

Article

Proteome Profiling of Brain Vessels in a Mouse Model of Cerebrovascular Pathology

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Simple Summary: We used genetically modified mice overexpressing the cytokine transforming growth factor beta 1 (TGF mice) that capture several aspects of the cerebrovascular pathology present in Alzheimer's disease and vascular cognitive impairment and dementia. To identify the proteins that demonstrated different levels in TGF mice compared to genetically unmodified mice, we performed mass spectrometry on surgically removed cerebral arteries to identify and compare proteins between the two groups. Out of the 3602 identified proteins in brain blood vessels, 20 had significantly different levels in TGF mice. We used multiple public databases to (a) characterize the identified proteins, (b) validate the presence of their RNA transcripts in cerebrovascular cells of mice and humans, and (c) validate their presence in extracellular vesicles (i.e., little pouches of cellular content that participate in cell-to-cell communication) present in human blood. Finally, using human blood, we demonstrated the presence of several of these proteins in blood and in extracellular vesicles isolated from blood. Our research provides protein-level insights into cerebrovascular pathology in age-related dementias. Identified proteins can potentially serve as markers of vascular cognitive impairment and dementia in humans.



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Abstract: Cerebrovascular pathology that involves altered protein levels (or signaling) of the transforming growth factor beta (TGF β) family has been associated with various forms of age-related dementias, including Alzheimer disease (AD) and vascular cognitive impairment and dementia (VCID). Transgenic mice overexpressing TGF β 1 in the brain (TGF mice) recapitulate VCID-associated cerebrovascular pathology and develop cognitive deficits in old age or when submitted to comorbid cardiovascular risk factors for dementia. We characterized the cerebrovascular proteome of TGF mice using mass spectrometry (MS)-based quantitative proteomics. Cerebral arteries were surgically removed from 6-month-old-TGF and wild-type mice, and proteins were extracted and analyzed by gel-free nanoLC-MS/MS. We identified 3602 proteins in brain vessels, with 20 demonstrating significantly altered levels in TGF mice. For total and/or differentially expressed proteins ($p \leq 0.01$, ≥ 2 -fold change), using multiple databases, we (a) performed protein characterization, (b) demonstrated the presence of their RNA transcripts in both mouse and human cerebrovascular cells, and (c) demonstrated that several of these proteins were present in human extracellular vesicles (EVs) circulating in blood. Finally, using human plasma, we demonstrated the presence of several of

these proteins in plasma and plasma EVs. Dysregulated proteins point to perturbed brain vessel vasomotricity, remodeling, and inflammation. Given that blood-isolated EVs are novel, attractive, and a minimally invasive biomarker discovery platform for age-related dementias, several proteins identified in this study can potentially serve as VCID markers in humans.

Keywords: cerebrovascular pathology; age-related dementia; mouse model; cerebrovascular proteomics; mass spectrometry; human plasma; protein biomarkers; extracellular vesicles

1. Introduction

With advancing age, blood vessels in the brain become increasingly vulnerable to pathologies. Damage to the brain's vasculature disrupts the neurovascular unit, a multicellular system in which neurons, vascular cells (smooth muscle, pericytes, and endothelial cells), astrocytes, and microglia collaborate to ensure proper brain function [1,2]. Cerebrovascular damage can cause and/or aggravate age-related cognitive decline and dementia [3–5]. Affecting an estimated 55 million people worldwide, age-related dementias are a major cause of disability and dependency among the elderly (World Health Organization). A cerebrovascular pathology partly involving altered signaling or increased levels of the cytokine transforming growth factor beta 1 (TGF β 1) has been associated with various types of dementias, including the two most frequent forms, namely Alzheimer's disease (AD) and vascular cognitive impairment and dementia (VCID) [6–10]. Additionally, hereditary small vessel diseases with cognitive deficits, namely cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), show loss of function of the HTRA1 or high-temperature requirement A serine peptidase 1 gene, which results in upregulation of TGF β signaling [11,12]. In particular, immunohistochemical analysis of the cerebral small arteries in CARASIL patients shows increased TGF β 1 protein expression in the tunica media [11]. Moreover, alterations in TGF β family signaling forms the basis for several vascular disorders in humans, including hereditary hemorrhagic telangiectasia [13–16] and primary pulmonary hypertension [17]. Importantly, accumulating lines of evidence point to a link between cerebrovascular health and TGF β family signaling [6,7]. Increased risk of sporadic vascular dementia has been associated with the Pro10Leu single-nucleotide polymorphism (SNP) in the *TGF β 1* gene [8], which is known to impact the TGF β 1 protein level [9].

TGF β family members, including TGF β 1, are multifunctional cytokines that bind to type I, II, and III receptors [18]. Mouse models lacking TGF β signaling components such as type I or II receptors (e.g., ACVRL1, TGF β RI, TGF β RII) die mid-gestation due to impaired vascular development [19]. Transgenic mice overexpressing a constitutively active form of TGF β 1 in the brain (TGF mice), originally developed to recapitulate the cerebrovascular pathology seen in AD [20], were later found to lack the cerebral amyloid angiopathy typical of AD [21,22] and, as such, were found to better recap the cerebrovascular pathology seen in VCID. Cerebrovascular structural abnormalities observed in TGF mice include thickened vascular wall; microvascular injury and degeneration, such as smaller capillary endothelial cells and pericytes, and abnormal chromatin condensation in endothelial cell nuclei; and string vessel pathology [20,21,23,24]. Functional abnormalities such as impaired vascular reactivity primarily related to endothelial-mediated dilatation, chronic cerebral hypoperfusion, and compromised neurovascular coupling are also hallmarks of TGF mice [23,24]. While TGF mice either do not develop cognitive deficits or do only in advanced age [25,26], they readily develop them when submitted to a comorbid cardiovascular risk factor for dementia, hence being a good model of VCID [27–29].

In recent years, -omics approaches, such as single-cell/nucleus transcriptomics and proteomics, have been used to profile the mammalian cerebrovasculature [30–33]. These approaches have provided insight into transcriptome- and proteome-level changes in brain

vessels from AD patients and mouse models [34,35]. However, to date, VCID-related pathology of brain vessels lacks similar profiling. Addressing this gap in knowledge, we set out to (a) characterize the cerebrovascular proteome of TGF mice using mass spectrometry (MS)-based quantitative proteomics, as well as (b) identify proteins with biomarker potential in humans using an *in silico* bioinformatics approach. Given that proteins are regarded as effectors of biological functions, proteomics findings are generally considered directly suited for biomarker and drug development work.

2. Materials and Methods

2.1. Mice

Six-month-old transgenic TGF mice and their C57BL/6J wild-type (WT) littermates were used in this study. TGF mice overexpress a constitutively active form of TGF β 1 under the control of the glial fibrillary acidic protein (GFAP) promoter on a C57BL/6J background (line T64) [36]. To eliminate sex-related differences in brain structures [37] and potentially in the vasculature [38], only males were used. A total of 18 mice, in particular 9 TGF mice and 9 WT littermates, were employed based on expected variances and differences between groups by our previous and others' studies [12,23,35]. Mice were housed under a 12 h light–dark cycle, in a room with controlled temperature (23 °C) and humidity (50%). Mice had access to tap water and food (Teklad Rodent chow, Research Diets Inc., New Brunswick, NJ, USA) ad libitum. All experiments were performed in compliance with the Animal Ethics Committee of the Montreal Neurological Institute and the Canadian Council on Animal Care guidelines, and complied with the ARRIVE 2.0 guidelines [39].

2.2. Surgical Extraction of Cerebral Arteries

The circle of Willis and major cerebral arteries along with their main branches were surgically removed from mice and individually stripped from the attached pia matter to obtain a clean preparation of vascular tissue, as previously described [33]. Arteries extracted from three mice were combined to constitute one biological replicate and stored at -80 °C. Three biological replicates (B1, B2, B3) were prepared for each of the two groups.

2.3. Cerebrovascular Proteomics Workflow

Protein extraction from mouse cerebral arteries was performed using a published and validated protocol [33]. Thereafter, the processing of samples for MS analysis, as well as the MS runs, preprocessing, and bioinformatics were performed without the knowledge of group allocation (i.e., WT or TGF). A brief description of the procedure is provided in Supplemental information.

2.4. Generation of Statistically Significant Protein Lists

Statistics were performed with the knowledge of group allocations using GraphPad Prism version 9.4.1. Specifically, proteins with altered levels between WT and TGF cerebral arteries were identified through parametric Student *t* and non-parametric Mann–Whitney U tests. The *p*-values were corrected for multiple testing using the Holm–Šidák method. Varying stringency was used to generate a database of two lists of proteins with altered levels between WT and TGF cerebral arteries. List 1 proteins were as follows: only proteins with $p < 0.01$, peptide score ≥ 35 ($< 1\%$ false discovery rate), and fold change ≥ 2 (up or down) were included, whereas peptides showing high variability ($> 55\%$) among replicates were excluded. List 2, more stringent, consisted of List 1 proteins identified with > 2 peptides.

2.5. Characterization and Classification of Proteins

The gene associated with each identified protein was queried using the UniProt database [40] employing the UniProtKB accession number of each protein. Cellular Component Analysis and the PANTHER Overrepresentation Test were conducted using the PANTHER Classification System (PANTHER version 17.0 Released 22 February 2022, ac-

cessed 24 and 26 April 2022, respectively) [41,42] on the UniProtKB accession number of identified proteins, and with *Mus musculus* selected for the organism. For Cellular Component Analysis, the PANTHER GO-slim ontology annotation set was used, which contains 3361 terms—namely 2267 biological process, 550 molecular function and 544 cellular component terms (<http://www.pantherdb.org/panther/goSlim.jsp>, accessed on 26 April 2022). Fisher's Exact test followed by False Discovery Rate (FDR) correction was used for PANTHER pathways overrepresented in genes associated with identified proteins in mouse brain vessels compared to the entire *Mus musculus* genome. The PANTHER pathway database consists of 177, mainly regulatory, signaling pathways. Protein interactors of TGF β 1 were identified by querying the BioGrid database, the IntAct Molecular Interaction database (<http://www.ebi.ac.uk/intact/>, accessed on 26 April 2022) [43], and an in-house database [35] that is a compendium of several databases, including BIND, BioGRID, HPRD, HMAP, and EcoCyc databases. Protein interactions were restricted to those observed in *Mus musculus* and/or *Homo sapiens*.

2.6. Comparison against Public Transcriptomics Datasets

Published single-cell transcriptomics datasets were examined to demonstrate that RNA transcripts of proteins identified were also present in mouse and/or human brain vascular cells. Analyses were performed on List 2 proteins only. For relative gene expression levels of proteins in mouse brain vascular cell types (e.g., endothelial, smooth muscle cells), two mouse cerebrovascular single-cell transcriptomics datasets [31,44] were downloaded from the NCBI GEO repository [45], and each gene was ranked by its relative abundance. For correlation between mouse and human relative gene expression in brain vascular endothelial cells, we used the dataset compiled by Yang and colleagues [34]. List 2 proteins were also compared for (a) common pathways across three publicly available databases, notably KEGG (<https://www.genome.jp/kegg/>, accessed on 16 November 2023), Reactome (<https://reactome.org/>, accessed on 16 November 2023), and PANTHER, and (b) molecular functions and biological processes, between humans and mice using the Uniprot database [40].

2.7. Comparison against the Public Human Extracellular Vesicles (EV) Database

Vesiclepedia [46], an extracellular vesicle (EV) database version 5.1, was used to identify List 1 and 2 proteins detected in the TGF cerebrovasculature and also detected in human EVs. These proteins constitute a rich source of brain vasculature-specific biomarkers, as well as receptors known for delivering molecules across the blood–brain barrier.

2.8. Proteomics of Plasma and Plasma EVs

Human plasma from 13 healthy consenting individuals (7 male and 6 female) was purchased from BioIVT (Westbury, NY, USA; bioivt.com) and used for proteomics analysis. Five hundred (500) μ L of each type of human plasma was precleared by centrifugations at $1500\times g$ for 10 min and at $10,000\times g$ for 10 min. Precleared plasma (supernatant) was used for abundant protein depletion or total EV isolation. For depletion of abundant proteins, 10 μ L of precleared plasma was loaded onto High-Select™ Top14 Abundant Protein Depletion spin columns (ThermoFisher, Waltham, MA, USA; catalog # A36369) and flowthrough collected as per manufacturer's instructions. For isolation of total EVs, 150 μ L of precleared plasma was loaded onto qEVsingle 35 nm sized exclusion chromatography columns (IZON, Westbury, MA, USA; product code # ICS-35), and total EVs were isolated as per manufacturer's instructions. The precleared (or undepleted) plasma, depleted plasma, and total EV fractions (200 μ L of fractions 6–8) were analyzed by proteomics as recently described [47]. EV isolation was confirmed by detection of proteins from the "Top 100 EV Proteins" list in the Vesiclepedia database. Additional MISEV guidelines were also followed.

2.9. Formatting Guideline Used for Genes and Proteins

For ease of reading, we used the gene symbol for proteins. In accordance with the organism-specific formatting guidelines, mouse proteins (indicated by their gene symbols as mentioned above) have the first letter in upper-case (e.g., *Acta2*), while human proteins were fully capitalized (e.g., *ACTA2*). When specifically indicating a gene, the symbols were italicized (e.g., *Acta2*, *ACTA2*).

3. Results

3.1. Characterization of Proteins Detected in Mouse Brain Vessels

We identified 3602 proteins from high-quality peptides (peptide score ≥ 35) in brain vessels of WT and TGF mice. Identified proteins included canonical vascular proteins, such as Claudin-5 (*Cldn5*), Solute carrier family 2 (*Slc2a1*), and von Willebrand factor (*Vwf*). Using the UniProt database, it was determined that the majority of proteins identified ($n = 3575$; 99.3%) were products of known genes. Cellular Component Analysis, conducted using the PANTHER Classification System, found 2785 component hits in two main categories: cellular ($n = 2167$ genes) and/or protein-containing complex ($n = 618$ genes). The top five cellular components were intracellular anatomical structures ($n = 1624$ genes), membrane ($n = 1495$ genes), organelle ($n = 1290$ genes), cytoplasm ($n = 1037$ genes), and cell periphery ($n = 754$ genes), the latter defined as part of a cell encompassing the (a) cell cortex cell region just beneath the plasma membrane and often containing a network of actin filaments and associated proteins, (b) the plasma membrane, and (c) any external encapsulating structures (Figure 1a). The top five protein-containing complex components were catalytic ($n = 181$ genes), nuclear ($n = 157$ genes), membrane ($n = 138$ genes), ribonucleoprotein ($n = 96$ genes), and intracellular ($n = 83$ genes) (Figure 1b).

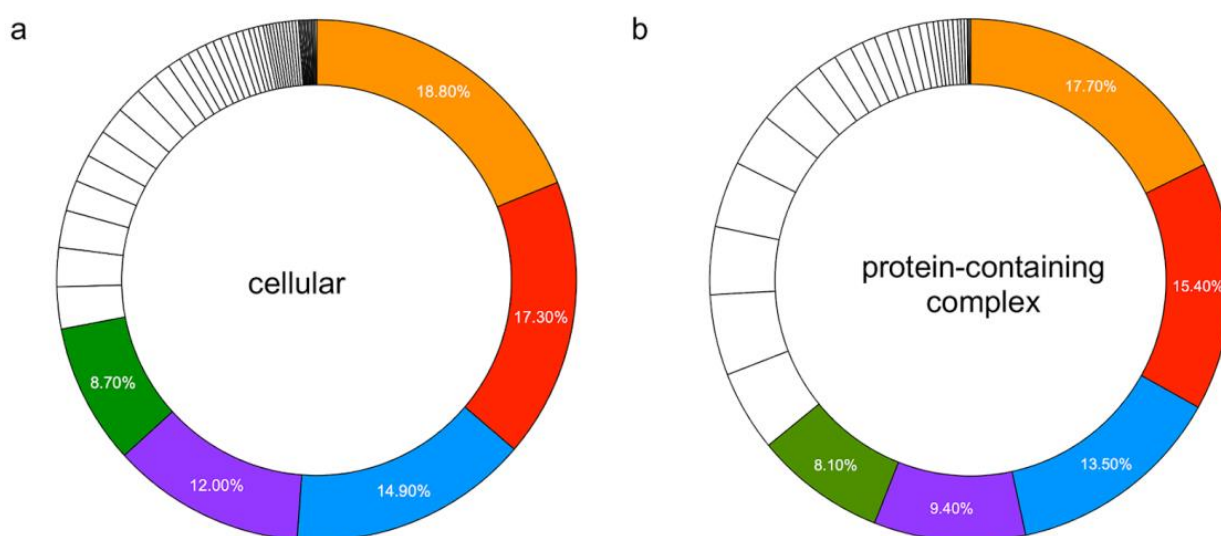


Figure 1. Percent distribution of the top 5 cellular and protein-containing complex components. Percentages represent the percent of gene hit against the total number of component hits in (a) cellular ($n = 69$ components): intracellular anatomical structures; membrane; organelle; cytoplasm; cell periphery; (b) Protein-containing complex ($n = 32$ components): catalytic; nuclear; membrane; ribonucleoprotein; intracellular.

Using the PANTHER Overrepresentation Test, we identified seven pathways to be significantly ($p < 0.05$, FDR corrected) overrepresented at a > 2 -fold enrichment (FEn) in mouse brain vessels, relative to the entire *Mus musculus* genome. These comprised pathways involved in (a) vasomotor regulation, namely endothelin signaling (FEn = 2.37) and 5-hydroxytryptamine/serotonin degradation (FEn = 3.21) pathways, the integrin signaling pathway (FEn = 2.79), and cytoskeletal regulation by Rho GTPase (FEn = 2.77), and (b) cellular metabolism/energetics, namely the tricarboxylic acid cycle (FEn = 4.10),

glycolysis (FEn = 3.59), and the insulin/IGF pathway–protein kinase B signaling cascade (FEn = 2.53).

In our list of 3602 proteins, we identified 102 (2.8%) direct interactors of TGFβ1, with ≥ 3 interactors detected from the following protein families: collagen (Col), heat shock proteins (Hsp), and large (Rpl) and small (Rps) ribosomal proteins (Figure 2). We also identified 1942 (53.9%) indirect interactors of TGFβ1.

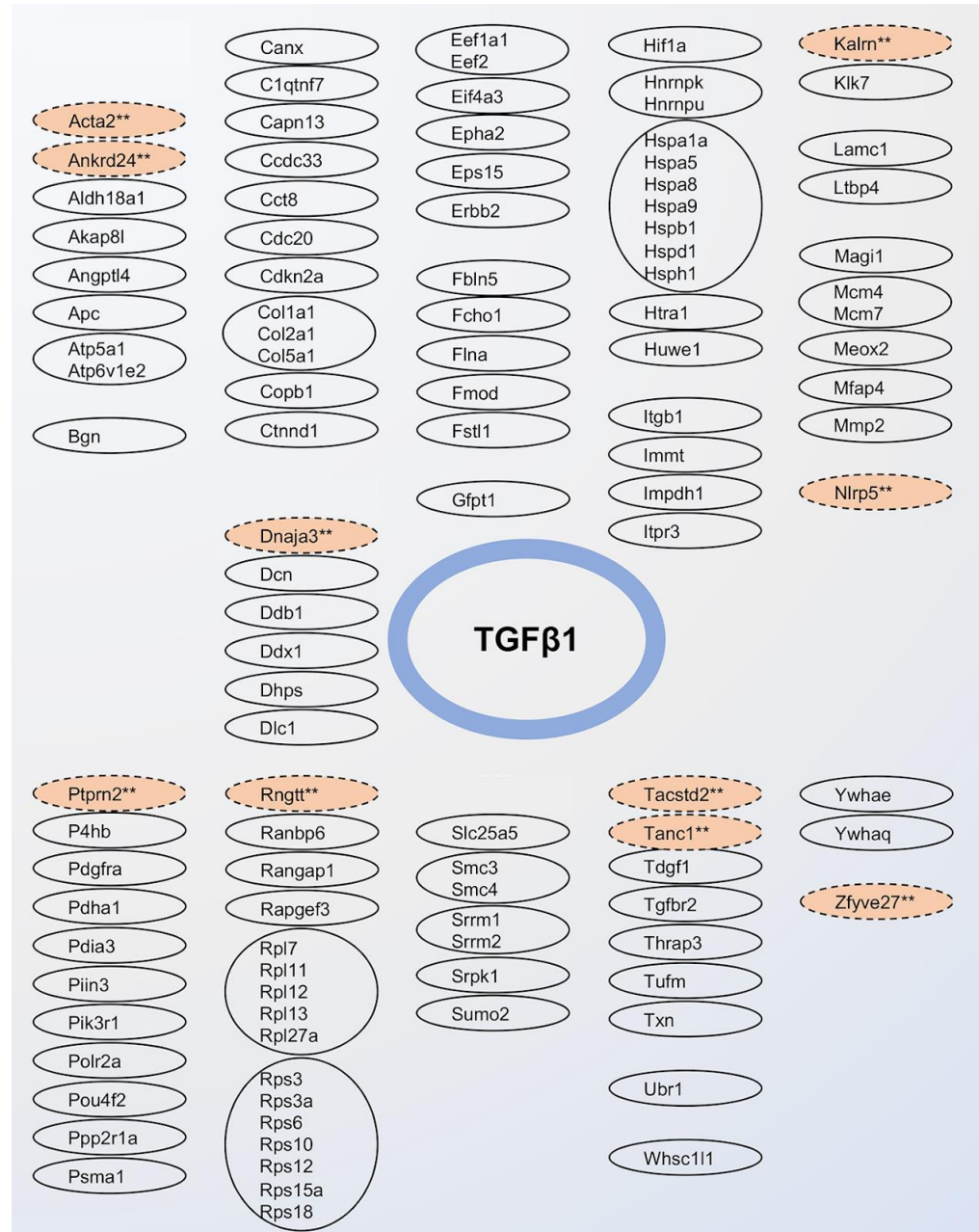


Figure 2. Protein interactors of TGFβ1. Solid circles indicate direct interactors, and dashed circles indicate indirect interactors. Protein interactors belonging to the same family of proteins are grouped together. Salmon colors depict TGFβ1 interactors with significant ($p \leq 0.01$, denoted as ** in the figure) 2-fold level change in the cerebrovasculature of TGF mice.

3.2. Proteins with Altered Levels in the Brain Vessels of TGF and WT Mice

Compared to WT mice, 60 proteins (1.7%) showed significantly altered levels ($p \leq 0.01$ and twofold change) in the arteries of TGF mice. These 60 proteins constituted List 1

proteins (Supplemental Table S1). Of these, 20 proteins were significantly ($p < 0.01$) detected with ≥ 2 peptides, with 10 showing level increases (Acta2, Ankrd24, Dll3, Adgrg2, Igdcc3, Kalrn, Nubpl, Ptprn2, Tanc1, Zfyve26), and 10 showing decreases (Ddx11, Dnaja3, Krt24, Nlrp5, Pof1b, Ptprd, Rngtt, Rp111, Tacstd2, Zfyve27) (Figure 3, Supplemental Table S1). These 20 proteins constituted List 2 proteins (see full names of proteins in Table 1). Half ($n = 10$) of List 2 proteins were indirect interactors of TGF β 1 (Figure 2).

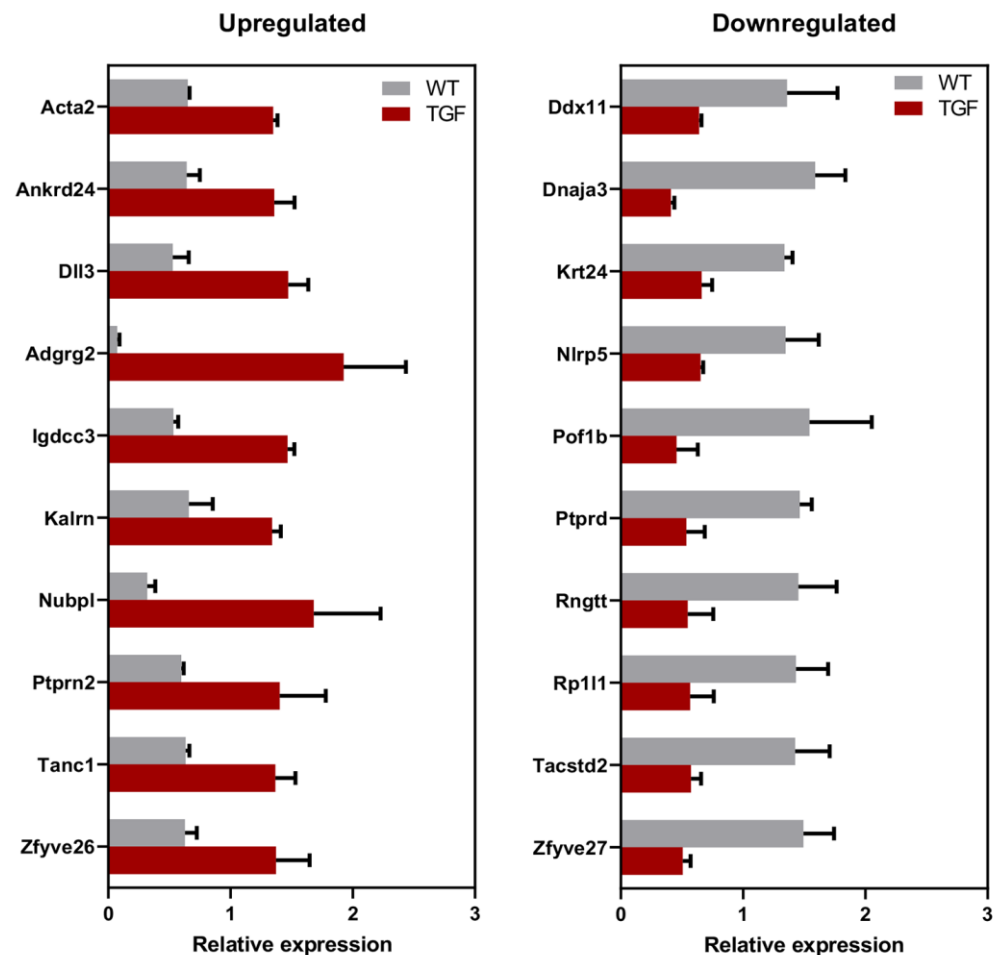


Figure 3. Relative expression of List 2 proteins in TGF brain vessels. Proteins with significantly altered levels in brain vessels of TGF mice relative to WT detected using the highest stringency in our database ($p \leq 0.01$, 2-fold change, and ≥ 2 peptides) constitute List 2 proteins ($n = 20$).

3.3. Comparison against Published Transcriptomics in Mouse Brain Vascular Cells

Examination of published single-cell transcriptomics data demonstrated that RNA transcripts of the above-mentioned 20 List 2 proteins are present in one or more cell types of the mouse brain vasculature. Abundance ranking of their gene expression in two separate single-cell transcriptomics databases, ranging from low ($< 30\%$) to very high ($> 90\%$), are shown in Figure 4a and Supplemental Figure S1. Comparison of transcripts in the arterial endothelial cell (aEC) and arterial smooth muscle cell (aSMC) categories identified 11/20 of these transcripts to be present in both cell types (Acta2, Ankrd24, Kalrn, Nubpl, Tanc1, Zfyve26, Ddx11, Dnaja3, Ptprd, Rngtt, Zfyve27) with an abundance ranking of $\geq 50\%$ (indicating moderate to high abundance). In contrast, 1/20 transcript (Igdcc3) and 2/20 transcripts (Ptprn2, Tacstd2) demonstrated an abundance ranking of $\geq 50\%$ in aEC and aSMC, respectively.

Table 1. Biomarker potential List 2 proteins detected in human extracellular vesicles. We note that the gene symbols of proteins are capitalized in accordance with the organism-specific formatting guidelines—in this case, indicating human proteins. The “X” denotes that a protein is present and/or has been identified. Abbreviations: EV, extracellular vesicle; HBEC, human brain endothelial cells; p, protein; r, mRNA.

ID (Human)	Common Name	Vesiclepedia Identified Molecule		Reported Detected in Vascular Cell and/or Blood, to Date		Type			
		p	r	Vascular [Ref. ID]	Blood [Ref. ID]	Primary HBEC	HBEC EV	Plasma (Depleted)	Plasma EV
ACTA2	Actin Alpha 2	X	X	Endothelial [48,49]	Plasma [50,51]	X	X	X	X
ANKRD24	Ankyrin Repeat Domain 24	X		-	-				
DLL3	Delta-Like Canonical Notch Ligand 3	X	X	-	-	X			
ADGRG2 (GPR64)	Adhesion G Protein-Coupled Receptor G2 (also known as G Protein-Coupled Receptor 64)	X	X	-	-				
IGDCC3	Immunoglobulin Superfamily, DCC Subclass, Member 3		X	-	-			X	
KALRN	Kalirin RhoGEF Kinase	X	X	-	Plasma [52]				X
NUBPL	Nucleotide Binding Protein-Like	X	X	-	-				
PTPRN2	Protein Tyrosine Phosphatase, Receptor Type N2		X	-	-				
TANC1	Tetratricopeptide Repeat, Ankyrin Repeat, and Coiled-Coil Containing 1	X	X	-	-	X		X	X
ZFYVE26	Zinc Finger FYVE-Type Containing 26	X	X	-	-				X
DDX11	DEAD/H-Box Helicase 11	X	X	-	-				X
DNAJA3	DnaJ Heat Shock Protein Family (Hsp40) Member A3	X	X	-	-				
KRT24	Keratin 24	X	X	-	Plasma [50]			X	X
NLRP5	NOD-like Receptor Family Pyrin Domain Containing 5			-	-	X			

Table 1. Cont.

ID (Human)	Common Name	Vesiclepedia Identified Molecule		Reported Detected in Vascular Cell and/or Blood, to Date		Type			
				Vascular [Ref. ID]	Blood [Ref. ID]	Primary HBEC	HBEC EV	Plasma (Depleted)	Plasma EV
		p	r						
POF1B	POF1B Actin Binding Protein	X		-	-				
PTPRD	Protein Tyrosine Phosphatase Receptor Type D	X		-	-	X			X
RNGTT	RNA Guanylyltransferase And 5'-Phosphatase	X	X	-	-				
RP1L1	Retinitis Pigmentosa 1-like 1			-	-	X			
TACSTD2	Tumor Associated Calcium Signal Transducer 2	X		Endothelial [53]	-				
ZFYVE27	Zinc Finger FYVE-Type Containing 27	X	X	-	-				

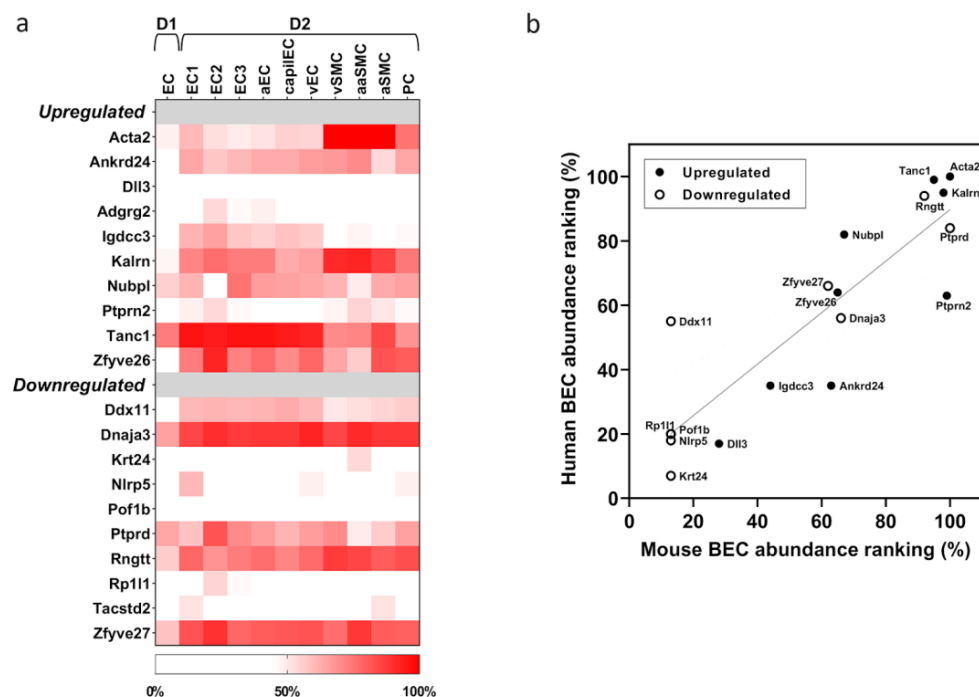


Figure 4. Relative gene expression of List 2 proteins in public vascular datasets. (a) Relative gene expression of the 20 List 2 proteins in mouse cerebrovascular single cell transcriptome databases D1 [44] and D2 [31]. Abbreviations: EC, endothelial cell; aEC, arterial EC; capilEC, capillary EC; vEC, venular EC; vSMC, venular smooth muscle cell; aaSMC, arteriole SMC; aSMC, arterial SMC; PC, pericyte; (b) Correlation between mouse and human relative gene expression of the 20 List 2 proteins in brain vascular endothelial cells. Upregulated and downregulated proteins are shown as closed and open symbols, respectively. Solid line represents the linear regression (Pearson $r^2 = 0.64$, $p < 0.001$) and dotted lines are 99% confidence bands.

3.4. Translatability of Findings to Human

To demonstrate the translatability of our findings (i.e., differentially expressed mouse proteins are also expressed in human brain vasculature), the relative gene expressions of our List 2 proteins were compared in public RNAseq databases between human and mouse brain vascular endothelial cells. Most ($n = 18$) of the proteins showed similar gene expression in the two species as demonstrated by a strong correlation (Pearson $r^2 = 0.64$, $p < 0.001$) in the expression ranking between humans and mice (Figure 4b). It was determined using three databases (KEGG, Reactome, PANTHER) that 11/18 proteins have common pathways (Table 2). In addition, we also compared molecular functions and biological processes between humans and mice using the Uniprot database and found that the majority of these functions and processes overlap between the two species (Supplemental Figure S4; Supplemental Table S3). Additionally, List 2 proteins were examined in proteomics datasets from human brain endothelial cells [54–56], which is part of the BBB Carta project [54]. Out of the 20 List 2 proteins, 6 were detectable in human brain endothelial cells, including ACTA2, DLL3, TANC1, NLRP5, PTPRD, and RP1L1.

Table 2. Summary of common pathways for the 20 List 2 significant proteins in mice and humans. The databases used for pathway comparison were KEGG (blue), Reactome (green), and PANTHER (orange). The colors of the arrows in the “Common” column identify a specific pathway. Italic writing indicates that the pathway is specific to humans (i.e., cellular responses to stimuli for DDX11) and underlined writing indicates that the pathway is specific to mice (i.e., disease for RNGTT) for the protein in question. Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; Reactome, Reactome Pathway Database; PANTHER, Protein Analysis Through Evolutionary Relationships.

Protein	KEGG	Reactome	PANTHER	Common
ACTA2	Vascular smooth muscle contraction Motor proteins Apelin signaling pathway Relaxin signaling pathway	Muscle contraction <i>Signal transduction</i>	Alzheimer disease—presenilin Signaling Inflammation Cytoskeletal regulation by Rho GPTase	✓✓✓
ANKRD24	-	-	-	
DLL3	Endocrine resistance Notch signaling pathway Th1 Th2 cell differentiation Pathways in cancer	Developmental biology - - -	Angiogenesis Notch signaling pathway - -	✓✓✓
ADGRG2	-	-	-	
IGDCC3	-	-	-	
KALRN	-	Developmental biology Signal transduction	-	✓
NUBPL	-	Metabolism	-	✓
PTPRN2	Type 1 diabetes mellitus	Immune system	-	✓✓
TANC1	-	-	-	
ZFYVE26	-	-	-	
DDX11	Cell cycle	<i>Cellular responses to stimuli</i>	-	✓
DNAJA3	Viral carcinogenesis	-	-	✓
KRT24	Estrogen signaling pathway Staphylococcus aureus infection	Developmental biology -	-	✓✓

Table 2. Cont.

Protein	KEGG	Reactome	PANTHER	Common
NLRP5	-	-	-	
POF1B	-	-	-	
PTPRD	Cell adhesion molecules	Neuronal system	-	✓✓
RNGTT	mRNA surveillance pathway	Gene expression Metabolism of RNA Disease	- - -	✓✓
RP1L1	-	-	-	
TACSTD2	-	-	-	
ZFYVE27	Endocytosis	-	-	✓

3.5. Blood Biomarker Potential in Human

Using a publicly available database, Vesiclepedia, we demonstrated that 80% ($n = 16$) of the 20 List 2 differentially expressed proteins in the TGF cerebrovasculature were detected in human EVs (Table 1), with several detected in easily accessible biofluids (e.g., plasma [50–52]). In addition, Acta2 and Tacstd2 were detected in EVs secreted by vascular endothelial cells [48,49,53]. Acta2 was also detected in EVs secreted by human blood–brain barrier cells [55]. Overall, 70% ($n = 42$) of the 60 TGF List 1 differentially expressed proteins (which includes List 2 proteins) were detected in human EVs in Vesiclepedia (Supplemental Table S1).

In addition, the 20 proteins in List 2 were also validated in the proteome of healthy human plasma to demonstrate their biomarker potential in humans. Depletion of abundant proteins from plasma was necessary to detect the presence of some of these proteins by proteomics. At least four of the List 2 proteins (ACTA2, KRT24, TANC1, and IGDC3) were detectable in depleted human plasma (Table 1). An example of an extracted ion chromatogram and an MS/MS spectrum for a TANC1 peptide in non-depleted and depleted plasma is shown in Supplemental Figure S2a,b.

Total EVs were also isolated from these plasma samples using size-exclusion chromatography and analyzed by proteomics. EV isolation was confirmed by detection of more than 60 EV proteins, which were mostly absent in the undepleted plasma samples (Supplemental Table S2). At least seven of the List 2 proteins (ACTA2, KALRN, TANC1, ZFYVE26, DDX11, KRT24, and PTPRD) were detectable in the total plasma EVs. Examples of extracted ion chromatograms and MS/MS spectra for the peptides of ACTA2 and KALRN in plasma and total EVs are shown in Supplemental Figure S3a–d.

4. Discussion

TGF mice overexpress a constitutively active form of TGF β 1 in brain astrocytes and recapitulate aspects of the cerebrovascular pathology seen in VCID and AD (but not the amyloid pathology). Particularly, they display thickened vascular walls due to accumulation of extracellular matrix proteins, string vessel pathology, impaired dilatory function, cerebral hypoperfusion, neurovascular uncoupling, and cerebral microhemorrhages [20,21,23,57,58]. Here, we demonstrate that the cerebrovascular proteome is significantly altered in TGF mice. Specifically, 60 proteins (1.7% of total identified proteins in WT mice) showed significant altered levels ($p \leq 0.01$ and twofold change) in the cerebral arteries of TGF mice compared to those of WT littermates. We focus our discussion on how level perturbations in List 2 proteins ($n = 20$), identified using stringent criteria from the 60 List 1 proteins, relate to the cerebrovascular abnormalities seen in TGF mice, in particular perturbation in vasomotricity, remodeling, and inflammation.

4.1. Perturbation in Vascular Tone and Vasomotricity

We identified three proteins with altered levels in the TGF cerebral vasculature, namely *Acta2*, *Tacstd2*, and *Tanc1*, that are known to influence vascular tone.

4.1.1. Increased Vascular Tone

The contraction of blood vessels in response to the stretch resulting from pulsatile blood flow is dependent on the interaction between thin and thick myofilaments, composed of *Acta2* (or α -2 actin) and β -myosin, respectively [59,60]. Increased *Acta2* level in the TGF cerebrovasculature likely increases vascular tone, hence contributing to the impaired vasomotricity observed in brain arteries of TGF mice [23]. Our finding of increased *Acta2* levels in the TGF cerebral arteries parallels observations in peripheral arteries, where the overactivation of TGF β 1 signaling stimulates the expression of smooth muscle contractile genes, including *ACTA2* [61,62]. Additional evidence linking *Acta2* to the regulation of vascular tone includes (a) the observation that *Acta2*-null-mice demonstrate compromised vascular tone, contractility, and blood flow [63], and (b) the dilative phenotype found in *ACTA2* missense mutations seen in cerebral arteriopathy in humans [64].

4.1.2. Attempt to Compensate by Promoting Vasodilation

In contrast to the vasocontractile phenotype promoted by upregulated *Acta2*, decreased *Tacstd2* protein levels in the TGF cerebrovasculature point to an attempt at promoting vasodilation via modulation of Ca^{2+} levels. *Tacstd2* is known to increase Ca^{2+} levels in the cytosol by inducing its release from internal stores [65,66], which increases vascular smooth muscle contractility [33]. Therefore, a decrease in *Tacstd2* level promotes vasodilation. *Tanc1*, demonstrating increased levels in TGF brain vessels, is an indirect interactor of TGF β 1 [67], and a scaffold protein associated with regulation of excitatory (e.g., glutamatergic) synapse strength in both brain and non-brain tissue [68,69]. It is, therefore, possible that increased levels of *Tanc1* is an attempt to promote blood vessel dilation via modulation of neurogenic signaling [70]. Indeed, *Tanc1* has been shown to interact with NMDA (N-methyl-D-aspartate) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) glutamate receptor subunits encoded by *GRIN2B* and *GRIA1* [71], and present in cerebral blood vessels [33,72].

In addition to the three proteins known to influence vascular tone, we also detected decreased levels of the ATP-dependent DNA helicase *Ddx11*, an alteration likely related to the contractile phenotype associated with chronic cerebral hypoperfusion in TGF mice [29,57,58]. Supporting this is the observation that in the vascular endothelium in humans, the *DDX11* gene is repressed under hypoxic conditions [73].

4.2. Cerebrovascular Remodeling

Remodeling of the cerebrovasculature is a key phenotypic feature of TGF mice [20,21,23,24]. In general, vascular remodeling is the process of altering blood vessel structure and arrangement via cell growth, proliferation, migration, death, and/or the production or degradation of the extracellular matrix [74]. Similar to the remodeling observed in TGF brain vessels, increase in the TGF β 1 level is known to promote aspects of vascular modeling, such as a proliferative phenotype, in peripheral arteries, as demonstrated (a) in cultured vascular smooth muscle cells from human pulmonary artery [75] and (b) in murine carotid artery vascular media due to localized overexpression of TGF β 1 in the endothelium [76]. We found that 11 of the 20 proteins with altered levels in the TFG cerebral vasculature were known influencers of vascular remodeling, namely *Dll3*, *Adgrg2*, *Krt24*, *Ptprd*, *Ptprn2*, *Igdcc3*, *Rngtt*, *Rp111*, *Tacstd2*, *Zfyve26*, and *Zfyve27*. Below, we highlight their specific links with processes contributing to vascular remodeling.

4.2.1. Vascular Remodeling via Promotion of Cell Proliferation

Recent literature suggests that TGF β 1 overexpression can upregulate the expression of several Notch signaling pathway proteins [77]. Indeed, an increase in *Dll3* level, a core

member of the canonical Notch signaling pathway, was observed in TGF vessels. DLL3 binds to the Notch family of receptors (Notch1 to 4) [78], of which Notch1 and Notch4 were detected in cerebral arteries of TGF and WT mice. In humans, an elevated DLL3 level has been linked with pathological angiogenesis via the DLL3/NOTCH4 signaling pathway [79]. *Adgrg2*, a G-protein coupled receptor, was increased in brain vessels of TGF mice. The literature indicates that G-protein coupled receptor signaling may play a role in arterial smooth muscle cell transformation [80]. In line with this, upregulation in *ADGRG2* transcripts has been reported during the transformation of quiescent human coronary artery smooth muscle cells to a proliferative and migratory phenotype observed in atherosclerosis [80]. Cytokeratin *Krt24*, a known anti-proliferative factor in the epidermis [81,82], was decreased in the TGF cerebrovasculature. Given that differential expression of cytokeratins has been observed in vascular smooth muscle cells, often in the setting of a proliferative state [83], a decreased *Krt24* level likely contributes to cell proliferation and remodeling. Decreased *Ptprd* and increased *Ptprn2* levels were also found in the TGF cerebrovasculature. These two proteins belong to the protein tyrosine phosphatase family of signaling molecules that regulate cellular processes, including differentiation and proliferation [84]. A decreased *Ptprd* protein level in a peripheral artery (i.e., pulmonary, rodent) was found to alter vascular smooth muscle cell morphology and migration, primarily via focal adhesion and cell cytoskeleton modulation [85]. Protein *Ptprn2* is a direct interactor of SMADs [86], the main downstream signal transducers for the TGF β superfamily receptors. Variations in the *PTPRN2* gene have been associated with calcified atherosclerotic plaque, a subclinical marker of atherosclerosis, in human peripheral arteries [87,88], and familial stroke [89]. Increase in *Ptprn2* (or human *PTPRN2*) protein level is also known to perturb the lipid-dependent sequestration of an actin-remodeling factor [90], a process capable of facilitating vascular remodeling. While the exact role of *Igdcc3* (or human *IGDCC3*) protein in brain vessels is unknown, its gene expression pattern strongly overlaps with regions of high Wnt activity during embryonic development [91], suggesting that its increase in the TGF cerebrovasculature may be associated with a proliferative phenotype [92].

4.2.2. Attempt to Attenuate Vascular Remodeling

Levels of proteins *Rngtt*, *Rp111*, *Tacstd2*, *Zfyve26*, and *Zfyve27*, which are known to promote a proliferative phenotype in various cancers, were decreased in the brain vasculature of TGF mice, likely as an attempt to attenuate vascular proliferation and remodeling. Reduction in the *Rngtt* level, a protein that caps nascent mRNA [93], has been shown to compromise the activity of Wnt3a/ β -catenin [94] and Hedgehog [95] pathways, both involved in a proliferative phenotype in the vessel wall [92,96]. Commonly associated with retinal degeneration, the *RPL11* gene has also been associated with brain arteriovenous malformations [97] and cancer [98,99] in humans. Similarly, expression of the TGF β 1 response gene, *TACSTD2* (or *TROP2*) [100], in cancer tissue (e.g., human glioma) has been shown to correlate with microvessel density—a commonly used marker for estimation of angiogenesis [101,102]. In addition, by conducting experiments in both humans and mice, Guo et al. demonstrated that *Tacstd2* (or human *TACSTD2*) promotes the expression of MMP13 and PECAM1, two well-known angiogenesis factors, via activation of ERK-1 and -2 signaling pathways [103]. With regard to *Zfyve26*, decreased levels in mice are known to disrupt endolysosomal membrane trafficking [104], which in turn can exacerbate vascular calcification [105]. Therefore, its increase in the TGF cerebrovasculature may be a compensatory attempt at attenuating remodeling. *Zfyve27* (or human *ZFYVE27*) was recently found to promote endothelial cell migration and angiogenesis in humans, with its knockdown affecting these processes [106].

4.3. Chronic Inflammation

While TGF β 1 has been known to function both as an anti- and a pro-inflammatory cytokine [107], a pro-inflammatory phenotype has been observed in TGF mice [24,108], including inflammation of brain vessels characterized by activated perivascular astrocytes and

microglial cells [21]. TGF β 1 is known to stimulate the production of reactive oxygen species (ROS) in vascular cell types, including endothelial and smooth muscle cells [109,110]. ROS are free radicals derived from molecular oxygen, and their overproduction in the cerebrovasculature contributes to the inflammatory response [111] and promotes dysfunction by shifting cell bioenergetics [112]. Six of the twenty proteins with altered levels in the TGF cerebral vasculature, namely Nlrp5, Pof1b, Kalrn, Nubpl, Dnaja3, and Ankrd24, had links to inflammation, as described below.

4.3.1. Proteins Indicative of an Inflammatory Process

Decreases in Nlrp5 and Pof1b levels were observed in TGF brain vessels. Nlrp5 (or human NLRP5) belongs to a class of cytoplasmic pattern recognition receptors [113] and is known to be expressed in both mouse and human cerebrovasculature, largely in endothelial cells [31,114] and pericytes [31,115]. Given that treatment with hydrogen peroxide (an endogenous ROS) decreases the expression of NLRP5 in human cerebral endothelial cells, its decrease in the TGF cerebrovasculature may indicate ROS overproduction and an inflammatory environment [114]. With regard to Pof1b (or human POF1B), its gene expression in blood has been associated with ischemic stroke in humans [116]. POF1B is also known to be highly expressed in epithelial cells with tight junctions, where it localizes at tight junctions and regulates cytoskeleton dynamics [117]. In the vasculature, *Pof1B*/*POF1B* expression has been observed in endothelial cells and pericytes [31,34,44], and may contribute to the regulation of the blood–brain barrier permeability, similar to its function in the epithelium. Its decrease in the TGF cerebrovasculature may indicate a compromised blood–brain barrier and an inflammatory environment.

4.3.2. Attempt at Reducing Inflammation

Altered levels of proteins Kalrn, Dnaja3, Nubpl, Spen, and Ankrd24 may suggest compensatory attempts at reducing inflammation in the TGF cerebrovasculature. An increased Kalrn level was found in TGF brain vessels. In humans, KALRN, a guanine nucleotide exchange factor, is known to associate with and down-regulate inducible nitric oxide synthase (iNOS) activity, and thereby the production of nitric oxide, a free radical [118,119]. Interestingly, polymorphisms in the *KALRN* gene have been associated with an increased risk of both cerebrovascular and cardiovascular diseases, including ischemic stroke [120] and coronary artery disease [119,121,122]. Perturbed levels of mitochondrial proteins Dnaja3 and Nubpl may also be an attempt at attenuating inflammation in the TGF cerebrovasculature via (a) decreased Dnaja3-associated dampening of innate immunity [123] and (b) increased Nubpl-associated dampening of ROS production [124]. While little is known about Ankrd24, it can interact with nuclear factor kappa B (NF- κ B) [43], a transcriptional activator of inflammatory mediators. Direct interaction of Ankrd24 with NF- κ B may serve a similar function as the interaction of the stress response protein Ankrd2 with NF- κ B repressor subunit p50, which results in potent repression of inflammatory responses [125]. Interestingly, vasomotor impairment in TGF cerebral arteries was restored by the nonsteroidal anti-inflammatory drug (NSAID) indomethacin and pioglitazone [108], the latter likely via activation of PPAR γ receptors in brain vessels and reduction in NF- κ B and IL-6 levels [126].

4.3.3. Blood Biomarker and Translatability to Humans

In our study, we confirmed the presence of four List 2 proteins (ACTA2, KRT24, TANC1, and IGDCC3) in depleted human plasma and seven List 2 proteins (ACTA2, KALRN, TANC1, ZFYVE26, DDX11, KRT24, and PTPRD) in total plasma EVs. These findings hold significant promise for blood biomarker research, diagnostics, and treatment, particularly in the investigation of vascular brain injury in age-related dementias. Notably, a recent review [127] underscores the emerging role of EVs in blood plasma as biomarkers in age-related dementias, further emphasizing the importance of our results.

5. Conclusions

By performing MS-based vascular proteome profiling in a mouse model of cerebrovascular pathology related to VCID, we identified multiple proteins demonstrating significantly altered levels. Level dysregulation in these proteins points to perturbations in brain vessel vasomotricity, remodeling, and inflammation. We further demonstrated that several of the differentially expressed mouse proteins are (a) expressed in human brain vasculature and (b) found as cargo proteins in EVs from human plasma. Given the growing popularity of EVs in blood as a novel and minimally invasive biomarker discovery platform for age-related dementias, several of the proteins identified in our study using MS can serve as VCID protein biomarkers in humans.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biology12121500/s1>, Figure S1: Relative gene expression of List 2 proteins in public vascular datasets; Figure S2: Examples of extracted ion chromatograms and MS/MS spectra in precleared plasma and depleted plasma; Figure S3: Examples of extracted ion chromatograms and MS/MS spectra in precleared plasma and total plasma EVs; Figure S4: The figure shows the reproducibility between two biological runs of control animals (top panel) and between control and TGFbeta runs (bottom panel). The median relative standard deviation among replicates of control and TGFbeta is 10.1% and 15.7%, respectively and between control and TGFbeta samples is 26%; Table S1: List 1 and List 2 proteins; Table S2: EV markers detected; Table S3: Molecular Functions and Biological Processes of List 2 Proteins in Mice and Human. References [128–130] are cited in the supplementary materials.

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