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## IDENTIFICATION AND EXPRESSION PROFILING OF BLOOD-BRAIN BARRIER MEMBRANE PROTEINS

Nitin Agarwal, Ethan S. Lippmann, and Eric V. Shusta\*

Department of Chemical and Biological Engineering, University of Wisconsin – Madison, Madison, Wisconsin, USA.

### Abstract

Blood-brain barrier (BBB) membrane proteins play crucial roles in the proper functioning of the BBB as well as in disease progression. Previously we developed a novel approach for identifying membrane proteins expressed at the BBB, which we referred to as Multiplex Expression Cloning (MEC). In the current study, the proteome coverage of the MEC approach was expanded to allow the identification of a total of 30 BBB membrane proteins that are diverse in function and abundance. To unveil those membrane proteins that are enriched at the BBB and hence partially responsible for some of its unique characteristics, the transcript abundance levels for all 30 BBB membrane proteins were compared to those found in microvessels derived from lung, liver, heart and kidney. Such quantitative PCR profiling (qPCR) of RNA samples from laser capture microdissected microvessels revealed that the transcripts for 5 membrane proteins, namely Lutheran glycoprotein, carbonic anhydrase IV, uncoupling protein 2 (UCP2), podocalyxin and solute carrier family 38, member 5 (SLC38A5) were BBB selective in that expression was elevated in brain microvessels when compared to all of the vascular beds tested. Many other membrane protein transcripts, while not as BBB-restricted, showed selective expression within subsets of tissues indicating other potential parallels and contrasts between vascular beds in the body. The identification of BBB membrane proteins could help better understand the molecular mechanisms responsible for BBB function and those with selective expression may have utility for BBB-targeted therapies.

### Keywords

Blood-brain barrier; Multiplex expression cloning; membrane proteomics; laser capture microdissection; quantitative PCR

## INTRODUCTION

Membrane proteins play a significant role in defining several functional aspects of the blood-brain barrier (BBB), ranging from its characteristic tight junctions that prevent paracellular transport of molecules to the highly specialized transport systems that regulate the exchange of nutrients and signaling agents across the BBB. Since the BBB only comprises 1/1000 the volume of the brain (Pardridge 2007), proteomic studies aimed at membrane proteomics of the whole brain, such as the ones described by Nielsen *et al.* (Nielsen *et al.* 2005) and Wang *et al.* (Wang *et al.* 2006), although quite comprehensive, typically underrepresent the BBB membrane proteome. To date, there are very few studies

\*To whom correspondence should be addressed: Dr. Eric V. Shusta, Associate Professor, Department of Chemical and Biological Engineering, University of Wisconsin-Madison, 1415 Engineering Drive, Madison, WI 53706, USA, shusta@engr.wisc.edu, Ph: (608) 265-5103, Fax: (608) 262-5434.

that have directly attempted moderate to high throughput analysis of the BBB proteome. Haqqani et al. (Haqqani *et al.* 2007, Haqqani *et al.* 2005) combined laser capture microdissection (LCM) isolation of brain microvessels with quantitative mass spectrometry (MS) to directly characterize the expression levels of 57 and 160 BBB proteins that were differentially expressed in blood vessels subjected to cerebral ischemia. More recently, the identification of 881 different proteins expressed at the mouse brain microvasculature was enabled by MS analysis of lysates obtained from brain microvessels selectively isolated by the use of LCM (Lu *et al.* 2008). In addition, absolute quantification of 34 known membrane resident transporters at the BBB has been performed using MS combined with isotope-labeled peptide standards (Kamiie *et al.* 2008). While these studies have broken ground in terms of BBB proteomics, they were not focused primarily on membrane proteins or as in the latter example, focused only on known BBB transporters. Moreover, use of the aforementioned MS-based methods tends to underrepresent membrane proteins as a result of their relative insolubility and / or low abundance levels *in vivo* (Grant & Wu 2007).

To complement these existing studies and MS-based proteomic techniques, we recently described a novel proteomics approach that is capable of sampling both highly hydrophobic and low abundance membrane proteins (Agarwal & Shusta 2009). Briefly, the approach referred to as Multiplex Expression Cloning (MEC) involves the expression of a BBB cDNA library in a mammalian host cell line where subsequently, with the aid of a BBB membrane protein specific polyclonal antiserum (BMSPA), BBB-resident membrane proteins can be identified. In this study, we have expanded the coverage of the MEC procedure by several technical adaptations leading to the identification of many more BBB membrane proteins. Although important, the identification of these proteins merely serves as a first step towards their characterization. In particular, the expression profile of these proteins in tissues and vascular beds other than the brain could be highly useful in understanding their significance at the BBB during health and disease. Thus, the transcript expression levels of BBB membrane proteins identified using the MEC approach were measured across several vascular beds by combining MEC with LCM and quantitative PCR. In this way, it was discovered that five membrane protein encoding transcripts, Lutheran glycoprotein, carbonic anhydrase IV, uncoupling protein 2 (UCP2), podocalyxin and solute carrier family 38, member 5 (SLC38A5), were selectively expressed at the BBB.

## MATERIALS AND METHODS

### Cell culture

The HEK293 T/17 (human embryonic kidney cell line) cell line was obtained from the American Type Culture Collection (Manassas, VA) and is a clone of the parent HEK293 cell line that has been stably transfected with the SV40 large T-antigen and hence supports efficient episomal replication of SV40-origin containing plasmids. HEK293 T/17 cells were cultured in a humidified incubator at 37°C with 5% carbon dioxide using DMEM media (Gibco, Invitrogen, Carlsbad, CA) buffered with 2.0 g/l sodium bicarbonate and 7.2 g/l HEPES to pH 7.2 and supplemented with 4.5 g/l dextrose and 10% fetal bovine serum (Gibco, Invitrogen).

### Multiplex expression cloning

The generation of the BBB cDNA library and the BBB membrane protein-specific polyclonal antiserum (BMSPA) has been described previously (Agarwal & Shusta 2009, Shusta *et al.* 2002b, Pardridge *et al.* 1990). Enrichment of the BBB cDNA library using the MEC approach was carried out as described previously (Agarwal & Shusta 2009), except for the following modifications (Figure 1). The clone recovery process after FACS isolation was greatly improved by switching the host cell line from HEK293 to HEK293 T/17. Due to

constitutive expression of the SV40 large T-antigen, the HEK293 T/17 cells allow the SV40 origin containing plasmids from the BBB cDNA library to replicate to greater numbers thus facilitating their recovery from cells. Additionally, the transfected cells were permeabilized by the use of 0.5% saponin in PBS prior to immunolabeling to allow the BMSPA to access intracellular components of the cell membrane (Figure 1, path 2). The preparation and sequencing of the cDNA pools and individual cDNA clones was conducted as described previously (Agarwal & Shusta 2009). The BMSPA was incubated with an untransfected HEK293 T/17 cell monolayer at 4°C for 1 hour to remove any background antigenicity prior to immunolabeling.

### **Generation of tissue acetone powders, BMSPA subtraction and Western blotting**

Fresh bovine lung, liver, kidney and heart tissue were obtained from a local meat processor. Tissues were cut into small pieces which were then homogenized using a Polytron tissue homogenizer (PowerGen 125, Fisher Scientific, Pittsburgh, PA). Ten volumes of ice cold acetone were added to the homogenized tissue and the precipitate was collected by filtering through a Whatman filter paper (Fisher Scientific). The semi-dry precipitate was blended again with the same volume of ice cold acetone used in the previous step and filtered again. The precipitate formed a cake which was pulverized with a mortar and pestle, spread out on a surface and left to dry overnight at room temperature. The dried powder was further pulverized the next day and stored at -20°C. The BMSPA was subtracted by the acetone powders by incubating it at a dilution of 1:150 in 10 mL PBSG (40 % goat serum in 0.01 M PBS, pH 7.4) with 0.5 g of acetone powder of the appropriate tissue for 1 hour at 37°C in a shaker. For subtraction by several tissues, this process was serially repeated for each individual tissue powder. Finally the subtracted BMSPA was filter sterilized and stored at 4°C for later use. Western blotting under reducing conditions was performed as described previously (Shusta et al. 2002b).

### **Protein expression profiling using tissue-subtracted BMSPA antisera**

HEK293 T/17 cells were transfected with individual membrane encoding cDNA clones using Lipofectamine 2000 per the manufacturer's protocol (Invitrogen). The transfection mix was replaced with fresh media 24 hours after the transfection step. At 60 hours post-transfection, the culture medium was aspirated and the transfected monolayers were washed once with PBSF (0.01 M PBS, pH 7.4 supplemented with 10% fetal bovine serum). The cells were then blocked for 30 minutes with PBSG at 4°C, washed once in PBSF and incubated for 1 hour at 4°C in HEK293 T/17-subtracted BMSPA or tissue-subtracted BMSPA, as appropriate, diluted 1:500 in PBSG. The cells were then washed twice in PBSF, incubated for 30 minutes in HEK293 T/17-depleted phycoerythrin (PE) conjugated anti-rabbit IgG (Sigma Aldrich) diluted 1:200 in PBSG at 4°C and then washed twice with PBSF. The cells were subsequently detached from the plates via gentle pipetting and subjected to flow cytometric analyses. Only live cells capable of excluding the nuclear stain, propidium iodide, were analyzed. A BD FACSCalibur instrument equipped with a 488 nm laser for excitation and a 670 LP filter for detection of Propidium Iodide and a 585/42 nm filter for detection of the PE signal was used for flow cytometry analyses. The mean fluorescence intensity of the immunolabeled cells was measured.

### **Preparation of tissue sections**

In order to preserve the integrity of mRNA, tissue specimens from bovine brain, lung, liver, kidney and heart were freshly isolated and immediately frozen by embedding in a cryomold using Tissue-Tek O.C.T Compound (Sakura Finetek, Zoeterwoude, The Netherlands), which was subsequently floated in a bath of isopentane (Sigma Aldrich, St. Louis, MO) cooled by liquid nitrogen. The tissue sections were handled using RNase free techniques. Seven micron thick tissue sections were generated using a Microm HM505E (Microm GmbH,

Walldorf, Germany) and placed onto MMI cell cut slides (Molecular machines & industries inc., Rockledge, FL) in an RNase free environment. The slides were stored at  $-70^{\circ}\text{C}$  until LCM was performed.

### Laser capture microdissection of blood vessels and RNA recovery

Tissue sections were removed from  $-70^{\circ}\text{C}$  storage and thawed at room temperature for 1 minute. All subsequent labeling steps were conducted in RNase free conditions using RNase free reagents at room temperature. The tissue section was fixed in 100% ethanol for 1 minute, air dried for 3 minutes, incubated in 50  $\mu\text{g}/\text{ml}$  of fluorescein-labeled *Griffonia simplicifolia agglutinin-I-B4* (Vector Laboratories, Burlingame, CA) for 10 minutes, washed three times in RNase free water, and dried for 5 minutes in a dessicator. The sample was then dehydrated by two 30 second incubations in 95% ethanol followed by another two in 100% ethanol and finally a 3 minute incubation in 100% isopropyl alcohol. The tissue section was air dried for 2 minutes and then placed in a dessicator for 10 minutes to remove residual moisture. The lectin labeled blood vessels were cut out from the sections and captured on an adhesive cap using the MMI Cell Cut instrument (Molecular Machines & Industries Inc.). The isolation was restricted to microvessels ( $\sim 10$  microns or less in size) while large vessels as well as other agglomerates such as the glomeruli in the kidney were avoided to prevent enrichment of other cell types such as epithelial cells. Each cap could accommodate 200–300 blood vessels and a minimum of 3 caps were obtained for each tissue in order to have a sufficient amount of RNA. Total RNA was extracted from the microdissected blood vessels with the RNeasy micro kit (Qiagen, Valencia, CA) using the manufacturer's protocol for purification of total RNA from microdissected cryosections. Carrier RNA was not used to avoid issues related to RNA amplification. RNA was also extracted from whole tissue sections by simply dissolving the section in the lysis buffer and following the same protocol used for the microdissected samples. Additionally, the RNA samples were subjected to a DNase I digestion during the extraction protocol to remove any contaminating genomic DNA. The recovered RNA was subsequently amplified using the Quantitect whole transcriptome kit (Qiagen) as per the manufacturer's recommendation for a high yield reaction (8 hours of amplification time). This kit utilizes the multiple displacement amplification technology and yields amplified cDNA. After amplification, the cDNA yields were about 0.8–1  $\mu\text{g}/\mu\text{l}$  based on optical density readings at 260 nm.

### Quantitative PCR

Quantitative PCR was carried out using a Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA) and the Quantitect SYBR Green PCR kit (Qiagen) following the manufacturer's protocol. 1–2 ng of cDNA (1:250 dilution of amplified samples) was used as the template per reaction. Gene-specific primers were designed using PerlPrimer v1.1.14 (<http://perlprimer.sourceforge.net/>) and the oligo design and analysis tools on the website of Integrated DNA technologies (<http://www.idtdna.com>). The primer sequences are listed in Supplemental Table 1. Whenever possible, the primers were designed to span intron-exon boundaries. Gene expression levels were measured by calculating the delta-delta cycle threshold values ( $\Delta\Delta C_t$ ), which represent the difference in gene expression levels in terms of PCR cycles.  $C_t$  represents the PCR cycle at which the fluorescence from the PCR product reaches a defined threshold value, which was selected at a point where the amplification process was exponential in nature.  $\Delta C_t$  represents normalization of the gene expression to a control gene such as  $\beta$ -actin. Thus  $\Delta C_t = C_{t,\beta\text{-actin}} - C_{t,\text{gene}}$ .  $\Delta\Delta C_t$  represents the normalized difference in gene expression levels between the sample and a control ( $\Delta\Delta C_t = \Delta C_{t,\text{sample}} - \Delta C_{t,\text{control}}$ ). Two genes were initially tested as the reference gene, namely  $\beta$ -actin and the 60S acidic large ribosomal protein (RPLP0) and it was found that the  $\Delta\Delta C_t$  values obtained within and across tissues were similar for both these genes. Hence all of the remaining  $\Delta\Delta C_t$

values were calculated using just the  $\beta$ -actin gene as the reference. Approximate fold differences in Table 2 are reported as  $2^{\Delta\Delta Ct}$ .

## RESULTS

### Multiplex Expression Cloning (MEC) of BBB Membrane Proteins

As described previously, the MEC approach involves the enrichment of a bovine BBB cDNA library for those clones that encode membrane proteins by using a BBB membrane protein-specific polyclonal antiserum (BMSPA) (Figure 1) (Agarwal & Shusta 2009). Briefly, the cDNA library is transfected into the HEK293 T/17 mammalian host cell line for membrane protein expression. HEK293 T/17 cells were chosen as they are unlikely to naturally express BBB-selective membrane proteins and hence can be used to expression clone these proteins from a cDNA library. Subsequently, the transfected HEK293 cells are probed with the BMSPA that was raised against membrane preparations of bovine brain microvessels and hence specifically recognizes a panel of BBB membrane proteins (Figure 2) (Agarwal & Shusta 2009, Partridge et al. 1990). Cells expressing BBB membrane proteins on the cell surface are thus tagged by the BMSPA and isolated via fluorescence activated cell sorting (FACS) (Figure 1, path 1). Subsequently the plasmid cDNA harbored by the BMSPA-labeled cells is recovered, pooled together and amplified in an *E. coli* host. This completes one round of enrichment for those cDNA clones that encode for BBB membrane proteins. Once the plasmid pool has been sufficiently enriched for membrane protein-encoding clones after several rounds of MEC, the clones can then be individually analyzed and their sequences determined.

In the current study we extended the MEC approach in three significant ways. First, we sampled about 5–10 million transfected cells in each round to oversample the diversity of the BBB cDNA library and give a higher probability for identification of rare membrane protein-encoding cDNA clones. Next, to allow the sampling of intracellularly-localized membrane-associated proteins or intracellular epitopes of integral membrane proteins that may be recognized by the BMSPA, the HEK293 cells were permeabilized with 0.5% saponin solution prior to immunolabeling with the BMSPA (Figure 1, path 2.). A total of 304 cDNA clones obtained after the fourth round were individually analyzed by flow cytometry, yielding 27 cDNA clones whose membrane protein products were specifically recognized by the BMSPA. Sequencing of the 27 cDNA clones revealed 15 nonredundant sequences of BBB membrane proteins (Table 1). Finally, in addition to identifying membrane proteins that are generally expressed at the BBB using paths 1 (Agarwal & Shusta 2009) and 2 (this study) (Figure 1), another key goal of the current study was to adapt the MEC approach for identification of those membrane proteins that possess a certain level of specificity to the BBB. Accordingly, the BMSPA was serially “subtracted” by adsorption against acetone powders of bovine lung, liver, kidney and heart to remove antigenicity against those proteins that are commonly expressed both at the BBB and these tissues (Figure 1, path 3). In principle, the subtraction renders the BMSPA brain-specific with respect to these organs. The acetone powder adsorption approach was validated by the Western blot of tissue lysates in Figure 2, which reveals that while the unsubtracted BMSPA recognizes several proteins in the tissue lysates of lung, liver, kidney and heart, the ‘brain-specific’ BMSPA demonstrates selectivity to several proteins present at the BBB. Four rounds of MEC utilizing the ‘brain-specific’ BMSPA (Figure 1, path 2) were conducted and 186 clones obtained after the final enrichment round were individually analyzed. Of these, 40 clones were found to encode for BBB membrane proteins representing 7 nonredundant clones. Of these 7 clones, Lutheran glycoprotein, RPLP1 and PODXL had already been identified by the unsubtracted BMSPA, whereas ARHGAP21, RPL10, LOC613429 and OCIAD1 were newly identified (Table 1). Table 1 also lists the nature of the membrane-association of these proteins, transcript abundance levels *in vivo* as determined in a previous BBB genomic study



(Enerson & Drewes 2006) and a list of previous reports in the literature that have identified these proteins to be expressed at the BBB. Most notably, previous MS analyses have only identified 2 of the 30 BBB membrane proteins (Haqqani et al. 2007, Haqqani et al. 2005, Lu et al. 2008) listed in Table 1, thus highlighting the complementary capability of the MEC approach to effectively sample those membrane proteins that are not particularly accessible to mass spectrometry.

### Expression Profiling of BBB Membrane Proteins Across Other Tissues

Next, to better understand the tissue distribution of these BBB membrane proteins, two different approaches were employed. First, the comparative protein expression profiles of individual BBB membrane proteins across whole tissues were evaluated by using single-organ subtracted BMSPA antisera as depicted in Figure 3A. If a BBB membrane protein that is expressed by the transfected cells is also present on another tissue, for example lung, it will be recognized by the unsubtracted BMSPA but will display a reduction in labeling when probed with the lung-subtracted version of BMSPA. Thus, by probing cells transfected with the cDNA of an individual BBB membrane protein with lung, liver, kidney or heart-subtracted BMSPA, one can generate an approximate expression profile for that protein. The bar graph in Figure 3B depicts the results of this tissue-based protein expression profiling approach for two representative genes, namely Lutheran glycoprotein and carbonic anhydrase IV (CAIV). As shown, there is a significant depletion in the antigenicity of the lung-subtracted and kidney-subtracted BMSPA for CAIV, indicating that CAIV is also expressed at lung and kidney in addition to the BBB. In contrast, Lutheran glycoprotein appears to be quite BBB-specific as no organ subtraction was capable of depleting the antigenic signal. In this way, qualitative tissue expression profiles were generated for 9 BBB membrane proteins as noted in Figure 3C. Notably, this analysis revealed that CD46, MHC1 and UCP2 were widely expressed across all tissues considered, whereas the expression of the Lutheran glycoprotein was more restricted to the brain. RACK1 and ITM2B were expressed in the brain and the liver, whereas LAMP2 had an elevated expression at the lung in addition to brain. FIH1-like protein was expressed in all organs tested except kidney.

Although this approach provides qualitative expression profiles for membrane proteins across *whole tissues* (as a result of the adsorption of antisera with *whole tissue* acetone powders), it does not reveal any information about the expression levels of these proteins within the *microvasculature* of these tissues. Moreover, statistically significant differences in antibody labeling upon depletion could only be distinguished for those BBB membrane proteins that display a high level of basal labeling with the unsubtracted antiserum as a result of the associated errors of the experiment. Hence this approach was limited to 9 of the 30 BBB membrane proteins identified in Table 1. Accordingly, we used a second, more quantitative approach and measured the relative abundance of the BBB membrane protein transcripts in the *microvasculature* of the peripheral tissues. As shown in Figure 4A, the microvessels in bovine tissue sections of brain, lung, liver, kidney and heart were fluorescently labeled using the endothelial binding lectin, *Griffonia simplicifolia agglutinin-I-B4*. Isolation of individual microvessels was then performed using laser capture microdissection (LCM) (Figure 4B). Total RNA was recovered from the microdissected blood microvessels and amplified in order to facilitate the measurement of transcript abundance levels via quantitative PCR analyses (Figure 4B). The LCM process led to selective isolation of blood vessels as indicated by substantial enrichment in endothelial markers von Willibrand factor (vWF) and PECAM1 in the microdissected samples compared to total tissue isolates (supplemental Figure 1B). To ensure that the RNA amplification step did not introduce bias, transcript abundance levels of amplified and unamplified RNA samples were compared. There was no difference ( $p > 0.1$ ) in the values obtained for amplified and unamplified samples either for endothelial markers, vWF and

PECAM1 (supplemental Figure 1A). Importantly, while a BBB marker, glucose transporter (GLUT1), was enriched in the BBB RNA isolates, transcripts from potential contaminating cells like neuron specific enolase (NSE, neurons) and glial fibrillary acidic protein (GFAP, astrocytes) are both decreased in the microvessel RNA isolates indicating the selective enrichment of BBB-derived transcripts (supplemental Figure 1A).

Using these LCM-derived isolates, the relative transcript abundance values for all 30 membrane proteins were measured and BBB expression compared to lung, liver, kidney and heart microvascular expression (Table 2). While transcripts for all membrane proteins were detected at the BBB as expected given the nature of the MEC approach that relies on membrane protein expression at the BBB, differential transcript expression profiles for these proteins across tissues were common. For example, SCP2 is more highly enriched in blood vessels of liver and heart compared with the BBB (Figure 5). Other proteins, such as MHC1 display similar expression levels across each vascular bed whereas transcripts encoding proteins such as ARHGAP21 are BBB enriched only when compared to certain vascular beds in the body like lung and liver. Notably, of the BBB membrane proteins listed in Table 1, Lutheran glycoprotein, CAIV, UCP2, PODXL and SLC38A5 all display elevated expression levels at the BBB as compared to all other vascular beds tested indicating a high level of BBB exclusivity for these membrane proteins (compare to BBB-resident GLUT-1 in Figure 5). Indicative of the vascular origin within the brain, each of these five transcripts was also enriched in the BBB isolates compared with total brain whereas non-vascular transcripts were decreased (compare to BBB-resident GLUT1 and contrast to non-resident transcripts NSE and GFAP in Supplemental Figure 1A).

## DISCUSSION

In the current study we have employed several variations of MEC to clone BBB membrane proteins. The process required *protein* presence in isolated BBB membranes used to raise the BMSPA, *transcript* presence in cDNA libraries derived from freshly isolated microvessels, and *transcript* presence in LCM-derived RNA samples. Thus, several redundant yet distinct approaches indicated the presence of these membrane proteins at the BBB. Indeed, several of the identified membrane proteins have been localized to the BBB by targeted experiments or generalized genomic studies. This study now confirms the presence of such proteins at the BBB while also providing new information regarding BBB membrane proteins that have not been previously ascribed to the BBB (Table 1).

A particular focus of this study was an attempt to identify membrane proteins with highly selective expression at the BBB to help elucidate the unique functional attributes of the BBB membrane proteome. First, using whole tissue antibody subtraction in the MEC process to help identify BBB-selective membrane proteins, Lutheran glycoprotein and podocalyxin were amongst the proteins identified. Further analysis using single-organ subtracted antiserum confirmed that Lutheran glycoprotein was highly specific to the BBB. While this approach of whole tissue antiserum subtraction may yield clones having BBB-selective expression like Lutheran glycoprotein, it will miss potentially BBB vascular-specific clones because while they may be expressed in a certain tissue (and hence subtracted), they may not be of vascular origin. For example, CD46 has a significant expression level in all the tissues tested (Figure 3B). However, the widespread expression of CD46 is not only vascular in origin (Perez de la Lastra *et al.* 1999) but also includes localization at the epithelium of lung and kidney (McQuaid & Cosby 2002). Therefore, to better compare expression of the membrane proteins on a vascular basis, we generated a quantitative transcript profile across 5 vascular beds, and vascular-enriched BBB membrane proteins were identified. In particular those membrane proteins that are specifically expressed at the BBB when compared to other vascular beds are of interest since they may contribute to the unique

properties of the BBB. Twenty-eight of thirty proteins were differentially expressed at the BBB versus at least one of the peripheral vascular beds indicating a unique “fingerprint” for membrane protein expression at the BBB.

Of special interest are the five BBB membrane proteins that have transcripts with highly specific BBB expression compared with all vascular beds tested. Namely, Lutheran glycoprotein, carbonic anhydrase IV, podocalyxin, uncoupling protein 2 (UCP2) and the amino acid transporter SLC38A5 are all preferentially expressed at the BBB with comparative elevation in transcript levels from 4–10<sup>5</sup> fold. The Lutheran glycoprotein is involved in the attachment of endothelial cells to laminin present in the basement membrane (Udani *et al.* 1998). Previous studies have confirmed the BBB-enrichment of the Lutheran glycoprotein compared with liver and kidney tissues (Shusta *et al.* 2002b) and it is upregulated in human brain tumor vessels (Boado *et al.* 2000). Carbonic anhydrase IV is a GPI-anchored protein that has been reported to be BBB-resident and localized to the luminal endothelial surface suggesting an important role in regulating the carbon dioxide-bicarbonate balance in the brain (Ghandour *et al.* 1992). PODXL is expressed in vascular endothelial cells throughout the body including in the brain (Testa *et al.* 2009, Horvat *et al.* 1986), glomerular podocytes (Kerjaschki *et al.* 1984), platelets and megakaryocytes (Miettinen *et al.* 1999). Interestingly, the podocalyxin protein acts both as an anti-adhesin in its role in keeping open the glomerular slits in podocyte foot processes in the kidney (Dekan *et al.* 1991), but has also been shown to act as an adhesin as a ligand for L-selectin and can support lymphocyte adhesion and rolling (Sasseti *et al.* 1998). Moreover, podocalyxin, when overexpressed ectopically in MDCK epithelial cells can disrupt tight junctions and relocalize tight junction proteins (Takeda *et al.* 2000). Our current results indicate the BBB-enrichment of PODXL, a finding also suggested in our previous suppression subtractive hybridization (SSH) genomics study (Shusta *et al.* 2002a). PODXL has also been shown to be overexpressed in proliferating endothelial cells found around glioblastomas indicating potential clinical importance for this membrane protein (Hayatsu *et al.* 2008). While its function at the BBB is unclear, the preferential expression of podocalyxin at the BBB suggests an important role. UCP2 is a mitochondrial uncoupling protein expressed in the brain that has been largely studied in terms of neuronal function (Horvath *et al.* 1999, Andrews *et al.* 2005). Interestingly, it has been shown that UCP2 helps maintain a low level of reactive oxygen species (ROS) thereby preventing oxidative stress to cells (Andrews *et al.* 2005), and as a result has been shown to serve as a neuroprotector in traumatic brain injury and ischemia (reviewed in (Paradis *et al.* 2003)). Thus, the cloning of UCP2 at the BBB and its moderate selectivity compared with peripheral vascular beds suggests a potentially intriguing role for the BBB in brain stress response. Finally, the most BBB-selective membrane protein identified was the amino acid transporter SLC38A5, a carrier of glutamine, asparagine, and histidine, among other amino acids (Nakanishi *et al.* 2001). A 2.2 kb transcript of SLC38A5 is abundant in human brain and lung, whereas a larger 2.6 kb transcript is expressed in liver and in kidney (Nakanishi *et al.* 2001). In the brain, SLC38A5 has been shown to be present in astrocyte processes (Cubelos *et al.* 2005), and very recently the transcript was shown to be one of the most highly expressed in freshly isolated and purified brain endothelial cells (Lyck *et al.* 2009). Our data confirms the expression of this membrane protein at the BBB and indicates the extremely restrictive expression when compared to the peripheral vasculature indicating a potential BBB biomarker. In conclusion, the BBB serves as a critical interface between the brain and the rest of the body. As a result, BBB membrane proteins play important roles in facilitating the exchange of material between the blood and brain, and the data presented in this study begin to unearth the membrane protein heterogeneity between the BBB and peripheral vascular beds.



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### List of abbreviations

<b>BBB</b>	Blood-brain barrier
<b>BMSPA</b>	BBB membrane protein-specific polyclonal antiserum
<b>FACS</b>	Fluorescence activated cell sorting
<b>MEC</b>	Multiplex expression cloning
<b>SV40</b>	Simian Virus 40
<b>LCM</b>	Laser capture microdissection

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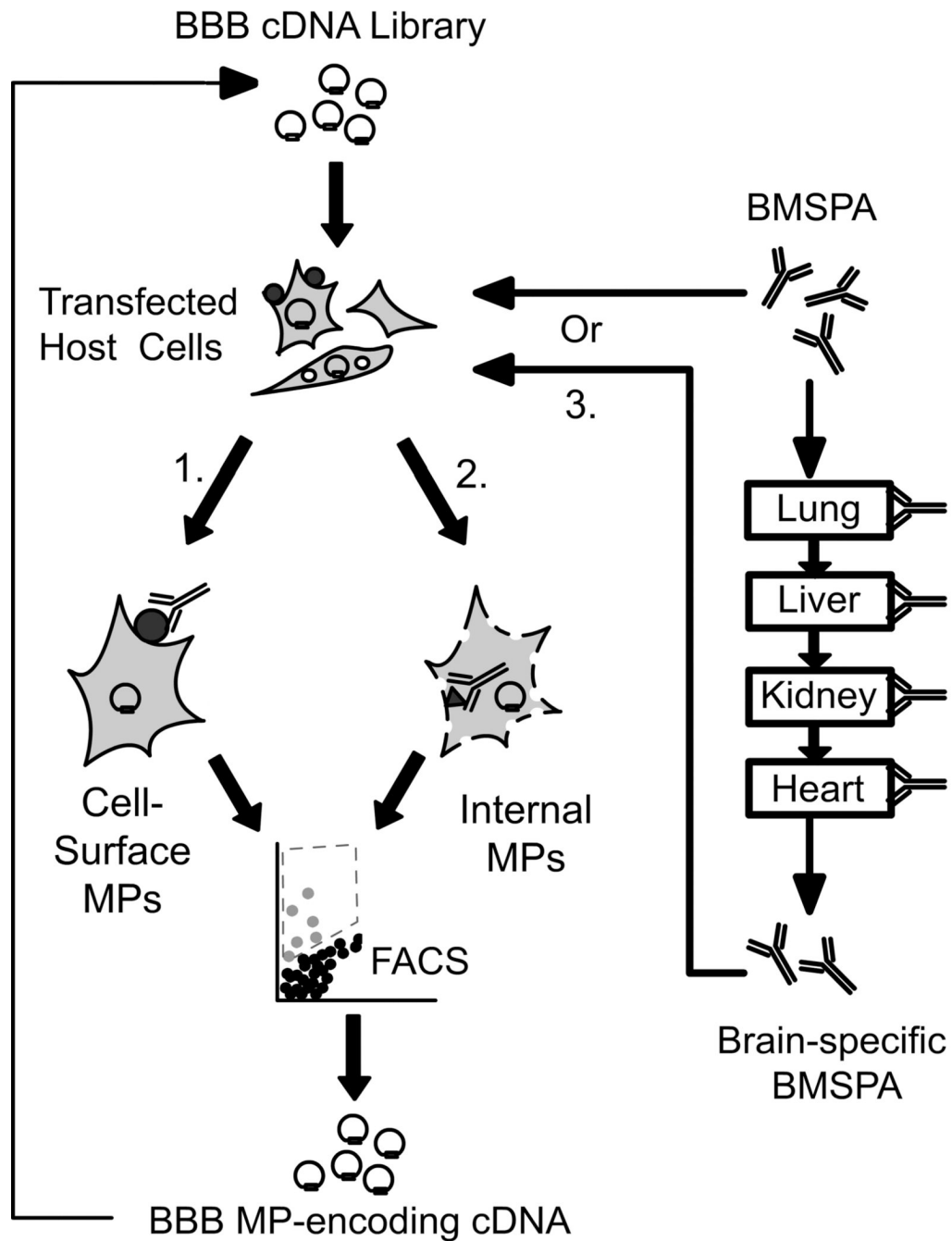
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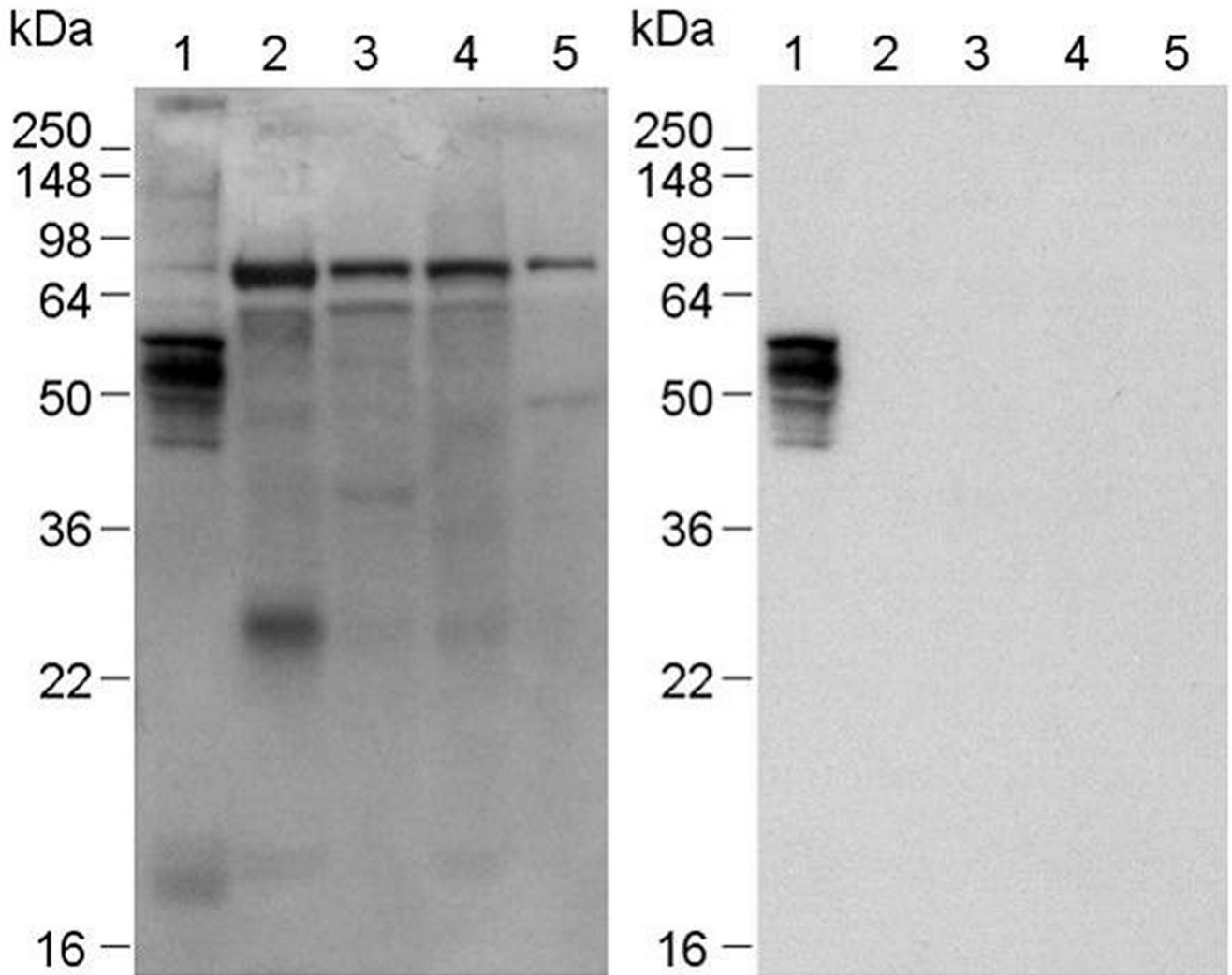
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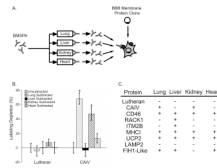
**Figure 1.** Schematic of multiplex expression cloning approaches. Host HEK293 T/17 cells are transfected with the BBB cDNA library and then probed with either the unsubtracted BMSPA or the ‘brain-specific’ BMSPA to identify extracellular (1. Unpermeabilized cells; antibody cannot access interior cellular compartments) and intracellular (2. Saponin permeabilized cells allowing intracellular access to the antibody) BBB membrane protein epitopes. The ‘brain-specific’ BMSPA (3.) was generated by serial subtraction against acetone powders of lung, liver, kidney and heart as shown to the right and used with saponin permeabilized cells (path 2.). The target cells are isolated via fluorescence activated cell sorting (FACS) and the plasmid they harbor is recovered. The process is repeated until the

pool is sufficiently enriched for cDNA that encode BBB membrane proteins (usually 4 rounds).



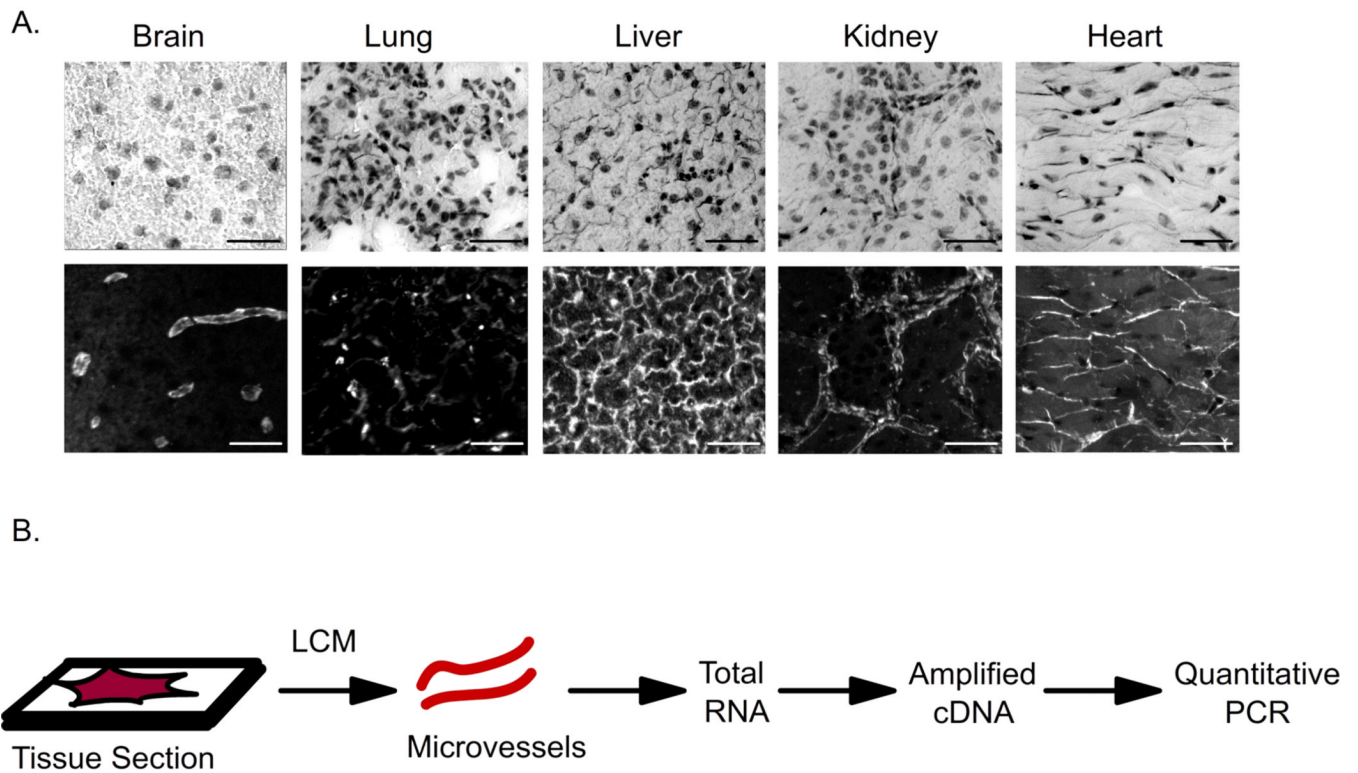


**Figure 2.** Antigens recognized by BMSPA. Western blot of bovine tissue lysates separated by reducing SDS-PAGE and stained with the un-subtracted BMSPA (left panel) or with the 'brain-specific' subtracted version of BMSPA (right panel). Lane 1: Brain microvessels, lane 2: total lung, lane 3: total liver, lane 4: total kidney and lane 5: total heart lysates. (Note: The blot exposure times for the panels were selected so that the intensity of the bands in lane 1 of each panel was comparable)

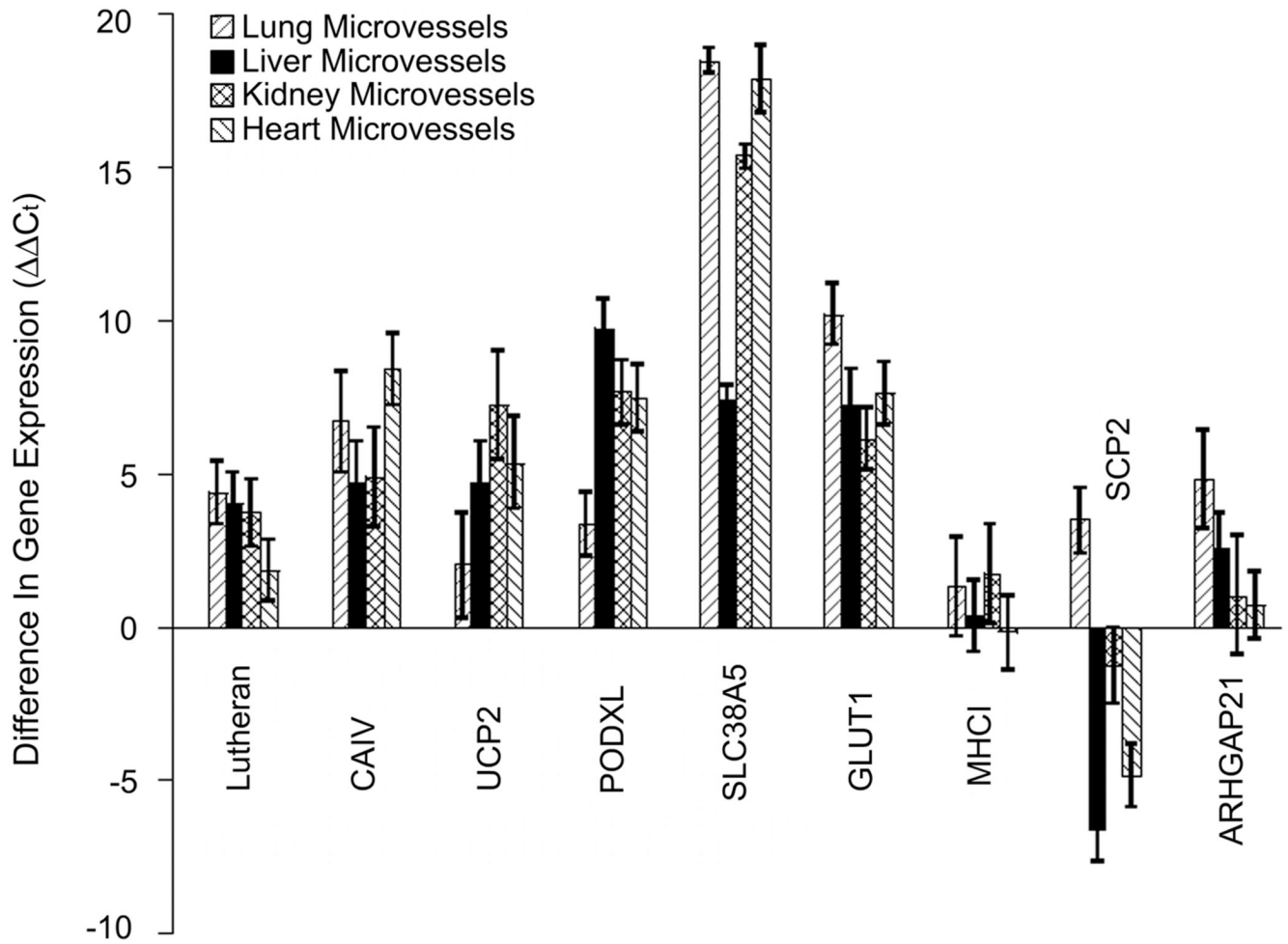


**Figure 3.**

Protein expression profiling across tissues. A. The BMSPA was subtracted by adsorption with acetone powders of lung, liver, kidney or heart to generate single tissue-subtracted versions of BMSPA, which were subsequently used to probe HEK293/T17 cells transfected with individual BBB membrane protein clones. B. Percent depletion in the cell surface immunolabeling intensity for HEK293/T17 cells transfected with individual cDNA clones. Cells were probed with single tissue-subtracted versions of BMSPA and immunolabeling intensity was measured using flow cytometry. Error bars denote standard deviation for 2 independent samples. C. Protein expression profiles across tissues based on percent depletion. A '+' sign indicates that the protein is "expressed" in the tissue indicated (vascular, avascular or both) in addition to the BBB since there is a statistically significant depletion in the level of subtracted BMSPA labeling as determined by a two-tailed student's t-test (100% in Figure 3B,  $p < 0.05$ , also indicated with a \*), whereas a '-' sign indicates negligible depletion suggesting that the protein is "not expressed" in the tissue or expression is at too low of a level to yield a detectable depletion in antiserum binding (0% in Figure 3B).



**Figure 4.** Microvessel processing for LCM and qPCR. A. Phase contrast image of hematoxylin-stained tissue sections (top) with the corresponding fluorescence image displaying fluorescent labeling of endothelial cells using a fluorescein-linked *Griffonia simplicifolia agglutinin-I-B4* lectin (bottom). B. The fluorescently labeled microvessels were subsequently excised using laser capture microdissection (LCM) as shown and the total RNA was extracted from these samples, amplified and subsequently used for transcript abundance profiling. (Scale bar = 20  $\mu$ m)



**Figure 5.** Expression profiles for selected genes from Table 2 comparing transcript abundance at the BBB when compared to microvessels in the tissue indicated. Positive  $\Delta\Delta C_t$  values represent enrichment of the gene at the BBB when compared to microvessels in the tissue indicated. Error bars denote standard deviation for duplicate PCR reactions of two independent PCR runs.

Table 1

BBB Membrane proteins identified using the MEC approach.

Symbol	Protein Name	Genbank <sup>a</sup>	Location <sup>b</sup>	Transcript Abundance <sup>c</sup>	BBB <sup>d</sup>
CD46	Membrane cofactor protein	NM_183080.1	TM	N.D.	(Shusta <i>et al.</i> 2002c)
CAIV	Carbonic anhydrase IV	NM_173897	GPI	4	(Enerson & Drewes 2006, Ghandour <i>et al.</i> 1992)
RACK1	Receptor for activated C-kinase	BC102286	MPA	2	(Enerson & Drewes 2006)
LAMP2	Lysosomal associated membrane protein 2	BC102819	TM	9	(Enerson & Drewes 2006)
LU <sup>e</sup>	Lutheran glycoprotein (Auberger B antigen included)	NM_174741	TM	4	(Shusta <i>et al.</i> 2002b, Enerson & Drewes 2006)
SPARC	Secreted protein acidic cysteine rich / osteonectin	BT030548	ECM	295	(Haqqani <i>et al.</i> 2007, Enerson & Drewes 2006, Pen <i>et al.</i> 2007)
ITM2B	Integral membrane protein 2B	XM_587730	TM	29	(Enerson & Drewes 2006, Calabria & Shusta 2008)
MHCI	Major histocompatibility complex class I, A	A Y960156	TM	4	(Pardridge 2007, Enerson & Drewes 2006, Shusta 2005)
SCAMP1	Secretory carrier membrane protein 1	NM_001076054	TM	N.D.	
UCP2	Mitochondrial uncoupling protein 2	NM_001033611	TM	4	(Enerson & Drewes 2006)
SVIL	Supervillin	BC113265	MPA	N.D.	
RPLP1 <sup>e</sup>	Ribosomal acidic protein large P1	BC102695	MA	116	(Enerson & Drewes 2006)
ND4	Mitochondrial NADH dehydrogenase subunit 4	YP_209214	TM	108	(Enerson & Drewes 2006)
CTNNA1	$\alpha$ -catenin related protein	XM_872743	MPA	3	(Enerson & Drewes 2006)
P85 $\alpha$	Phosphatidylinositol 3-kinase regulatory subunit 1	NM_174575	MPA	N.D.	
SDF1	Stromal cell derived factor 1	NM_001113174.1	MPA / MA	N.D.	(McCandless <i>et al.</i> 2008)
NDFI1 <sup>f</sup>	Nedd4 Family interacting protein 1	NW_001504192.1	TM	N.D.	
CHDI1	Chitinase Domain Containing 1	NM_001015515.1	MPA	N.D.	
SLC38A5	Solute carrier family 38, member 5	NM_001015580.1	TM	8	(Enerson & Drewes 2006, Lyck <i>et al.</i> 2009)
LMNA <sup>g</sup>	Lamin A/C	NW_001494724.1	MA	10	(Lu <i>et al.</i> 2008, Enerson & Drewes 2006)
DSTN	Destrin (Actin Depolymerizing Factor)	NM_001015586.1	MPA	20	(Enerson & Drewes 2006)
WAVE2	WAS protein family, member 2	NW_001494698.2	MPA	2	(Enerson & Drewes 2006)
PODXL <sup>e</sup>	Podocalyxin like protein	XM_868371.3	TM	12	(Enerson & Drewes 2006, Shusta <i>et al.</i> 2002a, Testa <i>et al.</i> 2009)
RIP11 <sup>f</sup>	RAB11 family interacting protein 5 (class I)	XM_616110.3	MA	N.D.	
FIH1-like	Similar to factor inhibiting hypoxia-inducible factor subunit $\alpha$	NW_001494359.2	?	N.D.	
SCP2	Sterol Carrier Protein 2	NM_001033990.2	MA	20	(Enerson & Drewes 2006)
ARHGAP21 <sup>f,e</sup>	Rho GTPase activating protein 21	XM_581232.3	MPA / MA	28	(Enerson & Drewes 2006)



Symbol	Protein Name	Genbank <sup>a</sup>	Location <sup>b</sup>	Transcript Abundance <sup>c</sup>	BBB <sup>d</sup>
RPL10, <i>f,e</i>	Ribosomal protein L10	NM_174760.2	MA	8	(Enerson & Drewes 2006)
LOC613429 <sup>e</sup>	Hypothetical protein LOC613429	BC103023.1	TM	N.D.	
OCTAD1 <sup>e</sup>	OCTAD1 Domain containing 1	NM_001015648.1	TM	N.D.	

<sup>a</sup> Accession numbers from a BLAST search against the nucleotide collection database (nr/nt) for *Bos taurus* derived sequences – underlined entries refer to the *Bos taurus* genomic sequence and represent novel mRNA transcripts, that were named based on homology to the corresponding protein from other mammals;

<sup>b</sup> TM-transmembrane, MPA-Membrane protein associated, MA-Membrane associated, ECM-Extracellular matrix, GPI-glycosylphosphatidylinositol anchored, ?-Not yet known;

<sup>c</sup> Transcript abundance reported as tags per 100,000, N.D. = Not detected [from (Enerson & Drewes 2006), GSE3696 record [GEO; NCBI]];

<sup>d</sup> References indicating BBB localization;

<sup>e</sup> Proteins identified to be BBB-enriched by MEC using 'brain-specific' BMSPA;

<sup>f</sup> Proteins identified by MEC that require cell membrane permeabilization by saponin for detection (intracellular epitope). The first 15 entries in this table were identified by our initial study using path 1 (Agarwal & Shusta 2009), many of which were again identified in the MEC processes performed in this study, the next 15 clones were newly identified in this study using path 2 (using either the unsubtracted or subtracted BMSPA).

Table 2

Gene expression profiles of BBB versus other microvasculatures.

Gene	Lung	Liver	Kidney	Heart
Lutheran <sup>a</sup>	4.4 ± 1.0 (21)	4.0 ± 1.0 (16)	3.7 ± 1.1 (13)	1.9 ± 1.0 (4)
CAIV <sup>a</sup>	6.7 ± 1.7 (104)	4.7 ± 1.4 (26)	4.9 ± 1.6 (30)	8.4 ± 1.2 (338)
CD46	4.5 ± 1.9 (23)	2.2 ± 1.1 (5)	N.D.	2.5 ± 1.2 (6)
RACK1	N.D.	-1.5 ± 1.3 (-3)	N.D.	-2.2 ± 1.8 (-5)
ITM2B	3.5 ± 1.5 (11)	N.D.	1.6 ± 1.2 (3)	N.D.
MHCI	N.D.	N.D.	1.7 ± 1.6 (3)	N.D.
UCP2	2.0 ± 1.8 (4)	4.7 ± 1.4 (26)	7.3 ± 1.8 (158)	5.4 ± 1.5 (42)
LAMP2	3.5 ± 1.4 (11)	-1.7 ± 1.2 (-3)	1.8 ± 1.3 (3)	N.D.
FIH1-like	2.4 ± 1.6 (5)	N.D.	N.D.	-4.1 ± 1.1 (-17)
RPLP1	2.8 ± 1.3 (7)	-2.8 ± 1.2 (-7)	N.D.	-3.3 ± 1.4 (-10)
ARHGAP21	4.8 ± 1.6 (28)	2.6 ± 1.2 (6)	N.D.	N.D.
SCP2	3.5 ± 1.0 (11)	-6.6 ± 1.1 (-97)	-1.3 ± 1.2 (-2)	-4.9 ± 1.0 (-30)
LOC613429	4.0 ± 1.1 (16)	N.D.	2.3 ± 1.1 (5)	N.D.
PODXL <sup>a</sup>	3.4 ± 1.0 (11)	9.7 ± 1.0 (832)	7.7 ± 1.1 (208)	7.5 ± 1.1 (181)
OCIAD1	5.7 ± 1.0 (52)	N.D.	N.D.	N.D.
SPARC	2.5 ± 1.2 (6)	3.5 ± 1.2 (11)	1.9 ± 1.1 (4)	1.3 ± 1.3 (2)
SCAMP1	5.0 ± 1.4 (32)	2.2 ± 1.1 (5)	N.D.	3.3 ± 1.1 (10)
SVIL	2.3 ± 1.1 (5)	N.D.	N.D.	-2.9 ± 1.2 (-7)
ND4	4.7 ± 1.2 (26)	-1.6 ± 1.2 (-3)	1.4 ± 1.1 (3)	-1.7 ± 1.2 (-3)
CTNNAL1	3.0 ± 1.5 (8)	1.6 ± 1.1 (3)	N.D.	N.D.
P85 <sup>a</sup>	3.9 ± 1.4 (15)	-1.2 ± 1.2 (-2)	N.D.	-3.0 ± 1.2 (-6)
SDF1	-1.3 ± 1.0 (-2)	-1.7 ± 1.0 (-3)	N.D.	N.D.
NDFIP1 <sup>b</sup>	N.D.	N.D.	N.D.	N.D.
CHID1	4.0 ± 1.2 (16)	N.D.	N.D.	-1.5 ± 1.0 (-3)
SLC38A5 <sup>a</sup>	18.5 ± 0.4 (4×10 <sup>5</sup> )	7.4 ± 0.5 (169)	15.4 ± 0.4 (4×10 <sup>4</sup> )	17.9 ± 1.1 (4×10 <sup>5</sup> )
LMNA	N.D.	4.0 ± 1.0 (16)	1.9 ± 1.1 (4)	N.D.
DSTN	2.5 ± 1.2 (6)	1.6 ± 1.1 (3)	N.D.	N.D.
WAVE2 <sup>b</sup>	N.D.	N.D.	N.D.	N.D.
RAB11FIP5	7.2 ± 1.8 (147)	4.5 ± 1.4 (23)	2.8 ± 1.4 (7)	N.D.
RPL10	0.9 ± 0.2 (2)	-3.3 ± 0.2 (-10)	N.D.	-1.6 ± 0.7 (-3)
vWF <sup>a</sup>	1.8 ± 1.2 (3)	5.4 ± 1.5 (42)	3.3 ± 1.2 (10)	2.7 ± 1.0 (6)
PECAM1	N.D.	3.3 ± 1.1 (10)	1.2 ± 1.0 (2)	N.D.
GLUT1 <sup>a</sup>	10.2 ± 1.0 (1176)	7.3 ± 1.2 (158)	6.2 ± 1.0 (73)	7.6 ± 1.0 (194)

The mean  $\Delta\Delta C_t$  values are given along with standard deviation for four measurements spanning two independent PCR experiments each. A positive value represents enrichment of the gene at the BBB when compared to microvessels from the tissue indicated and vice versa. Approximate fold differences are indicated in parentheses. N.D. = No difference in transcript abundance as determined by a 2-tailed student's t-test ( $p < 0.05$ ).

<sup>a</sup>Membrane proteins that have enriched transcript abundance at the BBB compared to peripheral vascular beds.

<sup>b</sup>Membrane proteins that have comparable transcript abundance levels at all vascular beds examined.

Note: In order to compare transcript abundance in other vascular beds, simply subtract the  $\Delta\Delta C_t$  values given in the table. For example, the average  $\Delta\Delta C_t$  value for Lutheran glycoprotein transcript in lung compared to heart microvasculature =  $4.4 - 1.9 = 2.5$  or approximately 6-fold, implying that the transcript abundance of Lutheran glycoprotein in lung microvessels is 6-fold higher than in heart microvessels.