive and PDPN-negative cell population using FACS.

3.6 Mouse tumor models

For subcutaneous inoculation (s.c), immunodeficient SCID mice (Charles River) harbouring the YFP transgene (YFP/SCID) (Yu et al., 2008b) were injected with single cell suspensions of indicated glioma cell lines in serum free DMEM medium at 3 x 10^6 cells per mouse in 0.2 ml volume, in the left flank. Viability of cells was tested and exceeded 90% according to trypan blue exclusion assay.

For intracranial inoculation (i.c), SCID/YFP transgenic mice were anaesthetized, surgically prepped, scull exposed, drilled and striatum stereotactically injected with 2 x 10^5 glioma cells per inoculum in 2 µl volume of serum free media, as described previously (Magnus *et al.*, 2014b) . The site of injection was standardized using Stoelting Stereotaxic Injector at coordinates (AP = +0.5; ML = +1.5.; DV = -3.0) of bregma and sagittal sutures. For systemic EV injection, the indicated cells were cultured and EVs isolated from conditioned media as described earlier (Choi *et al.*, 2018) and below. EV isolates were assessed for protein concentration using the BCA assay (Pierce Biotechnology, Rockford, IL) and the equivalent of 10 µg of intact EVs was injected into the tail vein (i.v.) of SCID/YFP mice. Within 15 minutes post injection, whole blood was collected via the inferior vena cava (IVC) (procedure outlined below) and platelet poor plasma was isolated and stored until used (Chennakrishnaiah et al., 2018).

Whenever possible and required, injections with cells engineered to express the luciferase gene were monitored by collecting bioluminescence data which was obtained using Xenogen (IVIS 200) bioluminescence scanner following the administration of D-Luciferin Firefly potassium salt (Caliper Life Science; 15ug/ml) substrate intraperitoneally. All procedures

involving animals were performed in accordance with the guidelines of the Canadian Council of Animal Care (CCAC) and the Animal Utilization Protocols (AUP) approved by the Institutional Animal Care Committee (ACC) at MUHC RI and McGill University.

3.7 Immunostaining

Tumors were resected and preserved in fresh 4% paraformaldehyde (PFA). Tissue processing was performed in an automated tissue processor unit (Leica TP 1050 tissue processor), followed by paraffin embedding. Tissue blocks were sectioned using American Optical microtome into 5 μm thick sections mounted on pre-coated glass microscope slides (Magnus *et al.*, 2014b). Prior to staining, sections were de-waxed in Xylene, followed by re-hydration in a series of alcohol washes (95% to 50% ethanol). For hematoxylin and eosin (H&E) staining, rehydrated slides were washed and incubated in Hematoxylin (1.5% Acid Solution, pH 2.5), then washed in water and dipped in the Blueing Solution. Partial dehydration (50% to 80% ethanol) was performed before proceeding to incubation in Eosin solution, which was then followed by three 5-minute washes in 99% ethanol and Xylene (Magnus *et al.*, 2014b). For immunostaining, first, antigen retrieval was performed using Vector Antigen Unmasking Solution heated to 95°C for 15 minutes. Primary antibodies used for these studies were specific for human PDPN (abcam Catalog No. 128994; 1:250 dilution), mouse Fibrin (abcam Catalog No. ab34269; 1:100 dilution), mouse CD31 (R&D Catalog No. AF3628; 1:20 dilution) and CD61 (Origene Catalog No. AP02622PU-N; 1:100). Incubations with primary antibodies were carried out overnight in a humidified chamber at 4°C. Thereafter, slides were washed three times in PBS (5 min each) and incubated with corresponding HRP-conjugated secondary antibodies, followed by final mounting in Vectastain Elite kit (PK-4006), ImPACT DAB (SK-4105), and VectaMount Mounting Medium (H-5000, Vector Labs,

Burlington, ON, Canada). For fluorescent staining (Fibrin/CD31) secondary antibodies (Invitrogen donkey Anti-Rabbit Alexa Flour 488(Green) Catalog No. A21206, Invitrogen donkey Anti-Goat Alexa Flour 594 (Red) Catalog No. A32758) were incubated with tumor sections in the dark at 37°C for 1 hour. After a series of 5-minute PBS washes, slides were mounted with cover slips using Vectashield Hardset DAPI (Vector) glue, allowed to dry before being visualized as indicated (Magnus *et al.*, 2014b). Quantification of fibrin occluded vessels was performed manually by the random selection of 7 vessel abundant fields within each slide and counting fibrin positive versus total vessels. Martius Scarlet Blue (MSB) staining was performed on tumor tissues, as per established institutional protocols (RIMUHC Pathology Labs). Representative MSB as well as CD61 stained slides were sent for whole slide scanning (Aperio ScansScope AT Turbo, Leica Biosystems). In each slide, 7 representative, equal and random fields were used to quantify fibrin occluded vessels.

3.8 Blood collection

Collection of whole blood was performed in 3.8% sodium citrate and Apyrase from the inferior vena cava (IVC), a 150ul sample was taken for complete blood count (CBC; Diagnostic and Research Support Service (DRSS) Laboratory at the Comparative Medicine and Animal Resource Center, McGill University, and the remaining sample was centrifuged at 1,500g for 15 minutes. Platelet-poor plasma (PPP) was collected and stored frozen at –80°C for further analysis.

3.9 EV isolation and analysis

EV isolation was performed as previously described (Choi *et al.*, 2018). Particularly, conditioned medium (CM) was collected from cultured cells grown for 72 h in media containing 10% of EV-depleted FBS (Ultracentrifuged at 150,000g for 18 h at 4 °C). CM was centrifuged at 400g for 10 min, supernatant recentrifuged at 2,000g for 15 min, and remaining supernatant quickly poured off into clean tubes and passed through 0.8 μm pore-size filter. The resulting filtrate was concentrated using Amicon Ultra-15 Centrifugal Filter Unit (EMD Millipore, Billerica, MA) with 100,000 NMWL cut-off. The concentrate was mixed with 50% of iodixanol solution (Sigma, St. Louis, MO) and processed for density gradient ultracentrifugation at 200,000g for 2 h (Choi *et al.*, 2018; Choi and Gho, 2015). Fractions were serially collected, and their density was determined by measuring the absorbance at 340 nm of sample volumes taken from the individual fractions using an ELISA plate reader (Choi and Gho, 2015). Calculations were made in reference to the generated standard curve. Individual fractions were also analyzed for EV concentration and size distribution using nanoparticle tracking analysis (NTA) system (NS500, NanoSight Ltd., UK). Three recordings of 30s at 37 °C were obtained and processed using NTA software (version 3.0). EVs from individual iodixanol fractions were subsequently collected by ultracentrifugation, protein extraction performed, and concentration of EV proteins was quantified using the microBCA assay (Pierce Biotechnology, Rockford, IL). For nano-flow cytometry the conditioned medium from cells grown for 72 h was collected and processed for EVs as described previously (Choi *et al.*, 2018). The concentrated supernatant was analyzed by NTA and diluted with PBS to the concentration of 10^{11} particles/ml. EVs were incubated with indicated fluorophore-conjugated antibodies at an antibody to EVs concentrate ratio of 1:50 for 2 h at room temperature in the dark (anti-PDPN Alexa 488, Cat. No. 337006; anti-TF PE, Cat. No. 365203; anti-CD81 APC, Cat. No.

349509; anti-CD9 FITC, Cat. No. 312103, all from BioLegend). In order to clear out the excess and unbound antibodies, EVs were re-isolated from staining mixtures using qEV size exclusion chromatography (SEC) columns (Izon Science, UK) according to the manufacturer's instructions. The EV containing fractions (0.5 ml) were identified by NTA. Parallel isotype controls adequately matched with the corresponding antibodies were similarly processed and all samples were read using CytoFLEX system (Beckman Coulter, Pasadena, CA) equipped with 3 lasers (405, 488, and 640 nm wavelength) (Choi *et al.*, 2018). Data were acquired and analyzed using Cytexpert 2.0 software (Beckman Coulter).

3.10 Transmission electron microscopy and immunogold staining

EVs were collected as described previously and washed using 0.1% sodium cacodylate buffer. Following ultracentrifugation, the pellet was resuspended in 2.5% glutaraldehyde fixative solution for 24 hrs at 4 degrees. Charged TEM grids were laid over with 10 µl drops of fixed EVs and contact was maintained for 20 minutes. Grids were washed twice with 0.02 M glycine (5-10min each). For immunogold staining, grids were blocked by overlaying with 10µl drops of BCO (2% BSA- 2% Casein and 0.5% Ovalbumin) blocking agent for 5 minutes followed by incubation with primary antibody (1:1 dilution) (anti-PDPN ab128994; anti-CD63 ab59479 - Abcam) overnight at 4 degrees. Grids were washed with DPBS 5 times (3min per wash), blocked again, and treated with 15µl drops of corresponding gold-conjugated secondary antibodies (1:20 dilution) for 30minutes. Grids were washed again, dried and negative staining was performed using 4% uranyl acetate, after which the grids were allowed to dry for 1hr. EV preparations were examined using FEI Tecnai 12 BioTwin 120 kV TEM (AMT XR80C CCD Camera System) at the Facility for Electron Microscopy Research (FEMR), McGill University.

3.11 ELISA

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to estimate systemic levels of PDPN (LSBio, LS-F6466), PF4 (LSBio, LS-5404), D-dimer (LSBio, LS-F6179), TF (IMUBIND American Diagnostica Inc, 845) in plasma of tumor bearing mice (PDPN, PF-4, D-dimer) and tumor homogenates (D-dimer), as well as plasma samples from GBM patients cared for at the University of Virginia (UVA - D.S; N.K) (PDPN and TF ELISA). Patient plasma sample analyses were conducted anonymously under the approval of the institutional Research Ethics Board (REB, MUHC # 2019-5493). Assays were conducted according to the manufacturers' protocols in duplicates, and their sensitivities as reported by the manufacturers were: 0.156 ng/ml for PDPN, 0.068 ng/ml for PF4, 243.7 pg/ml for D-dimer, and 10 pg/mL for TF. ELISA readings of OD were performed using TECAN Infinite 200 PRO multimode plate reader equipped with i-control™ software interface.

3.12 Platelet activation in the presence of GBM cells and respective EVs

To assess platelet activation in the presence of $PDPN^{high}$ vs $PDPN^{low}$ GBM cells or EVs, the respective U373P and U373vIII cells were cultured in 8 chamber Falcon Culture Slides. At 48-72 hours later, culture media was removed, and cells were washed three times with PBS. Platelets freshly extracted by differential centrifugation from blood of YFP-SCID mice in the presence of Apyrase (Millipore Sigma, Catalog No. A6237) in Tyrode's buffer and finally resuspended in pre-warmed PBS (37°C) were added on top of the cells and incubated for 10 min at 37°C. Following incubation, a fixative solution PAMFix (Platelet Solutions, Catalogue No. PSR-001) was added. Following fixation, chamber slides were spun down to allow the removal of the fixative and preparations were stained using monoclonal APC-conjugated anti-P-Selectin antibody (CD62-

P/APC, Psel.KO2.3). Similarly, platelet activation by EVs derived from either U373P or U373vIII cells was assessed via the co-incubation of freshly isolated platelets with respective EVs for 30 minutes with mild shaking. Following co-incubation, preparations were fixed, spun at 2000g and stained with APC-conjugated anti-P-Selectin. All preparations were visualized using the Zeiss LSM 780 confocal microscope.

3.13 Single-Cell RNAseq

Raw Single-cell RNA-seq data for GSE57872 (Patel *et al.*, 2014) were obtained from the Sequence Read Archive (*SRA*) data base. The data were aligned using HISAT2 and the obtained counts were applied to scImpute for the imputation of dropout event values. The imputed dataset was then normalized to obtain TPM (transcript per million) values, converted to logarithmic scale, and centered by subtracting the mean values of genes across samples. TCGA expression data for Glioblastoma (Brennan *et al.*, 2013) were downloaded as z-scores using the cBioPortal for Cancer Genomics (Gao et al., 2013a). Violin and TSNE plots were created in R environment using packages *ggplot2* and *Rtsne*, respectively. Expression data for EGFR and PDPN was scaled and used for k-means clustering of samples using *ComplexHeatmap* package from R/Bioconductor (Gu et al., 2016). PDPN and Cluster signatures were identified by extracting top 50 feature genes from GSEA analysis as indicated by comparing groups of samples (Subramanian et al., 2005). The roadmap plots were generated from an independent set of scRNAseq data as recently described (Couturier *et al.*, 2020).

3.14 TCGA Data analysis

TCGA expression data for Glioblastoma (Brennan *et al.*, 2013) were downloaded as z-scores using the cBioPortal for Cancer Genomics (Gao *et al.*, 2013a) . Expression data for EGFR and PDPN was scaled and used for k-means clustering of samples using the *ComplexHeatmap* package from R/Bioconductor (Gu *et al.*, 2016).

3.15 Identification of gene expression signatures for EGFR/PDPN-based clusters

Cluster signatures were identified by extracting the top 50 positively and negatively associated feature genes using GSEA (Subramanian *et al.*, 2005). Briefly, for each cluster, GSEA was used to rank the genes in order of their differential expression, using the signal-to-noise metric, between the cluster and the rest of the samples. From this list, the top 50 (POS, positive, upregulated) and the bottom 50 (NEG, negative, downregulated) genes were extracted as the signature genes for the cluster analyzed. This process was repeated for each cluster in the RNA-seq data set. Gene set enrichment analysis was also performed using GSEA, where each gene set (e.g., the POS signatures of a specific single-cell RNA-Seq cluster) was tested for its enrichment among up-regulated or down-regulated genes in another dataset (e.g. the TCGA bulk-tissue data) to identify the relationship between the EGFR/PDPN-based cell clusters and bulk tissue data.

3.16 Data analysis and statistics

Analysis of Pfister-46 GBM dataset was performed using the R2 Genomics Analysis and Visualization Platform and employed statistical one-way analysis of variance (ANOVA). TCGA dataset analysis for PDPN mRNA levels as function of IDH1 mutational status was performed using GlioVis Data Visualization Tools for Brain Tumor Datasets, which employed the paired t-

test statistical analysis. Statistical analysis of platelet counts, ELISA experiments, as well as fibrin and CD61 positive blood vessel counts was done using ANOVA and Tukey's multiple-comparison post-test. A *P* value of <0.05 was used as a measure of significance of difference between groups. GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) was used to perform the latter statistical analyses.

4 Patterns of PDPN Expression Among GBM Cell Subpopulations – The Interplay Between Oncogenic Mutations and the Epigenome

Tawil et al Thrombosis Research 2018; Tawil et al Seminars in Thrombosis and Hemostasis 2019; Tawil et al. 2020, Blood Advances

4.1 PDPN expression in GBM is non-random.

In this segment of our work, we aimed to explore the landscape of PDPN expression in GBM and glean some insight regarding the regulatory mechanisms that could alter its expression. In view of the association between PDPN expression and the risk of thrombosis in GBM (Riedl *et al.*, 2017) we wished to understand whether this coagulant effector is expressed across cancer cells in a random (non-specific) or selective manner.

Our first approach was to assess the patterns of PDPN mRNA expression between molecular subtypes of GBM as defined by TCGA (Verhaak *et al.*, 2010) and SNO-EANO consensus (Wen *et al.*, 2020) studies. To accomplish this, we extracted z-score expression values for PDPN from the TCGA database for the samples annotated for the respective proneural (PN), neural (NEU), classical (CL) and mesenchymal (MES) GBM subtypes (Fig. 4.1a). We also performed similar analyses for TF (Fig 4.1b) and the wider spectrum of cellular coagulome (Fig. 4.2) as well as genes related to angiogenesis (angiome; Fig. 4.2). In all these instances the profiles of vascular effector genes differed between GBM subtypes with PN tumors generally expressing lower levels than CL and MES lesions.

This pattern was especially striking for PDPN, the levels of which were considerably higher in MES than in PN tumors. Also, CL lesions exhibited increased levels of PDPN transcripts than

their PN counterparts (Fig 4.1a). Because CL GBMs harbor frequent amplifications of the EGFR gene often coupled with oncogenic mutation leading to expression of EGFRvIII and since in GBM cell lines overexpression of this oncogene drives a procoagulant phenotype *in vitro* (Magnus *et al.*, 2010) we asked whether EGFR levels also parallel the upregulation of PDPN. This was found not to be the case as levels of EGFR transcript were, as expected, the highest in CL GBM samples, while PDPN levels were the highest in MES subtype of tumors (Fig. 4.3 – EGFR/PDPN). Thus, while PDPN expression among GBM tumors is non-random and subtype specific it does not follow the expression pattern of oncogenic EGFR, a question that we subsequently analyzed in more detail (below).

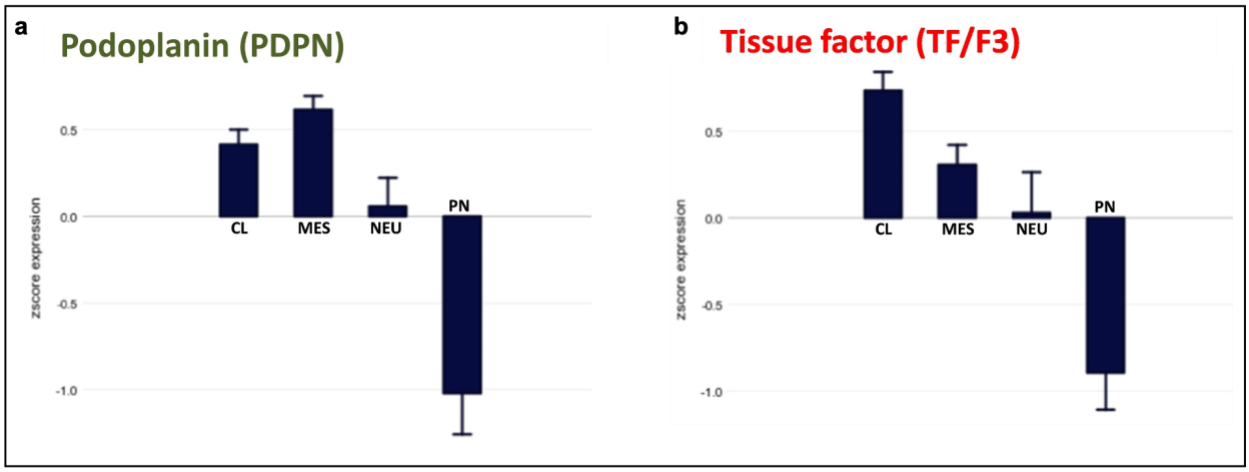


Figure 4.1 ***Subtype specific expression of podoplanin and tissue factor in glioblastoma.***

Average expression levels of **a.** podoplanin (PDPN) and **b.** tissue factor (F3) were extracted from subtype annotated TCGA dataset**.** The z-score (y axis) comparison was used to capture the relative expression levels of F3 and PDPN transcripts (bars) of each GBM subtype (PN, NEU, MES, CL) in comparison to all tumors

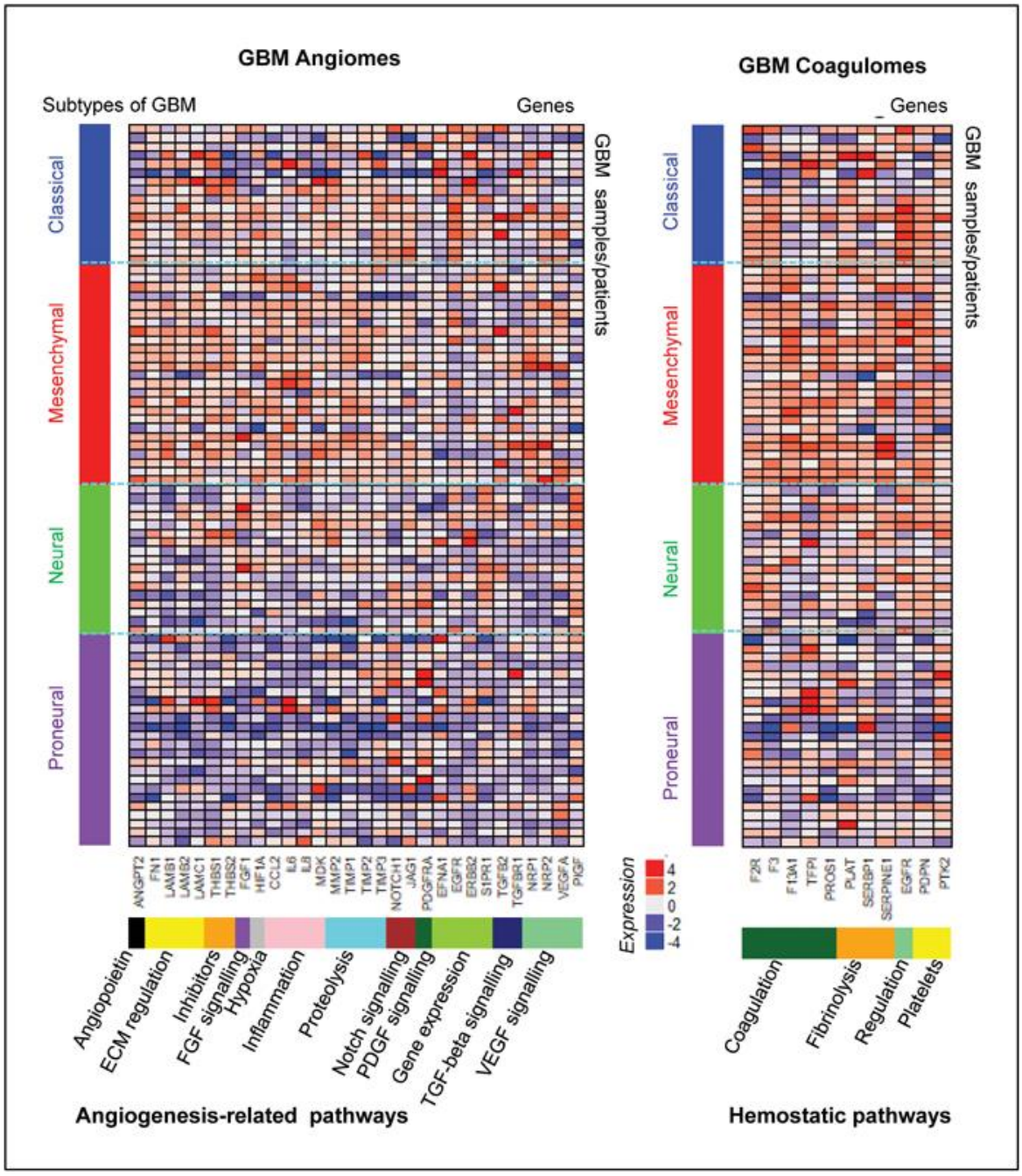


Figure 4.2. ***Angiomes and coagulomes of GBM subtypes*.**
Average expression levels of genes involved in angiogenesis regulation (left panel) and hemostasis was extracted from The Cancer Genome Atlas (TCGA) dataset and annotated for molecular subtypes of GBM (proneural, neural, mesenchymal, and classical GBM). Both angiogenesis-related (angiome) and coagulation-related (coagulome) genes (D'Asti *et al.*, 2014a) were manually audited based on the literature and found to exhibit differences defined by the GBM subtype, mostly higher expression levels in mesenchymal relative to proneural tumors. Heatmaps were generated from a matrix of the selected genes and their respective expression levels using the heatmap function of the R base stats package.

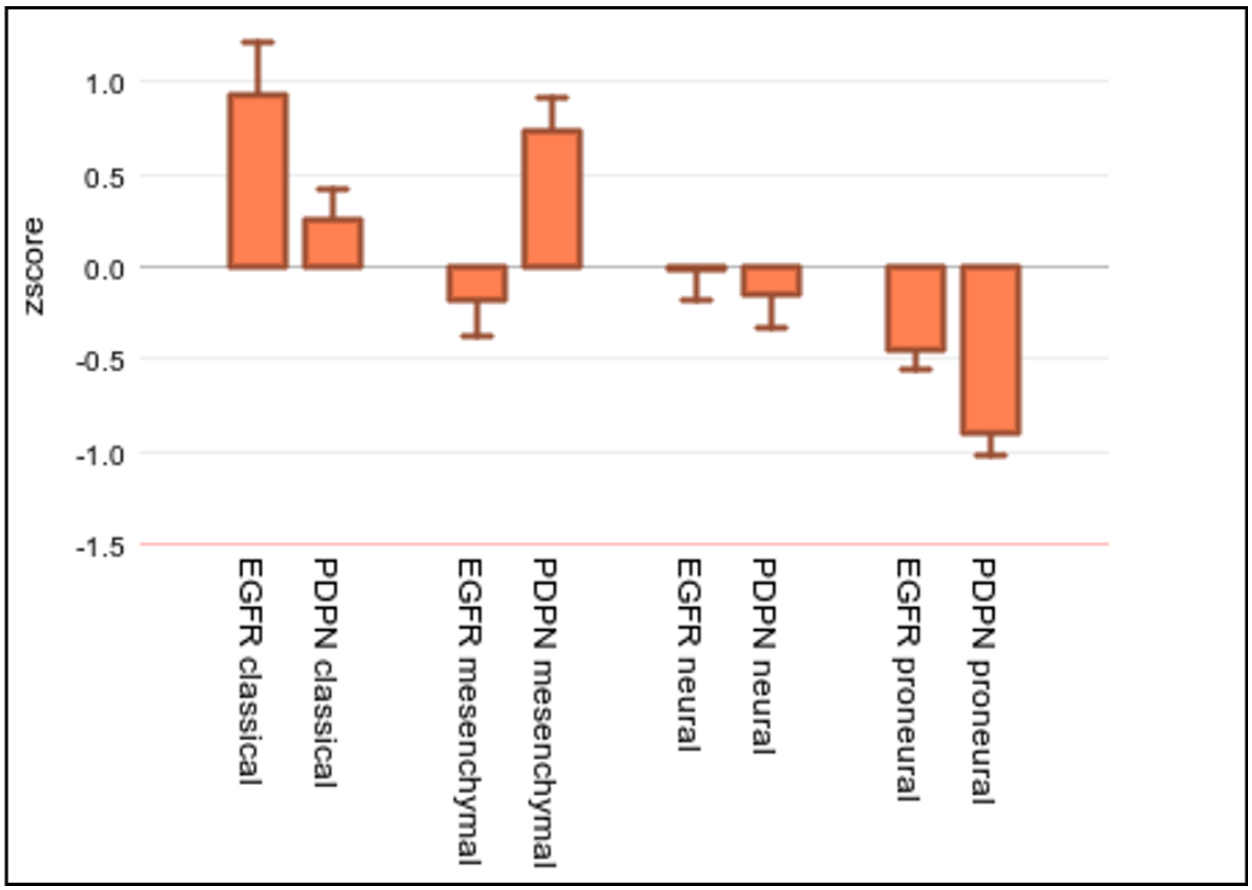


Figure 4.3. Expression pattern of PDPN and EGFR in GBM subtypes.
Average expression levels of EGFR and PDPN were extracted from subtype annotated TCGA dataset. The z-score (y axis) comparison was used to capture the relative expression levels of EGFR and PDPN transcripts (bars) within each GBM subtype

4.2 PDPN expression by distinct GBM cell subpopulations.

Single cell transcriptomics revealed that GBM subtypes are, in fact, reflections of cellular mosaics with individual cells with properties of all, as well as intermediate, subtypes one of which predominates in the equilibrium (Patel *et al.*, 2014). In this context it is reasonable to ask which of the constituent cellular subpopulations carries effectors of the coagulant phenotype, especially PDPN.

To explore this question, we performed initial analysis of single cell transcriptomes of five different GBM lesions (Patel *et al.*, 2014) that covered the spectrum of disease subtypes seeking to examine the distribution of PDPN mRNA. Interestingly, this analysis revealed differential contribution of PDPN expressing ($PDPN^{high}$) cells to individual tumors as revealed by the violin plots, with their lowest representation in proneural GBM (MGH26) (Fig. 4.4a). Further, we wanted to know whether the $PDPN^{+/high}$ cells represent an unspecific component of various GBM populations or form a particular population of cancer cells, that are closely related to one another. To assess this aspect of the cellular architecture we used t-distributed stochastic neighbor embedding (t-SNE), a dimensionality reduction tool that allows for the identification of significant features within a complex set of expression profiles. t-SNE involves the mapping of the high-dimensional state-vectors onto a low-dimensional space (2D or 3D), while preserving important information reflecting the relatedness of the samples constituting the data set. Remarkably, the resulting plots revealed a non-random distribution and clustering of high PDPN expression among GBM cells with their 3-4 transcriptionally distinct subpopulations present across all tumor samples (Fig. 4.4b).

Another approach to understand the heterogeneity of GBM cell subsets is the analysis of their relatedness to pathways (roadmaps) of neural stem cell differentiation (Couturier *et al.*, 2020). In a recent study involving single cell RNA sequencing, the cells emanating from a founder progenitor population harbouring a set of oncogenic mutations (e.g. EGFR) epigenetically acquire gene expression signatures of neural, astrocytic, oligodendroglial and mesenchymal progeny all of which populate individual human GBM tumors (Fig. 4.4c).

To this end a fetal brain roadmap was developed based on the scRNAseq analysis of cells isolated from the telencephalon of four human fetuses ranging from 13 to 21 weeks of gestation, and the determination of which fetal brain cells were most closely representative of each cancer cells. Couturier et al. found that 94% of whole tumor cells were captured by five distinct fetal brain cell types: glial progenitor cells, truncated radial glia, oligo-lineage cells, astrocytes, and neurons. Consequently, these were the five cell types that were used to build the roadmap where principal component analysis (PCA) was performed on equal number of cells corresponding to each cell type. This allowed to produce the PC space that represented the roadmap onto which equal numbers of cancer cells from each patient were projected. Such an approach combined with RNA velocity analysis allowed for the identification of a cell distribution pattern supportive of the existence of a hierarchical dynamics in GBM with GSCs positioned at the apex of the roadmap and constituting the origin of the aforementioned astrocytic, mesenchymal, oligodendrocytic and neuronal cancer cells with cells belonging to each type aggregating together at the periphery of the roadmap (Couturier et al., 2020).

In this context we asked whether PDPN expression will be randomly distributed among GBM cells or concentrate in one or more specific subpopulations, and how would this pattern relate to other coagulant mediators (e.g., TF) or oncogenic transcripts (EGFR). Interestingly, elevated PDPN mRNA was found predominantly in the mesenchymal subset of GBM cells with small contribution of astrocytic population (Fig. 4.4d). In contrast, TF was found mainly in astrocytic GBM cells, while EGFR was dispersed among all cellular populations (Fig 4.5). Thus, EGFR expression pattern was disentangled in patient derived GBM cell populations with the expression of PDPN, which was assigned to a specific cancer cell subset. In other words, this analysis suggests that while oncogenic pathways, such as EGFR, may prime GBM cells for certain gene expression profiles, including PDPN levels, the epigenetic differentiation programs impose the association of specific procoagulant effectors with distinct cancer cell subpopulations.

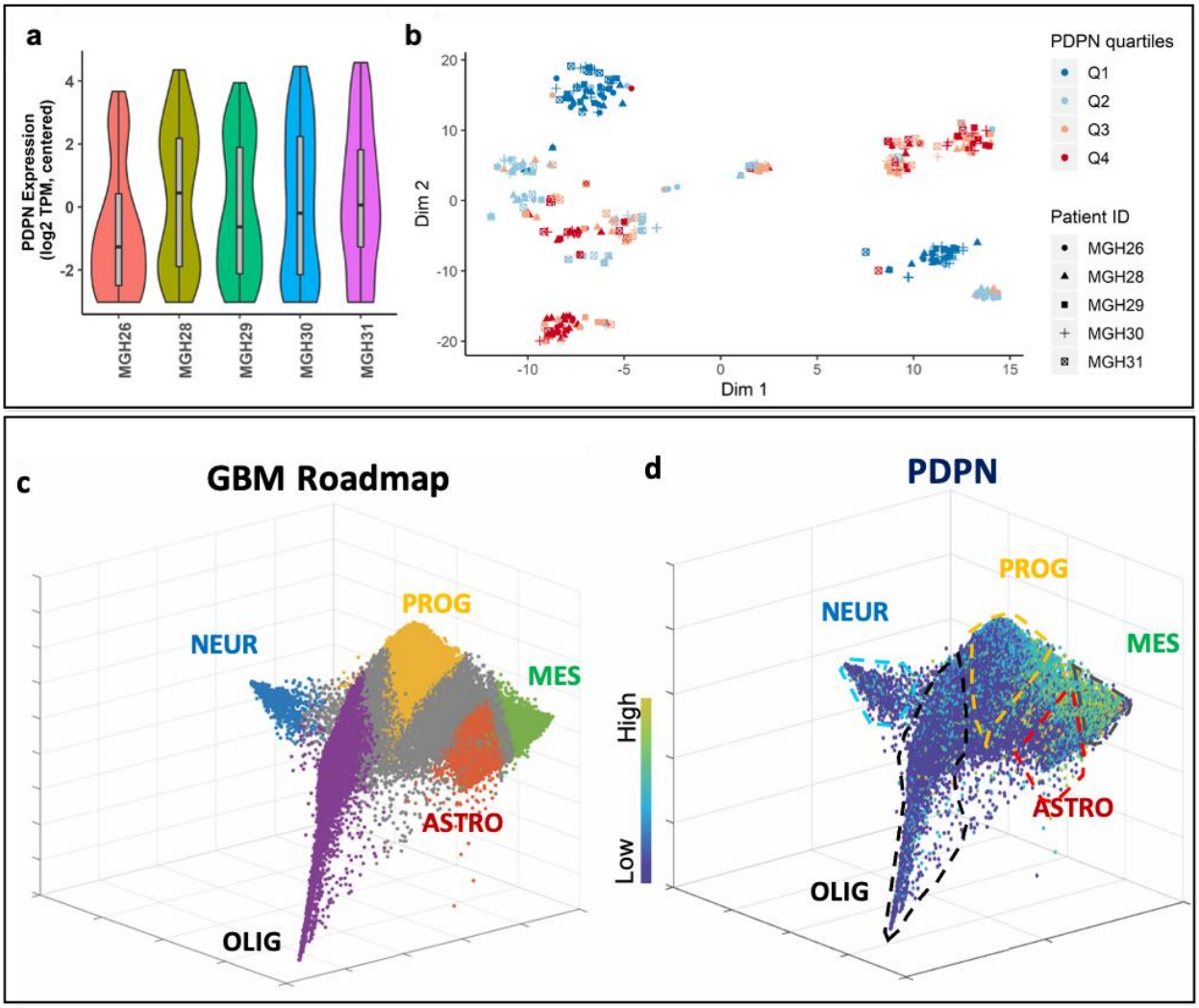


Figure 4.4 ***PDPN expressing cell populations in glioblastoma.***
a. Violin Plot demonstrates the heterogeneous expression of PDPN among cell populations within individual GBMs. **b**. t-SNE analysis captures the distinctiveness of PDPN-positive and PDPN-negative cell subpopulations of 5 individual GBMs. These cellular subsets comprising individual cells originating from different GBM tumors exhibit transcriptional similarities. PDPN expression was ranked by quartiles Q1 to Q4 in an increasing order of expression. **c.** Plot depicting the main differentiation roadmaps of glioblastoma cell subpopulations: PROG- progenitors, NEUR- neural, MES – mesenchymal, ASTRO – astrocytic, OLIG – oligodendrocytic, as revealed by single cell sequencing (Couturier *et al.*, 2020); **d.** PDPN high expressing cells (yellow/orange/red cells) cluster in the region of MES GBM cells, with some contribution of astrocytic and progenitor cells.

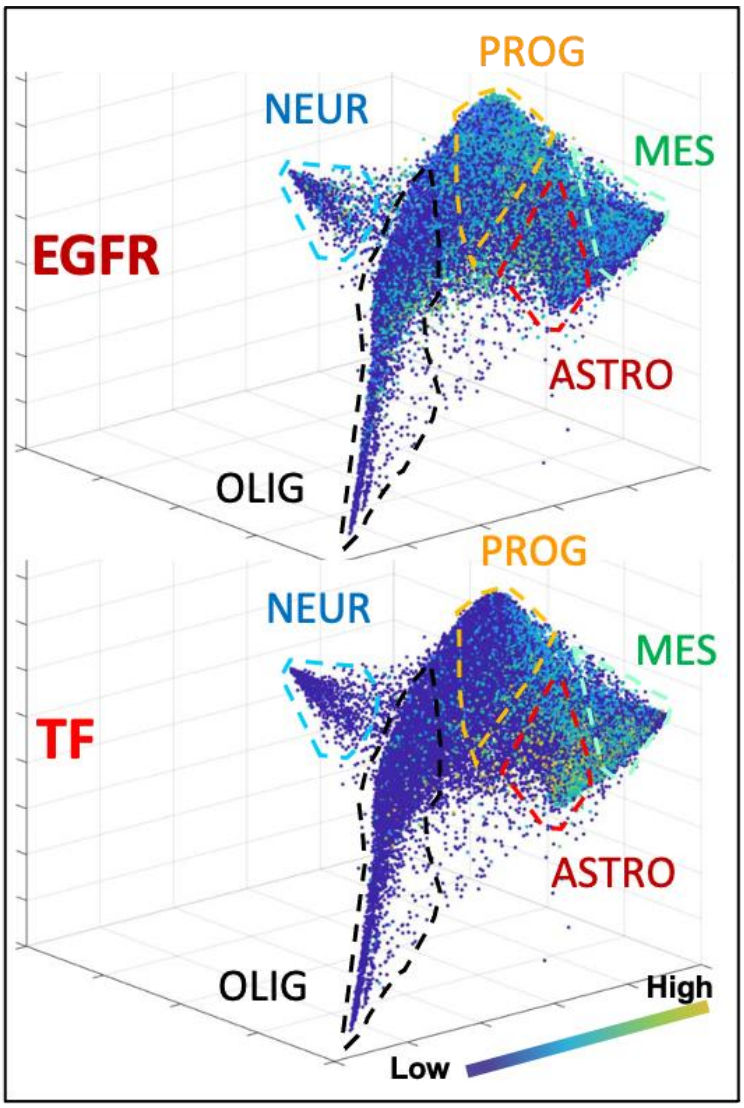


Figure 4.5. Distribution of EGFR and TF expression within GBM roadmaps. EGFR expressing cells (yellow/cyan cells) exhibit a dispersed pattern while TF expressing cells (yellow/orange/red cells) are found mainly concentrated in the area occupied by GBM cells conforming to the astrocytic lineage. In conjunction with Figure 4.4 these results suggest that coagulant effectors are differentially expressed between subsets of GBM cells where mesenchymal cells are enriched in PDPN and astrocytic cells are enriched in TF. This may suggest that these respective cell subsets may cooperate in certain coagulant processes (e.g., intratumoural microthrombosis) while playing unique roles in other events (e.g., systemic hypercoagulability – see subsequent chapters).

4.3 Characteristics of PDPN expressing GBM cells

We set out to characterize PDPN-expressing cells as implied 'cellular' effectors of CAT in GBM. To that end we used several computational tools to extract the information as to the regulatory program(s) that favors expression of PDPN by these specific cancer cells, including pathway enrichment analysis. Accordingly, extraction of gene expression Hallmarks and KEGG pathways associated with $PDPN^{high}$ cell subsets indicated the preponderance of genes involved in coagulation, inflammation, wound healing, mesenchymal transition and RAS signaling (Fig. 4.6a). Gene set enrichment analyses (GSEAs) of single cell transcriptomes also revealed that multigenic Coagulation Hallmark was also tightly correlated with PDPN expression (Fig. 4.6b), all of which suggested that $PDPN^{high}$ GBM cells possess a distinct coagulant phenotype amidst other cancer cell populations. Notably, a similar association between PDPN enrichment and the Coagulation Hallmark was also present in the Cancer Genome Atlas (TCGA) dataset representing a whole tumor mass, which may suggest that tumor positivity for PDPN mRNA may reflect tumor enrichment for a specific $PDPN^{high}$ cell subset (Fig. 4.6c).

a

	SIZE	ES	NES	NOM_p.val	FDR_q.val
HALLMARK_TNFA_SIGNALING_VIA_NFKB	177	0.658	2.13	0	0.0275
HALLMARK_KRAS_SIGNALING_UP	184	0.569	2.12	0	0.0143
HALLMARK_COAGULATION	127	0.564	2.12	0	0.0095
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	63	0.609	2.08	0	0.0161
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	178	0.591	2.07	0	0.0146
GO_EPITHELIAL_CELL_FATE_COMMITMENT	16	0.748	2.06	0	0.0142
GO_REGULATION_OF_COAGULATION	76	0.552	2.06	0	0.0123
GO_REGULATION_OF_THE_FORCE_OF_HEART_CONTRACTION	24	0.715	2.06	0	0.0121
GO_REGULATION_OF_WOUND_HEALING	115	0.529	2.05	0	0.0112
GO_PROTEIN_KINASE_A_SIGNALING	27	0.641	2.05	0	0.0101

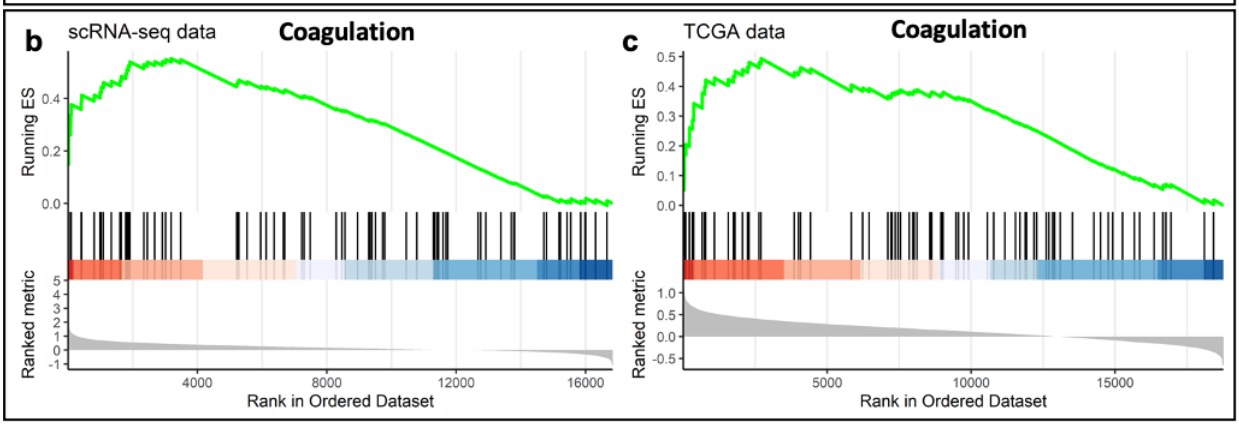


Figure 4.6 ***PDPN expressing cell populations exhibit a procoagulant and proinflammatory phenotype.***

a. Pathway enrichment analysis shows the top 10 enriched pathways in cells with highest PDPN expression (Q4), compared to those with the lowest PDPN expression (Q1). Of note is the preponderance of coagulation and inflammatory pathways according to Hallmarks and KEGG. **b**. GSEA plot showing pronounced enrichment of Hallmark Coagulation genes in cells with high PDPN expression (Q4) in the scRNA-seq data set, compared to those with low PDPN expression (Q1). **c.** This observation persists when analyzing TCGA bulk tumor samples (right panel), wherein Hallmark Coagulation genes are similarly enriched in tumors with high PDPN expression (Q4), compared to those with low expression (Q1).

4.4 The relationship between PDPN expression and the status of the oncogenic EGFR in GBM cells

Given the emerging link between oncogenic mutations and CAT (Dunbar *et al.*, 2020; Magnus *et al.*, 2013; Unruh *et al.*, 2016; Yu *et al.*, 2005) and the enrichment of PDPN expression in specific GBM subtypes (e.g. MES) we sought to reconcile the influence of genetic and epigenetic factors in PDPN regulation. In particular, we wished to explore the impact of oncogenic drivers on PDPN expression, especially EGFR/EGFRvIII that are paradigmatic for GBM progression. We approached this question bearing in mind the previously documented observations obtained with GBM cell lines which suggest that oncogenic EGFR (including EGFRvIII) drives the expression of coagulation related genes in GBM (Magnus *et al.*, 2010; Magnus *et al.*, 2013).

In patient derived single cell datasets annotated for differentiation roadmaps described earlier we did not observe a correlation between EGFR and PDPN levels (Fig 4.7). In order to further understand whether there is a relationship between EGFR/EGFRvIII-driven transformation and the expression of PDPN we interrogated in more depth other RNAseq datasets. These analyses revealed the unexpected level of expression complexity. Thus, in a subset of GBM cells low PDPN expression was correlated with elevated levels of EGFR (Fig. 4.8-4.9) while another cell subset displayed the exact opposite relationship (Fig 4.9a). On the surface these observations may suggest that either PDPN is not affected by the transforming action of EGFR/EGFRvIII oncogenes (at least insofar as this can be inferred from the expression level of EGFR transcript), or this effect is modulated by other factors, which may not be recapitulated by GBM model cell lines in culture.

To resolve this emerging conundrum, we revisited and extended our prior observations, both in patient samples and in GBM cell lines. As mentioned above, the analysis of well characterized GBM cell lines suggested that oncogenic activation of the EGFR signalling pathway leads to dysregulation of coagulome, including elevated expression of TF (Tawil *et al.*, 2019). While EGFR is amplified, mutated (EGFRvIII), or upregulated in a subset of GBMs (classical, to some extent mesenchymal) the impact of these events on the emerging mediators of VTE, such as PDPN (Costa *et al.*, 2019; Riedl *et al.*, 2017), has not been extensively explored in clinical samples especially in view of cellular heterogeneity underlying GBM progression (Patel *et al.*, 2014).

Thus, to explore further the possible relationships between EGFR and PDPN expression at the cellular level we first interrogated the single cell RNA-seq data set comprising subtype annotated GBM cell samples (Patel *et al.*, 2014) (Fig. 4.8 and 4.9). We employed the K-means clustering approach focusing on PDPN and EGFR expression to audit single cell transcriptomes pooled from 5 different GBM tumors. This analysis, as mentioned earlier, predictably revealed the existence of four different tumor cell phenotypes, including: $PDPN^{low}/EGFR^{high}$ (Cluster 1), $PDPN^{high}/EGFR^{high}$ (Cluster 2), $PDPN^{low}/EGFR^{low}$ (Cluster 3), $PDPN^{high}/EGFR^{low}$ (Cluster 4) (Fig. 4.9a). To assess whether this reflects a random distribution of PDPN and EGFR, or a cellular pattern, we developed extended gene expression signatures by comparing the genome-wide gene expression profile of each cluster to remaining cells within the dataset. Except for the $PDPN^{low}/EGFR^{low}$ cells (Cluster 3), which showed high number of antithetically common signature genes with the $PDPN^{high}/EGFR^{high}$ (Cluster 2) and $PDPN^{high}/EGFR^{low}$ cells (Cluster 4), other clusters showed little overlap in their signature genes (≤3 genes; Fig. 4.9b). In accordance with these findings, the expression profile of the obtained gene signatures (other than PDPN itself)

successfully differentiated between the four clusters, with the PDPN^{low}/EGFR^{low} cluster (Cluster 3) standing apart from the rest of the cells (Fig. 4.9c).

Again, PDPN^{high} cells (Clusters 2 and 4) were positively enriched for coagulation transcripts (Fig. 4.9d). Moreover, while PDPN^{low}/EGFR^{high} GBM cells expressed elevated transcripts linked to cell signalling, proliferation, and differentiation (OLIG1, DLL1), transcriptomes of their PDPN^{high}/EGFR^{low} counterparts were enriched for regulators of hemostasis and inflammation (C1R, C1S, CLEC2B) (Table 4.1). These observations further suggest that enrichment or depletion for PDPN is not a random effect and points to the involvement of distinct transcriptional programs among GBM cell subpopulations, in which this gene is switched on or off in the context of different levels of EGFR expression. In other words, as we earlier inferred from the correlative roadmap analysis, the EGFR expression per se does not define PDPN mRNA levels, but may, in specific contexts, coincide with suppression of PDPN as revealed by the sizable contribution of PDPN^{low}/EGFR^{high} cells in the dataset.

To account for the representation of these cell populations along with the associated stroma in global patterns of PDPN and EGFR expression in GBM we explored TCGA data set. This is useful for both technical and conceptual reasons, as single cell datasets contain reduced numbers of analyzable transcripts and EGFR may also impact GBM stroma through non-cell-autonomous mechanisms, possibly impacting PDPN. To gain related insights, we further clustered bulk tumor transcriptomes available through TCGA around PDPN and EGFR expression levels (Fig. 4.9e). This analysis revealed the existence of four tumor subgroups with distinct global phenotypes (Ph; Fig. 4.8 and 4.9e), including: EGFR^{low}/PDPN^{low} (Ph1), EGFR^{low}/PDPN^{high} (Ph2),

EGFR^{high}/PDPN^{low} (Ph3) and EGFR^{high}/PDPN$^{low/intermediate}$ (Ph4). Interestingly, these phenotypes only partially overlapped with TCGA mandated transcriptional subtypes (Verhaak *et al.*, 2010) in that Ph4 subgroup was enriched for classical GBM, while Ph2 contained several mesenchymal tumors (Fig. 4.9e). This data also suggests that PDPN^{low}/EGFR^{high} phenotype of single GBM cells observed earlier (Fig 4.9a, Cluster 1) may be enriched in Ph3 and Ph4 bulk tumor subsets, while PDPN^{high}/EGFR^{low} (Fig. 4.9a; Cluster 4) cells may dominate the Ph2 GBM subgroup.

If this were to be the case, the respective tumors would be expected to be enriched for the expression of the aforementioned 50 gene signatures, derived from transcriptomes of single cells with specific patterns of PDPN and EGFR expression. To test this possibility, we performed GSEA to examine whether the top 50 genes positively correlated with each single cell cluster are enriched in the gene signature of whole GBM tumors (TCGA) with a corresponding PDPN/EGFR expression pattern. This was, indeed, found to be the case (Fig. 4.9f). We therefore reasoned that distinctive gene expression signatures of GBM cell populations with antithetical expression levels of EGFR and PDPN may suggest a mechanistic link between these two genes and their impact on coagulant profiles of the corresponding tumors. The overall lesson from this analysis, however, was that there may be different regulatory relationships between EGFR and PDPN in different GBM cell subpopulations. In this regard tumors characterized by EGFR^{high}/PDPN^{low} attracted our special attention, as they accounted for almost half of all TCGA cases (Ph4) and they exhibited, what might be considered a counterintuitive phenotype, where low levels of EGFR corresponded to high expression of PDPN. This correlation is intriguing because it challenges the link between EGFR/EGFRvIII-driven oncogenesis and coagulant phenotype of GBM cells proposed earlier (Magnus *et al.*, 2010). If this were the case, the heterogeneity of GBM cell populations would

provide the basis for the coexistence of EGFR-expressing cells (possibly mitogenic drivers) and PDPN-expressing cells (putative thrombosis drivers) the genesis and interdependence of which would be of considerable interest. Therefore, we sought to evaluate a causal relationship between oncogenic EGFRvIII and PDPN expression.

4.5 PDPN as a regulatory target of oncogenic EGFR

EGFR and its mutant form (EGFRvIII) are thought to act as drivers of tumor progression in a subset of GBM cells (Liu *et al.*, 2015) and they also affect the cellular coagulome (Magnus *et al.*, 2010). In addition, in a large fraction of GBMs and in GBM cells we observed an antithetical expression patterns between EGFR and PDPN suggesting the existence of a regulatory link.

Therefore, we next explored more directly the potential impact of enforced expression of EGFRvIII oncogene on PDPN in a panel of human glioma cell lines. The respective cells included $EGFR^{low}$ parental U87P and U373P cells and their EGFRvIII overexpressing aggressive counterparts (U87vIII and U373vIII; Fig. 4.10a). Notably, while both parental glioma cell lines expressed appreciable levels of PDPN mRNA and protein, those signals were undetectable in variants harboring EGFRvIII (Fig. 4.10b, c). Moreover, in single cell transcriptomes of human gliomas, the limited number of which could be reliably verified for EGFR mutation status, we observed a trend for lower PDPN levels in EGFRvIII expressing cells, albeit below statistical significance (not shown). We also obtained aggressive variants of U373P cells lacking EGFRvIII and derived through a prolonged selection *in vivo* (Fig. 4.10a). These alternatively transformed cells (U373PT) retained their high PDPN expression (Fig. 4.10a-d) (Magnus *et al.*, 2014b). We next analyzed patient-derived proneural glioma stem cells (PN-GSC) devoid of EGFR expression

and positive for PDPN (Fig. 4.10e) (Mao *et al.*, 2013). When subjected to serum-induced astrocytic differentiation (Spinelli *et al.*, 2018) these PN-GSC cells (GSC157, GSC1079; Fig. 10e) triggered the expression of EGFR and down-regulated PDPN. Thus, while PDPN expression in glioma is compatible with aggressive growth it is suppressed in the context of cell subsets with activated EGFR expression.

Since the available data do not suggest that CAT is specifically suppressed in GBMs expressing high levels of EGFR/EGFRvIII (Unruh *et al.*, 2016) we would like to speculate that in the complex tumor milieu the EGFR expression/activity is modulated in specific subsets of cancer cells (as in the case of undifferentiated GSCs) which may allow PDPN expression resulting in thrombosis driven by this particular cell subset. This process cannot be recapitulated in cell lines engineered to constitutively express high levels of EGFRvIII.

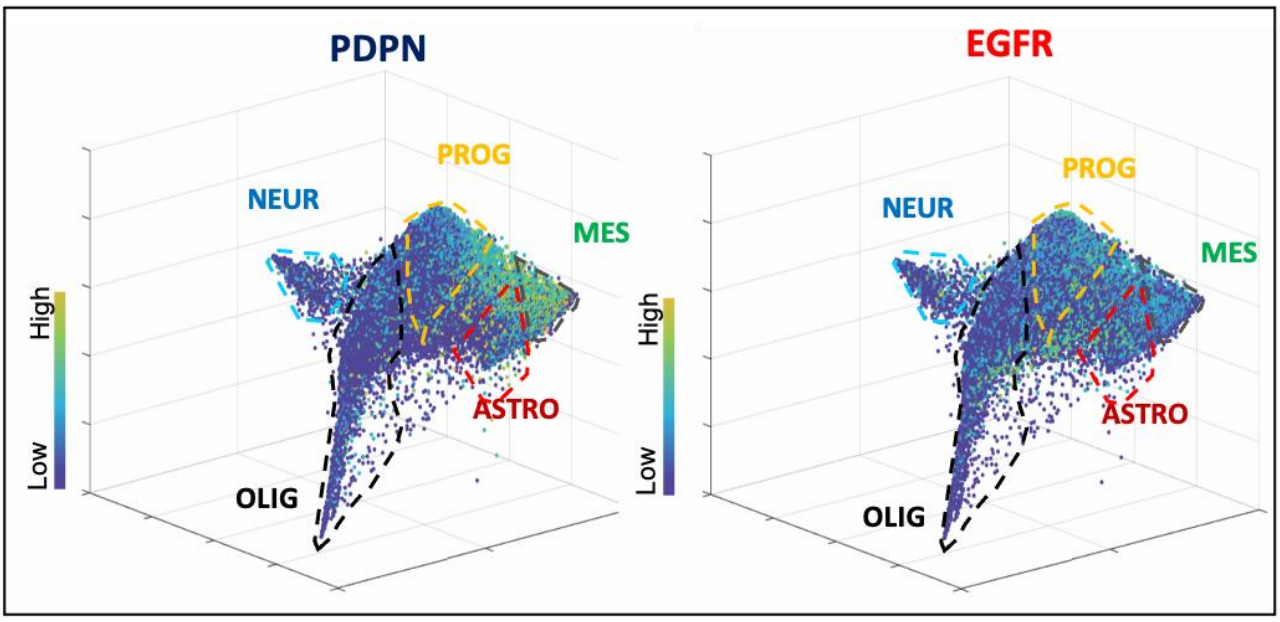


Figure 4.7 ***PDPN and EGFR expression patterns exhibit minimal overlap.***

Differentiation roadmap plot portraying the expression pattern of PDPN (left) and EGFR (right). EGFR expression is scattered between multiple cell populations with weak overlap with PDPN.

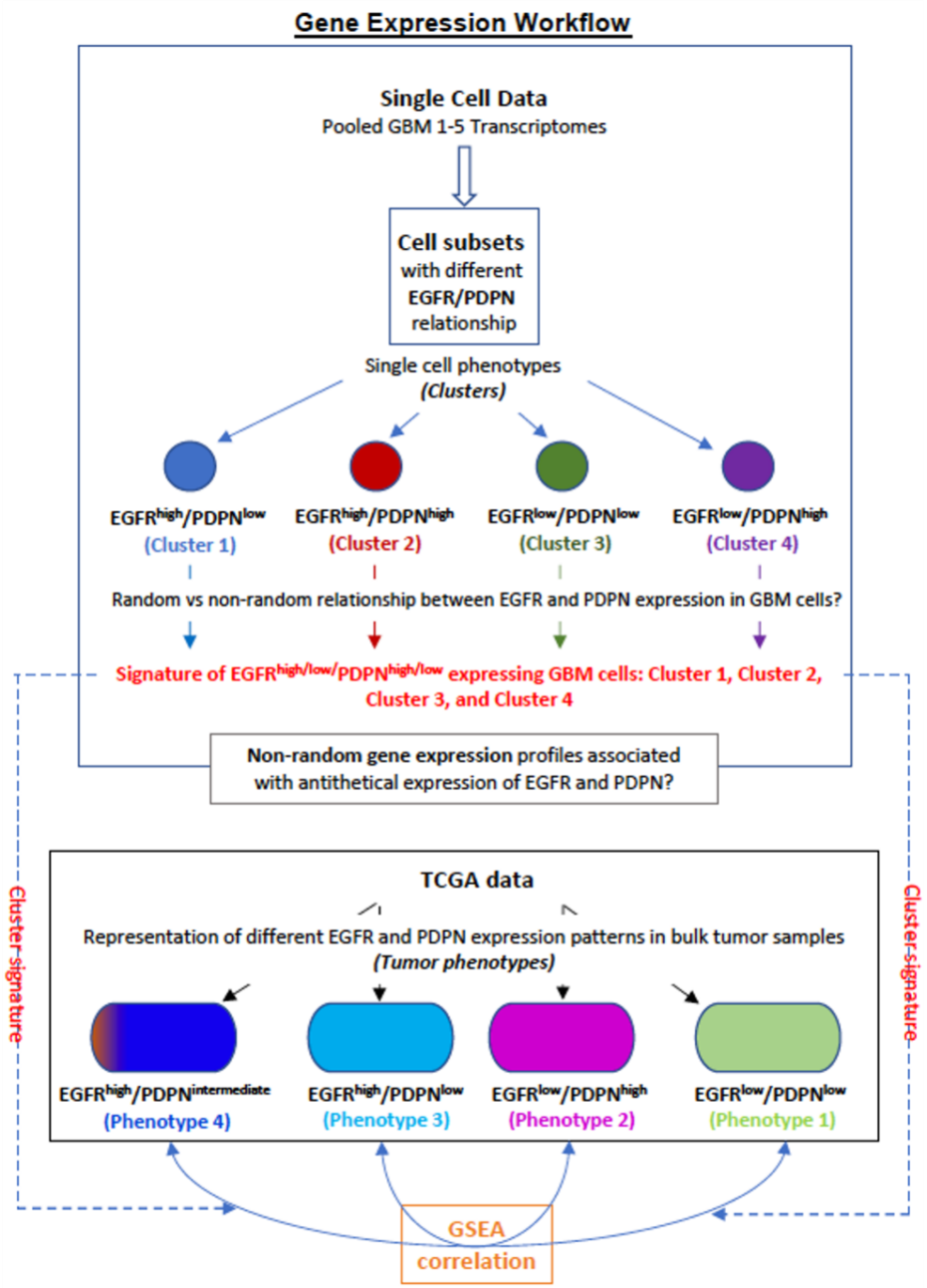


Figure 4.8 ***The gene expression analysis workflow.***

To characterize PDPN expressing GBM cells the single cell GBM transcriptomes(Patel *et al.*, 2014) were pooled and clustered around PDPN and EGFR expression patterns. The gene expression signatures of these clusters were used to establish the phenotypes of PDPN expressing GBM cells and to interrogate their presence in bulk transcriptomes of TCGA dataset. GSEA plots were developed to compare the PDPN phenotypes.

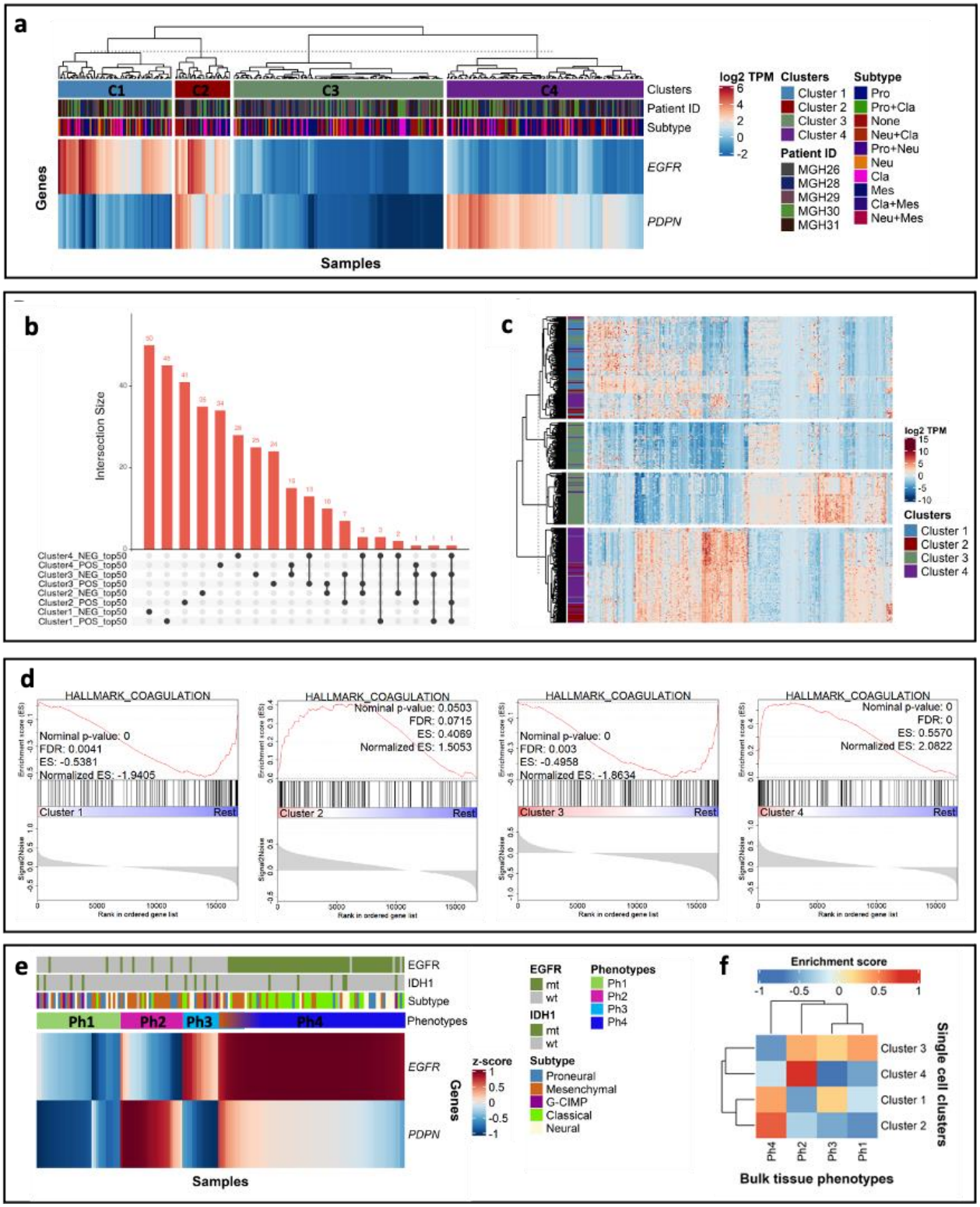

a
C1
C2
C3
C4
Clusters
Patient ID
Subtype
EGFR
PDPN
Genes
Samples
log2 TPM
Clusters
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Patient ID
MGH26
MGH28
MGH29
MGH30
MGH31
Subtype
Pro
Pro+Cla
None
Neu+Cla
Pro+Neu
Neu
Cla
Mes
Cla+Mes
Neu+Mes
b
Intersection Size
Cluster4_NEG_top50
Cluster4_POS_top50
Cluster3_NEG_top50
Cluster3_POS_top50
Cluster2_NEG_top50
Cluster2_POS_top50
Cluster1_NEG_top50
Cluster1_POS_top50
c
log2 TPM
Clusters
Cluster 1
Cluster 2
Cluster 3
Cluster 4
d
HALLMARK_COAGULATION
Nominal p-value: 0
FDR: 0.0041
ES: -0.5381
Normalized ES: -1.9405
Cluster 1
Rest
HALLMARK_COAGULATION
Nominal p-value: 0.0503
FDR: 0.0715
ES: 0.4089
Normalized ES: 1.5053
Cluster 2
Rest
HALLMARK_COAGULATION
Nominal p-value: 0
FDR: 0.003
ES: -0.4958
Normalized ES: -1.8634
Cluster 3
Rest
HALLMARK_COAGULATION
Nominal p-value: 0
FDR: 0
ES: 0.5570
Normalized ES: 2.0822
Cluster 4
Rest
Rank in ordered gene list
e
EGFR
IDH1
Subtype
Ph1
Ph2
Ph3
Ph4
Phenotypes
EGFR
PDPN
Genes
Samples
z-score
EGFR
mt
wt
IDH1
mt
wt
Subtype
Proneural
Mesenchymal
G-CIMP
Classical
Neural
Phenotypes
Ph1
Ph2
Ph3
Ph4
f
Enrichment score
Cluster 3
Cluster 4
Cluster 1
Cluster 2
Single cell clusters
Ph4
Ph2
Ph3
Ph1
Bulk tissue phenotypes

Figure 4.9 ***Interrelationship between PDPN and EGFR in glioblastoma cell populations.***

a. K-means clustering of single cells from five GBM patient-derived tumors using the expression profiles of EGFR and PDPN. Each column represents one single cell, with the centered log2 transcript per million (TPM) EGFR and PDPN expression shown in the heatmap. The ID of the patient from which each cell is derived, as well as the subtype classification of each cell according to Patel et al. 2014 (Patel *et al.*, 2014) is also shown on top. **b.** The extent of overlap between gene expression signatures of PDPN/EGFR-based single cell clusters. The first eight bars in the bar graph represent the number of unique genes of each cluster, whereas the remaining bars represent intersection size, with the intersecting clusters shown using the vertical lines that connect the cluster nodes. **c.** K-means clustering of GBM single cells based on the expression signatures of PDPN/EGFR-based single cell clusters. Cell cluster annotation corresponds to those represented in panel **a**. **d.** Gene Set Enrichment Analysis **(**GSEA) showing the expression distribution of hallmark genes of coagulation in PDPN/EGFR-based single cell clusters. In each panel, the x-axis represents the genes, sorted by their differential expression between the indicated clusters and the rest of the cells. Vertical black lines represent the genes that belong to the coagulation pathway, according to: [Molecular Signatures Database – Hallmark Coagulation; M5946]. The curve represents the GSEA running enrichment score (ES). **e.** K-means clustering of TCGA bulk glioblastoma samples using centered normalized expression of EGFR and PDPN (z-score). The four sample clusters are referred to as molecular phenotypes: $EGFR^{low}/PDPN^{low}$ (Ph1), $EGFR^{low}/PDPN^{high}$ (Ph2), $EGFR^{high}/PDPN^{low}$ (Ph3) and $EGFR^{high}/PDPN^{low/intermediate}$ (Ph4). **f.** GSEA enrichment score (ES) of the positive gene signature of each cell cluster (rows) across the TCGA molecular phenotypes (columns). Each ES represents the enrichment of the positive gene signature of a cell cluster (Single cell dataset; C1-C4) among genes that are up-regulated in a given TCGA molecular phenotype (Ph1-Ph4) (red) or down-regulated (blue).

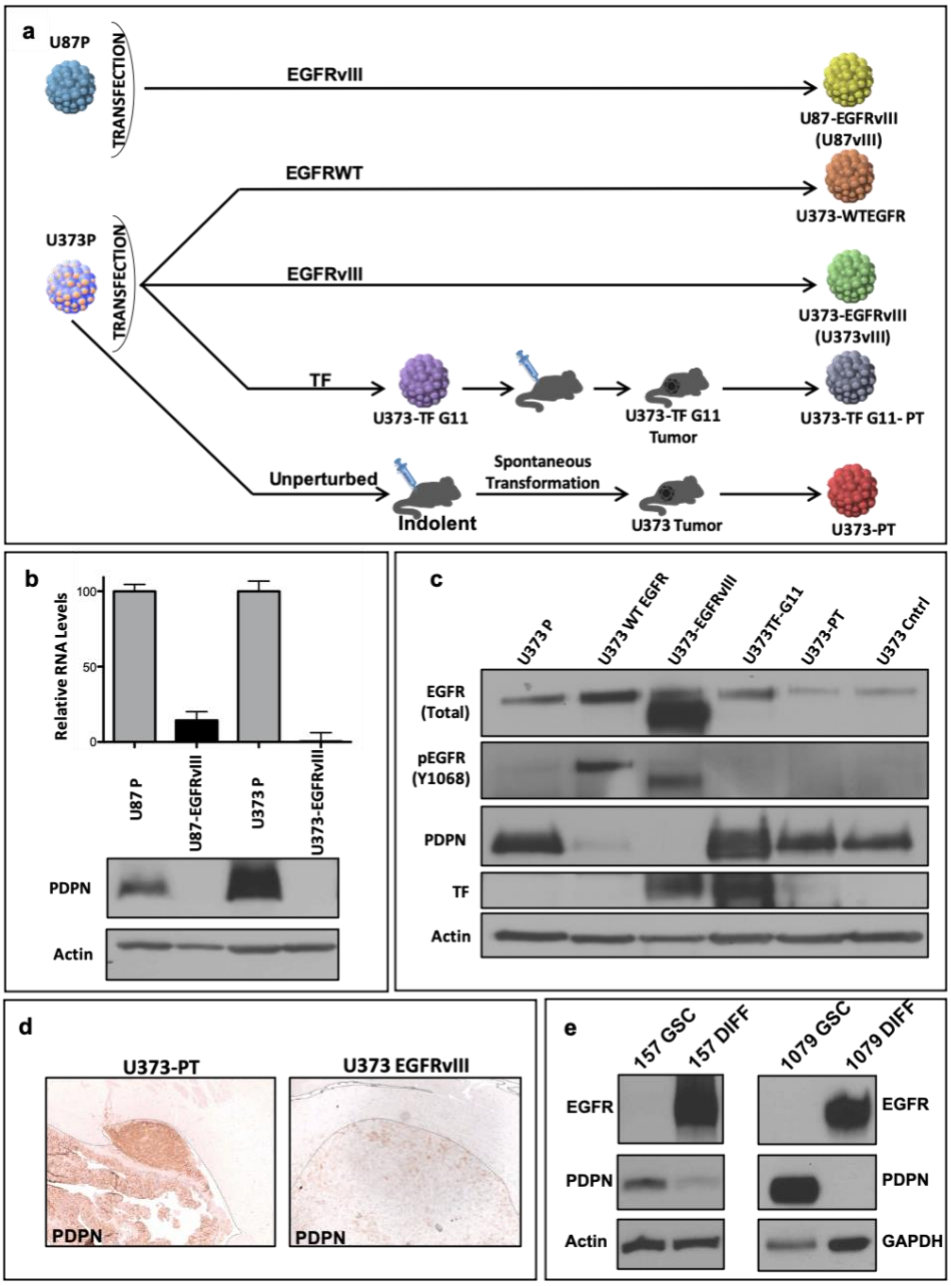


Figure 4.10 ***Down-regulation of PDPN in glioma cells expressing oncogenic EGFR.***

a. Derivation of U87 and U373 families of isogenic glioma cell lines driven by EGFRvIII-dependent and -independent pathways of tumorigenesis. **b.** Downregulation of PDPN mRNA and protein in U87 and U373 cell lines engineered to express oncogenic EGFRvIII. **c.** Expression levels of wild type (upper band) EGFR, EGFRvIII (lower band), PDPN and TF in isogenic variants of U373 glioma. **d.** Immunohistochemical staining for PDPN of glioma xenografts originating from intracranial injection of tumorigenic variants of U373 cells with (U373vIII) or without (U373PT) EGFRvIII expression. EGFRvIII-associated down-regulation of PDPN is maintained *in vivo*. **e.** Reciprocal changes in the expression of EGFR and PDPN in proneural glioma stem cell

lines (GSC157 and GSC1079) in stem cell (GSC) and differentiated (DIFF) cultures maintained in the presence of serum for 25-30 days.

CLUSTER 1		CLUSTER 2		CLUSTER 2		CLUSTER 4	
Cluster1_POS_top50	Cluster1_NEG_top50	Cluster2_POS_top50	Cluster2_NEG_top50	Cluster3_POS_top50	Cluster3_NEG_top50	Cluster4_POS_top50	Cluster4_NEG_top50
EGFR	SERPINE1	EGFR	ACTRT1	MAGEC2	DNAJC15	PDPN	RPS23
C2orf80	CCR7	PDZD2	MAGEB2	TMSB15A	BST2	CD68	RPLP1
PLPPR1	LYPD3	MEIS1	RPL3	MAGEA12	PGAM2	SERPING1	MCM10
LINC00266-1	PSMD3	AQP4	PHLDB2	ITIH6	SDC4	EFEMP1	RPL37A
OLIG1	LOXL2	PDPN	RPLP2	CD163L1	NRCAM	GFPT2	CD163L1
LMO1	ITGB5	ALDH1L1	NDUFB2	GALNT14	ATP13A4	CHI3L1	GPR153
ARL4A	HSPB3	LGI1	RNF144A	KLRC2	AGT	CCN1	PCDH15
GALR1	S100A4	ARHGAP1	RPL37A	MAGEB2	EGFR	EFNA1	EGFR
SHD	STC1	GRIA1	RPL10	RPL35	C4orf47	SULF1	RPL35
RPS4Y1	LCTL	BST2	SULT2A1	RPS20	ZNF516	CAV1	CENPV
CD82	AZIN2	TIMP3	UGT1A5	RPL27	PLAAT4	C1R	HELLS
PCDH17	GLIPR1	ATP13A4	MBNL3	RPL12	GADD45B	GAP43	MAGEA12
LINC01546	NR4A2	PTEN	OR5B3	CSAG1	EFEMP1	PCSK1	MAGEC2
SCG3	IL13RA1	ATP1A2	PRRT4	ADCK5	CHI3L1	PLA2G5	SULT2A1
CRISPLD2	C8orf88	RBP1	PTPA	MCM10	SERPINB6	SDC4	RPL27
RBP7	CTSH	ATP1B2	OR52E4	RPL21	DPF3	VWA5A	RPL23A
PTPRO	TGFBI	SLITRK5	TMEM98	CDC6	SPP1	EMP1	RPL21P7
ACTA1	S100A3	ALDOC	OR8G1	SIX6	TCEAL5	SPOCD1	RPS27L
APOD	DDR2	KLHL4	FBXL22	RPL26	RCAN2	TGFB2	ATP5MPL
NKAIN4	PRELID2	ADAMTS6	NUBP2	PHLDA2	SPOCD1	IFI35	RPL39
BEX1	SLC43A1	MBOAT2	PHLDA2	RPL39	SPARCL1	BCL6	RPL4
FSCN1	MGST1	RSRP1	RARG	RPL35A	CTSK	TCIM	TMSB15A
TSPAN7	OSR2	PCDHB11	NECAB2	MSC	DDX3Y	GPC1	RPS16
DLL1	SLC7A3	PRR13	DLX5	FGF13	NME5	CLU	LAMA1
LANCL2	ERICH2	KLF9	KLRC2	RPL32	SLC25A18	EFEMP2	HMGN2
TLE2	ITGA5	SMARCC2	COL2A1	SLFN11	PLA2G5	SRPX2	NPM1
OCIAD2	POLE4	SLC15A2	SHISAL1	C14orf39	ZBTB20	NAMPT	TMEM98
ATCAY	CTSC	SLC1A3	RPS11	NQO1	TGFB2	ELMOD1	SOX10
MOG	ZCCHC12	COLGALT2	C10orf120	OR5G5P	TMBIM1	C1S	STMN1
NCALD	SLC7A8	OLFM2	MSC	RPL23A	CAMK2N1	PPFIA2	BUB1B
FAIM2	TANGO2	PHYHIPL	KCNAB3	BUB1B	C1R	SOD2	CDC6
TSC22D4	OAF	C19orf18	GDPD3	SFRP2	PCDHB11	MAOB	FANCI
KCNQ2	BLVRB	SLC30A4	SLFN11	TMA7	CCDC152	TMBIM1	HMGB1
GPR37L1	SLC39A12	RAB3D	FCGR2B	RPL7	CCN1	HS3ST3B1	RPS21
TENM1	CALCB	S1PR2	ENAM	RPL19	SCN3A	CADPS	RAB33A
TMEM233	NABP1	SNX27	RPL7	KRTAP13-4	C19orf18	CHI3L2	COL9A3
FAM222A	SLC39A14	ZFHX4	FCRLA	HMGB2	ALDOC	CLEC2B	RPL26
NFIX	CNN1	AASS	ITIH6	NPM1	GAP43	UBD	OLIG1
CRIP1	ANXA1	ABCD2	OST4	HBQ1	DHRS3	TAGLN	KLRC2
POSTN	RGS10	UBE2B	GALNT14	RPL37A	ABCA1	BHLHE40	RPS6
CNTN1	GEM	SRGAP2C	RPL30	TAAR1	RNF180	SPP1	RPL21
NKD1	TFPI2	NFIA	LENG1	FBXL22	TCIM	PNPLA4	KLRC4
SOX8	ARHGDIB	TMED9	MOGAT1	RPS27	IRF9	TAGLN2	RPS17
OR6C1	ALPL	F11R	TMSB10	FCRLA	CD68	CES1	EMP2
SLAIN1	ST8SIA4	ARAP2	ATP5F1E	MYCNOS	ADM	S1PR1	ZDHHC22
PRELID3B	NRP2	RNF180	OR10A2	NID2	SERPING1	PGM2L1	LINC01546
HLA-F	C9orf50	KIF13B	LAYN	RPL29	IRS2	MAN1C1	TM4SF1
INSYN2A	A2M	ITGB8	RPL3L	NR0B1	ZFP36	DHRS3	MTRNR2L1
RTN1	LDLR	CCDC152	MAGEC2	RPL14	FOSB	MMP7	MTAP
DNAJC15	MICA	SLC35A4	ADCK5	SH3KBP1	PDPN	GBP2	APOD

Table 4.1 ***Top 50 gene signatures of clusters 1-4.***

Extended gene expression signatures for each cluster generated by comparing the genome-wide gene expression profile of that cluster to the rest of the cells within the single cell RNA-seq dataset.

4.6 EGFR impacts PDPN expression through a combined effect of epigenetic reprogramming and signalling

In spite of certain artificiality of GBM cell lines expressing EGFRvIII, their phenotypes have been informative as they suggested that this oncogenic alteration impacts PDPN levels. The nature of this effect is of considerable interest as therapies targeting EGFRvIII and its downstream effectors are being widely pursued to treat GBM, almost uniformly without consideration for the possible change (exacerbation) of the coagulant phenotype of cancer cells and thrombotic consequences.

To examine how activated EGFR may control PDPN levels, U373vIII cells were treated with blockers of the canonical EGFR signalling, such as pan-ERBB kinase inhibitor (dacomitinib/PF00299802), or a selective PI3K inhibitor (pictilisib/GDC-0941) for up to 72 hours (Fig. 4.11a, b). However, no rescue of PDPN expression was observed in these settings, arguing against a direct role of this receptor or downstream PI3K signalling alone.

Since oncogenic transformation may also affect the epigenome, both at the level of DNA methylation and chromatin architecture (Liu *et al.*, 2015), we also interrogated PDPN expression in U373vIII and U87vIII cells treated with DNA demethylating agent, 5-Azacytidine (5Aza) (D'Asti *et al.*, 2016) (Fig. 4.11c). While this treatment led to the expected re-expression of the known methylated microRNA-520g locus (D'Asti *et al.*, 2016), it failed to rescue the expression of PDPN in EGFRvIII-transformed cells.

We next considered changes in the chromatin architecture, as a known epigenetic factor involved in gliomagenesis (Reifenberger *et al.*, 2017), PDPN regulation and modulation of the

coagulome (Unruh *et al.*, 2019). One of the key elements in this regard is the polycomb repressive complex 2 (PRC2) involving gene silencing activity of the histone-lysine N-methyltransferase, known as enhancer of zeste homolog 2 (EZH2) that tri-methylates lysine 27 (K27) of histone 3 (Harutyunyan et al., 2019). Indeed, EGFR has been implicated in epigenetic gene repression through impact on EZH2 (Chen *et al.*, 2018). While exploring this thread through GSEA surveys of both single cell and bulk GBM datasets (Patel *et al.*, 2014; Verhaak *et al.*, 2010), we noticed that genes known to be positively correlated with EZH2 expression in a meta-analysis of thousands of bulk-tissue RNA-seq datasets [https://www.nature.com/articles/ s41467-018-03751-6] also positively correlated with EZH2 in the singe-cell GBM dataset (Fig. 4.11d left), and negatively correlated with PDPN across the GBM cell data (Fig. 4.11d, right). This observation suggests that $PDPN^{low}$ cells exhibit high EZH2 regulon activity, and inversely $PDPN^{high}$ cells exhibit low EZH2 regulon activity.

To further assess this linkage, U373vIII cells were cultured in the presence of varying concentrations of the EZH2 inhibitor (UNC1999) over a period of 25 days to allow cellular reprogramming, followed by the assessment of PDPN protein expression. Indeed, UNC1999 completely blocked the H3K27 tri-methylation and the resulting repressive state gradually leading to a partial recovery of PDPN expression in U373vIII cells (Fig. 4.11e). Moreover, the combined treatment with both UNC1999 and dacomitinib resulted in a further increase in PDPN protein levels (Fig. 4.11f). These results suggest that the epigenetic silencing may play an important role in PDPN regulation in a subset of glioma cells, thereby enabling additional layer of PDPN control executed partially through oncogenic EGFR signaling. This is a novel observation, important to consider as EZH2 and PRC2 directed therapeutics that increasingly capture interest in GBM

research field may exacerbate the pro-thrombotic phenotype in a subset of tumors (Grinshtein et al., 2016; Suter et al., 2020).

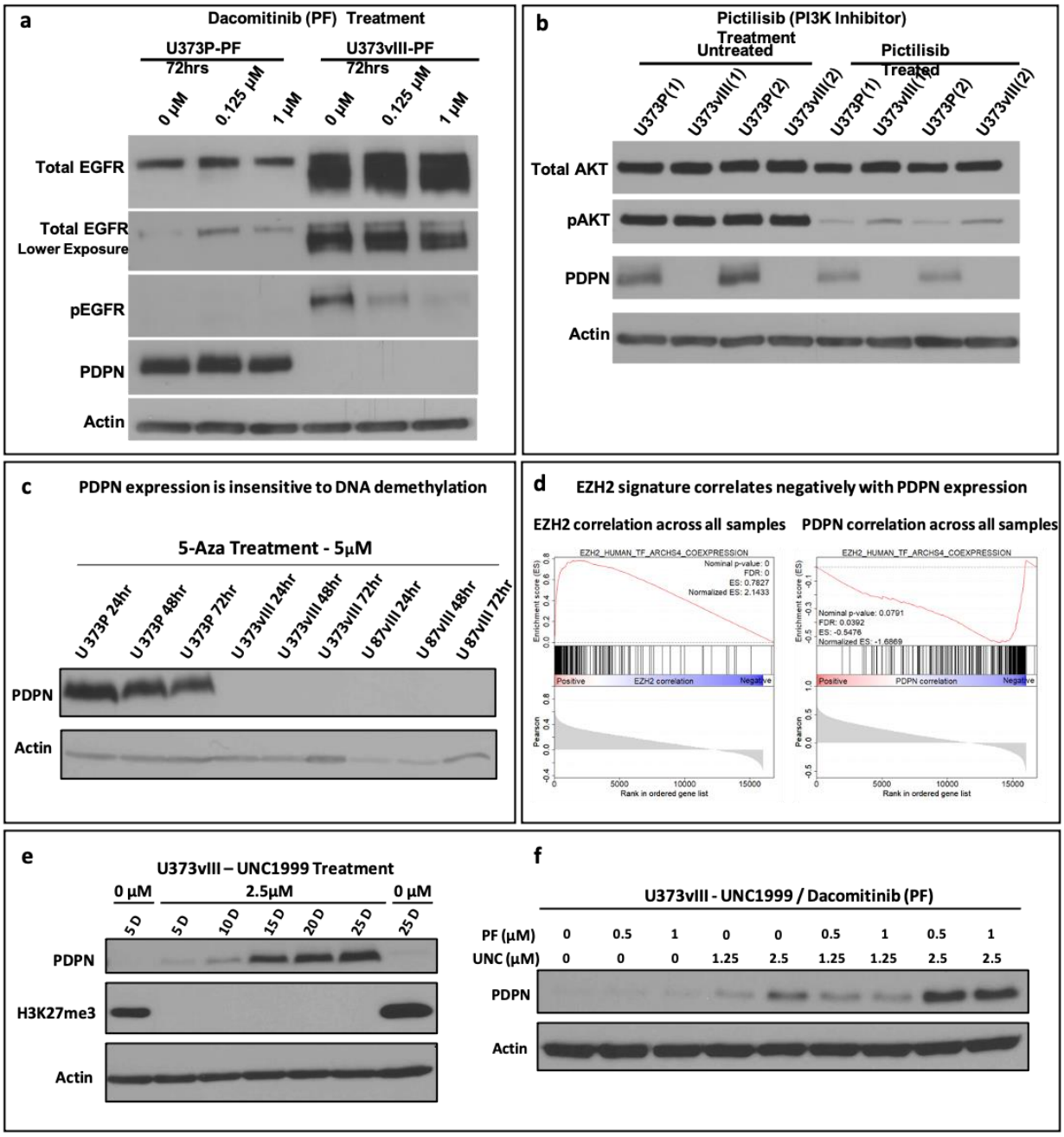


Figure 4.11 ***Histone methylation and oncogenic signalling impact PDPN expression.***

a. Irreversible blockade of EGFR phosphorylation by dacomitinib (PF) does not rescue PDPN expression in EGFRvIII-driven U373vIII cells. **b.** Inhibition of PI3K activity (downstream effector of EGFR) by pictilisib fails to restore PDPN expression in U373vIII cells. **c.** Treatment with 5-Aza cytidine does not lead to re-expression of PDPN protein in U373vIII. **d.** Relationship between known EZH2-correlated genes and EZH2 (left) or PDPN (right) across GBM cells. In each GSEA plot, the x-axis shows the ranking of genes based on their correlation with EZH2 (left) or PDPN (right) across the single-cell GBM dataset[33]. The vertical black lines denote the set of genes known

to be positively correlated with EZH2, based on a meta-analysis of publicly available bulk-tissue RNA-seq datasets [https://www.nature.com/articles/s41467-018-03751-6]. **e.** Treatment of U373vIII cells with the EZH2 inhibitor (UNC1999) leads to gradual and partial re-expression of PDPN over 25 days (25D). **f.** Enhancement of PDPN protein expression in U373vIII glioma cells co-treated with inhibitors of EZH2 (UNC1999) and EGFR (dacomitinib) suggests the role of histone H3 trimethylation and chromatin modification, as well as EGFR kinase signalling in PDPN regulation.

4.7 Epigenome-impacting mutations of IDH1 oncogene down-regulate PDPN expression in glioma

IDH1 R132H mutation are paradigmatic for the link between genomic lesions and the epigenome in GBM. Of importance is also the fact that patients with IDH1 R132H are protected from both microvascular and systemic thrombosis (Unruh *et al.*, 2016), which further suggests a potential role of the epigenome in CAT. For these reasons we investigated the regulation of PDPN transcript in GBMs expressing mutant or wild type IDH1.

While the influence of EGFR on PDPN may depend, at least in part, on the concomitant EZH2 activity, other mutant oncogenic drivers, such as IDH1 R132H, may directly impact cellular epigenome (Liu *et al.*, 2016; Sturm *et al.*, 2014). IDH1 mutations drive a distinct (proneural-like) subset of high grade glioma (recently distinguished from GBM (Wen *et al.*, 2020), influence coagulant phenotype of glioma cells and their tissue factor (TF) levels (Unruh *et al.*, 2019) and are associated with low incidence of VTE (Unruh *et al.*, 2016). Since PDPN is an important correlate and possibly an effector of VTE in GBM (Costa *et al.*, 2019; Riedl *et al.*, 2017), and has been linked to IDH1 (Sun *et al.*, 2020) we set out to compare the levels of its transcript in subsets of GBM lesions expressing mutant or wild type IDH1(Reifenberger *et al.*, 2017) (Fig. 4.12a-f).

Indeed, GSEA revealed a negative association between IDH1 mutations and the expression of genes characterizing the $PDPN^{high}/EGFR^{low}$ GBM phenotype (Cluster 4) in the scRNA-seq dataset. These genes (and phenotype) positively correlated with the wild type IDH1 status in GBM (Fig. 4.12a). The differentials in PDPN transcript levels were also captured through the analysis of two independent data sets (Pfister-46 MAS5.0-u133p2 and TCGA) using the R2 and Glio-Vis Genomics Analysis and Visualization platforms, respectively (Fig. 4.12b and d).

Unlike in the case of EGFRvIII this influence correlated with DNA methylation levels affecting PDPN gene locus in IDH1 mutant tumors relative to their IDH1-wild type counterparts, as revealed by multiple probes specific for this genomic region (Fig. 4.12c). In contrast, the GBM subset expressing wild type IDH1 exhibits upregulated PDPN mRNA, including in an independent dataset (Fig. 4.12e, f). Thus, in glioma *PDPN* locus is a target of at least two different epigenetic mechanisms: one operating at the level of DNA methylation and controlled by oncogenic IDH1, while the other is executed by chromatin modifications in association with EZH2 and modulated by oncogenic EGFR.

Thus, our studies bring together the genetic, epigenetic, and coagulant mechanisms that emerge during progression of GBM. These relationships are relatively complex in the case of IDH1 wild type (bone fide) GBMs and result in co-existence of PDPN-expressing and – non-expressing cell populations even within the same lesion, which may also harbor cells enriched for other effectors of the hemostatic system. Our study suggests that while EGFRvIII status functionally impacts PDPN expression, it does not predict its levels across GBM cell populations due to coexistent epigenetic influences. While IDH1 wildtype GBMs are associated with a high risk of VTE (~25%) not all patients develop systemic thrombosis, a circumstance that necessitates more personalized approaches to thromboprophylaxis. It is also of note that while high expression of PDPN correlates with the VTE risk, this does not exclude accessory roles of other factors (e.g. TF) that could be co-expressed with PDPN in the same, or different GBM cells. It is possible that studies on EZH2 levels or DNA methylation patterns may separate GBMs with multifactorial propensity to cause thrombosis, from those less likely to do so. To conceptualize further studies in

this domain it would be important to understand the functional contribution of PDPN to activating local and systemic thrombosis in GBM.

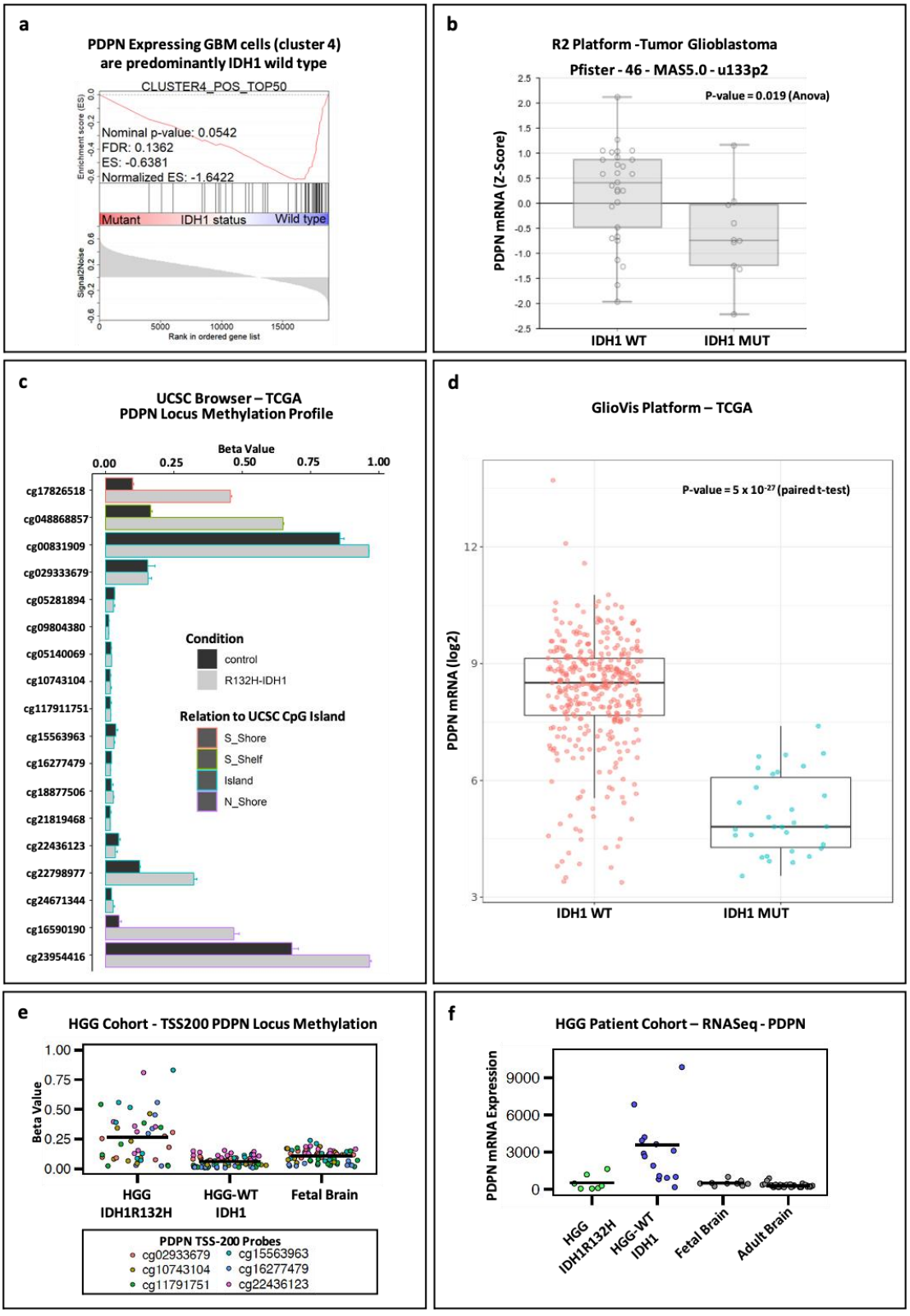

a
PDPN Expressing GBM cells (cluster 4)
are predominantly IDH1 wild type
CLUSTER4_POS_TOP50
Nominal p-value: 0.0542
FDR: 0.1362
ES: -0.6381
Normalized ES: -1.6422
Mutant
IDH1 status
Wild type
Enrichment score (ES)
Signal2Noise
Rank in ordered gene list
b
R2 Platform -Tumor Glioblastoma
Pfister - 46 - MAS5.0 - u133p2
P-value = 0.019 (Anova)
PDPN mRNA (Z-Score)
IDH1 WT
IDH1 MUT
c
UCSC Browser – TCGA
PDPN Locus Methylation Profile
Beta Value
cg17826518
cg048868857
cg00831909
cg029333679
cg05281894
cg09804380
cg05140069
cg10743104
cg117911751
cg15563963
cg16277479
cg18877506
cg21819468
cg22436123
cg22798977
cg24671344
cg16590190
cg23954416
Condition
control
R132H-IDH1
Relation to UCSC CpG Island
S_Shore
S_Shelf
Island
N_Shore
d
GlioVis Platform – TCGA
P-value = 5 x 10-27 (paired t-test)
PDPN mRNA (log2)
IDH1 WT
IDH1 MUT
e
HGG Cohort - TSS200 PDPN Locus Methylation
Beta Value
HGG
IDH1R132H
HGG-WT
IDH1
Fetal Brain
PDPN TSS-200 Probes
cg02933679
cg15563963
cg10743104
cg16277479
cg11791751
cg22436123
f
HGG Patient Cohort – RNASeq - PDPN
PDPN mRNA Expression
HGG
IDH1R132H
HGG-WT
IDH1
Fetal Brain
Adult Brain

Figure 4.12. ***IDH1 mutation correlates with epigenetic silencing of the PDPN gene in glioma.***

a. GSEA analysis associates the signature of PDPN expressing cells (cluster 4) with the wild type and not mutant IDH1 gene in the single cell GBM dataset (Patel *et al.*, 2014). **b.** Independent dataset analysis of bulk GBM samples (Pfister-46-MAS5.0-u133p2 - GSE36245) suggests lower PDPN expression in IDH1 mutant *versus* IDH1 wild-type tumors. **c.** Preferentially methylated PDPN gene locus in IDH1 mutant bulk glioblastoma samples (TCGA; x-axis) as detected using multiple independent probes (y axis). **d.** Independent analysis of TCGA bulk GBM samples using GlioVis platform indicates downregulation of PDPN transcript in tumors with mutant IDH1. **e.** Independent cohort of high-grade glioma (HGG) samples (Jabado - internal dataset (Fontebasso et al., 2014; Harutyunyan *et al.*, 2019; Krug et al., 2019)) reveals increased PDPN TSS200 locus methylation in IDH1 R132H mutant tumors and its reduced methylation in IDH1 wild type (IDH1-WT) tumors; color denotes different probes (bottom box). **f.** Corresponding PDPN mRNA expression (as in **e**) shows increased levels of PDPN transcript in IDH1 wild type brain tumors compared to IDH1mutant ones.

5 PDPN is Released from GBM Cells as Cargo of Pro-thrombotic Extracellular Vesicles

Tawil et al. 2021, Blood Advances

5.1 PDPN is detected in EVs derived from GBM cell lines

CAT is thought to be influenced by systemically released cancer derived coagulants, including EVs (Hisada and Mackman, 2017). Given the emerging role of PDPN in VTE risk prediction in GBM and its platelet activating properties (Riedl *et al.*, 2017; Tawil *et al.*, 2021), it is reasonable to suggest that this protein could act as a systemically acting cancer coagulant. However, PDPN is a transmembrane protein associated with GBM cells, which normally do not circulate in peripheral blood in meaningful numbers and don't form distant metastases (Krol et al., 2018). Therefore, a mechanism would need to exist to allow shedding of bioactive PDPN into the general circulation. One of such mechanisms described in the case of other membrane-associated coagulants in other cancers (Garnier *et al.*, 2012; Geddings and Mackman, 2013; Hisada and Mackman, 2017) is the release of membrane associated receptors as cargo of extracellular vesicles (EVs).

In view of these findings GBM-EVs (GBM-MPs) are of interest as carriers of circulating coagulants such as TF, PDPN and others. There is a well-documented lack of correlation between levels of circulating TF-MPs and VTE risk in brain tumors (Thaler *et al.*, 2012) and, to the best of our knowledge, the evidence that PDPN is secreted as GBM-EVs has been lacking. Therefore, we wished to explore whether GBM cells do release PDPN as cargo of EVs and whether such EVs retain the ability to activate platelets *in vitro* and *in vivo* and can be implicated in CAT. To this

end, we purified EVs from conditioned media of glioma cells (Choi *et al.*, 2018) and characterized them for size (by NTA) and purity (electron microscopy). A subsequent western blot analysis of corresponding protein lysates revealed the presence of PDPN signal in EVs of high PDPN expressing cells (U373P) (Kowal *et al.*, 2016) (Fig. 5.1a).

The cell surface localization of PDPN as a transmembrane protein, prompted us to question whether PDPN is also present on the surface of EVs released by the PDPN expressing U373P cells. To address this question, we first performed immunogold staining and electron microscopy (EM) of EVs isolated from PDPN expressing U373PT cell population (a tumorigenic variant of parental U373P cells). EM confirmed the presence of PDPN on the surface of U373PT EVs and the colocalization of PDPN and CD63 on the same small vesicles of ≤100 nm size range (Fig. 5.1b).

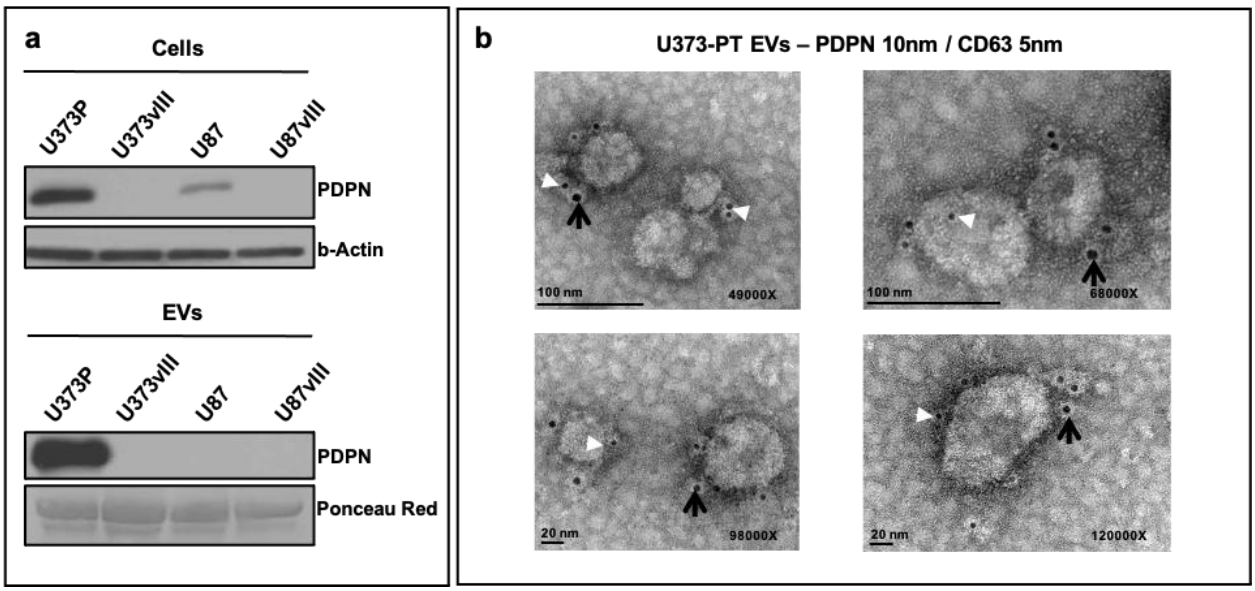


Figure 5.1 ***PDPN is released from glioma cells as cargo of extracellular vesicles.***

a. Detection of PDPN immunoreactivity in glioma cells and EVs; highly positive U373P cells release ample PDPN-positive EVs relative to PDPN-downregulated U373vIII and U87vIII cell lines. Lower levels of PDPN expression in U87P cells result in the absence of PDPN signal in EVs. **b.** Immunogold staining and electron microscopy of U373P EVs for PDPN (10 nm gold particles, black arrows) and exosomal marker CD63 (5 nm gold particles, white arrows); Multiple small EVs (≤100 nm) stain for both PDPN and CD63.

5.2 EVs carrying PDPN exhibit exosome-like characteristics

The morphology and size of glioma cell derived small EVs carrying PDPN corresponded to that of exosomes (50-150 nm) (Kowal *et al.*, 2016). To further characterize this EV population, we subjected EV isolates to iodixanol density gradient (OptiPrep) resolution. Subsequently, after collecting the corresponding fractions, EVs from each fraction were isolated, counted (NTA), lysed and immunoblotted to analyze for the presence of coagulation (PDPN and TF) and EV markers (flotillin 1, CD81, Syntenin and CD9) (Kowal *et al.*, 2016). As expected, a subset of EVs isolated from cultures of primary tumor-derived (PT) cell lines (U373PT and U373vIII PT) floated at the exosomal density of iodixanol (~1.1gmL, fractions 4-5) and exhibited corresponding patterns of canonical EV proteins (CD81, CD63, syntenin).

As expected, the PDPN and TF expression patterns in these exosome-like EV were mutually exclusive (as in respective cancer cells (Magnus *et al.*, 2010); Fig. 4.6b, c) in that U373vIII PT-EVs were $PDPN^{negative}/TF^{high}$, whereas U373PT-EVs were $PDPN^{high}/TF^{negative}$ (Fig. 5.2a). Encouraged by the results of density gradient fractionation analysis, we wanted to confirm the coexistence of PDPN/TF and EV markers on individual EVs as well as explore the possibility of PDPN and TF being present on the same or different vesicles. Accordingly, we performed single EV immunoprofiling analysis using nano-flow cytometry (Choi *et al.*, 2018) to compare individual EVs for PDPN, TF and EV markers between donor glioma cells expressing either PDPN alone (U373PT), TF alone (U373vIII PT) or both (U373-PT-G11(Magnus *et al.*, 2014b); Fig. 5.2b). Again, EVs matched the PDPN/TF profiles of their parental cells, noting that double positive U373-PT-G11 cells produced a proportion of EVs harboring both PDPN and TF, in addition to single positives (Fig. 5.2b). Collectively, these results suggest that GBM cells exteriorize their pro-

thrombotic effectors in a form of exosome-like EVs, in a manner reflective of their cellular phenotype and oncogenic status.

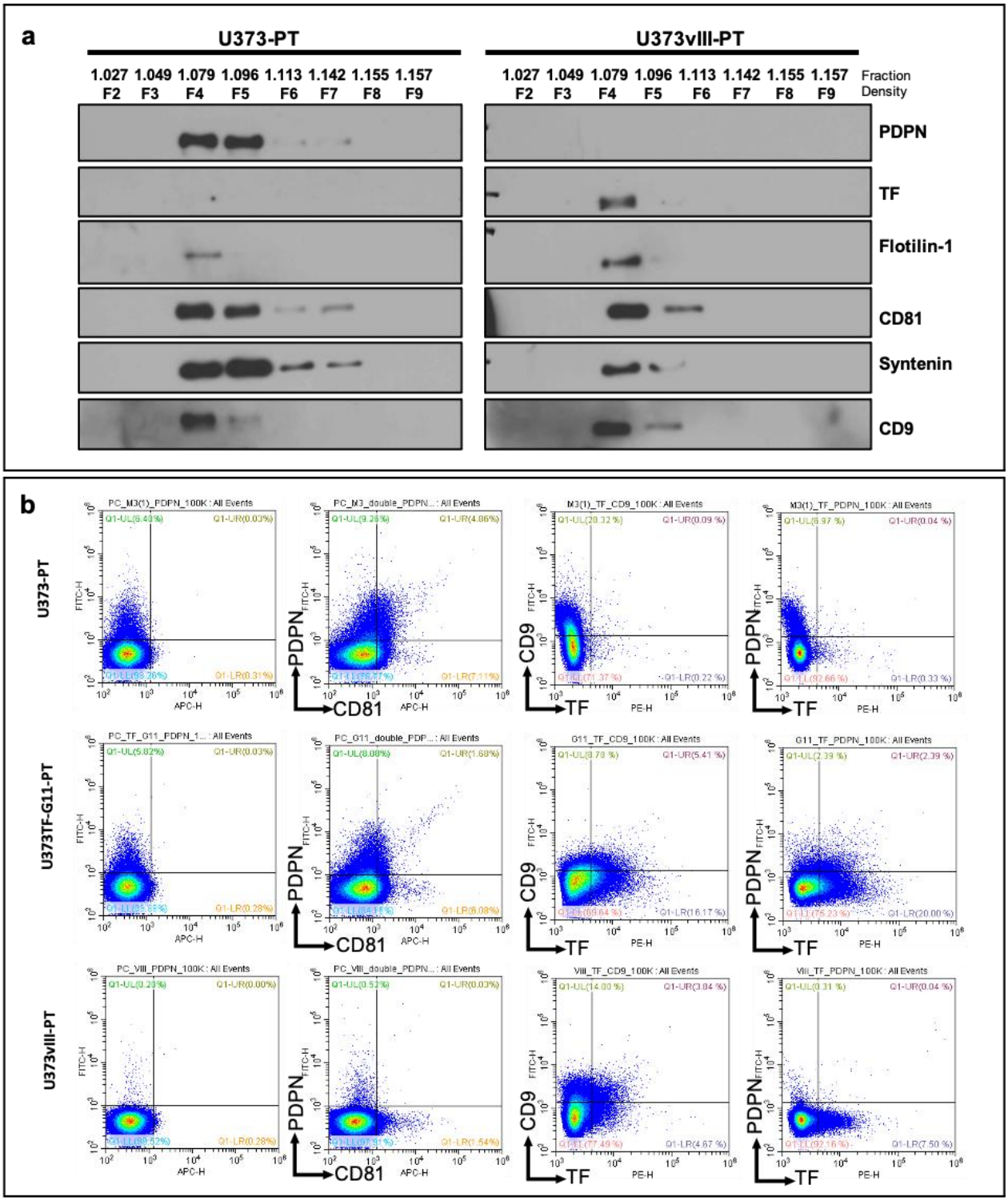


Figure 5.2 ***PDPN carrying extracellular vesicles exhibit exosome-like features.***

a. Glioma EVs float at exosomal density in the iodixanol gradient (F4-F5) and commonly express multiple EV markers (Flotillin 1, CD81, Syntenin, and CD9); EVs released from EGFRvIII-negative (U373PT) cells carry PDPN, but not TF, while their isogenic counterparts from

EGFRvIII-positive cells (U373vIII) carry TF, but not PDPN. **b.** Single EV nano-flow cytometry of EV populations from glioma cells expressing PDPN alone (U373PT), PDPN and TF (U373TF-G11-PT) and TF alone (U373vIII-PT); EVs are heterogenous but their subsets co-express CD81, PDPN and CD9, TF.

5.3 PDPN-carrying EVs are capable of inducing platelet activation

Having established the presence of PDPN on the surface of EVs exteriorized by GBM cells, we wanted to assess the capacity of PDPN carrying EVs to exert an activating impact on platelets. Hence, we took advantage of YFP mice as a source of YFP-labelled platelets. We isolated fresh YFP-labelled platelets from citrated blood collected through the inferior vena cava (IVC) and co-incubated them with GBM cells and their respective EVs. Following a 15-minute co-incubation with mild shaking, we proceeded to assess platelet activation using P-selectin, as a marker. Confocal microscopy revealed that the incubation of YFP-labelled platelets with $PDPN^{high}$ GBM cells or their EVs *ex vivo* led to the expression of P-Selectin at sites of surface contacts (Fig. 5.3). On the contrary U373vIII cells and their EVs, devoid of PDPN, failed to induce any comparable platelet activation in this assay.

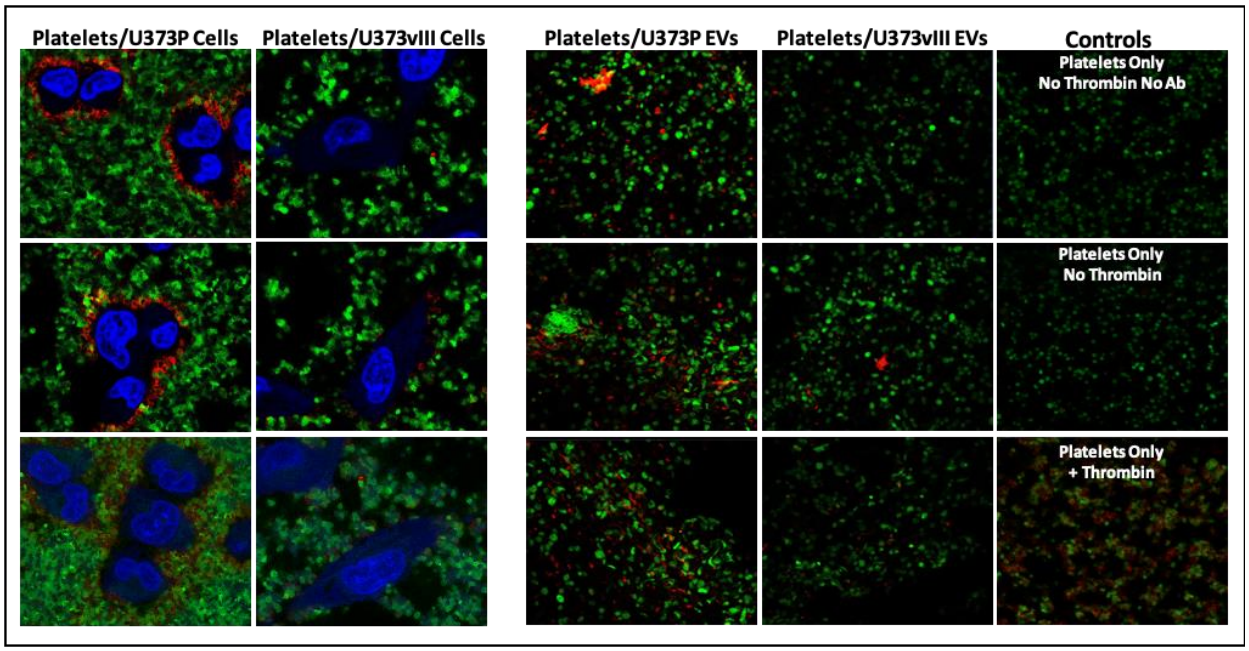


Figure 5.3 ***Platelet activation by PDPN expressing glioma cells and their corresponding extracellular vesicles.***

PDPN-expressing (U373P) and non-expressing (U373vIII) cells (left panels) and their EVs (middle panels) exhibit differential ability to trigger P-selectin exposure (red) by fluorescent mouse platelets (green). Platelets were isolated form mice harboring YFP transgene and incubated with intact cancer cells (left panel), their EVs (right panel) or controls (right panels); only PDPN-expressing U373P cells efficiently triggered P-selectin exposure and platelet activation.

6 Coagulant Consequences of the Expression and Release of GBM-associated PDPN

Tawil et al. 2021, Blood Advances

6.1 PDPN is detected in the peripheral blood of tumor-bearing subjects

Having established that GBM cells release PDPN as cargo of the EVs, we wished to assess whether this process occurs *in vivo*. Such findings would be important for at least two reasons. First, if circulating PDPN corresponds to the levels of this protein found in GBM tissues and predictive of high VTE risk, measuring PDPN in the EV fraction of blood could potentially be developed into a biomarker of high risk, or impending thrombosis. Second, since PDPN-EVs are capable of activating platelets ex vivo, these vesicles could serve as triggers of the hypercoagulable state in GBM patients and be eventually amenable to therapeutic mitigation with anti-platelet or anti-PDPN agents.

The challenge associated with detection of informative cargo in cancer related EVs stems from their low abundance amidst circulating particles emanating from host cell populations (Abels and Breakefield, 2016; Arraud et al., 2014). Therefore, we chose to initially evaluate total amounts of PDPN released into the blood in the presence of PDPN expressing tumors, in both mice and GBM patients. In order to address this question, we implanted mice subcutaneously with xenografts of U373PT ($PDPN^{high}/TF^{low}$) and U373vIII ($PDPN^{low}/TF^{high}$) cells. Subcutaneous inoculation was chosen to allow a larger tumor mass to develop so as to render PDPN detection more feasible using the available immunodetection assays. Mice were sequentially sacrificed when sufficiently large tumors developed (~ 0.7-1 cm^3) and blood was drawn from the IVC. A 150µl

sample of whole blood was initially sent for complete blood count (CBC) and platelet counts and the rest was utilized for plasma extraction. Assessment of human PDPN levels in plasma of tumor bearing mice was carried out using a specific anti-human PDPN ELISA to minimize the contribution of non-cancer cells. This analysis revealed that circulating human PDPN was readily detectable in blood of mice harbouring human U373PT ($PDPN^{high}/TF^{low}$) xenografts, while similar U373vIII tumor burden produced minimal PDPN signal, with tumor-free mice being negative (Fig. 6.1a, b).

We next wished to account for the discrepancy between the ratio of tumor : body mass : blood volume between our mouse model and the reality in GBM patients whose lesions are considerably smaller relative to body mass. Along these lines we wished to ascertain that under these conditions PDPN could still be detected in plasma of GBM patients. Our collaborative access to a small cohort of plasma samples from GBM patients enabled us to analyze for the presence of PDPN in this material by ELISA. Indeed, we were able to detect variable levels of PDPN, mostly well above the normal level of 1.31 ± 0.13 ng/ml reported in literature (Zhao *et al.*, 2018), in line with the heterogeneous nature of PDPN expression across GBM tumors (Fig. 6.1c). While we were not able to correlate levels of PDPN in plasma with the corresponding tumor PDPN expression or to carry out EV isolation, both due to limited access to samples, these results corroborate the feasibility of circulating PDPN detection in both mice and patients with GBM. We believe it would be essential to perform such a retrospective study in the future in a larger cohort if the sample access could be secured (efforts are ongoing).

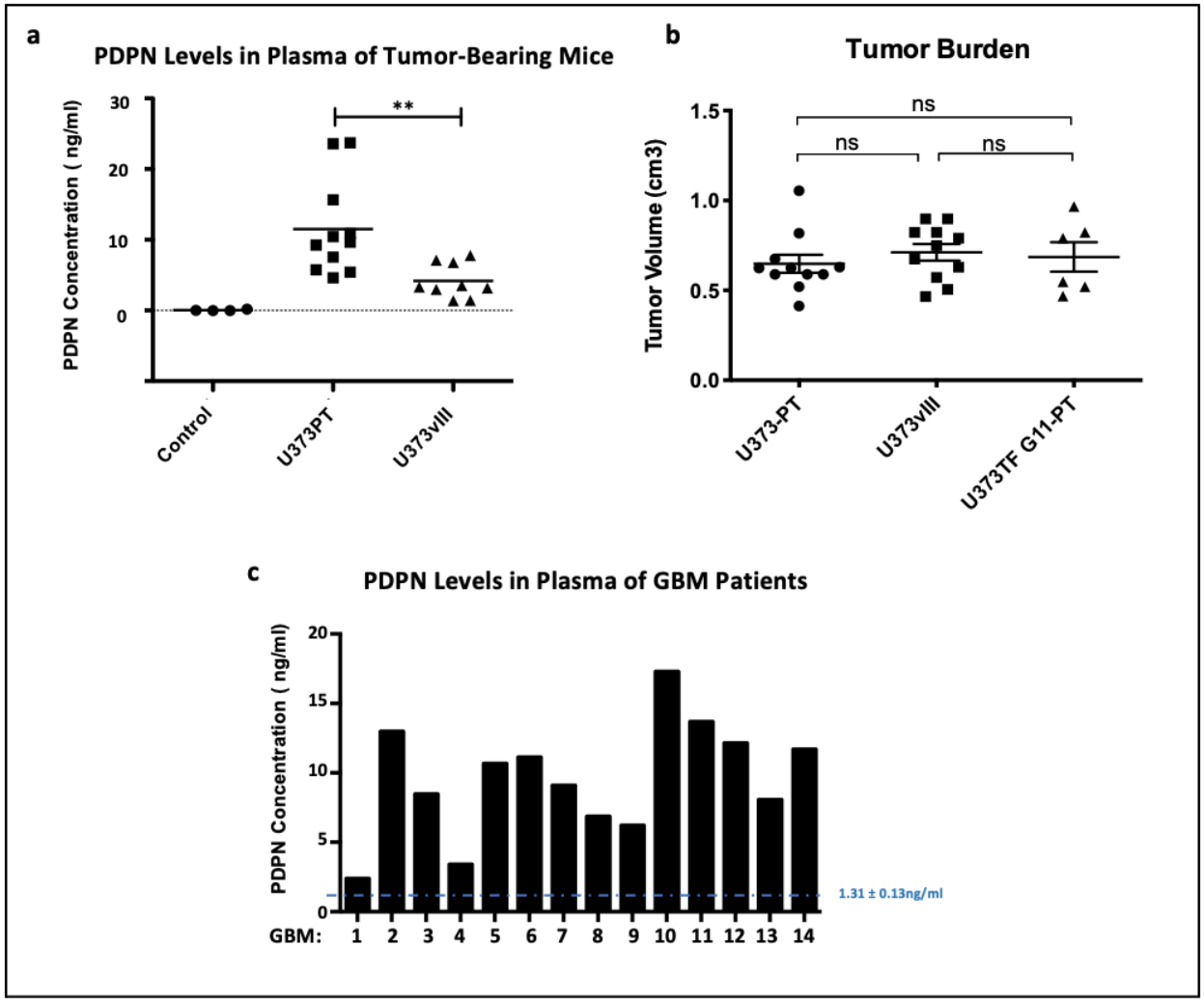


Figure 6.1 ***PDPN levels in plasma of tumor-bearing subjects.***

a. Immunodetection (ELISA) of circulating human PDPN in plasma of mice with U373PT and U373vIII glioma xenografts; suppression of PDPN in EGFRvIII-driven U373vIII cells leads to low PDPN levels in blood (control - plasma of tumor-free mice). **b** Tumor sizes were comparable across the entire panel. **c**. Detection of variable PDPN levels in plasma of GBM patients (human PDPN ELISA), mostly above normal levels of 1.31 ± 0.13ng/ml reported in literature (Zhao *et al.*, 2018). It is noteworthy that ELISA readings may, at least in theory, detect both soluble fragments of PDPN (if any) and PDPN released as EVs from cancer cells. Our analysis of GBM EVs confirms the latter possibility (below).

6.2 PDPN release into systemic circulation results in platelet activation and consumption

With evidence of PDPN release into the systemic circulation at detectable levels, we set out to explore the functional consequences. We surmised that if circulating PDPN (as EVs) retains its ability to activate platelets the corresponding markers would be expected to arise in blood such as P-selectin-positive platelet pool and soluble platelet 4 (PF4) elevation. An additional question of great interest was whether such events are also triggered by circulating TV-EVs. In theory, TF exposed on the surface of circulating GBM-derived EVs would be expected to trigger thrombin generation which could be expected to activate platelets, as well fibrin formation, followed by fibrinolysis, all of which would lead to rise of circulating coagulation markers such as fibrin D-dimer (DD), prothrombin fragment 1.2 (F1.2) and thrombin-antithrombin complexes (TATs). Such effects of TF, though plausible, would overlap with those of PDPN and contravene the disconnect between TF expression and VTE risk in GBM patients. Finally, we were interested in investigating the potential for PDPN/TF cooperation in promoting a procoagulant phenotype and as such potentially setting the stage for the development of GBM associated VTE.

To explore these questions experimentally, mice were xenografted with glioma cells expressing either PDPN (U373PT), TF (U373vIII), or both (U373TF-G11-PT) and tested for activation of platelets (for PDPN) and the clotting cascade (for TF). Interestingly, in absence of signs of myelosuppression as indicated by comparable RBC counts (Fig. 6.2a), tumors expressing high levels of PDPN (U373PT, U373TF-G11-PT) triggered a significant reduction in overall platelet counts (Fig. 6.2b) as revealed by complete blood count (CBC). This observation coincided with the upregulation of the circulating platelet factor 4 (PF4) detected upon analyzing the corresponding plasma samples using a PF4 ELISA. Both of these observations suggested the

aberrant and systemic platelet activation and consumption, which was in line with recent clinical results in GBM (Riedl *et al.*, 2017; Unruh *et al.*, 2016).

Notably, the co-expression of high PDPN and high TF levels in U373TF-G11-PT cells did not significantly change platelet-related experimental endpoints. This was in spite of the fact that we confirmed that TF was present on the surface of these cells, and was successfully released into pericellular space in a biologically active form as EVs (Garnier *et al.*, 2012), as demonstrated by the analysis of MP-TF (TF-EV) procoagulant activity both *in vitro* and *in vivo* (Fig. 6.3a-f). This result is surprising given the expected thrombin generating potential of the TF pathway, which could activate platelets, but once again, also resembles recent clinical findings, which did not reveal a strong association between TF expression and the VTE risk in GBM patients (Thaler *et al.*, 2012).

Additional analysis showed that TF-expressing xenografts did exhibit coagulant properties, as revealed by increased D-dimer levels in plasma of animals with U373TF-G11-PT and U373vIII tumors relative to that of their counterparts with $PDPN^{high}/TF^{low}$ expression profiles (U373PT). In the latter case D-dimers were only slightly (insignificantly) above the baseline (Fig. 6.4a). This result is both intriguing and counterintuitive as it suggests that TF associated with GBM cells does trigger coagulation cascade, as evidenced by the rise in D-dimer, but in such a fashion that peripheral platelets are not activated. The later may indicate that there is a spatial separation between circulating platelets and sites of thrombin generation by TF pathway on cancer cell or EV membranes.

In order to understand whether these results are triggered by cancer cells expressing PDPN and/or TF, both associated with EVs, or are unspecific in nature we interrogated the ability of GBM EVs to trigger various arms of the hemostatic system. Indeed, some of these coagulant responses observed in mice harboring GBM xenografts were recapitulated by intravenous injection of the respective EVs in the absence of the tumor mass. Notably, increase in PF4 levels, could be induced following intravenous injection of PDPN carrying GBM-EVs. In contrast, TF-carrying U373vIII EVs failed to increase PF4 in blood of tumor-free mice, while PDPN-enriched U373PT and U373TF-G11 EVs elevated PF4 in the circulation (Fig. 6.4b). This is understandable as EV-associated PDPN could mediate direct and single-step interaction with the CLEC2 receptor on platelets and mediate their thrombin-independent activation.

It is noteworthy that injection of glioma TF-EVs failed to trigger a statistically significant upsurge in D-dimer levels (Fig. 6.5a) suggesting that in this case TF-EVs may be insufficient to trigger strong coagulant response in the peripheral circulation. This is in spite of the robust TF procoagulant activity (TF-PCA) associated with TF-EVs *in vitro* and *in vivo,* as measured by factor Xa generation assay (Fig. 6.3). Clearly such TF activity present in the peripheral circulation does not translate into systemic activation of the clotting cascade, plausibly due short half-life of EVs in plasma (Mackman, 2012) and their dilution rendering the multistep process of thrombin generation dispersed and spatially suboptimal. In contrast, in the tumor microcirculation the exposure of plasma carrying coagulation zymogens (FVII, FX, prothrombin) to TF concentrated on the surfaces of densely packed cancer cells surrounding permeable blood vessels would be expected to be more efficient and persistent.

We reasoned that the elevated D-dimers observed in plasma of mice xenografted with U373TF-G11-PT and U373vIII could be a result of D-dimer generation within the tumor mass (rather than circulating blood) and its subsequent release into the blood stream. To test this hypothesis, we compared the total D-dimer content of tumors to that of corresponding plasma in the same mice. This analysis (ELISA) revealed a marked (10^4-fold) higher content of D-dimers within the whole tumor mass relative to total volume of plasma (Fig. 6.5b). This observation suggests a possibility that peripheral D-dimer may come from thrombosis occurring within the tumor microcirculation, rather than, as presently believed, being a marker of systemic hypercoagulability. Of course, given the wide-spread use of D-dimer as a marker of cancer coagulopathy, this notion deserves an independent investigation. Overall, these results suggest that activation of different components of the hemostatic machinery could be tumor (and cancer cell population)-specific. Moreover, EV-mediated release of PDPN appears to be sufficient to systemically upregulate platelet activation markers in a mouse model of GBM.

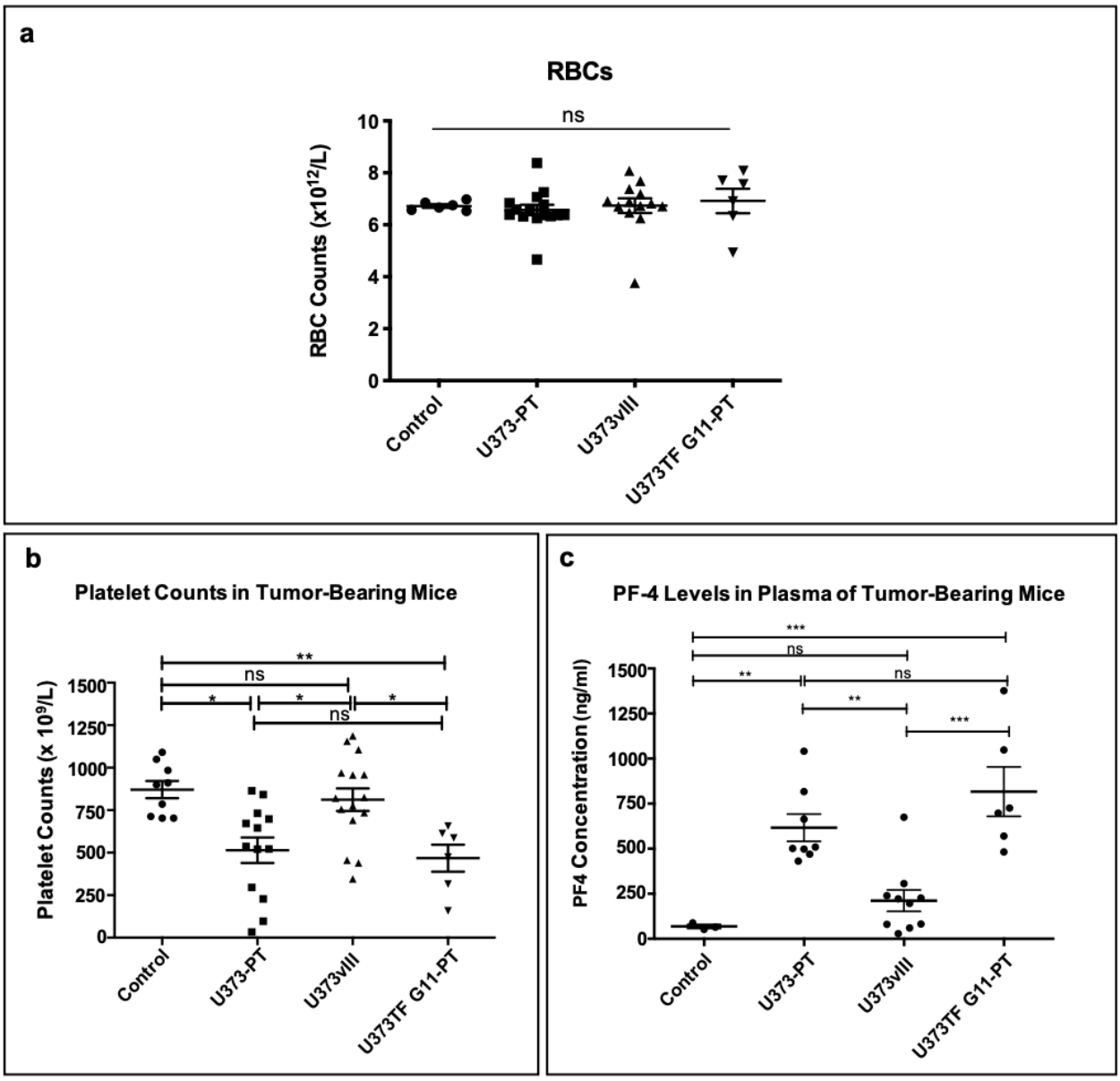
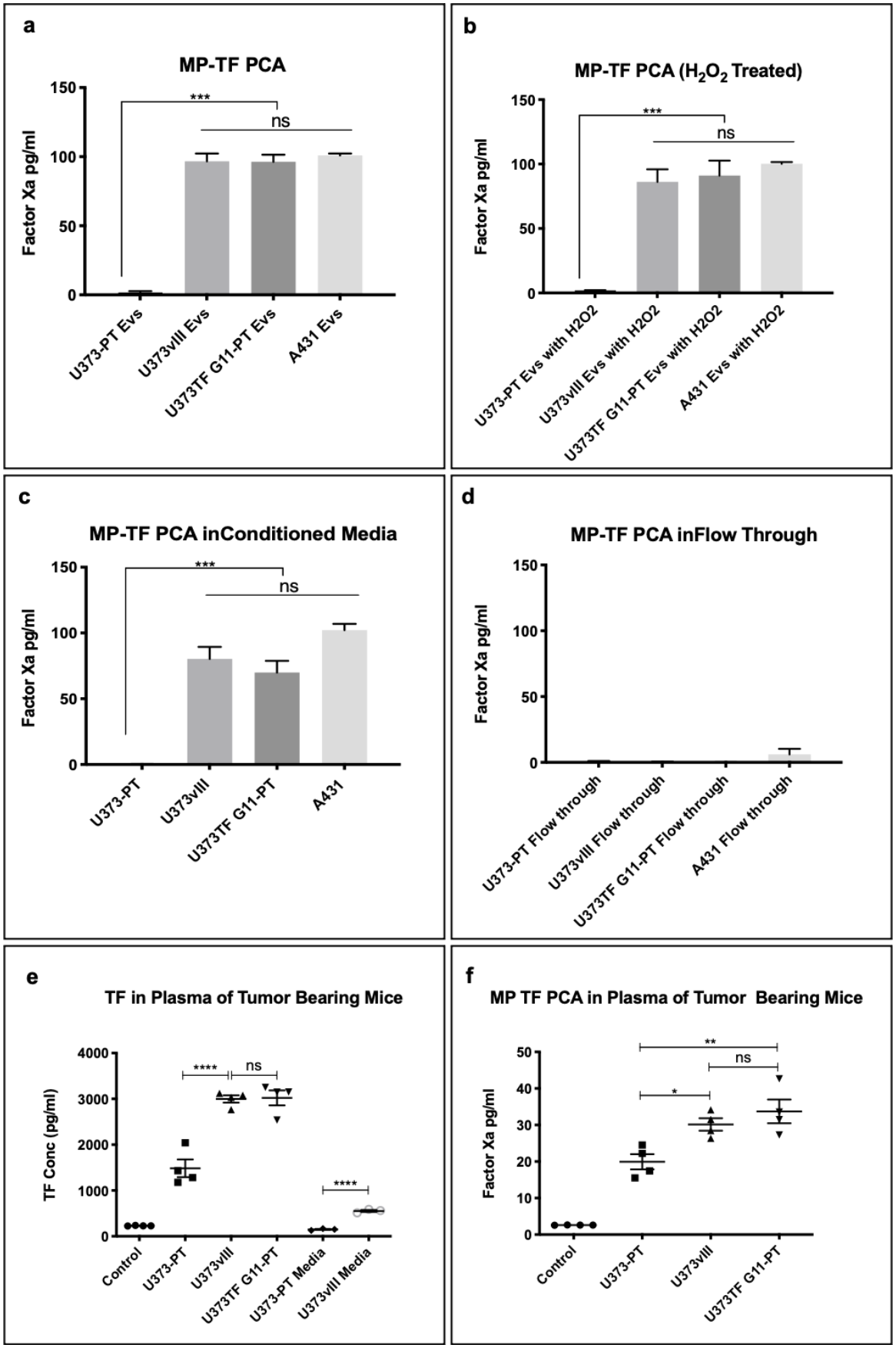


Figure 6.2 ***Systemic platelet activation as function of tumor-expressed and released PDPN.***

a. RBC counts reflective of normal bone marrow function and absence of suppression **b.** Reduced platelet counts in mice harboring PDPN-high glioma xenografts (U373PT, U373TF-G11-PT) *versus* PDPN-low tumors (U373vIII) and tumor-free controls. **c.** Increased PF4 levels in plasma of mice with PDPN-high glioma xenografts versus those with PDPN-low tumors and controls (as in **b**).


a
MP-TF PCA
Factor Xa pg/ml
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ns
U373-PT Evs
U373vIII Evs
U373TF G11-PT Evs
A431 Evs
b
MP-TF PCA (H2O2 Treated)
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ns
U373-PT Evs with H2O2
U373vIII Evs with H2O2
U373TF G11-PT Evs with H2O2
A431 Evs with H2O2
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MP-TF PCA inConditioned Media
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U373TF G11-PT
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U373vIII Flow through
U373TF G11-PT Flow through
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TF in Plasma of Tumor Bearing Mice
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Control
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U373vIII
U373TF G11-PT
U373-PT Media
U373vIII Media
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MP TF PCA in Plasma of Tumor Bearing Mice
Factor Xa pg/ml
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40
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Control
U373-PT
U373vIII
U373TF G11-PT

Figure 6.3 ***Microparticle-TF procoagulant activity (MP-TF PCA) in the U373P-related models of glioblastoma and plasma of tumor-bearing mice.***

a. MP-TF PCA assessment in EVs isolated from U373, U373vIII and U373TF G11 cells (A431 EVs used as positive control). **b.** MP-TF PCA assessment in peroxide (20μM) treated EVs isolated from U373, U373vIII and U373TF G11 cells; peroxide was used to cause TF decryption (Bach, 2006). **c**. Assessment of MP-TF PCA in conditioned unfractionated media from U373P, U373vIII and U373TF-G11 cells. **d.** Assessment of TF PCA in soluble fraction of conditioned media. Amicon concentration column flow-through fraction following EV concentration step was assayed for U373P, U373vIII and U373TF G11 cells conditioned media. **e**. TF levels and MP-TF PCA (**f**) in plasma of U373-PT, U373vIII, and U373TF G11-PT tumor-bearing mice.

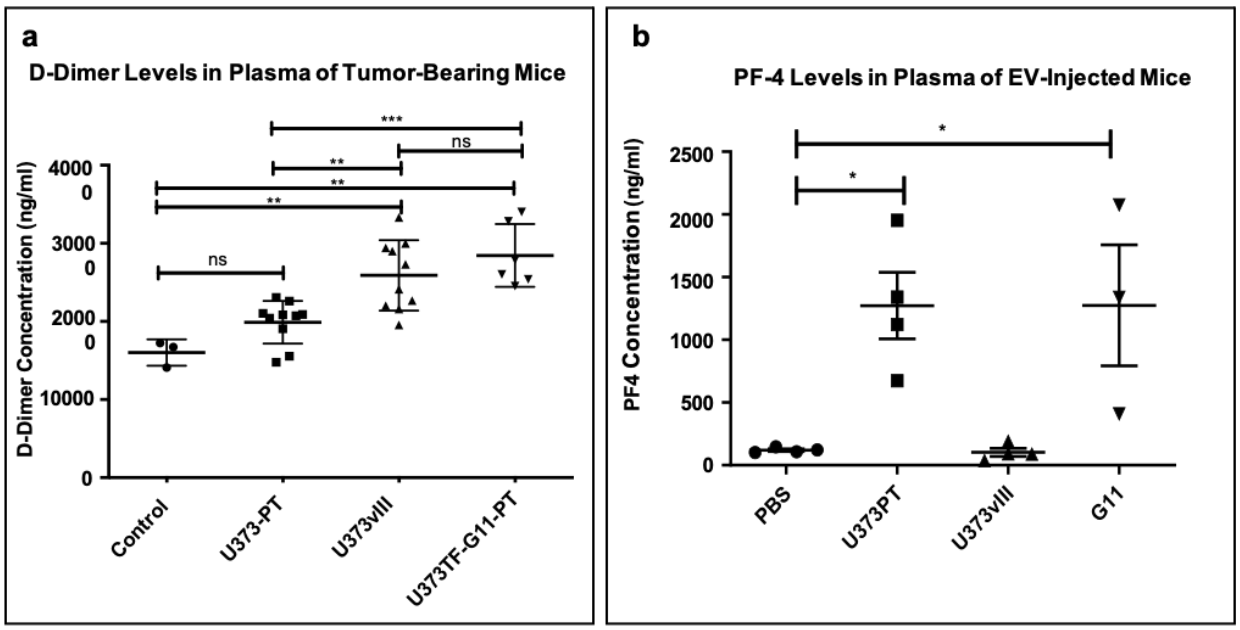


Figure 6.4 ***D-Dimer and PF4 levels in plasma of tumor-bearing and EV-injected mice respectively.***

a. D-dimer levels in plasma of mice bearing glioma xenografts; D-dimers in the case of tumors expressing PDPN (U373PT) were not significantly different than those in controls. D-dimers were elevated in TF-expressing U373vIII tumors and U373TF-G11-PT tumors with high levels of both TF and PDPN. **b**. PF-4 levels in plasma of mice injected *i.v.* with glioma EVs (10 μg/mouse); PF-4 elevation occurred in mice injected with PDPN carrying EVs (U373TF-G11-PT and U373PT) regardless of TF status, while PDPN-negative and TF-positive EVs (U373vIII) produced no such increase above the background (PBS). ns – not significant, P values: * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001

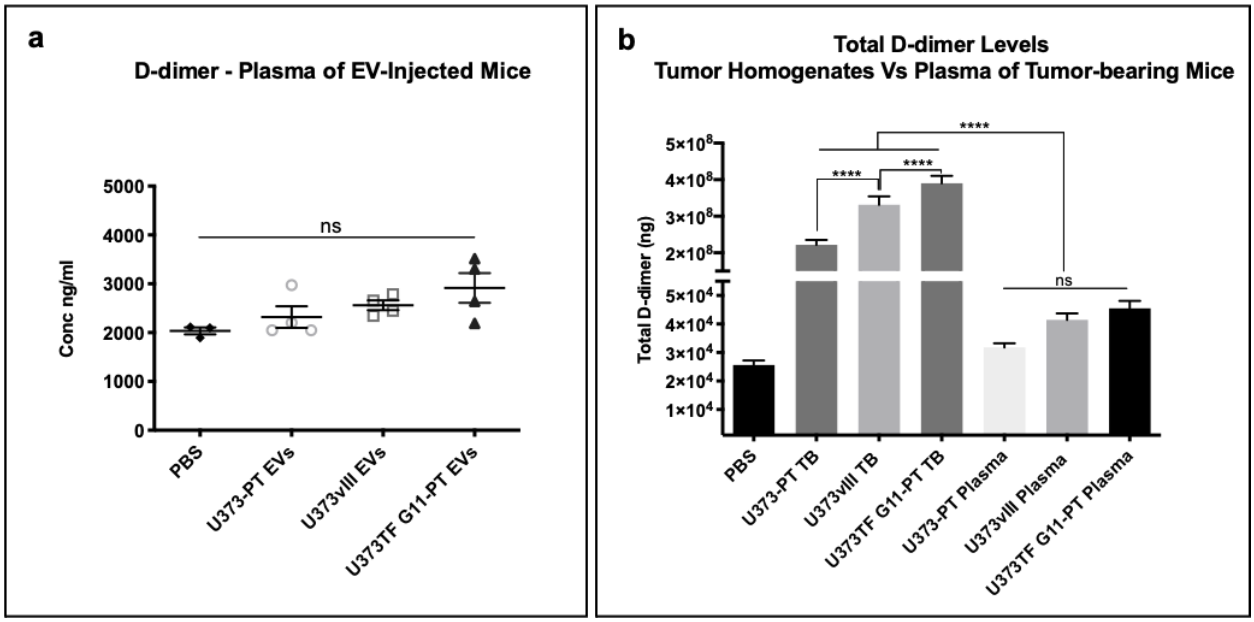


Figure 6.5 ***D-dimer levels in mice exposed to glioma tumors and EVs.***

Plasma of tumor-bearing and EV-injected mice as well as total D-dimer in whole tumor mass versus total plasma were assayed as indicated. **a.** D-dimer levels in plasma of EV-injected mice**. b.** Total D-dimers in the whole tumor mass versus plasma of mice with the respective tumors (ELISA); ns – non-significant, P value: ** - 0.01; *** - 0.001; **** - 0.0001

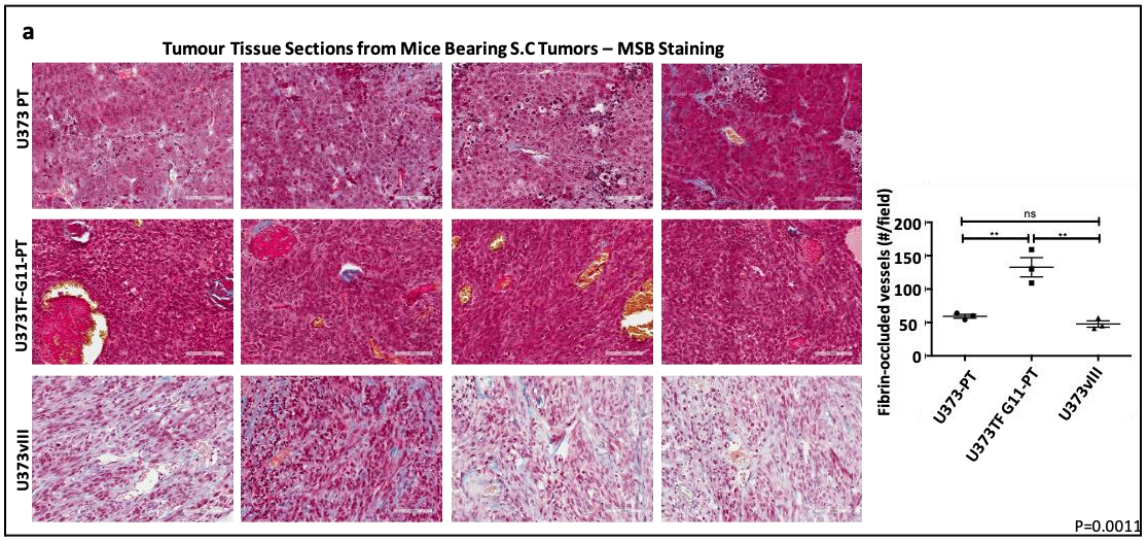
6.3 TF and PDPN cooperate in promoting intra-tumoral microthrombosis

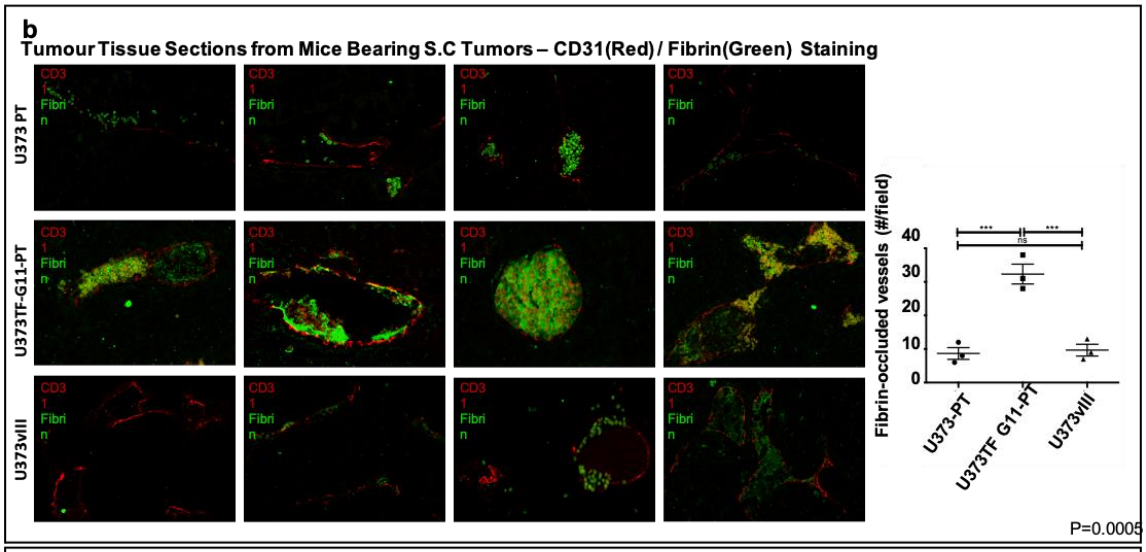
Our previous results pointed towards the idea that coagulant effects of PDPN and TF in cancer may exhibit spatial differences impacting mechanisms that may influence VTE risk versus microvascular thrombosis. As mentioned earlier previous work by Riedl et al. indicated the preponderance of platelet thrombi within PDPN expressing GBM tumors when compared with $PDPN^{low/deficient}$ ones (Riedl *et al.*, 2017). At the same time PDPN was found in the peripheral circulation and EV-associated PDPN was sufficient to trigger systemic activation of platelets in experimental models (Tawil *et al.*, 2021). While TF was also expressed in a subset of GBMs and released into plasma of GBM patients (Tawil *et al.*, 2021), this circumstance did not predict VTE risk in GBM patients (Thaler *et al.*, 2012). Moreover, TF expressing GBM xenografts did not efficiently trigger systemic platelet activation markers (PF4), but instead supported intratumoral coagulation as evidenced by elevated expression of D-dimer.

These observations amount into a suggestion that the nature of systemic thrombosis and local microvascular thrombosis may be mechanistically different. For this reason, we wanted to explore in more detail the impact of different profiles of PDPN and TF expression on the intratumoral microthrombosis, which appears to be affected by both PDPN and TF. In order to gain insights regarding the possibility of PDPN/TF cooperation in driving thrombosis at the intratumoral level, GBM xenografts were investigated for vascular morphology, fibrin deposition and extent of intraluminal platelet-rich thrombi (Figs. 6.6a-c). Interestingly, staining with MSB dye revealed that the highest intravascular fibrin content was in tumors arising from U373PT-G11-PT cells ($PDPN^{high}/TF^{high}$), with considerably lower signal for both U373vIII ($PDPN^{low}/TF^{high}$) and U373PT ($PDPN^{high}/TF^{low}$) lesions (Fig. 6.6a). This observation was corroborated by

immunofluorescent staining for intravascular fibrin combined with CD31 staining in order to have a clear delineation of the vascular lumen (Fig. 6.6b). We further tested xenografts for the presence of platelet thrombi within the vessels of these tumors, and similarly we also found that tumors arising from the $PDPN^{high}/TF^{high}$ U373PT-G11-PT cells had the highest number of vessels with occluding thrombi positive for CD61, a marker of platelets (Fig. 6.6c).

Thus, while systemic activation of platelets (PF4) could be linked to PDPN expression by cancer cells and tumor-derived systemically circulating EVs, the extent of intra-tumoral microthrombosis appeared to be a function of TF and PDPN co-expression. This observation may suggest that in the tumor microenvironment a uniquely high concentration of these cell membrane-associated pro-thrombotic effectors may lead to their cooperation. The possibility that different sites of CAT (peripheral large vessels, tumor microvessels, vascular sites of tumor dissemination) may exhibit different mechanisms of thrombosis has rarely (if ever) been considered in GBM. If proven correct, such spatial and mechanistic diversity would have profound diagnostic, therapeutic and biological consequences. For example, a better understanding of the part of the CAT process that is of medical concern in a given patient (VTE or micro-thrombosis) would need to be considered to accurately interpret diagnostic findings such as D-dimer or PF4 elevation. It may also be the case that the biological impact of microthrombosis on cancer cells and stroma (angiogenesis, inflammation) may depend on whether PDPN, TF, both or none are involved (Magnus *et al.*, 2014a). Additionally, the host-related factors such as age-related vascular damage, micro-strokes, or inflammation related, or unrelated to cancer may compound these effects further. Future studies may shed more light on these important questions and lead to more targeted interventions.


a
Tumour Tissue Sections from Mice Bearing S.C Tumors – MSB Staining
U373 PT
U373TF-G11-PT
U373vIII
Fibrin-occluded vessels (#/field)
ns
**
**
200
150
100
50
0
U373-PT
U373TF G11-PT
U373vIII
P=0.0011


b
Tumour Tissue Sections from Mice Bearing S.C Tumors – CD31(Red) / Fibrin(Green) Staining
U373 PT
U373TF-G11-PT
U373vIII
CD3
1
Fibri
n
Fibrin-occluded vessels (#/field)
***
ns
***
40
30
20
10
0
U373-PT
U373TF G11-PT
U373vIII
P=0.0005

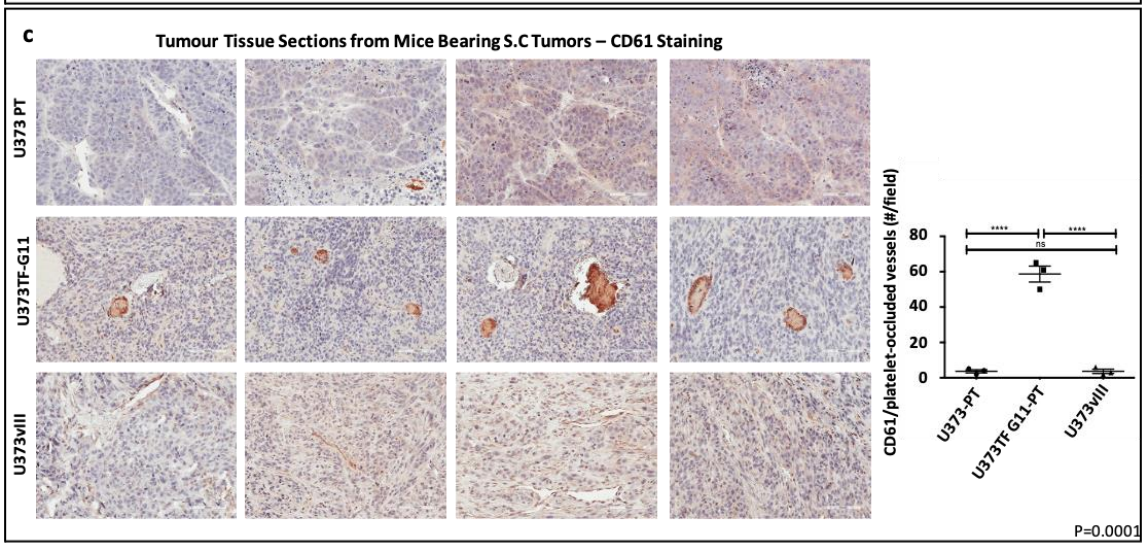

c
Tumour Tissue Sections from Mice Bearing S.C Tumors – CD61 Staining
U373 PT
U373TF-G11
U373vIII
CD61/platelet-occluded vessels (#/field)
****
ns
****
80
60
40
20
0
U373-PT
U373TF G11-PT
U373vIII
P=0.0001

Figure 6.6. ***Cooperation between PDPN and TF in tumor microthrombosis in glioma xenografts.***

a. MSB staining for thrombin in four different representative xenografts expressing PDPN alone (U373PT), PDPN and TF (U373TF-G11-PT) or TF alone (U373vIII); quantification of occluded vessels containing fibrin thrombi (right panel) indicates significant elevation in the case of U373TF-G11-PT (PDPN/TF positive) tumors. **b.** Immunofluorescent staining of tumors for endothelial cells (CD31 - red) and fibrin (green); fibrin-occluded vessels predominated in U373TF-G11-PT tumors ($PDPN^{pos}/TF^{pos}$). **c.** Immunohistochemical staining of glioma xenografts for mouse platelet marker CD61; platelet-rich thrombi were most abundant in U373TF-G11-PT (PDPN and TF expressing) tumors (right panel). ns – non-significant, P value: ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001

7 Studies on Biological Effects of PDPN in Glioblastoma Progression

7.1 PDPN expressing subsets of GSCs exhibit enhanced tumorigenic potential

As mentioned earlier, thrombosis worsens prognosis in a wide spectrum of cancers (Blom *et al.*, 2005; Timp *et al.*, 2013). In many instances this effect is not only a consequence of superimposed comorbidity but also a function of changes in the disease biology. For example, vaso-occlusive microthrombosis associated with GBM was implicated in formation of hypoxic regions and their impact on invasive features of tumor cells (Brat *et al.*, 2004). In addition, coagulation effector molecules and platelets possess non-hemostatic activities that may alter cellular signaling properties (Albrektsen *et al.*, 2007; Ruf *et al.*, 2011) and biological responses (Labelle *et al.*, 2011) and have been long implicated in promoting various aspects of cancer progression such as survival (Versteeg *et al.*, 2013), angiogenesis (Belting et al., 2005), immunomodulation and metastasis (Gil-Bernabé et al., 2012; Gil-Bernabé et al., 2013; Haemmerle *et al.*, 2018; Key et al., 2016). For example, earlier work from our group revealed the ability of TF to re-shape the tumor microenvironment and mediate the escape of indolent GBM cells from dormant state in mice (Magnus *et al.*, 2014b).

While thrombosis and its mediators contribute to tumor-promoting effects of the microenvironment (Magnus *et al.*, 2014a; Magnus *et al.*, 2014b; Magnus *et al.*, 2014c; Versteeg et al., 2008) it is unclear whether this non-cell autonomous mechanism could also apply to PDPN. In this regard, of particular interest is the question as to whether interactions with the coagulation system and platelets could select for PDPN expressing (coagulant) GBM cell populations, or

whether other selective processes impose PDPN expression and pro-thrombotic properties on cancer cells. Since PDPN is associated with tumor cell aggressiveness, stemness and may participate in signaling processes (Quintanilla *et al.*, 2019), both coagulant and non-coagulant pathways of cellular enrichment are conceivable in the context of GBM.

To begin to address some of these questions, we have taken advantage of the existence of patient-derived GSC lines containing natural mixtures of PDPN-positive ($PDPN^{pos}$) and PDPN-negative ($PDPN^{neg}$) cellular subpopulations. These cells could be sorted by FACS and tested for functional properties (GSC528; Fig. 7.1). Interestingly, $PDPN^{pos}$ subset of GSC528 cells exhibited an increased clonogenicity in culture (Fig. 7.1b, c) and elevated ability to initiate tumor growth in mice (Fig. 7.1d). These observations suggest that the overexpression of PDPN in subsets of GBM cells may be associated with (conceivably drive) their intrinsic aggressiveness and impact procoagulant microenvironment in this disease. However, these correlative experiments do not establish causation and are inconsistent with recent studies in which PDPN disruption in GBM cells did not dimmish their aggressive potential (Eisemann *et al.*, 2019).

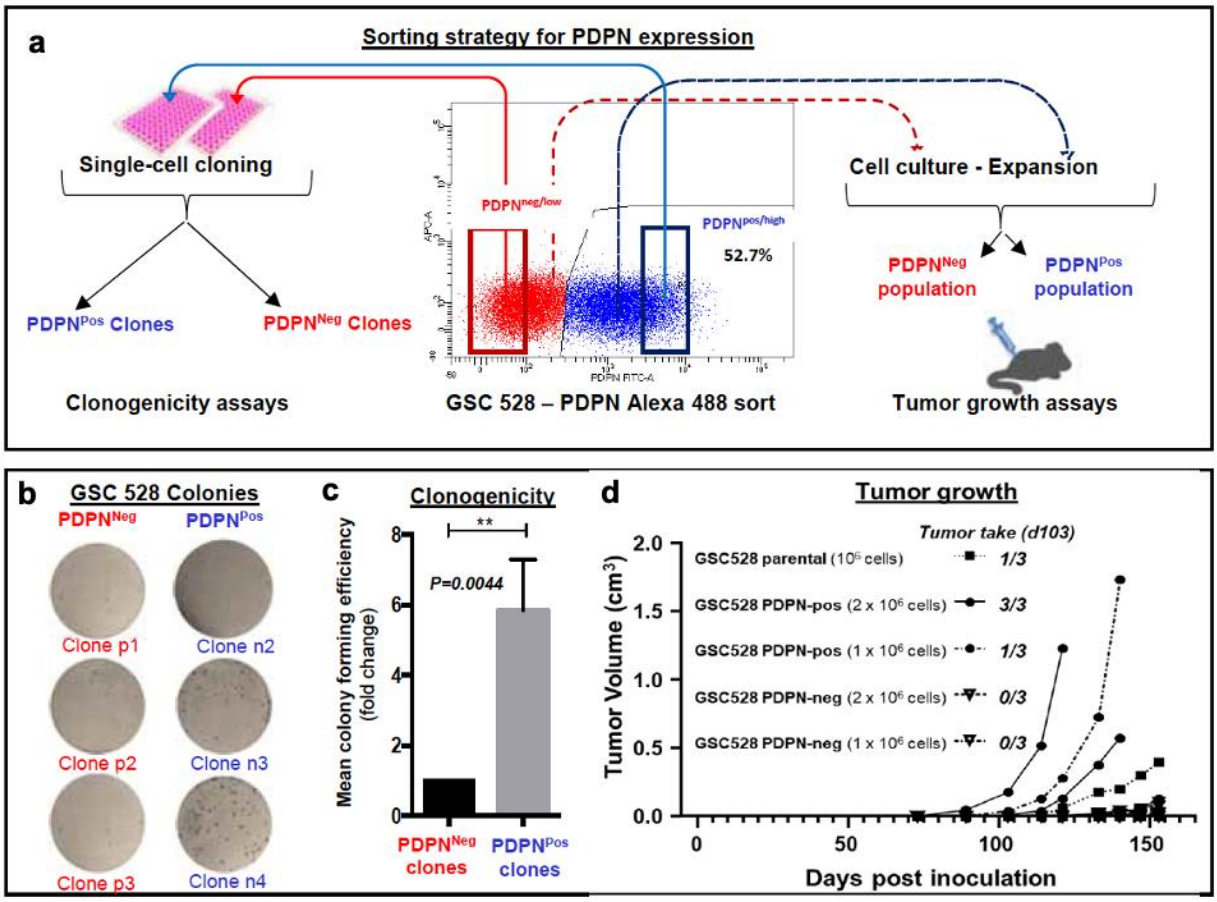


Figure 7.1 ***PDPN expressing glioma stem cells exhibit intrinsic aggressiveness.***

a. Strategies to separate PDPN-positive ($PDPN^{pos}$) and PDPN-negative ($PDPN^{neg}$) subpopulations coexistent within patient derived glioma stem cells (GSC528). The cells were sorted into indicated subsets or autocloned by FACS. **b-c.** Differential clonogenic efficiency of $PDPN^{pos}$ and $PDPN^{neg}$ GSC subpopulations. d. Elevated tumor take and growth rate of $PDPN^{pos}$ GSC subset; ** $p < 0.05$.

7.2 PDPN expression potentially contributes to decreased survival in mouse intracranial GBM model

In view of the existing contradictory data regarding the contribution of PDPN expression to tumorigenicity and reduced survival in GBM (Eisemann *et al.*, 2019; Ernst *et al.*, 2009; Yao et al., 2015), we sought to develop a more direct evidence using the orthotopic (intracranial) implantation model of GSCs. To that end used a random cloning approach to generate 3 $PDPN^{pos}$ and 3 $PDPN^{neg}$ luciferase tagged clones of GSC528 cells and injected them intracranially into NSG mice. Tumor growth and survival of tumor bearing mice were monitored over several weeks. Interestingly, although individual clones showed some variability, our luciferase bioluminescence imaging (Fig. 7.2), as well as survival data (Fig. 7.3) supported our previous observation of the enhanced tumorigenic potential in the case of $PDPN^{pos}$ subsets of GSC528 cells.

While the emerging correlation between PDPN expression and aggressiveness of, at least, some of the GSC lines is intriguing it does not prove causal or mechanistic involvement. In search of further evidence, we generated PDPN-deficient (KO) variants of two different $PDPN^{pos}$ clones of GSC528 cells. We chose to target PDPN gene in clonal populations due to co-existence of $PDPN^{pos}$ and $PDPN^{neg}$ cells among the parental GSC528 cells, a property that would compromise the screen for authentic PDPN-KO sublines. PDPN gene targeting was accomplished using CRISPR technology (guide plasmids generously provided by Dr. Sid Huang), followed by primary sorting for successful infectants (FACS) expected to carry GFP tag. The resulting clones were verified for PDPN expression using western blotting and immunofluorescence (FACS). After the successful generation of the corresponding PDPN-KO subclones for each of the two $PDPN^{pos}$ GSC528 clonal cell lines and validating their stability for several passages *in vitro* (Fig 7.4 a) we

proceeded to inject these cells intracranially to establish whether the absence of PDPN alters tumor aggressiveness.

These *in vivo* experiments revealed some ambiguities and variability (Fig 7.4b, c). Thus, the KO of PDPN in PDPN^{pos} GSC 528 clone 3 resulted in prolonged survival from a median of 73 days post injection in controls to 104 days in one its PDPN-KO variants. However, PDPN-KO variants of PDPN^{pos} GSC 528 clone 2 did not exhibit a similar impact (Fig 7.4 c). On the contrary, mice with tumors originating from PDPN^{pos} GSC 528 clone 2 survived longer (146 days post injection - median survival) than their PDPN-KO injected counterparts (116.5 days post injection - median survival) (fig 7.4b). While we noted a difference in survival between these two random clones (2 and 3) of PDPN^{pos} GSC528 cells the less aggressive of which (clone 2) was insensitive to PDPN deletion, these data failed to demonstrate a strong causative linkage with, or requirement for, PDPN expression and brain tumor progression.

To exclude the possibility of contamination with PDPN-expressing cells or revertants, the tumors formed by PDPN^{pos} GSC 528 clone 2 cells and their PDPN-KO variants were examined for PDPN expression by immunohistochemistry (IHC). Surprisingly, while the latter cells passed a rigorous screen for PDPN prior to injection, tumors originating from this cell line were highly PDPN-positive. We interpret this observation as a result of the existence of a minor clone in the GSC528-PDPN^{pos}-clone2-PDPN-KO population, which remained undetectable during extended period in culture, but expanded only under conditions of *in vivo* growth upon inoculation. Thus, while this observation depletes our pool of true PDPN-KO cell lines it may suggest that the expression of PDPN may afford GSCs an *in vivo* growth advantage in the mixed population. These

preliminary, unpublished findings require extensive verification that extends beyond this thesis project. This is especially important since our screen of GSC lines with different subtypes and aggressiveness reveal that, contrary to reports and speculations in the literature (Quintanilla *et al.*, 2019) only a subset of these patient-derived isolates express PDPN (FACS data). This observation is especially striking in the case of mesenchymal GSCs.

Since in GBM tissues PDPN is often expressed at high levels (Riedl *et al.*, 2017) the scarcity of this marker in stem cells driving these tumors (GSCs) suggests that, as we suggested earlier (Tawil *et al.*, 2021), differentiation pathways and microenvironment may trigger PDPN expression in specific subsets of GBM cells *in vivo* as progeny of GSCs assumes different phenotypes. This circumstance suggests that, regardless of whether isolated GSCs express PDPN constitutively, this protein may still have a role *in vivo*, including control of processes beyond thrombosis, as long as cellular subsets are generated in which the *PDPN* gene is turned on by its epigenetic control mechanisms (Chapter 4, (Tawil *et al.*, 2021)). However, the absence of PDPN expression is not rate limiting for GBM progression.

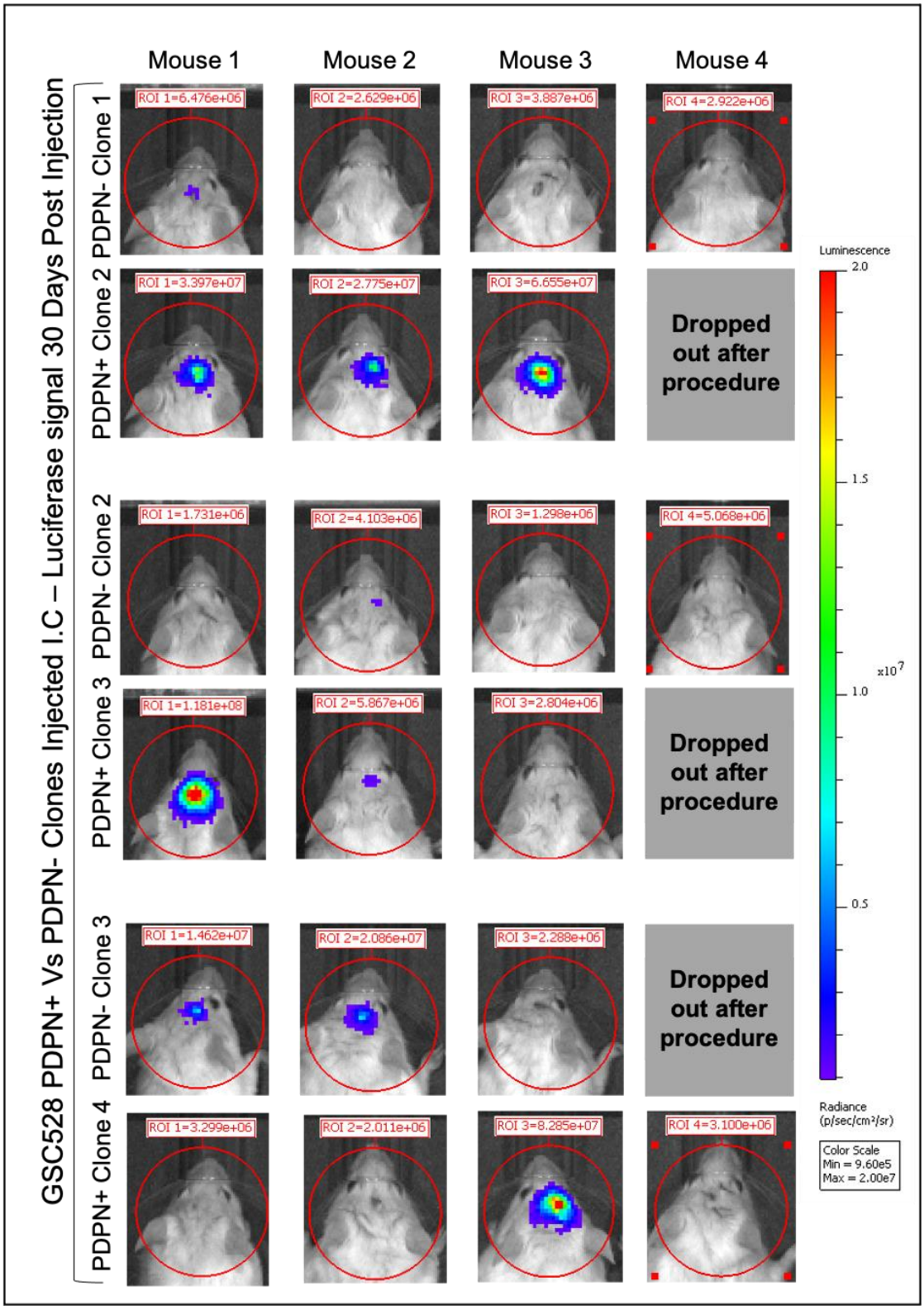

GSC528 PDPN+ Vs PDPN- Clones Injected I.C – Luciferase signal 30 Days Post Injection
Mouse 1
Mouse 2
Mouse 3
Mouse 4
PDPN- Clone 1
ROI 1=6.476e+06
ROI 2=2.629e+06
ROI 3=3.887e+06
ROI 4=2.922e+06
PDPN+ Clone 2
ROI 1=3.397e+07
ROI 2=2.775e+07
ROI 3=6.655e+07
Dropped out after procedure
PDPN- Clone 2
ROI 1=1.731e+06
ROI 2=4.103e+06
ROI 3=1.298e+06
ROI 4=5.068e+06
PDPN+ Clone 3
ROI 1=1.181e+08
ROI 2=5.867e+06
ROI 3=2.804e+06
Dropped out after procedure
PDPN- Clone 3
ROI 1=1.462e+07
ROI 2=2.086e+07
ROI 3=2.288e+06
Dropped out after procedure
PDPN+ Clone 4
ROI 1=3.299e+06
ROI 2=2.011e+06
ROI 3=8.285e+07
ROI 4=3.100e+06
Luminescence
2.0
1.5
1.0
0.5
x10^7
Radiance (p/sec/cm²/sr)
Color Scale
Min = 9.60e5
Max = 2.00e7

Figure 7.2 ***PDPN-positive clones of glioma stem cells exhibit intrinsic enhanced tumorigenicity in orthotopic injection model.***

Luciferase imaging of $PDPN^{pos}$ and $PDPN^{neg}$ GSC clone-injected mice monitored for tumor growth (30 days post injection time point).

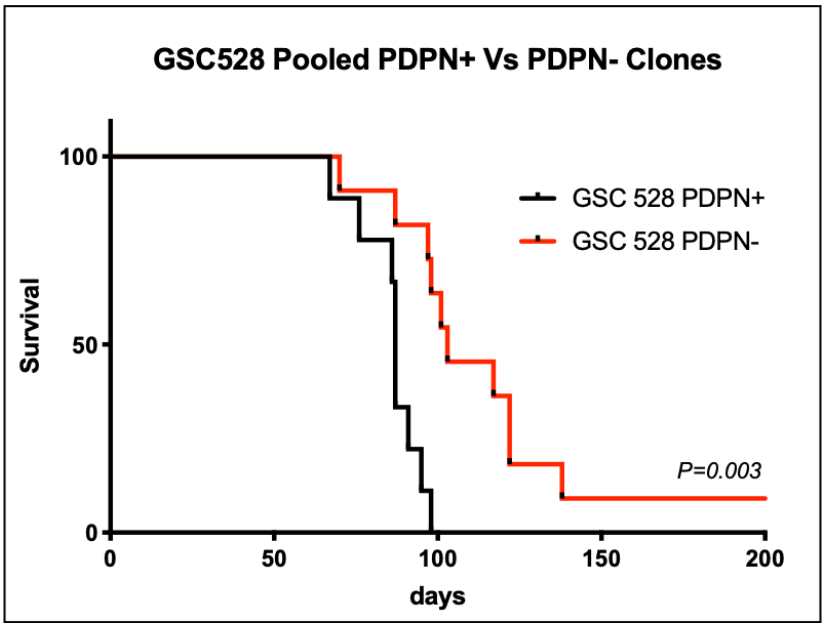


Figure 7.3 ***Mice intracranially injected with PDPN-positive clones of glioma stem cells exhibit decreased survival.***

Kaplan-Meier survival shows mice intracranially injected with PDPN-positive clones of glioma stem cells exhibit decreased survival compared to those injected with PDPN-negative clones; ** p=0.003.

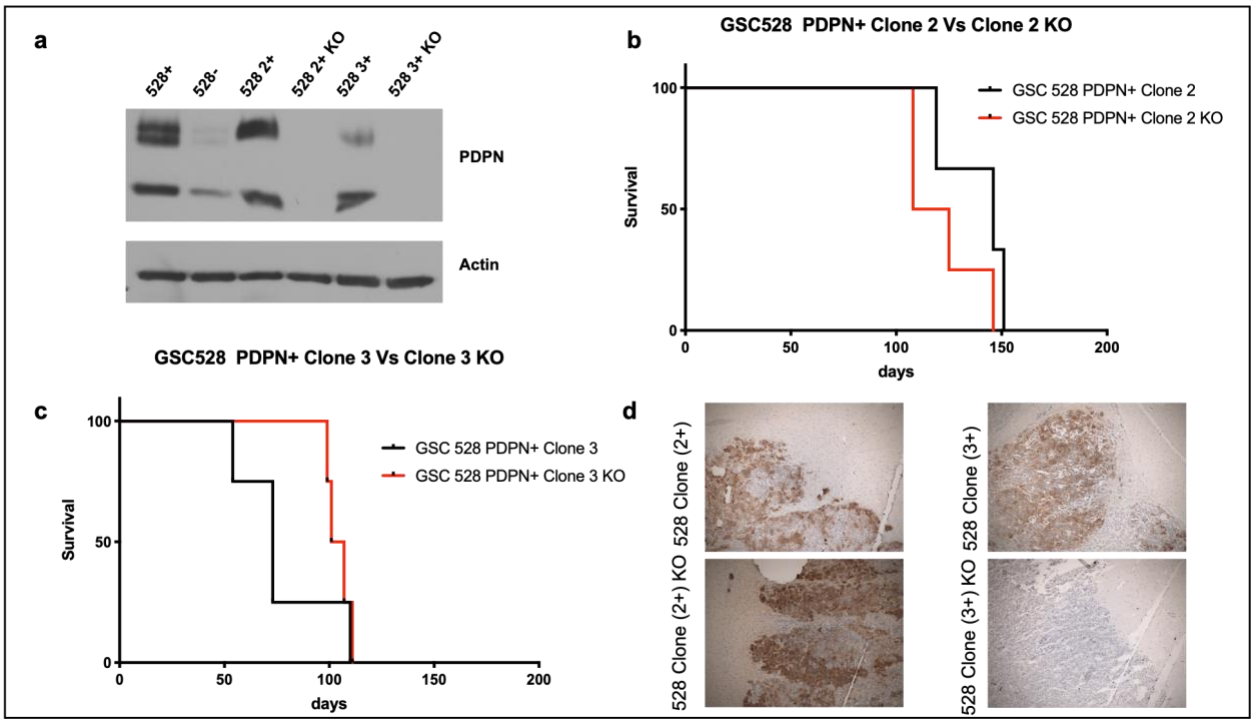


Figure 7.4 ***Survival of PDPN knock out and PDPN⁺ GSC528 clones injected mice.***

a. Generation and validation of PDPN KO cell lines of GSC 528 PDPN⁺ (clones 2 and 3) following several passages *in vitro*. **b.** Survival percentage of mice intracranially injected with PDPN⁺ GSC 528 clone 2 and its corresponding PDPN KO. **c.** Survival percentage of mice intracranially injected with GSC 528 clone 3 its corresponding PDPN KO. **d.** Representative images (IHC) of intracranial tumors generated following the injection of GSC 528 PDPN⁺ clones 2 and 3 and their corresponding PDPN KOs

7.3 Impact of PDPN expression on tumor responses to chemotherapy

In view of our preliminary data suggesting that PDPN-expressing cells may possess a growth advantage under clonal growth conditions and in restrictive microenvironments *in vivo*, but not in standard cell culture, we reasoned that this property may also impact the responses to therapy. In GBM the standard care involves a course of temozolomide (TMZ) chemotherapy combined with surgical and radiation treatment (Wen *et al.*, 2020). An earlier study from our laboratory documented a divergent evolution of mesenchymal GSCs following nearly total depopulation of tumor (beyond clinical detection) by a single injection of TMZ (Garnier *et al.*, 2018). Since the relapse of tumors in this model occurs from residual tumor estimated to contain less than a few thousands of cells (as judged by BLI measurement) the process is likely (oligo)clonal and we surmised this may create conditions under which PDPN effects, e.g., through recruitment of platelets could alter the disease trajectory. It is worth noting that accurate assessment of size or extent of residual tumor mass is hardly possible using BLI as many factors could introduce variability. These include variabilities in luciferase expression levels, cell viability, and perfusion, all of which could impact photon generation (following luciferin administration) which is used as an inferential read out of existing LUC-positive malignant cells. While the exact extent of residual tumor mass is not an acute point of our investigation, other tools that equally have their limitations but could permit more accurate assessment are MRI, computed tomography (CT) scan and positron emission tomography (PET) In regard to MRI, the image corresponds to distribution of water in the tissue (brain) and may be affected by changes in vascular permeability, similar to clinical settings. CT scans on the other hand are anatomically accurate, however the failure to generate sufficient contrasts between tumour versus normal brain tissue could hinder the accurate identification of tumor mass boundaries. Other technologies such as dynamic contrast-enhanced

magnetic resonance imaging (DCE-MRI), fluid-attenuated inversion recovery (FLAIR), or PET are designed to observe specific features of lesions and their readings could be best interpreted if considered in that context or collectively in combination with other imaging modalities.

Another component of our hypothesis rests on the observation made under another project ongoing in our laboratory (Meehan et al – in preparation) whereby the effect of TMZ would differ dramatically between mice with different status of NK cell activity. Thus, in standard NSG mice MES-GSC xenografts regress but eventually relapse as TMZ-resistant tumors, while in SCID mice the latter phase is abrogated leading to frequent long-term cures. While this result does not reflect common clinical experiences with GBM it reveals the potential of NK cells of cooperating with TMZ-mediated tumor depopulation (unlike their NSG counterparts SCID mice retain intact or elevated NK cell activity (Okada et al., 2019)).

Platelets are increasingly considered to act as innate immunomodulators with the ability to release multiple effectors that can blunt activity of cytotoxic effectors, including NK cells (Eriksson et al., 2019). If so, cancer cells that could recruit, retain and activate platelets, for example through expression of PDPN, may be immunoprotected. We reasoned that if this was the case, the ability of GSCs to repopulate the tumor following TMZ therapy could be enhanced in the presence of PDPN, including the resulting effect of activated platelets on NK cell-mediated cytotoxicity.

To examine this prediction, we constructed an experimental model based on GSC1123 cells, which were previously characterized for TMZ responses (Garnier *et al.*, 2018). SCID mice injected

with GSC1123 are completely cured following 1 injection of TMZ administered 2 weeks post subcutaneous implantation and the emergence of palpable tumors of ~0.52cm^3 in volume. Tumors shrink over the course of 3 to 4 weeks until they completely disappear to leave only impalpable lesions that could only be detected using bioluminescence when using a LucBFP transduced GSC1123 system.

A completely different scenario unfolds when tumors are implanted in NSG mice devoid of NK cells, although tumors initially start to shrink dramatically, they never shrink beyond the palpable volume of ~0.02-0.05 cm^3 and shortly resume their growth to reach the end point of ~2-2.5 cm^3 within 3 weeks, at which point mice have to be euthanized. Again, these results point at a crucial role for NK cells in contributing to tumor response to TMZ and/or the subsequent control of the tumor mass following successful response to TMZ induction. In this setting the presence of NK cells facilitates maintaining GSC1123 xenografts in a dormant-like phase where viable tumor cells clearly persist as indicated by bioluminescence imaging, but the lesions never give rise to full blown tumors.

As mentioned earlier, the underlying rationale behind our interest in a potential impact of PDPN expression on tumor progression post TMZ therapy is centered around the capacity of PDPN to activate platelets. Platelets are the richest source of transforming growth factor beta (TGFβ) , they possess the highest cellular concentrations of TGFβ and approximately 40% of all TGFβ found in peripheral blood plasma is released by platelets (Karolczak and Watala, 2021). Platelets contain 40 to 100 times more TGFβ , stored in their alpha-granules, than any other non-neoplastic cell (Meyer et al., 2012), which they readily release following their activation (Blobe et

al., 2000; Meyer *et al.*, 2012). PDPN-mediated release of TGFβ by platelets has been implicated by a number of studies in various aspects of tumorigenesis including tumor cell extravasation and pulmonary metastasis (Takemoto et al., 2017b), as well as EMT and migration of urinary bladder tumor and lung SCC cells *in vitro* (Hu et al., 2017; Suzuki-Inoue, 2018). More importantly, platelet derived TGFβ released following contact with tumor cells has been shown to inhibit NK antitumor activity (Cluxton et al., 2019; Knudson et al., 2017; Kopp *et al.*, 2009; Otegbeye et al., 2018).

In view of these observations, we surmised that if NK cell-mediated antitumor activity plays a central role in response of GSC xenografts to TMZ and if this response can be blunted by platelets following ligation of their CLEC2 receptor, tumor cells expressing PDPN may be prone to evade the combined NK/TMZ cytotoxicity, grow more aggressively and relapse in NK-cell competent hosts. Therefore, the cures we earlier observed in the GSC1123 model can simply be related to the lack of PDPN expression by these cells.

In order to explore this possibility, we generated stable PDPN-expressing variants of GSC1123LucBFP cells (Fig 7.5) and proceeded to inject them into NSG or SCID mice subcutaneously. Our experimental schema included: a total of 40 mice, 20 SCID and 20 NSG injected with $1x10^6$ cells / mouse. Within each group of 20 mice, animals were further divided into 2 groups of 10, one group was injected with GSC1123LucBFP WT (the original cell line, only transduced with luciferase and BFP), and the other received the GSC1123LucBFP PDPN cell line (stably PDPN-expressing GSC1123LucBFP). Furthermore, each group of 10 mice was divided into two groups of 5, where one group received a TMZ injection (120mg/kg) two weeks post tumor inoculation, and the other was left untreated (Fig 7.6).

At the time of this writing this is still an ongoing experiment, and to date, we have made several preliminary observations. First, at variance from our observations with GSC528, enforced PDPN expression in GSC1123 cells did not result in an enhancement of tumorigenic potential (already relatively high). This is illustrated by statistically insignificant fluctuations in tumor growth rates within the first two weeks post inoculation. Second, contrary to our predictions, with the passage of time, we observed a lower (rather than higher) tumor growth rate in mice injected with GSC1123LucBFP PDPN cells (relative to control), in both NSG and SCID mice. In particular, mice of both strains injected with GSC1123LucBFP PDPN cells exhibited significantly lower tumor volume at day 18 post injection compared to GSC1123LucBFP WT controls (Fig 7.7 and 7.8). Third, no obvious impact of PDPN expression was observed on the initial response of tumors to TMZ. Thus, all GSC1123LucBFP tumors responded similarly to the treatment, regardless of their PDPN status of the injected cells and the strain of the mice (Fig 7.7 and 7.8). As expected, in the TMZ-treated NSG group all tumors shrunk to a similar size of ~0.03-0.04 cm^3, only to stop responding and then growing back, to reach the volume of ~0.4-0.5cm^3 within a period of 25 days post TMZ administration (Fig 7.9).

Mice were euthanized shortly after reaching a volume of 1-1.3 cm^3 (day 33 post TMZ administration; data not shown), and tumors were collected, and formalin fixed. We verified PDPN expression pattern and by performing the respective IHC staining. Indeed, tumors originating from GSC1123LucBFP cells remained devoid of PPDN expression, while tumors arising from GSC1123LucBFP PDPN cells exhibited ample PDPN expression. The results of PDPN expression

assessment remained consistent irrespective of the strain of mice or whether TMZ treatment was administered or not (Fig 7.10).

Within the SCID group, tumors initiated by either PDPN-expressing or PDPN-deficient GSC1123LucBFP cells shrunk completely to the level of impalpable lesions by day 14 post TMZ injection (Fig 7.9) and were only detectable by bioluminescence imaging. Follow up assessment of these lesions 2 months post injection (45 days post TMZ administration) in SCID mice revealed that 3 mice within the GSC1123LucBFP PDPN had detectable small lesions in their flanks, compared to 2 mice with such lesions within the GSC1123LucBFP group (Fig 7.11). These mice are currently under observation and the status of their lesions will be periodically surveyed by bioluminescence imaging in order to investigate whether PDPN expression has any long-term impact on the dormant-like phenotype induced by TMZ in SCID mice. It should be mentioned that, as described in previous chapters SCID mice have normal number and function of platelets which can be activated by PDPN expressing cancer cells and their derived EVs (Tawil *et al.*, 2021). Therefore, our data obtained thus far do not support a major role of PDPN and platelet activation in regulating post TMZ responses of GS1123 xenografts in the presence or absence of NK cell-mediated cytotoxicity.

Taken together, these results are consistent with those of others (Eisemann *et al.*, 2019) and suggest that PDPN acts mainly as pro-thrombotic effector in subsets of GBM cells and its enrichment in tumors may be achieved through association with other genes defining the phenotype of PDPN-positive GBM subpopulations we described. We cannot, however, exclude the possibility that PDPN and platelets may have other roles in GBM, and those may not be

accessible to our analysis due to limitations of our present assays, and models. One such possibility is the role of the PDPN/CLEC2 system in shaping vascular morphogenesis and integrity in the brain (Lowe *et al.*, 2015). Finally, the reduced growth rate of GSC1123 tumors overexpressing PDPN is intriguing, as it may suggest unappreciated roles of PDPN with the tumor microenvironment.

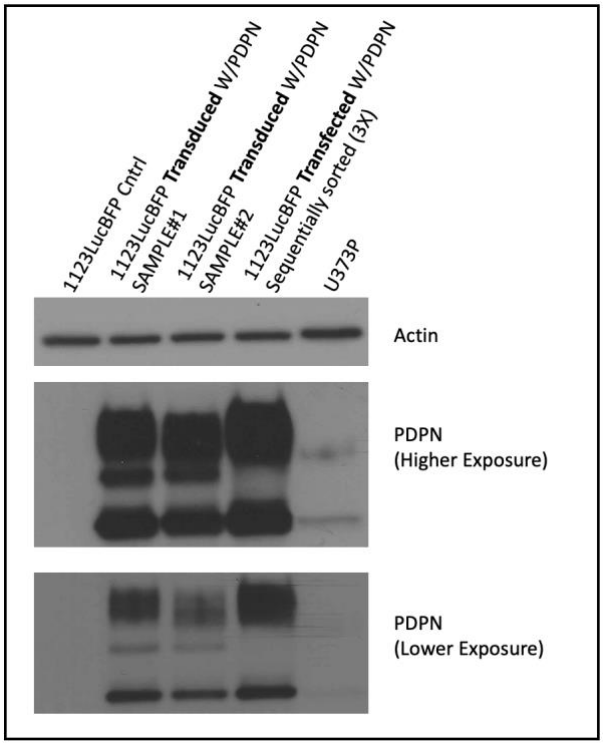


Figure 7.5 ***Generation of PDPN expressing GSC1123LucBFP system.***

Two approaches were utilized: generation of a lentiviral construct followed by the transduction of GSC113LucBFP and direct transfection using a non-lentiviral vector followed by three rounds of sequential sorting of PDPN-expressing cells over a period of ~1month. Transfected system was eventually used due to the presence of an additional unrecognized band in the transduced cells.

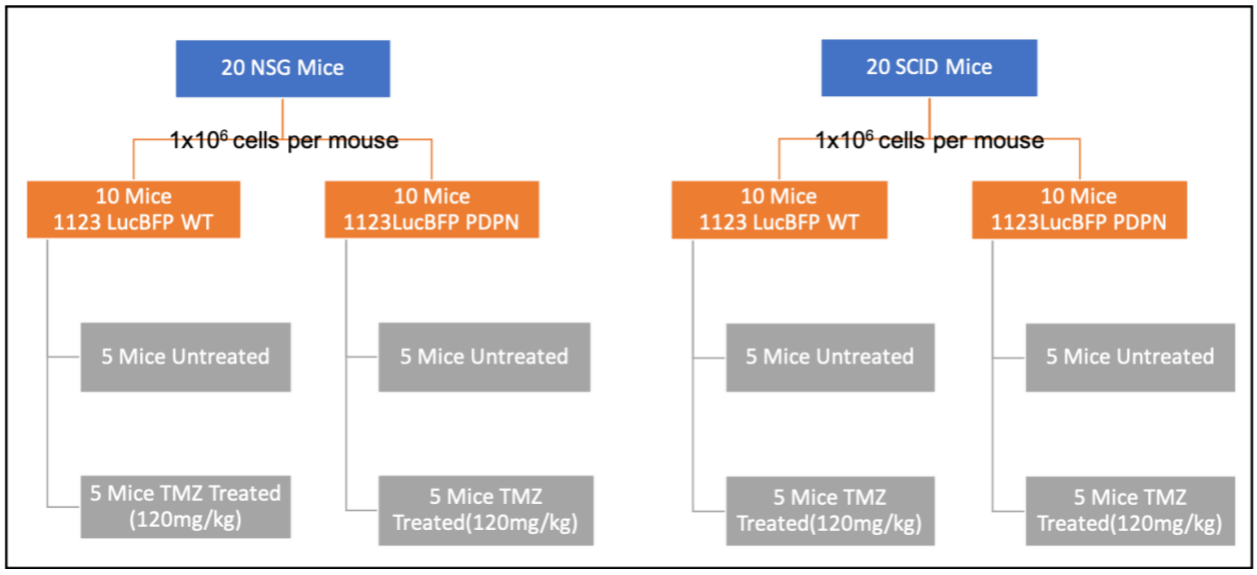


Figure 7.6 ***GSC1123 subcutaneous injection schema.***
Guiding schema describing the layout and the distribution of experimental groups within the *in vivo* experiment aiming to assess the impact of PDPN expression on the response to TMZ as well as recurrence post TMZ therapy

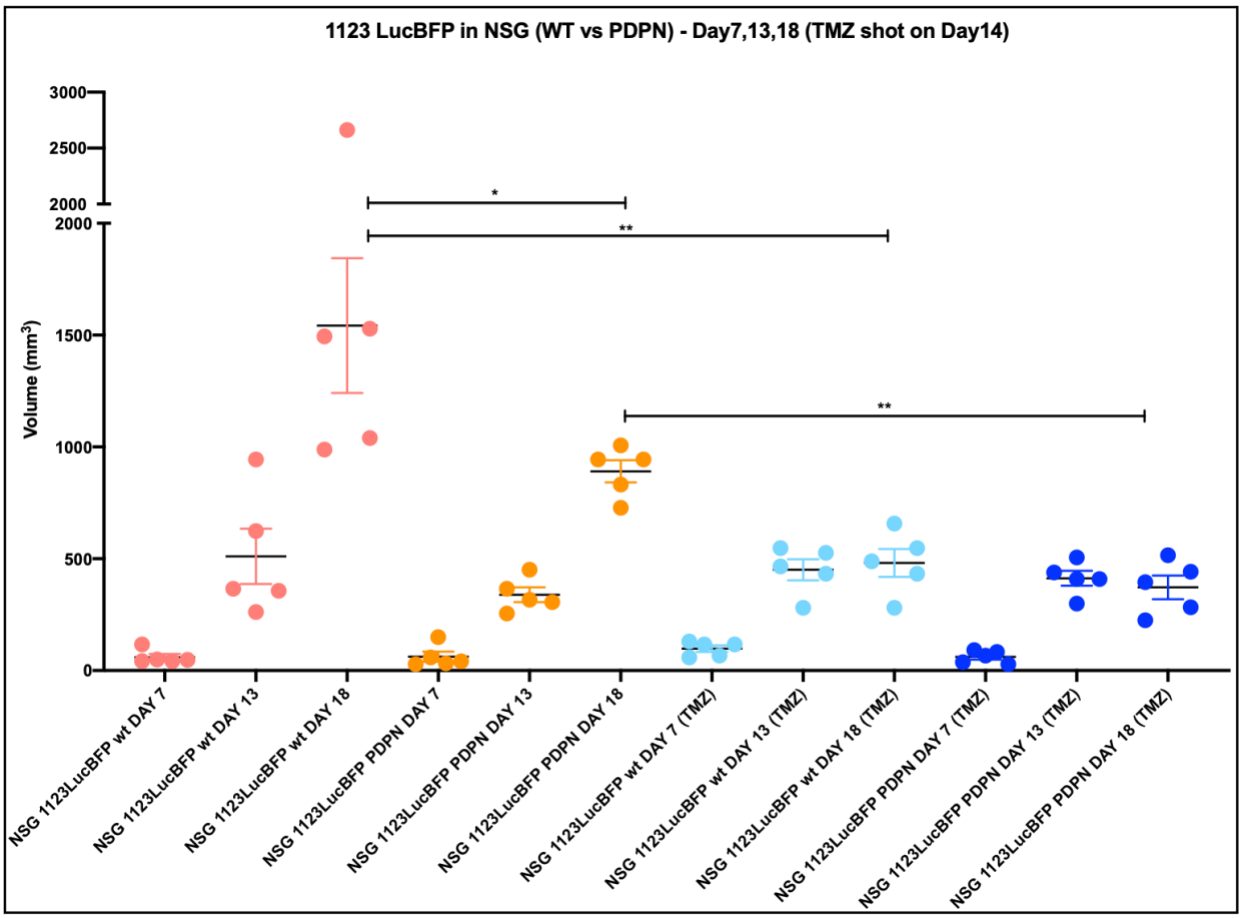


Figure 7.7 ***GSC1123 tumor growth and initial response to TMZ within NSG group.***

Tumor volume was periodically assessed following the subcutaneous injection of GSC1123LucBFP and GSC1123LucBFP PDPN cells. TMZ was administered on day 14 post injection and initial response to TMZ is represented by tumor volumes at day 18 post injection. It is worth noting that tumour formation patterns related to PDPN status and depicted by these results are inconsistent with those pertaining to PDPN-expressing and non-expressing clones of GSC528 cell line described earlier. This may reflect subtype- or GSC-specific differences and in a broader sense reflects the unexpected weakness of GSC-based models of CAT. Indeed, most of the available patient derived GSC lines do not recapitulate the patterns of PDPN, or TF expression found in GBM (Chapter 4) in vitro and in vivo. However, targeted modifications of these models may enable their application in experimental analysis of CAT in mice.

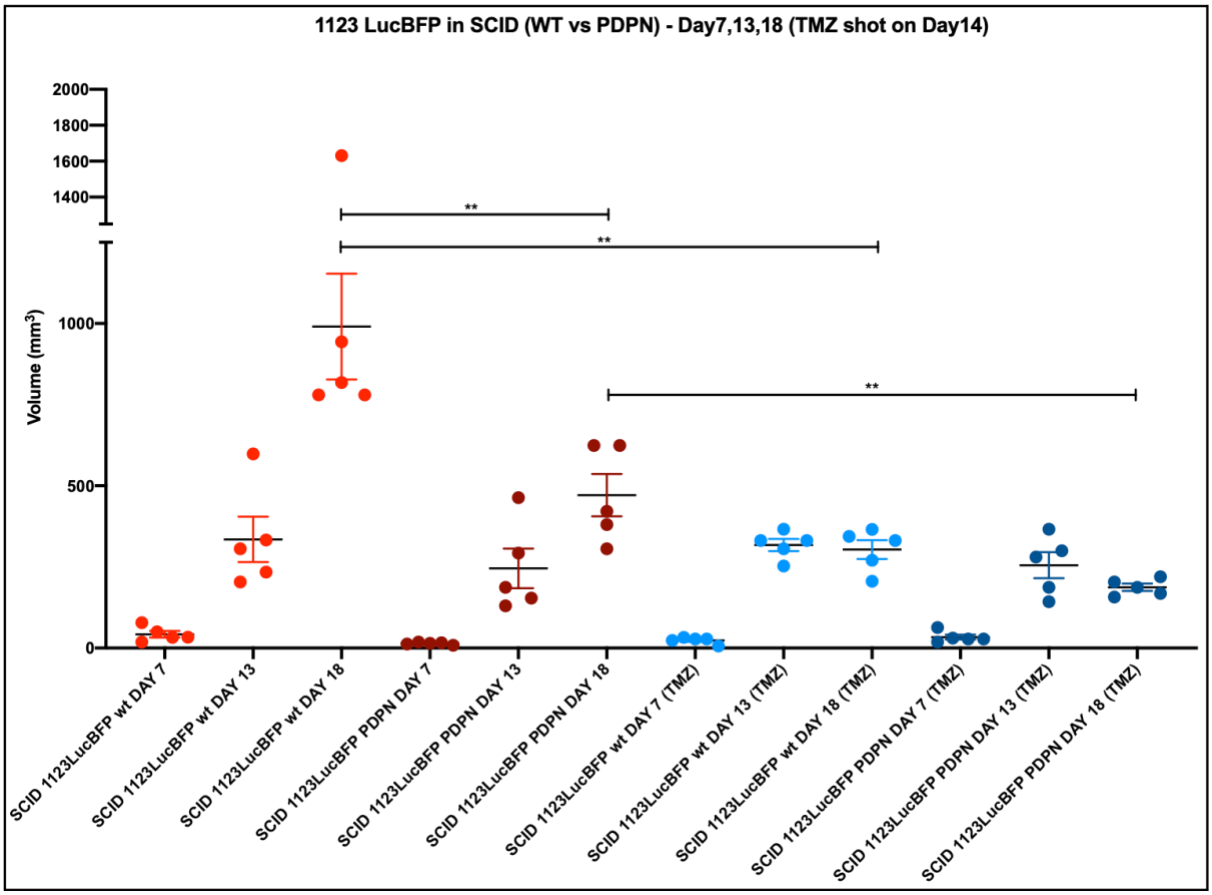


Figure 7.8 ***GSC1123 tumor growth and initial response to TMZ within SCID group.***

Tumor volume was periodically assessed following the subcutaneous injection of GSC1123LucBFP and GSC1123LucBFP PDPN cells. TMZ was administered on day 14 post injection and initial response to TMZ is represented by tumor volumes at day 18 post injection.

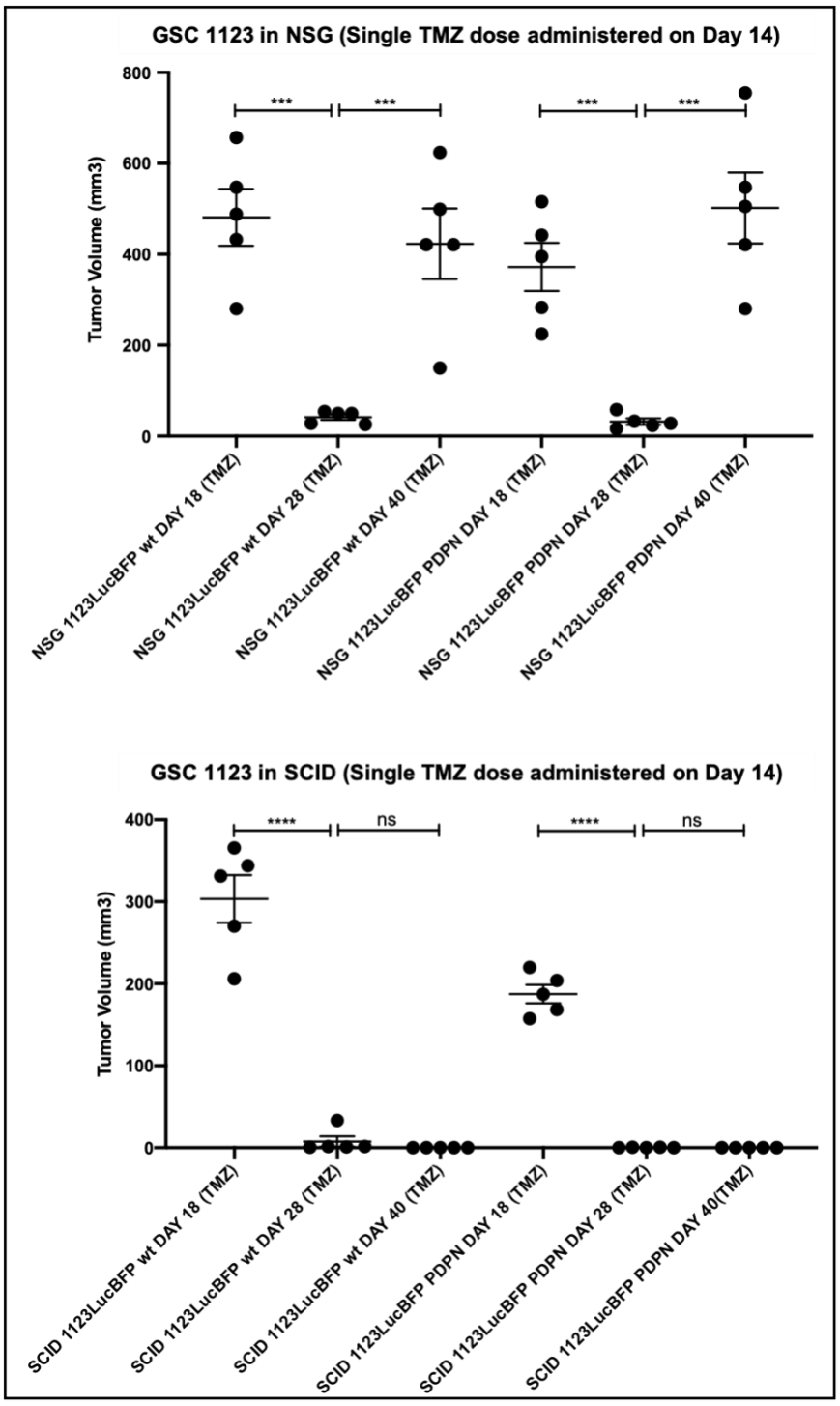


Figure 7.9 ***GSC1123 tumor growth and long-term response to TMZ in NSG and SCID groups.***

Tumor volumes were continuously monitored, and the graphs show the long(er) term response in both NSG and SCID groups injected with GSC1123LucBFP and GSC1123LucBFP PDPN with tumor volumes reported for day18, 28, and 40 post injection (4, 14, and 26 days post TMZ administration respectively).

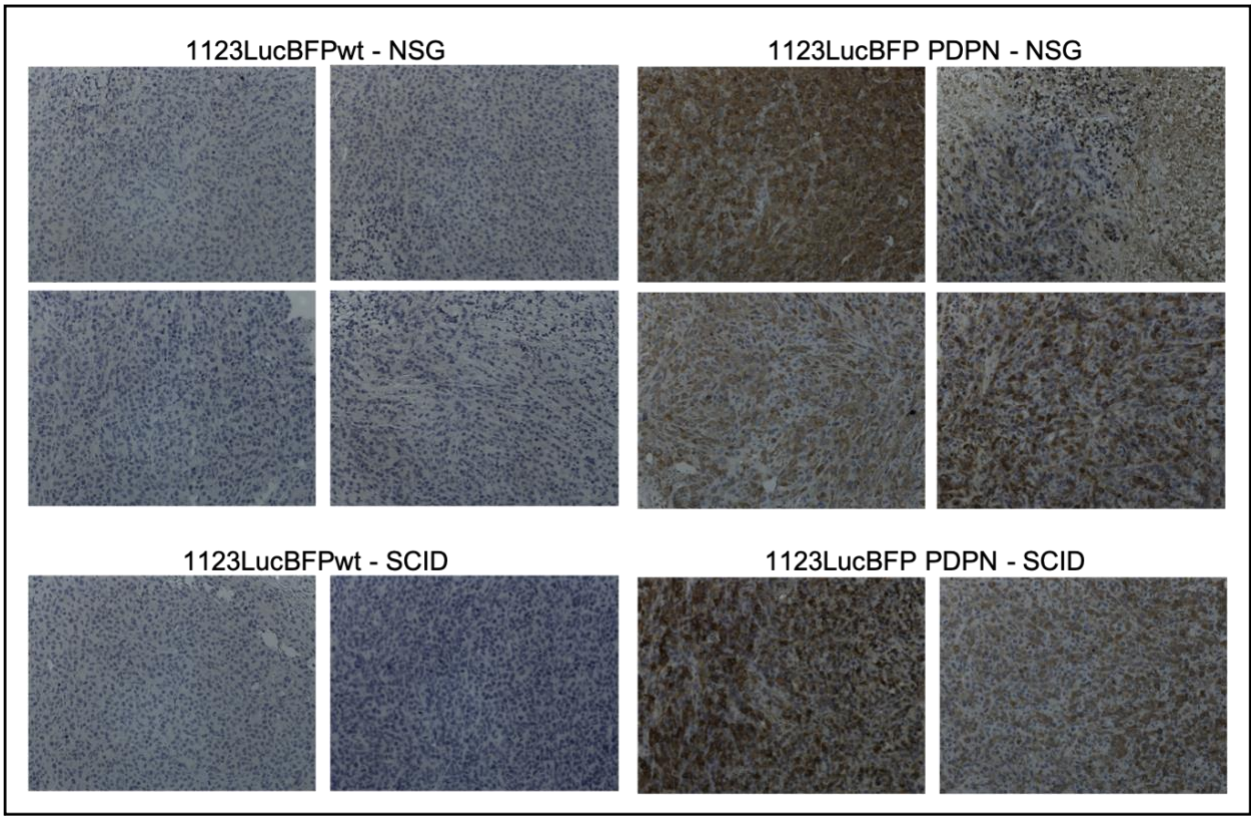


Figure 7.10 ***PDPN expression in GSC1123 subcutaneous tumors.***

Representative IHC staining for PDPN photographs of sections of subcutaneous tumors from all the experimental groups.

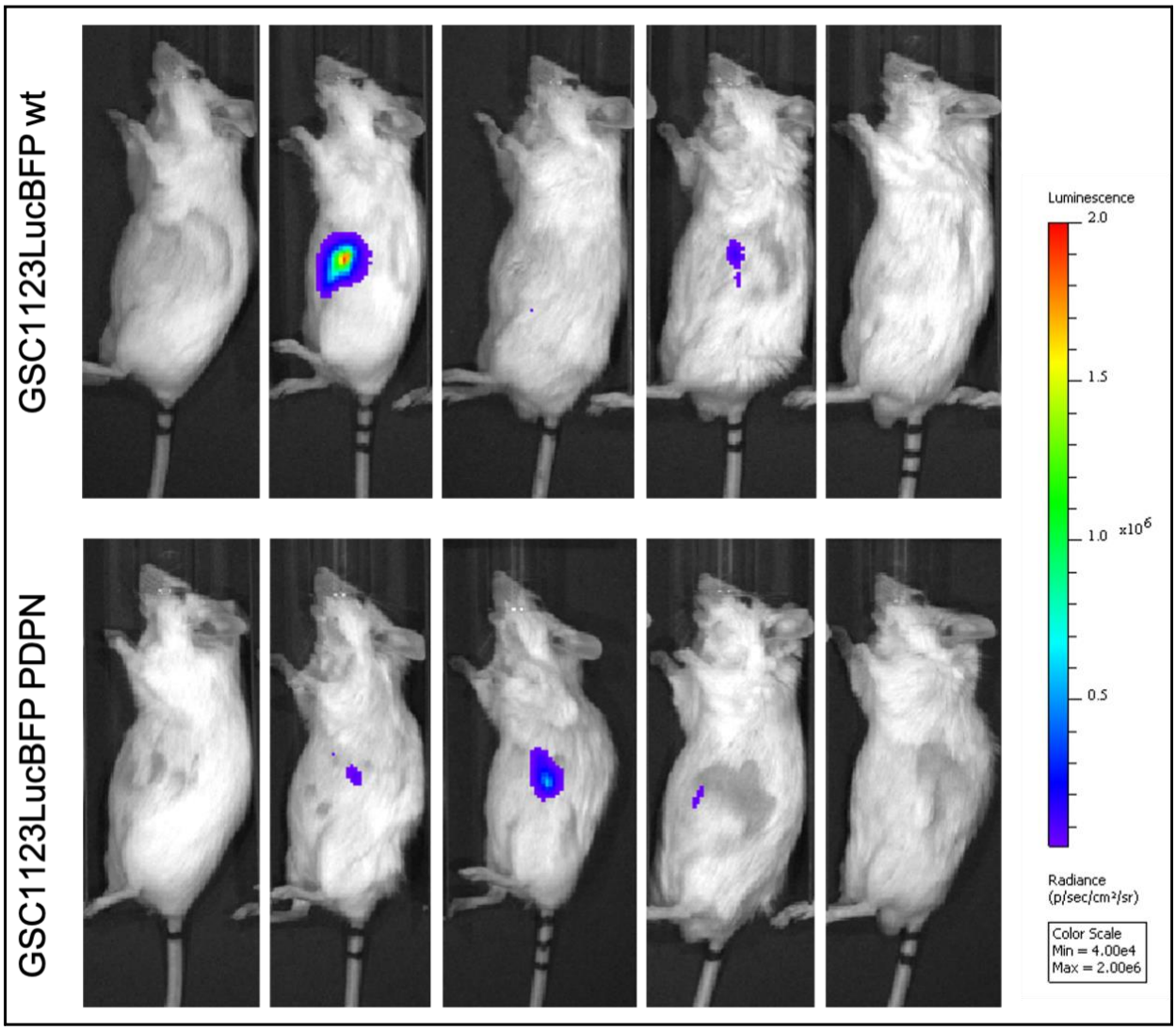


Figure 7.11 ***Bioluminescence imaging of persistent post TMZ lesions.***

Luciferase bioluminescent imaging (performed 45 days post TMZ administration) on surviving GSC1123LucBFP and GSC1123LucBFP PDPN injected SCID mice 32 days after the complete tumor regression in response to TMZ treatment administered 14 days post injection of corresponding GSC lines.

8 General Discussion and Future Directions

8.1 Thesis Summary and general discussion

The current PhD thesis unveils a number of novel findings that transpired throughout my efforts to understand the place of PDPN in the emerging complexity of GBM and related vascular pathologies (Spinelli *et al.*, 2021). This work focused on two main questions: First, we explored the cellular population context in which PDPN is expressed in GBM. This led us to investigate the possible mechanisms whereby PDPN could participate in promoting CAT in GBM, in both local and systemic realm. In this regard the predominant aim here was to uncover the route through which tumor associated PDPN could contribute to systemic hypercoagulability and ultimately to GBM-associated peripheral VTE. Second, we interrogated the possible non-canonical effects of PDPN through which this platelet activating protein could impact the biology of GBM irrespectively of, or through, hemostatic mechanisms.

This effort was driven by the lack of clarity around putative effectors of VTE in one of the most procoagulant cancers, such as GBM (BLOM et al., 2006; Diaz et al., 2021; Saidak et al., 2021; Yust-Katz et al., 2015). In this regard, studies usually remain agnostic as to specific molecular mediators, or presume those vascular aberrations in the tumor mass, brain microenvironment as such, or therapeutic interventions are sufficient to explain the high incidence of thrombosis in GBM patients. On the other hand, studies have been underway to explain the uniquely high VTE risk in GBM by unique coagulant aspects of GBM cells.

In this regard our initial interests were directed by the notion that, in GBM the occurrence of VTE may be a function of the aberrant expression of TF, which is often overexpressed in GBM (Sartori *et al.*, 2013), and has a unique and potent role in initiating the coagulation cascade (Adams and Bird, 2009). In spite of this compelling logic recent studies failed to establish an epidemiological association between TF expression and VTE incidence in this setting (Thaler *et al.*, 2012). Indeed, this is a puzzling observation as brain tissue is used as a standard for TF activity assays and TF is upregulated in GBM cells on which it confers a procoagulant activity (Magnus *et al.*, 2010; Rong *et al.*, 2005). Yet this did not seem to translate into a predictor of VTE. In this regard our study, places GBM-associated TF in a new light (Chapter 6 and below).

On the other hand, a strong association between PDPN expression within GBM tissue and the VTE risk in patients was documented by Riedl et al. albeit without mechanistic explanation (Mir Seyed Nazari *et al.*, 2018; Riedl *et al.*, 2017). As such, we expanded on the work initiated by this group by taking it further and exploring GBM cell subpopulations that express this mediator and by examining the possibility of PDPN being released from GBM cells into the systemic circulation as cargo of GBM-derived EVs. Thus, EVs from specific cancer cells may play a key role in promoting systemic coagulopathy via a previously unaccounted for process: direct platelet activation by PDPN carrying, circulating tumor-EVs (Tawil *et al.*, 2021).

This thesis also critically examines, in ways that, to our knowledge, has never been done before, the cellular sources and regulators of coagulant phenotypes of GBM cells. Our laboratory, and others, had previously documented that oncogenic driver mutations in GBM influence the profile of coagulation related molecules expressed by cancer cell (coagulome) and implicated TF

as one of the key targets of such regulation. For example, TF expression is demonstrably influenced by such oncogenic mutations as EGFRvIII and IDH1 (D'Asti et al., 2014b; Magnus *et al.*, 2010; Tawil *et al.*, 2019; Tawil *et al.*, 2020; Unruh *et al.*, 2019; Unruh *et al.*, 2016). While this holds true for isolated cancer cell lines, we reasoned that additional factors ought to be considered in complex cell populations *in vivo* where the expression of common oncogenes may be superimposed with different cellular positions in the stem cell hierarchy, multicellular equilibria or differentiation roadmaps, all shaped by the cellular epigenome (Couturier *et al.*, 2020; Neftel *et al.*, 2019). Remarkably, we found that TF and PDPN are expressed by distinct subsets of GBM cells with their enrichment in astrocytic and mesenchymal cell subsets, respectively (Chapter 3).

These observations suggested to us that the epigenome may override the impact of mutant driver genes on the GBM coagulome, but they provided little insights as to the underlying mechanisms. To glean more understanding of this regulation we first focused on the relationship between PDPN and EGFR expression in GBM datasets. It caught our attention that, while one would expect EGFR/EGFRvIII expressing tumor cells to be more aggressive and possibly more procoagulant, marked with higher levels of PDPN, this profile was more complex. Notably, a prominent population of GBM cells in single cell RNAseq datasets exhibited antithetical expression profile of EGFR and PDPN, as if the former suppressed rather than upregulated the latter. This is counterintuitive as EGFR pathway activates AKT, which was implicated in PDPN upregulation (Peterziel *et al.*, 2012). As described in Chapters 3 and 4 our analysis led to the conclusion that it is an impact of EGFR on chromatin architecture (through EZH2) that suppresses PDPN in GBM cells. We speculate that on this background, pathways of mesenchymal differentiation are permissive for PDPN expression through mechanisms that still need to be

elucidated while other cellular states (including progenitors) are not. The latter is borne out in our analysis of at least 6 different glioma stem cell lines the majority of which did not express PDPN and in some cases such expression could only be induced by serum-induced differentiation (Tawil *et al.*, 2021).

To recapitulate, our work brings forward several new elements. In brief, in chapter 4 we documented a non-random expression of PDPN among GBM cells and the association of this prothrombotic marker with cell subpopulations enriched for inflammation- and coagulation-related genes, as well as mesenchymal differentiation signature. We also delineated the counterintuitive influence of two oncogenic drivers: EGFR and IDH1 R132H on the expression of PDPN in two distinct GBM subgroups and through two different epigenetic mechanisms (chromatin modification and DNA-methylation, respectively). We posit that the related epigenetic mechanisms operating during the process of glioma stem cell differentiation may override the signalling cues regulating PDPN and other coagulation-related genes. In chapter 5 and 6 we demonstrated that GBM cells expressing PDPN produce a distinct form of systemic pro-thrombotic perturbation *in vivo,* which is, at least in part, attributable to EVs carrying PDPN (relative to coagulation driven by TF). Additionally, we suggested that microthrombosis within the tumor mass may be a function of cooperation between two major cancer coagulants (PDPN and TF) rather than PDPN alone. Finally, in chapter 7 we lay the foundation for an investigation of the impact of PDPN expression on GBM progression and response to TMZ therapy.

The relationship between genetic and epigenetic cancer progression on the one hand and thrombosis on the other is fascinating, but also perplexing. Genetically defined brain tumor

subtypes exhibit distinctive coagulomes (D'Asti *et al.*, 2014b; Magnus *et al.*, 2010), and similar linkages are also found in other cancers. For example, our group discovered that in a model of colorectal cancer (CRC), mutant KRAS was found to lead to upregulation of TF (Yu *et al.*, 2005) and in CRC patients with mutant KRAS the VTE risk is known to be 2-3 fold higher than in patients with wild type KRAS tumors (Ades *et al.*, 2015). While full consensus have not been reached yet in regard to the connection between tumor TF expression and VTE in CRC, and despite the debate in regard to their origin, higher levels of TF MPs in plasma of CRC patients were found to correlate with higher incidence of VTE (Geddings and Mackman, 2013; Hron et al., 2007).

Notably, in a subset of high-grade glioma with oncogenic mutations in the *IDH1* gene VTE is extremely rare, the extent of microvascular thrombosis very limited and the levels of TF-carrying EVs in blood tend to be low suggesting a link between this genetic event and thrombosis (Unruh *et al.*, 2016). A recent retrospective study by Dunbar et al. involving the genomic profiling of over 14,000 solid tumors identified somatic mutations of KRAS, particularly prevalent in colorectal and pancreatic cancers, to be associated with an increased risk for VTE confirmed by the link between RAS and VTE (hazard ratio [HR], 1.34; 95% confidence interval (CI), 1.09-1.64; adjusted P = .08) (Dunbar *et al.*, 2020). Indeed, this study firmly established a link between several mutant genes (STK11, KEAP1, MET, CTNNB1, CDKN2B) and the VTE risk across cancer spectrum (Dunbar *et al.*, 2020; Rak, 2021). Interestingly, IDH1 mutation does not stand out in this analysis largely because its predictive role for low VTE risk in high grade brain tumors is overshadowed by hepatobiliary cancer in which this mutation had no effect (Dunbar *et al.*, 2020). This is a startling example of the interplay between molecular context and transforming genes. Still, taken

together, these studies support the notion that the cancer cell genotype is an important factor for the VTE risk.

However, in GBM the clarity is still lacking, as TF involvement at a systemic level remains questionable and the same work by Dunbar and colleagues documented an absence of a correlation between oncogenic EGFR and the VTE risk (Dunbar *et al.*, 2020). Moreover, oncogenic EGFR in GBM is documented as an often occurring genetic aberration within the classical subtype, (Zhang et al., 2020), which was found to have no predictive value for the VTE risk (Diaz *et al.*, 2021). These observations, in combination with our data outlined in chapter 4 of this thesis suggest that in GBM other forces could be at play when it comes to modulating effectors of VTE, especially in IDH1 wild type tumors, and point largely to a potential involvement of the epigenome (Tawil *et al.*, 2021).

Our ongoing efforts are directed at further delineating and characterizing $PDPN^{+}$, TF^{+} and double-positive GBM cells using single cell RNAseq data and developmental roadmap analysis initiated by Couturier et al. (Couturier *et al.*, 2020). We have also employed other transcriptomic deconvolution approaches applied to TCGA data to generate further confirmation of cellular programs associated with PDPN expression and mesenchymal gene expression signature. Similarly of interest are programs that couple TF expression with the astrocytic signature with surprisingly limited overlap between the two. Thus, a single tumor may contain distinct cell subsets expressing different potent coagulants the effects of which may vary and occasionally converge (e.g. involving microthrombosis).

Why and how oncogenic EGFR turns down PDPN expression remains intriguing and incompletely resolved. We observed that while in GBM cell lines there is an unambiguous antithetical relationship between EGFRvIII status and PDPN expression, this is applicable to some, but not all subsets of GBM cells in situ. Clearly the regulatory wiring involved in PDPN expression is highly heterogeneous and the selective pressures that result in formation of these different cellular subsets is far from understood, especially in light of consistent preponderance of both EGFR and PDPN in a substantial subset of GBMs. It is possible that PDPN and related coagulopathy represents another case of cell-cell cooperation described by Furnari group where EGFRvIII positive and negative cells coexist in the tumor in a quasi-symbiotic manner (Inda *et al.*, 2010). The presence of PDPN-expressing cellular subpopulation would add prothrombotic dimension to this multicellular ecosystem. Interestingly, in the roadmap analysis EGFR is somewhat more correlated with the astrocytic signature and its expression declines as the analysis moves further towards GBM cells of the mesenchymal signature (data in preparation). Going forward, this analysis is poised to provide insight into the expression spectrum of prothrombotic effectors as a function of the developmental progression within GBM cellular ecosystem and, if successful, could be applied to a numerically adequate patient cohort. Such extension may allow us to shed more light on a potential correlation between the preponderance of intratumoral PDPN, and possibly TF, VTE risk and local microthrombosis.

In the context of GBM the occurrence of CAT represents a spectacular example of systemic vascular pathology associated with a localized cancer (Perry, 2012). Indeed, while mostly intracranial, GBM lesions trigger clotting in the peripheral vasculature, including life threatening pulmonary embolism (Cavaliere and Schiff, 2005; Jenkins *et al.*, 2010; Perry, 2012), in conjunction with activation of platelet-dependent mechanisms previously correlated with PDPN

expression (Riedl *et al.*, 2017). While, as mentioned earlier, TF is often expressed in GBM cells (Sartori *et al.*, 2013) its role in systemic aspects of CAT remains debated (Thaler *et al.*, 2012). Our data, presented in chapters 5 and 6 of this thesis, suggest that in GBM the effects of PDPN and TF, and those of their carrying EVs, may differ between settings of systemic and local thrombosis. There is certainly the possibility that in our system the amount of TF is not high enough to contribute to the systemic prothrombotic state and in sufficient amounts could similarly cooperate with PDPN at a systemic level, but this is an avenue that requires a separate line of investigation.

We also postulate that while tumor-derived PDPN associated with circulating EVs may directly interact with platelets in the peripheral blood, as indicated by the rise in PF4 upon direct EV injection, the effects of TF-carrying EVs are more complex. We observed only a mild activation of peripheral markers of coagulation, especially D-dimer, upon intravenous injection of EVs carrying active TF. This is in contrast with the potent ability of TF-expressing tumors to generate D-dimer in the tumor microenvironment. While this aspect requires further study the ability of EV-associated TF to generate thrombin to be able to activate platelets may be limited by spatial considerations and EV half-life in the circulation (Hyenne et al., 2019).

Our ongoing efforts, with preliminary results presented in chapter 7, pertaining to the investigation of a potential role of PDPN expression and GBM progression, including response to TMZ and post-therapy recurrence. While the rationale for this study is rather compelling given the mounting literature on the role of platelets in cancer (Haemmerle *et al.*, 2018), our experiments have so far generated conflicting results. PDPN seemingly contributes to enhanced tumorigenesis and shorter survival in the GSC528 xenograft model (proneural-like) while having no apparent

impact in the GSC1123 model (mesenchymal-like). While further studies will be certainly required to validate these observations, a potential for context specific impact of PDPN seems possible, whereby the co-expression of PDPN with other markers could be the underlying culprit behind the promotion of an enhanced tumorigenic phenotype in GBM.

One possible scenario involving PDPN effects independent of platelets could be the involvement of Wnt signaling potentiation through the co-expression and/or interaction of PDPN with CD44. There are several lines of evidence, including our own data, that the two proteins are often co-expressed with CD44 being a marker of the mesenchymal subtype of GBM (Ranjit et al., 2015; Tawil *et al.*, 2021). PDPN interaction with CD44 has been documented and was reported to promote directional cell migration (Martín-Villar *et al.*, 2010). Additionally, PDPN has been implicated in mammary stem cell function and tumorigenesis via Wnt/β-catenin signaling (Bresson *et al.*, 2018). Schmitt et al. have previously shown that CD44 is capable of positively regulating Wnt signalling in the developing brain of Xenopus laevis embryos (Schmitt et al., 2015). More recently and in corroboration of PDPN/CD44 interaction documented by Martin-Villar et al., in a model of chemically induced squamous cell carcinoma model, PDPN and CD44 were found to be co-expressed, colocalized at the plasma membrane, and interaction was documented by co-immunoprecipitation analysis (Montero-Montero et al., 2020). As such, in combination with findings by Bresson et al., it could be postulated that in GBM enhanced Wnt activation could be similarly modulated through a co-expression and/or interaction between PDPN and CD44. This possibility, however, remains to be explored.

In regard to our efforts to elucidate the potential role of PDPN in response to TMZ therapy, as mentioned earlier, our work is in progress, but it sets the ground for a potential contribution towards the understanding of the lack of curative effect of TMZ and the eventual relapse and disease recurrence frequently observed in GBM. One aspect considered in our study was the potential role of PDPN in activating immunosuppressive activity of platelets. While our data, so far, did not provide conclusive answers, this direction is of long-term interest.

A paradigm for the interplay between systemic immunosuppression and poor outcome in GBM is the wide-spread use of Dexamethasone (DEX). While it could be argued that DEX is used in response to, and to alleviate, the intracranial pressure and symptoms, it may also have causative role in the biology of GBM. Indeed, DEX still represents the drug of choice among synthetic glucocorticoids (GCs) and is the one most frequently used throughout the course of therapy with the main goal to reduce vasogenic cerebral edema associated with GBM and alleviate resulting headaches and neurological deficits (Cenciarini et al., 2019). While there is ample evidence for the effectiveness of GCs in reducing cerebral edema through multiple mechanisms including modulation of gene expression and function of occludins, claudins and vascular endothelial (VE)-cadherin that regulates endothelial permeability as well as modification of capillary bed permeability within the tumor mass (Gu et al., 2009; Hughes et al., 2005; Sinha et al., 2004; Swaroop et al., 2001), controversies also exist (Cenciarini *et al.*, 2019). Recent work by Shields et al. documented that DEX administration in patients treated with TMZ was a poor prognostic indicator of both overall survival (OS) and progression-free survival (PFS) (Shields et al., 2015). A similar observation was made by Wong and colleagues who noticed a significantly shortened

OS in patients treated with higher DEX doses (> 4.1mg/day) when compared to those receiving lower doses (Wong et al., 2015).

Interestingly, in two independent GBM cohorts, DEX-responsive gene expression alterations were found to be prognostic of poor outcome and comparison between patients with mesenchymal and proneural GBM showed that DEX administration within the former subgroup resulted in a significant upregulation of DEX-responsive genes amounting to significant triggering of proliferation, invasion and angiogenesis pathways (Luedi et al., 2017). Dubinski et al. also reported that reduced OS with DEX administration was observed in a particular group of patients who showed DEX-induced leukocytosis (DIL) (Dubinski et al., 2018).

For over 40 years, GCs generally and DEX in particular, have been known to reduce and blunt the activity of NK cells (Capellino et al., 2020). Despite being documented as the least abundant immune cell population to infiltrate glioma (2.11%), the most abundant phenotype is the $CD56^{dim}CD16^{-}$ previously reported to show significant activation and cytotoxicity in other tumors (Kmiecik et al., 2013; Levy et al., 2011). The NKG2D receptor of NK cells is a potent activating receptor and in GBM, TMZ therapy was found to induce the expression of its cognate ligand (NKG2DL) by tumor cells (Weiss et al., 2018). This process was highlighted to be crucial for the survival benefit conferred by TMZ whereby the inhibition of NKG2D system resulted in a significant decrease in survival (Weiss *et al.*, 2018). Inflammatory cells are particularly enriched in mesenchymal GBM and interestingly, a study looking at long term survivors in GBM (over 30 months) found that the corresponding tumors are very rarely classified as mesenchymal (Pineda et al., 2019). In fact, patients with predominantly mesenchymal tumors showed generally increased

treatment resistance (Vaillant et al., 2012) and a trend for worse survival (9.6 months vs 15 months) (Pineda *et al.*, 2019).

Our unpublished data provide direct evidence that NK cells are crucial for responsiveness of mesenchymal GSC xenografts to TMZ (Meehan et al. – in preparation). As a part of a different project in the laboratory we observed that mesenchymal GSC tumors regress completely upon a single dose of TMZ, but subsequently relapse as drug resistant and incurable lesions (Garnier *et al.*, 2018). We observed that this outcome can be mitigated by NK cells. Implicating NK cells in successful response to TMZ *in vivo* outlined in chapter 7 of the current thesis (Meehan et al. - in preparation), rekindles the previously mentioned considerations of how platelet releasate can alter NK cell activity. One mechanism of this inhibition is mediated via the NKG2D activation system (Kopp *et al.*, 2009), and the strong association of PDPN expression with the mesenchymal signature in GBM (Tawil *et al.*, 2021) formed the basis behind our hypothesis that a 'PDPN-platelets-NK' axis could play a role in impeding the efficacy of TMZ therapy. This remains an active experiment, but initial data with PDPN-overexpressing mesenchymal GSC xenografts does not strongly support a major role of this pathway in TMZ response. While early data from our ongoing experiment is contrary to our predictions, it adds to the discussion of potential roles platelets may play in the context of GBM.

One of the intended major objectives of our study was to expand on the emerging link between oncogenic transformation (genetic or epigenetic) and thrombosis in GBM (Tawil *et al.*, 2019; Yu *et al.*, 2005). Indeed, the risk of VTE varies between cancer patients and their subgroups in an apparently non-stochastic manner. While in some cases CAT correlates with specific

oncogenic drivers (Ades *et al.*, 2015; Unruh *et al.*, 2016), or molecular profiles of cancer cells (Unlu et al., 2018), better and biologically based predictive algorithms are presently lacking. Our study aims to narrow this gap by suggesting that the properties of PDPN-expressing cells and their ability to shed EVs may be relevant to the VTE risk in GBM patients which is, as our data suggests, influenced by the interplay of cancer cell genome and epigenome.

It should be noted that the recruitment of inflammatory cells, release of cellular chromatin, angiogenesis and other aspects of the tumor microenvironment are also orchestrated by cancer-related genetic and epigenetic transformation pathways and their related changes in the cellular secretome. It could be suggested that in GBM a better characterization of a broader phenotype of additional coagulant activities acting in concert with PDPN expressing cells (with and beyond PDPN levels) may provide additional clues as to their role in VTE and in biological processes involved. A better understanding of these cellular interrelationships, beyond single markers, may offer a novel path toward more personalised management of both VTE and non-coagulant effects of the hemostatic system in high grade brain tumors.

8.2 Future Directions

One open question in CAT is the spatial and temporal heterogeneity of mechanisms leading to activation of various facets of the hemostatic system and its interfaces with angiogenesis, inflammation and immunoregulation. As certain cancers progress toward metastasis through a succession of genetic and epigenetic transitions, this too would change their coagulomes as exemplified by an earlier study from our group suggesting that sequential hits on KRAS and TP53 genes drive progressive upregulation of TF in human colorectal cancer cell lines (Yu *et al.*, 2005).

In the realm of brain tumors, analogous transitions are to be expected during the onset of secondary GBM in a small subset of younger patients with preceding lower grade gliomas. Perhaps more importantly such coagulant evolution may accompany the change in biology of GBM observed at relapse, where additional molecular events may occur spontaneously or following therapy induced mutagenesis (Wang *et al.*, 2016), and mesenchymal features are frequently detected (Phillips et al., 2006). It is presently unknown how these changes may impact the extent and nature of coagulant properties of cancer cells and their microenvironment and whether these effects contribute to comorbidities and disease dynamics. Indeed, at least some coagulant transitions trigger accompanying inflammatory and angiogenic events in experimental gliomas and lead to accelerated tumor growth (Magnus *et al.*, 2014b). Therefore, the role of GBM evolution in progression of the associated CAT deserves further study in the future.

Our work established that cancers, including GBM, are coagulant mosaics with different cellular populations contributing different prothrombotic activities, for example TF and PDPN concentrated in astrocytic and mesenchymal cells respectively (Tawil *et al.*, 2021). It is of considerable interest to understand whether GBM infiltration throughout the brain leads to formation of spatially distinct domains with different coagulant properties. While the role of such a process in VTE risk may be extremely difficult to deconvolute, the biological effects of coagulation factors expressed locally may have a role in the expansion and vascular interactions of some but not other deposits of GBM cells, as observed clinically. For example, recurrent GBM occurs in nearly 78% of cases within a two-centimeter radius from the primary lesion (Kirkpatrick et al., 2017), but may also occur at a distance or contralaterally. Whether this represents the

stochastic consequence of tumor cell penetration into the brain parenchyma or impact of surgical injury, hemostasis and wound healing responses, remains an open and intriguing question.

Likewise, pre-existing vascular lesions in the brain, post-injury responses and glial scarring may represent a fascinating area of future exploration. Coagulation system is intricately interlinked with inflammation and wound healing cellular programs, as exemplified by the upregulation of IL-8 expression by signalling through the TF/VIIa complex (Albrektsen *et al.*, 2007). Our initial efforts to establish a model of brain-injury driven gliomagenesis have been mired in technical difficulties and were eventually abandoned, but the rationale is compelling. GBM is mostly an old age disease when cerebrovascular impairments due to atherosclerosis, ischemia and subclinical vascular events are expected to be common. It is notable that the time to onset of full blown GBM in patients with prior negative MRI is surprisingly short (often several months) (Chittiboina *et al.*, 2012) suggesting an acute trigger for 'awakening' of dormant disease (Lee *et al.*, 2018). It could be speculated those cerebrovascular alterations, including thrombosis, ischemia and inflammation, may contribute to such developments. It is of note that in certain genetic thrombophilias, such as homozygous factor V Leiden, the incidence of colorectal cancer is markedly elevated, which is consistent with inflammatory underpinnings in this disease (Vossen *et al.*, 2011). The incidence of GBM was not studied in this context and is worthy of exploration.

Our ongoing efforts along the lines of investigating the plausible pro-tumorigenic potential of PDPN in GBM are of great interest. While our analysis of PDPN as contributing factor to GBM progression and therapeutic response resulted in some ambiguities, we have not investigated the compounded impact of multiple hemostatic mechanisms likely to co-exist in any given tumor. A

paradigm for such studies would have been GSC models composed of cells expressing both PDPN and TF to mimic more closely the complexity of the GBM coagulome we described (Tawil *et al.*, 2021). It is possible that combined effects of platelets and the coagulation system effectors are involved in impacting the disease biology, and similarly diverse could be the countermeasures required to mitigate these influences (anticoagulants, antiplatelets).

In the future the thrust of this work could be directed towards untangling of whether this impact of hemostasis is direct and intrinsic, or mediated via an indirect sequence of events with an implication of the GBM-triggered coagulopathy mediated by PDPN and modulated by other factors. Conversely, some aspects of GBM-associated VTE could impact the course of GBM progression, potentially in a manner modulated by tumor subtypes, oncogenic drivers, and epigenetic states, none of which has been extensively studied to our knowledge.

Our observation that oncogenic EGFRvIII downregulates PDPN in conjunction with engendering a high level of tumorigenicity may suggest that the effects of hemostatic effectors may not always be pro-tumorigenic. Similarly, our ongoing work so far suggests that GSCs engineered to overexpress PDPN do not acquire greater aggressiveness. While TF, APC, PAR-1 and other hemostatic proteins have been implicated in function of hematopoietic stem cells (Nguyen et al., 2018), or cancer stem cells (Milsom *et al.*, 2007) this may not apply to GBM. In fact, the hemostatic system evolved as a primitive form of immunity and some of its components, such as TF bear resemblance of interferon receptors. While inhibitory effects of hemostatic mechanisms on certain functions of cancer cells is counterintuitive, it would be worthy of some scrutiny.

Finally, the investigation of the correlation between blood circulating PDPN and incidence of VTE in GBM patients carries a paramount importance and potential for the development of a reliable, VTE risk biomarker, which could pave the way for a better management of cancer-associated coagulopathy in GBM.

In light of the emerging understanding of the EV biogenesis, complexity and function, a related investment in studies on PDPN-EVs is warranted and ongoing. Several outstanding questions exist in this domain, including the packaging of PDPN via either exosomal or microvesicle pathway (Van Niel *et al.*, 2018), the molecular mechanism involved, and its cancer-dependent regulators, both genetic and epigenetic (Al-Nedawi *et al.*, 2008; Spinelli *et al.*, 2018). This work will equally include the comprehensive characterization of PDPN-carrying EVs in blood and their interactions with cellular targets and hemostatic mechanisms. Collectively, added biological depth may help understand whether thromboprophylaxis in GBM should be tailored according to underlying triggers and biologically based biomarkers rather than purely traditional clotting tests. We hope that findings and ideas collected in this thesis will contribute in some small measure to progress in care for patients with GBM.

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