Characterization of Cre Mouse Models to Target CNS Barriers for Generating Conditional Knockouts of ABC Transporters

Rachel L. Scheib
University of Tennessee Health Science Center

Follow this and additional works at: http://dc.uthsc.edu/dissertations
Part of the Medical Molecular Biology Commons

Recommended Citation
Characterization of Cre Mouse Models to Target CNS Barriers for Generating Conditional Knockouts of ABC Transporters

Document Type
Thesis

Degree Name
Master of Science (MS)

Program
Biomedical Sciences

Track
Cancer and Developmental Biology

Research Advisor
Erin Schuetz, Ph.D

Committee
Rennolds Ostrom, Ph.D. Radhakrishna Rao, Ph.D.

DOI
10.21007/etd.cghs.2014.0280

Comments
One year embargo expired May 2015

This thesis is available at UTHSC Digital Commons: http://dc.uthsc.edu/dissertations/235
Characterization of Cre Mouse Models to Target CNS Barriers for Generating Conditional Knockouts of ABC Transporters

A Thesis
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
From The University of Tennessee

By
Rachel L. Scheib
May 2014
ACKNOWLEDGEMENTS

I would like to first thank everyone in the Dr. Erin Schuetz laboratory for the help and guidance while maneuvering through graduate school with its many hills and valleys. I especially have to thank Dr. Ranjit Thirumaran and Cynthia Cline for their constant support and positivity, even when experiments or life never seems to go as planned. Thanks, Ranjit and Cynthia.

I also have to thank all the friends that have supported, experienced, and rationalized with me along the way. There’s no way to say how each one of you has contributed in helping me get where I am today, but I hope you at least know that I am forever grateful, thank you.

This journey could not have been possible without the love and support of my fiancé Jason Workman, and my family. I cannot express how grateful I am to have these people in my life. Jason, you were at all times my rock and cheerleader, love you more than anything. And to my Mom and Dad, thanks for always being there and understanding. If it’s possible to love you even more, I do. Thank you for making me the independent and caring woman I am today.

Lastly, I give this advice to current and upcoming graduate students: be honest and true to yourself and do what makes you happy. Sometimes this is not the easiest road, as some make it seem, it takes courage and strength. There’s a difference between pursuing a dream because you were always good at it and pursuing a dream because you are passionate about it. Life is a series of choices never really set in stone.
ABSTRACT

The central nervous system (CNS) includes the brain and spinal cord, where both possess a blood to brain and a blood to cerebrospinal fluid (CSF) barrier. The blood-brain barrier (BBB) and blood-CSF barrier (BCSFB) regulate the passage of many molecules to maintain and protect these sensitive organs from harmful xenobiotics (i.e. drugs, pollutants, etc.) or physiologic changes (i.e. glucose, ion, or water composition). These barriers also express ABC transporters, including P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP), which are known to contribute to efflux of endogenous toxins and therapeutics from the CNS. Pgp and BCRP expression and activity are a crucial determinant of drug efficacy in the CNS and have been studied using global transporter knockout mouse models. Since the CNS compartment and location of transporters is unique at each barrier site, a global knockout model does not address questions of how each barrier site contributes to ineffectiveness of drugs getting into the CNS. This unique composition also makes developing therapeutic strategies more difficult. In order to look at each barrier independently, we have attempted to generate conditional knockout mouse models of each transporter and in each barrier site. Our BBB and BCSFB models will provide insight into the biology of drug movement within the CNS while considering each barrier site’s contribution.
# TABLE OF CONTENTS

## CHAPTER 1. INTRODUCTION .....................................................................................1

The Blood-CSF Barrier ............................................................................................1  
Arachnoid ................................................................................................................1  
Choroid plexus .........................................................................................................3  
The Blood-Brain Barrier ..........................................................................................3  
Pgp and BCRP: Background and Clinical Relevance in the Brain .........................4  
P-glycoprotein (Pgp) ...............................................................................................4  
Breast cancer resistance protein (BCRP) ...............................................................5  
Drug administration into the CNS and transporter protein effects on disease ......5  
Animal models used to study drug concentrations in the CNS ...........................5  
Experimental Approach: Generating Transgenic Animals .................................6  
Cre-loxP System ....................................................................................................6

## CHAPTER 2. MATERIALS AND METHODS ................................................................7

Animals .....................................................................................................................7  
Generation of \textit{Ptgds-Cre} Mice .......................................................................7  
Detection of the Zygosity of \textit{Ptgds-Cre} Transgene Alleles by Multiplex Ligation- 
Dependent Probe Assay .........................................................................................7  
Transgene Localization in \textit{Ptgds-Cre} Mice Using Fluorescence \textit{In Situ} Hybridization (FISH) ..................................................................................................................9  
Generation of Pgp-Floxed Mice .................................................................9  
Generation BCRP-Floxed Mice ........................................................................11  
Whole Organ Fluorescence Analysis ..................................................................11  
Immunofluorescence on Frozen Tissue and Image Analysis .............................11  
Immunohistochemistry on Paraffin Embedded Tissue and Image Analysis ........12

## CHAPTER 3. RESULTS .................................................................................................14

Characterization of Mice Expressing Cre/tdTomato in AB Epithelial Cells ..........14  
Characterization of Mice Expressing Cre/tdTomato in Endothelial Cells ............24  
vWF-Cre model targeting brain endothelial cells ................................................24  
Cdh5-Cre model targeting endothelial cells ..........................................................24  
Characterization of Mice Expressing Cre/tdTomato in CP Epithelial Cells ..........30  
Characterization of Pgp and BCRP Conditional Knockout Mice ......................40  
Analysis of immunohistochemistry from Pgp conditional knockout mice ........40  
Analysis of immunohistochemistry from BCRP conditional knockout mice ......44

## CHAPTER 4. CONCLUSION ........................................................................................50

\textit{Ptgds-Cre} as a Model of Arachnoid Barrier Deletion of Pgp and BCRP ..........50  
\textit{Cdh5-Cre} as a Model of Endothelial Cell Deletion of Pgp and BCRP ..............50  
\textit{LPV-Cre} as a Model of Choroid Plexus Deletion of Pgp and BCRP ...............50  
Significance of Using Conditional Knockouts of Pgp and BCRP in the Brain ....51
LIST OF TABLES

Table 2-1. MLPA Cre 5’ and 3’ probe set sequences based on the mouse transgene. .....8

Table 2-2. Primary antibody dilutions, host species, and manufacturer information for immunofluorescence and immunohistochemistry. .................................13
LIST OF FIGURES

Figure 1-1. Localization of Pgp and BCRP in barrier cells within the CNS....................2

Figure 2-1. Genomic targeting scheme for generating Pgp-floxed and BCRP-floxed mice. .................................................................10

Figure 3-1. TdTomato reporter expression in Ptgds-Cre/tomato mice.........................15

Figure 3-2. Immunohistochemistry with NVU markers to validate tdTomato expression in the brain of Ptgds-Cre/tomato mice. ......................18

Figure 3-3. Immunohistochemistry with CD31, Pgp, and BCRP markers in comparison to native tdTomato expression in Ptgds-Cre/tomato mice......21

Figure 3-4. TdTomato reporter expression in vWF-Cre/tomato mice. .........................25

Figure 3-5. Immunohistochemistry with CD31 in comparison to native tdTomato expression in vWF-Cre/tomato mice. .................................................28

Figure 3-6. TdTomato reporter expression in Cdh5-Cre/tomato mice. .........................31

Figure 3-7. Immunohistochemistry with CD31, Pgp, and BCRP markers in comparison to native tdTomato expression in Cdh5-Cre/tomato mice........34

Figure 3-8. TdTomato reporter expression in LPV-Cre/tomato mice..........................37

Figure 3-9. Immunohistochemistry with CD31, Pgp, and BCRP markers in comparison to native tdTomato expression in LPV-Cre/tomato mice. .......41

Figure 3-10. Immunohistochemistry of cKO brain tissue from Cre/Pgp-floxed mice.....45

Figure 3-11. Immunohistochemistry of cKO brain tissue from Cre/BCRP-floxed mice. .............................................................................................................47
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>arachnoid barrier</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABI</td>
<td>allen brain institute/allen brain atlas</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>BCSFB</td>
<td>blood-CSF barrier</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Cdh5</td>
<td>vascular E-cadherin 5</td>
</tr>
<tr>
<td>cKO</td>
<td>conditional knockout</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>choroid plexus</td>
</tr>
<tr>
<td>Cre</td>
<td>cre recombinase enzyme</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>JAM</td>
<td>junction adhesion molecules</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LPV</td>
<td>lymphotropic pavova virus</td>
</tr>
<tr>
<td>MLPA</td>
<td>multiplex ligation probe assay</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>NVU</td>
<td>neurovascular unit</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein or MDR1 or ABCB1</td>
</tr>
<tr>
<td>Ptgds</td>
<td>L-prostaglandin D synthase</td>
</tr>
<tr>
<td>SAS</td>
<td>sub-arachnoid space</td>
</tr>
<tr>
<td>TJ</td>
<td>tight junction</td>
</tr>
<tr>
<td>vWF</td>
<td>von willebrand factor</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

The central nervous system (CNS) includes the brain and spinal cord, where both possess a blood to brain and a blood to cerebrospinal fluid (CSF) barrier. The blood-brain barrier (BBB) and blood-CSF barrier (BCSFB) regulate the passage of many molecules to maintain and protect these sensitive organs from harmful xenobiotics (i.e. drugs, pollutants, etc.) or physiologic changes (i.e. glucose, ion, or water composition).

The Blood-CSF Barrier

The blood-CSF barrier (BCSFB) contains two different types of barrier cells, choroid plexus (CP) epithelial cells present within the brain interior, and arachnoid barrier (AB) epithelial cells surrounding the cranial and spinal CSF that flows around the CNS.

Arachnoid

Among the brain barriers, the AB has been the least characterized. Meningeal tissue, consisting of three layers, covers the brain surface. The most exterior layer is the dura mater, followed by an inner layer of arachnoid mater, and then finally the pia mater that physically covers the brain. The sub-arachnoid space (SAS), where CSF flows around the brain and spinal cord, lies between the AB and pia layers. Another type of arachnoid cell extends from the AB layer into and through the SAS creating a web-like connection between the AB and pia. These web-like cells are called the arachnoid trabeculae [1]. It should be made clear that arachnoid trabeculae cells do not have barrier functions and only the single layer of AB cells create a physical barrier between blood and CSF fluids. Literature often uses the term “leptomeninges”, which includes the AB and pial layers of the meningeal tissue, but again only the AB has barrier properties. The AB functions as a physical barrier as evidenced by the exclusion of an intravascular dye from the CSF, pia, and brain parenchyma by the AB [1, 2]. Similar to the CP and BBB, AB cells express tight junction proteins, such as claudin-1, zona occludin-1 (ZO-1), and junction adhesion molecules (JAMs), to create a physical barrier. In addition, the AB expresses the epithelial markers desmoplakin, vimentin, and cytokeratin which most likely play a role in stabilization of tight junction formation by acting as intracellular links between junctions and the cell cytoskeleton [3, 4]. The AB also possesses a chemical barrier via expression of major ATP-binding cassette (ABC) transporters on the cell membrane surface (Figure 1-1A). Within the family of ABC transporters, Pgp and BCRP expression have only been recently elucidated at the AB [5], therefore there is much more to be discovered about this barrier.
Figure 1-1. Localization of Pgp and BCRP in barrier cells within the CNS.

A. Arachnoid barrier cells.  B. Brain endothelial cells.  C. Choroid plexus epithelial cells. The asterisk (*) signifies that Pgp is not expressed in mouse CP, but other species may express Pgp. Arrows indicate direction of efflux by the transporters.
Choroid plexus

The CP is located inside each of the four brain ventricles, functioning to produce CSF for the CNS. CSF is crucial for preventing brain injury, sustaining the buoyancy of the brain, and maintaining chemical homeostasis of factors circulating within the CSF [6]. Unlike the BBB, the CP has fenestrated capillaries without any barrier properties, meaning these capillaries do not exclude any substances from reaching the CP epithelial cells. A single layer of epithelial cells forms the barrier layer of the CP. These CP cells express tight junction (TJ) proteins providing a physical barrier between the blood and CSF. Claudin-3 is the most abundant, but CP cells also express claudin-1, claudin-2, claudin-11, JAMs and supporting proteins ZO-1, ZO-2, ZO-3 and cingulin [7]. In addition to these tight junctions, CP epithelium expresses cadherin-10, an adherens junction protein, and α, β-catenin scaffolding proteins to support and maintain the boundary between blood and CSF fluids [7].

Like the BBB, the CP also expresses drug transporters and drug-metabolizing enzymes creating a transport and enzymatic barrier. Pgp, BCRP, multidrug resistance protein (MRP) 1, and MRP4 are members of the ABC transporter family and are the most highly expressed within the CP epithelium [6, 7]. Pgp has been reported to be expressed on the apical (CSF-facing) surface or sub-apically within the CP epithelium; however, the exact membrane localization and/or presence of Pgp at the CP have been controversial in the literature [7-10]. BCRP is present on the apical surface of CP cells (Figure 1-1C), while MRP1/4 are localized to the basolateral surface of the CP epithelium [8]. Localization of BCRP at the CP suggests it is involved in facilitating substances into the CSF contrary to its function at the BBB where BCRP prevents substances getting into the brain from the blood. Since BCRP is known to transport other endogenous compounds, it is possible that BCRP transports an endogenous substance required into the CSF, or that this is a mechanism for removing cellular waste products due to the lack of a blood barrier. For those data that support Pgp expression on the apical CP surface, these explanations for why BCRP is located there may also apply.

The Blood-Brain Barrier

The blood-brain barrier (BBB), also called the neurovascular unit (NVU), is composed of brain endothelial cells, pericytes, neurons, astrocytes, and microglia [11]. Within the NVU, each cell type plays a specific role to support and form the BBB. Brain endothelial cells express several tight junction and adherens junction proteins to create a physical barrier between the blood compartment and the brain parenchyma [7]. Tight junctions, such as claudin-3, claudin-5, occludin, and JAMs are key proteins that eliminate the paracellular passage of substances. Many of these tight junction proteins are linked to cytoplasmic scaffolding and regulatory proteins such as ZO-1, ZO-2, ZO-3, and cingulin to ensure maintenance of the BBB [12]. Disruption of claudin-3 or claudin-5 has been shown to cause a severely compromised BBB, even causing death in mice that lack claudin-5 [12, 13]. Adherens junctions are also very important in maintaining an intact BBB, specifically vascular E-cadherin (VEC or Cdh5), α, β, and γ-catenin
scaffolding proteins. These proteins work to hold endothelial cells together, giving the BBB structure and support as well as making it possible to form the essential tight junctions for barrier function [12]. Therefore, disruption of adherens junctions can also result in a compromised or leaky barrier.

In addition to a physical barrier, brain endothelial cells possess a chemical barrier between the brain and the blood. This chemical barrier is established by the expression of many transport proteins and enzymes on the BEC membrane surface. These proteins maintain brain homeostasis by restricting access of xenobiotics, while simultaneously facilitating transport of compounds required for brain cell nutrition and survival (i.e. glucose, amino acids, lipids). ABC transporters such as Pgp, BCRP, and various MRPs are expressed on the apical or blood-side of endothelial cell membranes of the BBB and are major contributors in efflux of substances back into the blood (Figure 1-1B) [6-8, 14-18]. These neuro-protective proteins are efflux pumps and remove endogenously generated neurotoxic compounds back into the blood. Brain endothelial cells are considered to be the primary barrier between the brain and the blood and if this is dysfunctional or leaky, transporters present on other cell types of the BBB (pericytes and astrocytic end feet) can act as a second line of defense [12].

Pgp and BCRP: Background and Clinical Relevance in the Brain

Pgp and BCRP are two of the most studied efflux transporters in brain barriers. Pgp was the first ABC transporter to be discovered and BCRP followed over 20 years later [19-22].

P-glycoprotein (Pgp)

The multidrug resistance (MDR) transporter, also known as Pgp, MDR1, or ABCB1, is encoded by the ABCB1 gene. Humans have one isoform, ABCB1/MDR1, while rodents have two isoforms, Mdr1a and Mdr1b [22, 23]. Expression levels of Mdr1a and Mdr1b have been characterized to be tissue and age dependent, where Mdr1a is highly expressed in brain endothelial cells while Mdr1b is not [24, 25]. Mdr1a is expressed at the brain endothelial cell membrane as early as embryonic day 18 (E18) in mice and continues to increase in its expression until plateauing at post-natal day 28 (P28) [12]. While Pgp is located and functions in the brain barriers, this protein is not expressed exclusively in the brain. Pgp is highly expressed on the bile canalicular membrane in the liver, proximal tubules of the kidney, and apical surface of the intestinal epithelium [26, 27]. Substrates for Pgp are amphipathic and lipid-soluble, ranging in molecular weight between 300 and 1000 Daltons [22, 23]. Pgp is considered a promiscuous transporter because it transports a large variety of substrates that can include anticancer drugs, steroids, fluorescent dyes, and peptides [22]. Some of these compounds are also substrates for other ABC transporters such as BCRP. For example, topotecan (topoisomerase I inhibitor) can be a substrate for three ABC transporters, Pgp, BCRP, and MRP4 [10].
Breast cancer resistance protein (BCRP)

Unlike Pgp, BCRP is a half transporter encoded by the ABCG2 gene, where each half produced must homodimerize with another to create a functional protein to transport its large range of substrates. These compounds are predominantly, but not limited to, chemotherapeutic agents. BCRP is most well known for transporting the chemotherapeutic drug mitoxantrone [21, 28] as well as some endogenous physiological molecules like protoporphrin IX and pheophorbide a, a chlorophyll breakdown product [29]. Similar to Pgp, BCRP is also expressed in multiple organs, including the bile canalicular membrane in liver, proximal tubule in kidney, and apical surface of the intestinal epithelium.

Drug administration into the CNS and transporter protein effects on disease

Both Pgp and BCRP block drug penetration into the CNS, decreasing the efficacy of drug treatments developed to target the CNS. Therefore, drug treatment of diseases such as brain cancer, Parkinson’s disease, Alzheimer’s disease, and even fatal brain metastasis from other cancers (i.e. breast cancer) extremely difficult [30, 31]. Most chemotherapeutics or drug treatments are given through intravenous (IV) infusion while other treatment regimens require treatment to be given intrathecally (IT) by intralumbar injection in the lower spine. While IV injections are the primary means for drug delivery into the CNS, the BBB and BCSFB hinder the distribution of therapeutics to the intended target. A growing number of drugs are being given IT to better treat CSF disease [32], however, the contribution of AB drug transporters to CSF drug distribution has not been studied.

Instead of directly affecting drug penetration, Pgp and BCRP have been implicated in modulating aspects of Alzheimer’s and Parkinson’s disease. Alzheimer’s patients suffer from accumulation of β-amyloid proteins in the brain, but it has been reported that Pgp and BCRP may efflux these proteins to protect the brain from its toxic accumulation [7]. Other literature has also shown that brain capillaries from Alzheimer’s patients have lower expression of Pgp resulting in decreased β-amyloid clearance [33-35]. Parkinson’s disease has also been linked to a reduction of Pgp in brain endothelial cells [36-38].

Animal models used to study drug concentrations in the CNS

Many studies have utilized global transporter knockout (KO) animal models and analyzed the CSF as a surrogate for the amount of unbound drug available to the brain [39-42]. However, this approach does not address the question of how much each barrier contributes to the distribution of a drug within the CNS. The location of each transporter within the AB, BBB, and CP barriers must be considered as they may have different efflux directions and in some cases functionally opposing one another. For example, BCRP at the CP faces the CSF, facilitating drug entry into the CSF, while BCRP at the
BBB faces the blood, preventing drug penetration into the brain. An alternative method would be to use mice with conditional KO (cKO) of transporter proteins at each barrier to estimate the functional contribution of that drug transporter at the AB, BBB, and CP. This experimental design is especially relevant for drugs given IT, since they do not come into contact with the BBB until exported into the blood by transporters in the BCSFB. Understanding the independent roles each barrier has in protecting the brain from toxicities may give insight towards the more efficient treatment of CNS illness. New evidence using fluorescent tracer injections into the CSF suggests administration of compounds through the CSF can potentially expose the brain to a greater amount of compound (i.e. drugs). This fluorescent tracer was shown to actually bathe or “wash out” the brain parenchyma and has been implicated as a main route for waste disposal for the CNS, including possible β-amyloid clearance [43, 44]. Maybe this same principle applies to drugs injected into the CSF.

**Experimental Approach: Generating Transgenic Animals**

The goal of this study was to generate mice with conditional deletion of Pgp or BCRP in each of the barrier cell types; AB, BBB, and CP. Using the Cre-loxP system, we used tissue specific promoters to drive Cre expression within the AB [45], BBB [46], or CP [47]. Each model’s Cre expression was then characterized by mating the Cre mouse to a tdTomato fluorescent reporter mouse [48]. After characterization, each of the three Cre models were crossed with Pgp and BCRP-floxed mice to generate a cKO where only cells expressing the Cre enzyme (AB, BBB, or CP) would lose expression of Pgp or BCRP protein in that cell.

**Cre-loxP System**

Cre recombinase (Cre) is a 38kDa enzyme that recognizes loxP sites to mediate site-specific recombination between two loxP markers. Each loxP site is 34 base pairs (bp) in length and consists of two 13 bp inverted repeats with an 8 bp spacer that provides orientation for the overall sequence. If two loxP sites are in the same orientation within a linear DNA sequence, recombination initiated by Cre results in excision of the loxP-flanked DNA sequence [49]. Using the Cre-loxP system allows for flexibility in that a floxed target mouse line can be bred with any type of Cre expressing mouse line. As an example, breeding a Cre line that expresses the enzyme ubiquitously with a target floxed mouse can generate a global knockout mouse model. It is also possible to use an inducible Cre line to study deletion of a target gene at different time points in development. Using the Cre-loxP approach to generate global knockouts addresses issues traditional knockouts may have with lethality or infertility when the gene is deleted in the whole animal. In spite of all its advantages, the Cre-loxP system has some disadvantages such as the difficulty in finding a promoter to drive Cre expression in a specific tissue or cell and as with all transgenic models, expression level and site of integration of the Cre transgene can affect its success [50].
CHAPTER 2. MATERIALS AND METHODS

Animals

Mice were used according to protocols approved by the St. Jude Children’s Research Hospital Committee on the Use and Care of Animals. Cdh5-Cre [46, 51] (JAX stock #006137), vWF-Cre [47], LPV-Cre [47], and Ptgds-Cre mice were crossed to ROSA-tdTomato reporter (referred to as tdTomato) (JAX stock #007914) mice to validate the targeting of Cre expression in specific cell types such as endothelial cells, choroid plexus epithelial cells, and arachnoid barrier cells. These three cell types represent all the brain barriers in the central nervous system, and eventually Cre mice will be crossed to mice carrying a specific floxed gene of interest.

Generation of Ptgds-Cre Mice

The mouse prostaglandin D2 synthase (mPtgds) promoter was PCR amplified from the BAC Clone RP23-389G18 (Gensat BX1999; purchased from Children’s Hospital Oakland Research Institute) with restriction sites MluI in the forward primer (5’-gaCGCGTtggtccttccaagaggactg-3’) and EcoRI in the reverse primer (5’-ccgGAATTCttgctcagagcagagcagg-3’). The 2018 bp amplified product was subsequently cloned into CL20MCremCherry vector (received from Dr. John Gray at St. Jude Vector Lab) at the MluI and EcoRI restriction sites. This CL20MCremCherry (8971 bp) is a lentiviral vector that expresses Cre from a promoter that we replaced with the mPtgds promoter. The cloned mPtgds-CremCherry construct (9519 bp; to provide tissue specific expression of Cre and this construct also express mCherry so the tissue specificity could also be characterized in the pups) was then sequenced to validate its identity using the following sequencing primers (Forward 5'-aggcagggatattcaccatt-3' and Reverse 5' -gcaaacggacagaagcattt-3'). Further, this construct was linearized using MfeI and SfiI restriction enzymes to remove the bacterial sequences. Finally, this 5629 bp transgene was submitted to our transgenic core facility to microinject the transgene into FVB mouse oocytes to generate transgenic founders. The founder lines were bred until the mice were homozygous for the Cre transgene.

Detection of the Zygosity of Ptgds-Cre Transgene Alleles by Multiplex Ligation-Dependent Probe Assay

Multiplex ligation-dependent probe assay (MLPA) was used for determination of the zygosity of transgene alleles. During MLPA, an oligonucleotide ligation reaction was performed, followed by PCR using fluorescein-conjugated primer, such that the amount of PCR product generated for each genomic sequence is directly proportional to the number of input copies. For this assay, the genomic DNA samples were isolated from mouse tail using DNeasy Blood and Tissue Kit (QIAGEN #69506) and adjusted to approximately 10 ng/μL using DNA suspension buffer (TEKnova #T0221). The mice
bearing Cre transgene alleles were analyzed by using the MLPA Cre probe set to generate an amplification product of 117 bp. Three control probes were used to amplify control sequences elsewhere in the mouse genome were used with amplification products ranging in size from 108 bp, 114 bp and 136 bp [52]. Each probe set was composed of a 5’ and a 3’ half-probe, each containing unique target specific sequence, stuffer sequence, and universal primer sequences on their 5’ and 3’ ends, respectively (Table 2-1) [52]. All probes were synthesized at the standard 25nmol (25N) scale of synthesis and purified by polyacrylamide gel electrophoresis (Invitrogen Life Technologies). Synthesis scales refer to the amount of starting material present, not the amount of product produced. 3’ half-probes were synthesized with a 5’ phosphate to facilitate ligation.

MLPA was performed by incubating 50 ng DNA in 5 μL at 98°C for 5 min; after cooling to room temperature, it was mixed with 1.5 μL of Cre transgene specific probe mixture (containing 1.5 fmol each probe) and 1.5 μL SALSA hybridization buffer, then denatured at 95°C for 2 min and hybridized at 60°C for 16 hours. Hybridized probes were then ligated at 54°C for 15 min by addition of 32 μL ligation mixture. Following heat inactivation, 40 μL ligation reaction was mixed with 10 μL PCR mixture (SALSA polymerase, dNTPs, and universal primers, one of which was labeled with fluorescein (FITC)), and subjected to PCR amplification for 35 cycles. Primer sequences used for SALSA PCR were 5’-*GGGTTCCTAAGGGTTGGA-3’ for the forward primer (*FITC labeled) and 3’-GTGCCAGCAAGATCCAATCTAGA-5’ (unlabeled) for the reverse primer.

All reagents except probe mixes were from MRC-Holland (Amsterdam, The Netherlands). Amplification products were diluted in water (1:10) and then 1:9 in Hi-Di™ formamide (Applied Biosystems, USA) containing 1/36 volume of GeneScan 500 LIZ size standard (Applied Biosystems), to a final dilution of 20 to 200 fold, and then were separated by size on an 3730XL DNA Analyzer (Applied Biosystems / Life Technologies). Electropherograms were analyzed by GeneMapper® v5 (Applied Biosystems), and peak height data were exported to an Excel table. Normalization of peak height data was done by dividing each Cre transgene peak height by the average signal from three control probes, followed by division by a similar value calculated from a set of reference samples known to be heterozygotes for the transgene allele. This ratio reflects the copy number of the Cre transgene. Control probe sets were used exactly as previously described [52]. The probe set consists of a 5’ half-probe and a 3’ half-probe. Each probe contains universal primer sequence, stuffer sequence, and target sequence, the latter of which is specific for the transgene being assessed. Total length of PCR product assessed by capillary electrophoresis is shown by the total product length.

<table>
<thead>
<tr>
<th>Probe Sequence</th>
<th>5’ Half Probe</th>
<th>3’ Half Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal</td>
<td>GGGTTCCCTAAGGGTTGGA</td>
<td>TCTAGATTGGATCTTGCTGCGC</td>
</tr>
<tr>
<td>Stuffer</td>
<td>CGCTACTACTATTAGT</td>
<td>AACTAATATCCTAC</td>
</tr>
<tr>
<td>Target</td>
<td>ATGGACATGTTCAGGGATCGCA</td>
<td>GGCCTTTTCTGAGCATAACCTGGA</td>
</tr>
</tbody>
</table>
Transgene Localization in \textit{Ptgds-Cre} Mice Using Fluorescence \textit{In Situ} Hybridization (FISH)

The St. Jude Cytogenetic Shared Resource Laboratory isolated lungs from a \textit{Ptgds-Cre} mouse carrying two copies of the Cre transgene (determined by MPLA). Purified \textit{Ptgds-Cre} plasmid DNA was labeled with a green-dUTP (Alexa Fluor 488, Molecular Probes) by nick translation. The labeled transgene probe was combined with sheared mouse DNA and hybridized to metaphase and interphase nuclei derived from the transgenic mouse lung fibroblast culture in a solution containing 50% formamide, 10% dextran sulfate, and 2X SSC. The initial FISH assay indicated that the sample had a very weak homozygous insertion into a probable chromosome 1 location. For the confirmation assay, the previous process was repeated with the labeled transgene probe and a red-dUTP (Alexa Fluor 594, Molecular Probes) labeled chromosome 1 control probe (Pax3/RP23-260F1/1C4). Both probes were combined with sheared mouse DNA and hybridized to interphase nuclei and metaphase chromosomes derived from the transgenic mouse lung fibroblast culture in a solution containing 50% formamide, 10% dextran sulfate, and 2X SSC. The chromosomes were then stained with 4,6-diamidino-2-phenylindole (DAPI) and analyzed. Results indicated that the \textit{Ptgds-Cre} transgenic mice have the \textit{Ptgds-Cre} transgene homozygously inserted into chromosome 1. The transgene insertion site is at a position that is 47% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome 1, an area that corresponds to band 1D-E.

Generation of Pgp-Floxed Mice

The University of Connecticut Health Center Gene Targeting and Transgenic Facility made the targeting construct and produced mice carrying one floxed allele. The targeting construct contains \textit{loxP} sites flanking exon 4 of the \textit{ABCB1a} gene as well as a PGKNeo cassette for embryonic stem (ES) cell selection (Figure 2-1A). ES cells were cultured in selective media containing G418 and gancyclovir to find targeted clones. Surviving clones were then confirmed by long range PCR to have the correct targeted allele. These targeted clones were subsequently injected into mouse blastocysts and implanted into a surrogate mother to generate chimeric mice. Chimeric males were bred with wild-type female mice to check for germ line transmission of the targeted construct representing the F2 generation (coat colors blended if transmission successful). The F2 generation of mice were interbred until offspring became homozygous for the Pgp-floxed alleles. We received sperm of the homozygous Pgp-floxed offspring from transgenic facility in Connecticut, which was used for in-vitro fertilization in C57BL/6 wild-type female mouse by the St. Jude Animal Research Center staff. Offspring from this cross were interbred until again producing homozygous Pgp-floxed mice assessed by PCR. Deletion of exon 4 of \textit{ABCB1a} gene, upon introduction of Cre, results in the lack of Pgp protein due to disruption of its transmembrane domain.
Figure 2-1. Genomic targeting scheme for generating Pgp-floxed and BCRP-floxed mice.

A. Pgp-floxed mice.  B. BCRP-floxed mice.
Generation BCRP-Floxed Mice

ES cells containing a targeting construct for BCRP were obtained from Dr. Mike Dean from the National Cancer Institute (NCI). The targeting construct has loxP sites flanking exon 2 of the ABCG2 gene as well as a Neo cassette for ES cell selection (Figure 2-1B). Upon arrival, ES cells were cultured in selective media containing G418 and surviving clones were analyzed by Southern hybridization to confirm a correctly targeted allele. Four correctly targeted clones were sent to the St. Jude Transgenic Core Facility where these clones were injected into individual mouse blastocysts, then implanted into a surrogate mother to generate chimera mouse offspring. Chimeric males were bred with C57BL/6 wild-type female mice to check for germ line transmission of the targeted construct representing the F2 generation (coat colors blended if transmission successful). The F2 generation of mice were interbred until offspring became homozygous for the BCRP-floxed alleles as accessed by PCR and MLPA. Deletion of exon 2 of ABCG2, in cells expressing Cre, results in the lack of BCRP mRNA or protein being made.

Whole Organ Fluorescence Analysis

Offspring from all Cre-tomato crosses were collected at two different ages, early post-natal (P5-7) and adult (6-10wks). Animals were subsequently anesthetized by isofluorane and perfused through the heart with cold 1x phosphate buffered saline (PBS) followed by cold 4% paraformaldehyde (PFA, Electron Microscopy Sciences #15710) diluted in 1xPBS, which was performed by St. Jude Veterinary Pathology Core staff. Following perfusion, brain, liver, kidneys, and small intestine were removed for whole tissue fluorescent analysis on a stereoscope (Nikon SMZ1500 stereoscope with Nikon Intensilight CGFI fluorescence source) where the TRITC filter was used to examine tdTomato expression. Tissues from all Cre-tomato crosses were processed for frozen or paraffin slides and further examined by immunofluorescence (IF) or immunohistochemistry (IHC) respectively after whole organ images were captured.

Immunofluorescence on Frozen Tissue and Image Analysis

Mouse tissues were fixed in 4% PFA overnight at 4°C, then sucrose protected by incubating them in 15% sucrose in 1xPBS overnight at 4°C, then 30% sucrose in 1x PBS overnight at 4°C. The next day, tissues were equilibrated in OCT compound (Tissue Tek #4583, Electron Microscopy Sciences) for 30 minutes, embedded in a mold with OCT, then frozen in an ethanol bath chilled on a metal block on dry ice. Frozen blocks were then transferred to the -80°C freezer for storage until sectioning. Blocks were removed from the freezer and allowed to equilibrate inside the cryostat (Leica) -20°C chamber for at least 1 hour before sectioning at 15μm thickness. Slides were stored at -80°C until IHC could be performed. Slides thawed on a slide warmer at 37°C for 15-30 minutes followed by two 10-minute washes with 0.3% Triton X-100 in 1xPBS (PBST). Slides were blocked with 3% normal donkey serum diluted in PBST (PBST+) for 60 minutes
followed by an overnight incubation in desired primary antibody (diluted in PBST+) at 4°C (Table 2-2). After primary antibody incubation, slides were washed in PBST three times for 10 minutes each. Slides were then incubated with appropriate secondary Alexa Fluor 488, 555 or 647 antibodies (Invitrogen), diluted 1:500 in PBST+, for 2-3 hours. After secondary incubation, slides were washed in PBST four times for 10 minutes each followed by mounting with Prolong Gold anti-fade with DAPI (Invitrogen #P36935) and #1 cover-slips (Thermo Scientific #102450). Images were captured on a Nikon E800 using the 20x dry objective or Marianas confocal microscope using the 40x oil objective. After capture, images were processed by ImageJ software (64bit, version 1.46r) to remove non-specific background by adjusting the color range using color balance tools provided by the ImageJ software.

**Immunohistochemistry on Paraffin Embedded Tissue and Image Analysis**

Tissues from conditional knockout mouse models were perfused and left to fix in 4% PFA overnight at 4°C, then processed for paraffin slides by the St. Jude Veterinary Pathology Core. Tissues were sectioned at 4μm thickness and slides were stored at room temperature until IHC could be performed. Slides were deparaffinized and hydrated through a series of xylene and alcohols according to typical histology practices. Slide underwent antigen retrieval through incubation in Target Retrieval Buffer, pH 6.0 (DAKO #S1699) for 15 minutes at high pressure within a pressure cooker system. After retrieval, tissues on slides were peroxidase blocked with 0.3% peroxide and protein blocked with Sniper Block reagents (Biocare Medical #BS966L). Slides were subsequently stained with Pgp or BXP-53 primary antibody overnight at 4°C. After primary antibody incubation, slides were washed in PBST and then incubated with their respective secondary HRP-polymer (Rabbit on Rodent, Biocare Medical #RMR622H; Rat on Rodent, Biocare Medical #RT517H) for 30 minutes. Slides were washed in PBST and incubated with 3,3’-diaminobenzidine chromagen (DAB; Thermo Scientific #TA-125-HDX) for 3 minutes. Slides were washed in PBST and then counterstained with Mayer’s hematoxylin (Thermo Scientific #TA-125-MH) that was diluted 1:5 in distilled water and filtered prior to applying to slides for 3 minutes. Slides were washed in PBST and dehydrated through a series of alcohols and xylene. Slides were then mounted with Permount (Fisher #SP15-500) and coverslipped. Slides were left on a flat surface until dry, then examined on a Nikon Eclipse Ti microscope with a 10-20x dry objective.
Table 2-2. Primary antibody dilutions, host species, and manufacturer information for immunofluorescence and immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IF Dilution</th>
<th>IHC Dilution</th>
<th>Host Species</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgp</td>
<td>1:50,000</td>
<td>1:400,000</td>
<td>Rabbit</td>
<td>Dr. John Schuetz, SJCRH</td>
</tr>
<tr>
<td>BXP-53</td>
<td>1:2,000</td>
<td>1:2,000</td>
<td>Rat</td>
<td>Enzo #ALX-801-036</td>
</tr>
<tr>
<td>CD31</td>
<td>1:300</td>
<td>n/a</td>
<td>Goat</td>
<td>R&amp;D Systems #AF3628</td>
</tr>
<tr>
<td>GFAP</td>
<td>1:500</td>
<td>n/a</td>
<td>Rabbit</td>
<td>DAKO #Z0334</td>
</tr>
<tr>
<td>Desmin</td>
<td>1:200</td>
<td>n/a</td>
<td>Mouse</td>
<td>Novus Biologicals #NBP1-42133</td>
</tr>
</tbody>
</table>

Notes: BXP-53 (BCRP antibody), CD31 (endothelial surface marker), GFAP (glial filament associated protein), Desmin (pericyte marker). The Pgp antibody was obtained from Dr. John Schuetz at St. Jude Children’s Research Hospital (SJCRH) in Memphis, TN. A peptide (SALDTESEKVVQEALDKAREG) coupled to KLH was synthesized and sent to Rockland Immunochemicals for injection into rabbits to generate the IgG antibody. The Pgp antibody in the rabbit serum was purified by Invitrogen (previously Research Genetics) in 2000. The epitope of this antibody recognizes an internal and highly conserved amino acid sequence on the C-terminal region of Pgp. The classical MDR1 antibody is the monoclonal C219, but this antibody detects a 200kDa protein migrating in the same position as myosin, cross-reacts with cerbB2 protein (p185cerbB2), and recognizes MDR1 and MDR3 isoforms of Pgp.
CHAPTER 3. RESULTS

Characterization of Mice Expressing Cre/tdTomato in AB Epithelial Cells

Since there is no Cre transgenic mouse model that targets AB cells, a new AB-Cre model was generated. The aim was to find a promoter that would drive expression of Cre in arachnoid cells but not in endothelial cells, CP epithelial cells, or other organs. After surveying published literature and expression analysis through databases such as the Allen Brain Institute (ABI) and Gensat, these sources indicated that since both AB and CP epithelial cells are from a common embryonic origin, it would be possible to generate AB/CP specific cKO mice representing the BCSFB. The ABI uses in situ hybridization to look at mRNA expression of genes in the developing and adult mouse brain, while Gensat uses transgenic mice where promoters of various genes drive green fluorescent protein (GFP) expression to look at their localization in the mouse developing and adult brain. L-prostaglandin D synthase (Ptgds) was one of the most highly expressed genes in AB cells and never indicated to be expressed in brain endothelial cells per evidence described in published literature and databases described above. Ptgds was chosen to generate the new AB-Cre model. Ptgds has a dual function in the CNS, one where it is secreted into the CSF, presumably from AB or CP cells, and another serving as a transporter protein that binds lipophilic ligands and retinoid molecules [53]. In regards to its localization, Ptgds has been reported in the leptomeninges (arachnoid and pia meninges), CP, and oligodendrocytes within the CNS as well as the pigment layer of the retina, and leydig cells of the testis using in-situ hybridization (ISH) and IHC techniques [45, 54-57]. An example of Ptgds mRNA expression in mouse brain from ABI can be seen in Figure 3-1B.

After producing a founder that gave viable offspring and maintained Cre expression, Ptgds-Cre mice were bred to maintain homozygosity of the Cre transgene and subsequently crossed with the tdTomato reporter (Figure 3-1A). Seven out of eight (88%) offspring were positive for tdTomato fluorescence and gender did not influence fluorescence expression. However, increasing age seemed to correlate with increase in tdTomato signal meaning older mice had more tdTomato expression than young mice (Figure 3-1C). Ptgds-Cre/tomato offspring expressed tdTomato in a variety of cell types including, AB cells, some cell in the NVU (possibly endothelial cells), CP epithelium, liver hepatocytes, kidney tubules, and intestinal epithelium (Figure 3-1D).

IHC was used to elucidate which cells of the NVU were expressing tdTomato. The NVU is composed of endothelial cells, pericytes, and astrocytes. Using the vascular marker CD31 we concluded that tdTomato, driven by the Ptgds promoter, was likely expressed in endothelial cells of the brain due to the overlapping pattern seen between CD31 (green) and tdTomato (red) in Figure 3-2A. Co-localization of colors green and red result in orange color, but sometimes intensity of one color is greater than the other resulting in a more red or a more green overlap. We ruled out Cre expression in other cell types that contribute to the NVU, such as pericytes and astrocytes, using their respective markers desmin and glial fibrillary acidic protein (GFAP). These IHC studies
Figure 3-1.  TdTomato reporter expression in *Ptgds*-Cre/tomato mice.

A. Activation of the tdTomato reporter when bred with *Ptgds*-Cre mice. B. ISH (left panel) and expression (right panel) analysis of endogenous *Ptgds* mRNA in adult mouse brain from the Allen Brain Atlas. The colored scale bar represents level of expression; blue being the lowest expression and red being the highest expression. Clearly, the leptomeninges and choroid plexi are highly expressing Ptgds. C. Representative images of tdTomato expression of whole brain, liver, kidney, and intestine from age matched *Ptgds*-Cre/tomato and control (Cre negative) tomato mice (pups n = 6, adults n = 10). D. Representative images of frozen sections of *Ptgds*-Cre/tomato (n = 8) and control (Cre negative) tomato brain, liver, kidney, and intestine at 20X magnification. Dashed lines indicate the edge of the arachnoid barrier tissue on the surface of the brain.

Notes: Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. C, brain cortex. CB, cerebellum. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
A

B

C

<table>
<thead>
<tr>
<th>Brightfield</th>
<th>tdTomato Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre: +</td>
<td>Pup: + Adult: -</td>
</tr>
<tr>
<td>Brain (dorsal view)</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-1. Continued.
Figure 3-2. Immunohistochemistry with NVU markers to validate tdTomato expression in the brain of Ptgds-Cre/tomato mice.

A. Frozen brain sections stained with CD31 (endothelial cells in green), while Cre is expressed by tdTomato in red. B. Frozen brain sections stained with pericyte marker Desmin (green), while Cre is expressed by tdTomato in red. C. Frozen brain sections stained with antibodies to GFAP (astrocytes in grey) and CD31 (endothelial cells in green), while Cre is expressed by tdTomato in red.

Notes: Cell nuclei (blue) are stained with DAPI and all images were captured at 40X magnification. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
showed little overlap between pericyte and astrocyte markers with tdTomato fluorescence (Figure 3-2B and C). This result further supported tdTomato expression within brain endothelial cells of the Ptgsds-Cre model. TdTomato expression was seen in brain endothelial cells of seven out of eight animals characterized. The remaining animal was the only negative under stereoscope and tissue section analysis. AB expression of tdTomato in the Ptgsds-Cre/tomato mice was also varied yielding five positive animals out of eight. The remaining three animals were either negative from stereoscope analysis or at a young postnatal age (P4) with tdTomato expression only in the brain endothelial cells. According to the ABI, Ptgsds expression begins at embryonic age 13.5 (E13.5) and should be fully expressed in mice at E18.5, so these young pups should be positive for tdTomato at P4. Other published literature also supports expression of Ptgsds in 8-13 week old rats [55].

Another striking result was the inconsistency of CP expression in our Ptgsds-Cre model. CP expression of tdTomato was seen in only one out of eight (12.5%) animals, whereas the literature indicated the Ptgsds gene would also be highly expressed in CP. Animals negative for CP expression of tdTomato were of varying ages P4 through 7 weeks old, resulting no correlation with age. The animal with positive tdTomato in CP was 6 weeks old. This unexpected expression may be due to the Ptgsds-Cre transgene insertion location within the mouse genome. FISH analysis revealed insertion of the Ptgsds-Cre transgene into chromosome 1 within band regions D-E, but this location is not specific enough to say whether or not the insertion is interfering with any essential genes. Specifically genes or genetic elements that cause reduced expression of tdTomato in the CP or cause tdTomato expression to be in the brain endothelial cells.

The tdTomato expression in liver, kidney, and intestine was also analyzed by the same IHC method, where CD31 and transporter markers were used. This was done to compare native tdTomato expression in respect to Pgp and BCRP localization in the brain, liver, kidney, and intestine as well as vasculature sites within these organs. Slides with sequential frozen sections of brain, liver, kidney, and intestine tissue from Ptgsds-Cre/tomato mice were used. Using sequential sections allows for comparison of similar areas when looking at expression of different proteins. For example, brain sections stained for Pgp, CD31, and BCRP were only 15 μm apart making it easier to correlate a location relationship between all three markers. As stated previously, CD31 and tdTomato seem to co-localize in endothelial cells of the brain, but there seems to be a lack of co-localization in the vasculature of peripheral tissues, except for in the intestine and possibly the liver (Figure 3-3B). The tdTomato expression in liver, kidney, and intestine also seemed to vary animal-to-animal. We confirmed Pgp and BCRP expression in the AB and BBB, while Pgp and BCRP had differing expression patterns in the CP. Pgp is not expressed in mouse CP, but BCRP was expressed on the apical (CSF-facing) surface of CP epithelial cells (Figure 3-3A). IHC analysis confirmed the presence of Pgp and BCRP within the bile canaliculi of liver, kidney tubules, and apical surface of intestinal epithelium seen in Figure 3-3B. By comparing transporter localization at each tissue site with tdTomato expression, we concluded that Pgp and BCRP will be deleted in the AB and BBB of the brain, as well as kidney tubules of Ptgsds-Cre mice when bred with transporter floxed mice.
Figure 3-3. Immunohistochemistry with CD31, Pgp, and BCRP markers in comparison to native tdTomato expression in Ptgds-Cre/tomato mice.

A. Sequential frozen brain sections stained with CD31 (endothelial cell marker), Pgp, and, BXP-53 (BCRP) antibodies. Images captured at 20X magnification. B. Sequential frozen liver, kidney, and intestine sections stained with CD31 (endothelial cell marker), Pgp, and, BXP-53 (BCRP) antibodies. Images captured at 20X magnification.

Notes: Arrows indicate the edge of the AB layer. Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. Green color indicates antibody staining for CD31, Pgp, or BCRP. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
Figure 3-3. Continued.
Characterization of Mice Expressing Cre/tdTomato in Endothelial Cells

Two mouse models targeting endothelial cells were analyzed, one using the vWF promoter and another using the vascular E-cadherin (Cdh5 or VEC) promoter to drive Cre expression in mouse endothelial cells. The vWF-Cre model was the first to be characterized, but due to heterogeneous results an alternative model was needed and Cdh5-Cre was chosen.

vWF-Cre model targeting brain endothelial cells

The previously characterized mouse model vWF-Cre, was generated by Dr. Rodney Ho and had shown positive Cre expression specifically in brain endothelial cells [47]. Von Willebrand factor (vWF) is large glycoprotein produced by endothelial cells that binds and stabilizes Factor VIII (clotting cascade component) in the circulation [58]. After breeding vWF-Cre mice with the tdTomato reporter (Figure 3-4A), the resulting offspring showed that Cre, as expressed by tdTomato fluorescence, was not uniform in any tissues or cell types, varied among animals, and was not confined to the brain shown by whole organ stereoscope and frozen tissue sections (Figure 3-4B and C). Some brains were completely negative for tdTomato while others expressed the fluorescent reporter in only one brain hemisphere (Figure 3-4B). Tissue sections also revealed cellular heterogeneity of Cre expression in brain endothelial cells, choroid plexus epithelial cells, intestinal epithelium, and hepatocytes (Figure 3-4C).

IHC with the vascular marker CD31 was the final test to see how well the vWF-Cre model would work for us. The conclusion was that CD31 hardly ever co-localizes with the tdTomato expression due to the lack of tdTomato expression in endothelial cells of the vWF-Cre/tomato model (Figure 3-5). Co-localization of CD31 (green) and tdTomato (red) should result in an orange color, in which analysis of the vWF-Cre/tomato model showed little to none. Arteries at the brain surface were also negative for tdTomato expression indicating this was not just a phenomenon of small vessels or capillaries. No correlation could be concluded about any expression difference between genders due to its highly sporadic expression pattern. Based on age, generally, adult mice (6-10wks) positive for tdTomato had a slightly higher expression level than pups (P5), but ultimately our findings indicate that this model is not be suitable for targeting endothelial cells in hopes of generating a conditional knockout mouse. It has been reported that the portion of the promoter used to make the vWF-Cre mouse does not target all endothelial cells, which supports what we have seen in the brain vasculature [59, 60].

Cdh5-Cre model targeting endothelial cells

Characterization began again with the Cdh5-Cre mouse model distributed by Jackson Laboratories. The Cdh5 gene encodes a transmembrane vascular E-cadherin protein also known as CD144, which has been shown to be required for the maintenance
**Figure 3-4. TdTomato reporter expression in vWF-Cre/tomato mice.**

A. Activation of the tdTomato reporter when bred with vWF-Cre mice. B. Representative images of tdTomato expression of whole brain, liver, kidney, and intestine from age matched vWF-Cre/tomato and control (Cre negative) tomato mice (pups n = 8, adults n = 9). Multiple animals are included to show the heterogeneity of tdTomato expression for this model. C. Representative images of frozen sections of vWF-Cre/tomato (n = 7) and control (Cre negative) tomato brain, liver, kidney, and intestine at 20X magnification.

Notes: Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
**A**

![Diagram showing genetic regulatory elements](image)

**B**

<table>
<thead>
<tr>
<th>Brightfield</th>
<th>tdTomato Output</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cre:</strong></td>
<td>Pup</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- **Brain (dorsal view)**
- **Liver**
- **Kidneys**
- **Intestine**
<table>
<thead>
<tr>
<th></th>
<th>Brain</th>
<th></th>
<th></th>
<th>Liver</th>
<th>Kidney</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>BBB</td>
<td>CP</td>
<td>Liver</td>
<td>Kidney</td>
<td>Intestine</td>
<td></td>
</tr>
<tr>
<td>Rosa Tomato</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF-Cre/tomato (P5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF-Cre/tomato (6wks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-4. Continued.
Figure 3-5. Immunohistochemistry with CD31 in comparison to native tdTomato expression in vWF-Cre/tomato mice.

Frozen brain, liver, kidney, intestine sections stained with CD31 (endothelial cell marker) antibody. Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. Green color indicates antibody staining for CD31 as indicated. Images captured at 20X magnification.

Notes: AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
<table>
<thead>
<tr>
<th>tdTomato</th>
<th>Brain</th>
<th>BBB</th>
<th>CP</th>
<th>Liver</th>
<th>Kidney</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AB</td>
<td>BBB</td>
<td>CP</td>
<td>Liver</td>
<td>Kidney</td>
<td>Intestine</td>
</tr>
<tr>
<td>cdTomato</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdTomato</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
of selective properties of the endothelial cells of the BBB [46, 51]. \( \text{Cdh5-Cre} \) mice were crossed with \text{tdTomato} reporter mice (\textbf{Figure 3-6A}) and all resulting offspring were positive for \text{tdTomato} fluorescence, an output of \text{Cre}, in endothelial cells. \text{tdTomato} fluorescence was not different between male and female mice, but did seem to increase (more intense \text{tdTomato} fluorescent signal) with increase in age from P7 to adult (6 weeks) by whole organ stereoscope analysis (\textbf{Figure 3-6B}).

Upon examining tissue sections for this difference, all ages seemed equal in intensity (\textbf{Figure 3-6C}), so the differential may be an artifact of looking at whole organs versus thin tissue slices. \text{Cdh5-Cre/tomato} mice show fluorescent signal in the brain vasculature including choroid plexus fenestrated capillaries as well as endothelial cells in liver, kidneys, and intestine shown in \textbf{Figure 3-6C}. Its important to note that this model does not express \text{tdTomato} in the other CNS barrier cells, such as AB or CP epithelium; as our goal is to target one barrier at a time. IHC with endothelial cell marker \text{CD31} was performed on frozen tissue sections to again look at the co-localization of \text{CD31} (green) and \text{tdTomato} (red). We expected to see co-localization (yellow/orange color) in endothelial cells from all organs examined and our results show this (\textbf{Figure 3-7A} and \textbf{B}).

In addition to \text{CD31} IHC, we also performed this experiment with antibodies to our ABC transporters of interest, \text{Pgp} and \text{BCRP}, to show their location in comparison to the \text{tdTomato} expression. \text{Pgp} and \text{BCRP} are expressed at the luminal surface of brain endothelial cells and play a major role in drug efflux from the brain parenchyma into the blood. However, these ABC transports are not found in peripheral endothelial cells, for example in the vasculature of organs such as liver, kidneys, or intestine. We found in our \text{Cdh5-Cre/tomato} mice that the brain endothelial cells, which make up a portion of the BBB, express \text{tdTomato}, \text{Pgp}, and \text{BCRP} as seen by the similar vascular staining pattern in \textbf{Figure 3-7A}. Using this model we can conditionally knockout \text{Pgp} or \text{BCRP} in the brain endothelial cells exclusively since \text{Pgp} or \text{BCRP} are not expressed in any endothelial cells outside the CNS.

\textbf{Characterization of Mice Expressing Cre/tdTomato in CP Epithelial Cells}

In addition to \( \nu\text{WF-Cre} \), Dr. Rodney Ho generated a \text{LPV-Cre} mouse model to target the choroid plexus epithelial cells to express \text{Cre} [47]. This model differs from others in that it uses a viral control region, specifically a lymphotropic papova virus, to drive \text{Cre} expression in the transgenic animal. It has been reported in the literature that by using this control region to drive SV40 expression, CP specific tumors arise in mice [61, 62]. In order to validate this finding and analyze other brain barriers, we crossed the \text{LPV-Cre} mice with the \text{tdTomato} reporter (\textbf{Figure 3-8A}). Resulting offspring should express \text{tdTomato} in the CP epithelium only, indicating those cells express the \text{Cre} enzyme. The \text{tdTomato} expression could not be visualized by whole organ stereoscope due the internal location of the CP in the brain, but stereoscope analysis did confirm all other organs to be negative for \text{tdTomato} (\textbf{Figure 3-8B}). Tissue sections were examined to look for \text{tdTomato} expression in the CP, where we found the CP was positive but very
Figure 3-6. TdTomato reporter expression in Cdh5-Cre/tomato mice.

A. Activation of the tdTomato reporter when bred with Cdh5-Cre mice. B. Representative images of tdTomato expression of whole brain, liver, kidney, and intestine from age matched Cdh5-Cre/tomato and control (Cre negative) tomato mice (pups n = 9, adults n = 9). C. Representative images of frozen sections of Cdh5-Cre/tomato (n = 7) and control (Cre negative) tomato brain, liver, kidney, and intestine at 20X magnification. Dashed lines in the AB column indicate the edge of the arachnoid barrier tissue on the surface of the brain. Dashed lines in the CP column indicate to boundary of the brain ventricle that contains the CP.

Notes: Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
A

B

<table>
<thead>
<tr>
<th>Brightfield</th>
<th>tdTomato Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre:</td>
<td>Pup</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- **Brain (dorsal view)**
- **Intestine**
- **Kidneys**
- **Liver**
<table>
<thead>
<tr>
<th></th>
<th>Brain</th>
<th>Liver</th>
<th>Kidney</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>BBB</td>
<td>CP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosa Tomato</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdh5-Cre/tomato (P7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdh5-Cre/tomato (6wks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-6. Continued.
Figure 3-7. Immunohistochemistry with CD31, Pgp, and BCRP markers in comparison to native tdTomato expression in Cdh5-Cre/tomato mice.

A. Sequential frozen brain sections stained with CD31 (endothelial cell marker), Pgp, and, BXP-53 (BCRP) antibodies. Images captured at 20X magnification. B. Sequential frozen liver, kidney, and intestine sections stained with CD31 (endothelial cell marker), Pgp, and, BXP-53 (BCRP) antibodies. Images captured at 20X magnification.

Notes: Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. Green color indicates antibody staining for CD31, Pgp, or BCRP. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
Figure 3-7. Continued.
Figure 3-8. TdTomato reporter expression in LPV-Cre/tomato mice.

A. Activation of the tdTomato reporter when bred with LPV-Cre mice. B. Representative images of tdTomato expression of whole brain, liver, kidney, and intestine from age matched LPV-Cre/tomato and control (Cre negative) tomato mice (pups n = 9, adults n = 15). C. Representative images of frozen sections of LPV-Cre/tomato (n = 4) and control (Cre negative) tomato brain, liver, kidney, and intestine at 20X magnification. Dashed lines indicate the boundary of the brain ventricle that contains the CP.

Notes: Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
**A**

![Diagram showing the process of Cre recombinase activity](image)

**B**

<table>
<thead>
<tr>
<th>Brightfield</th>
<th>tdTomato Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre: +</td>
<td>Pup +</td>
</tr>
<tr>
<td>Brain (dorsal view)</td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>Intestine</td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>Kidneys</td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>Liver</td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 3-8. Continued.
weak. TdTomato expression was not visualized in any endothelial or AB cells, and did not differ between males and females, but mice did seem to show a correlation of increasing fluorescent signal with increase in age from P8 to adult (7 weeks) seen in Figure 3-8C.

For further characterization, we also performed IHC with CD31, Pgp, and BCRP markers. TdTomato expression did not co-localize with CD31 (no yellow/orange color) indicating this LPV-Cre model does not have any expression within endothelial cells. The staining of BCRP and tdTomato fluorescence seems to be overlapping within the CP epithelium (Figure 3-9A). BCRP is expressed on the apical (CSF-facing) surface of mouse CP epithelial cells whereas Pgp is not expressed in mouse CP. Expression of Pgp at the CP is currently a source of contention within the literature [7-10], but our results conclude that mice do not express Pgp at the CP. Overall, these results suggest this LPV-Cre model is suitable for conditionally deleting BCRP in the mouse CP, but will make no impact on Pgp due to lack of expression at this site. The LPV-Cre/tomato mice also do not show any tdTomato expression in other organs (Figure 3-9B).

**Characterization of Pgp and BCRP Conditional Knockout Mice**

*Ptgds-Cre, Cdh5-Cre, and LPV-Cre* mice homozygous for the Cre transgene were bred with Pgp-flox and BCRP-flox mice homozygous for the floxed allele. Offspring produced from these crosses were interbred to obtain mice that were confirmed to be homozygous for both the Cre transgene and the conditional knockout allele, which is determined by MLPA and PCR genotyping. These homozygous Cre/flox mice are the conditional knockout (cKO) mice and were used to characterize the status of Pgp and BCRP proteins in the AB, BBB, and CP of the brain by using traditional colorimetric IHC. Liver, kidney, and intestine tissues were also analyzed, but the wild-type control did not stain well and therefore makes it difficult to compare the control and cKO groups. The same antibody dilution and experimental conditions are not always the same for all tissues and each tissue may not express the same amount of Pgp or BCRP protein, so more optimization will be required for these tissues in order to correctly compare control and cKO groups.

**Analysis of immunohistochemistry from Pgp conditional knockout mice**

As discussed previously, Pgp can be found at the AB and BBB within the CNS. Although Pgp’s expression at the CP is controversial, our results consistently prove that Pgp is not located within the CP of mice. Wild-type mouse brain stained with our Pgp antibody was used as a positive control, while an isotype control antibody was used for a negative control. Isotype antibodies are used to control for background issues sometimes seen when other regions of the antibody or its epitope bind other proteins or structures non-specifically. Wild-type mouse tissues showed strong Pgp expression in the brain specifically in the AB and BBB while the CP was negative, as expected.
**Figure 3-9.** Immunohistochemistry with CD31, Pgp, and BCRP markers in comparison to native tdTomato expression in LPV-Cre/tomato mice.

A. Sequential frozen brain sections stained with CD31 (endothelial cell marker), Pgp, and, BXP-53 (BCRP) antibodies. Images captured at 20X magnification.  
B. Sequential frozen liver, kidney, and intestine sections stained with CD31 (endothelial cell marker), Pgp, and, BXP-53 (BCRP) antibodies. Images captured at 20X magnification.

Notes: Cell nuclei (blue) are stained with DAPI. Red color represents the tdTomato fluorescent reporter, which is an indicator of Cre expression. Green color indicates antibody staining for CD31, Pgp, or BCRP. AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
Figure 3-9. Continued.
For the Pgp-AB KO (Ptgs-Cre x Pgp-flox) mouse, deletion of Pgp at the AB and possibly at the BBB was expected due to evidence from the Ptgs-Cre/tdTomato characterization for Cre expression. This ablation pattern of Pgp expression in the brain was confirmed by IHC (Figure 3-10). It was clear that the AB layer did not express any Pgp, but the BBB compartment was a little more difficult to interpret. It seems that some vessels were negative for Pgp staining, while others were positive for Pgp. Characterization of more animals will help determine if this is a true phenotype or just a consequence of the genetics of one animal.

In the Pgp-BBB KO (Cdh5-Cre x Pgp-flox) mice, Pgp deletion was expected in only the endothelial cells due to strong evidence from the Cdh5-Cre/tdTomato characterization data previously shown above. The AB and CP sites of the cKO brain looked identical to the wild-type mouse as expected; positive AB and negative CP. The brain endothelial expression of Pgp in this cKO was present but fainter in comparison to wild-type (Figure 3-10). This result was very surprising given the strong Cre expression seen in endothelial cells of the Cdh5-Cre/tomato mouse. There is a possibility that the floxed allele is not being excised fully for some cells, or Cre is not being expressed in 100% of the endothelial cells of the brain – although the Cdh5-Cre/tdTomato characterization data suggests Cre/tdTomato is expressed in at least the majority of endothelial cells of the brain.

The expectation for the Pgp-CP KO (LPV-Cre x Pgp-flox) mouse was that it would be identical to wild-type due to the lack of Pgp expression in the CP of mice. This cKO resulted in a Pgp staining pattern identical to a wild-type mouse brain; positive AB and BBB, while CP was negative (Figure 3-10).

Analysis of immunohistochemistry from BCRP conditional knockout mice

As discussed previously, BCRP can be found at the AB, BBB, and CP within the CNS. BCRP is also known to be located in bile canaliculi of liver, kidney tubules, and the surface of intestinal epithelium. Wild-type mouse tissues stained with BXP-53 antibody for BCRP served as a positive control, while an isotope control antibody was used as a negative control. Wild-type mouse tissues showed strong and consistent BCRP expression in all three CNS barrier compartments as well as liver, kidney, and intestine as expected.

For the BCRP-AB KO (Ptgs-Cre x BCRP-flox) mouse, deletion of BCRP at the AB and possibly at the BBB was expected due to evidence from the Ptgs-Cre/tdTomato characterization for Cre expression. BXP-53 staining showed no ablation of BCRP at the AB or BBB. Deletion of BCRP was only seen in the CP, but as an incomplete deletion (Figure 3-11). This partial ablation could be due to this mouse still having one wild-type (non-floxed) BCRP allele, as we have not yet reached homozygosity of the BCRP-floxed alleles yet in our breeding scheme. Recalling the results for the Pgp-AB KO, the difference in phenotype between the Pgp-AB KO and the BCRP-AB KO is actually quite telling. As reported above, the Ptgs-Cre mouse has some variation in its offspring.
Figure 3-10. Immunohistochemistry of cKO brain tissue from Cre/Pgp-floxed mice.

Brain sections from each cKO model stained with our Pgp antibody at 1:400,000 dilution. Wild-type tissue was from Pgp-floxed mouse (Cre negative). The Pgp-AB KO (n = 1) is the result of breeding $Ptgds$-Cre and Pgp-floxed mice. The Pgp-BBB KO (n = 2) is the result of breeding $Cdhl$-Cre and Pgp-floxed mice. The Pgp-CP KO (n = 1) is the result of breeding $LPV$-Cre and Pgp-floxed mice.

Notes: AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
Figure 3-11. Immunohistochemistry of cKO brain tissue from Cre/BCRP-floxed mice.

Brain sections from each cKO model stained with our BXP-53 antibody for BCRP at 1:2,000 dilution. Wild-type tissue was from BCRP-floxed mice (Cre negative). The BCRP-AB KO (n = 1) is the result of breeding *Ptgds*-Cre and BCRP-floxed mice. The Pgp-BBB KO (n = 2) is the result of breeding *Cdh5*-Cre and BCRP-floxed mice. The Pgp-CP KO (n = 2) is the result of breeding *LPV*-Cre and BCRP-floxed mice.

Notes: AB, arachnoid barrier. BBB, blood-brain barrier. CP, choroid plexus.
which resulted in mice not being genetically identical even though all mice stem from the same transgenic founder. This may be why we are seeing the deletion of transporters in different compartments between the Pgp and BCRP cKOs generated using the Postds-Cre model. BCRP expression in liver, kidney, and intestine from this cKO was identical to wild-type indicating no peripheral deletion of BCRP outside of the brain.

BCRP deletion was expected to be in the endothelial cells of BCRP-BBB KO (Cdh5-Cre x BCRP-flox) mice, due to strong evidence from the Cdh5-Cre/tdTomato characterization data previously shown above. This cKO showed positive BCRP staining in the AB and CP, which is identical to wild-type, and partial deletion of BCRP in the brain endothelial cells (Figure 3-11). This deletion within the endothelial cells is different from the Pgp-BBB KO in that some vessels are completely negative (indicating full deletion) and other vessels were identical to wild-type stained vessels. There seemed to be no correlation to vessel size or location. This result again was very surprising given the strong Cre expression seen in endothelial cells of the Cdh5-Cre/tdTomato mouse. There is a possibility that the floxed allele is not being excised fully in some cells or Cre is not being expressed in all of the endothelial cells of the brain. BCRP expression in liver, kidney, and intestine from this cKO was identical to wild-type indicating no deletion of BCRP outside of the brain.

In the BCRP-CP KO (LPV-Cre x BCRP-flox) mice, BCRP deletion was expected in only the CP, while the AB and BBB should look identical to wild-type. However, IHC staining with BXP-53 antibody for BCRP showed no CP ablation, resulting in all compartments staining identical to wild-type mouse brain (Figure 3-11). It is possible that the viral control region is not driving enough Cre for exon 2 of BCRP to be excised. No differences were discovered between the CP KO transporter models since the BCRP-CP KO is the only CP KO model for mice (due to the lack of Pgp expression in the CP). BCRP expression in liver, kidney, and intestine from this cKO was identical to wild-type mice indicating no peripheral deletion of BCRP outside of the brain.
CHAPTER 4. CONCLUSION

Ptgds-Cre as a Model of Arachnoid Barrier Deletion of Pgp and BCRP

Although Ptgds-Cre mice have shown to be capable of deleting ABC transporters from select brain compartments, results have been inconsistent and varied between animals. The expectation of Ptgds localization in the brain from literature supports targeting the AB and CP. Originally this model was meant for AB specific deletion of ABC transporters but upon learning of Ptgds localization in the CP, it could serve as an ideal BCSFB model. We have shown deletion of Pgp in the AB and partial deletion of BCRP in the CP (recall this model only had one BCRP-floxed allele), but this has raised the question of why do we not see the same pattern of ablation when using the same Cre line. The genotyping was confirmed several times to ensure homozygosity of Cre and the transporter floxed allele. Currently, these cKO mice, homozygous for Cre and floxed alleles, are being maintained for continued study. Multiple rounds of breeding could possibly clear up this variation seen with initial animals, possibly due to an unpure genetic background of the F1 generation of cKOs. This model can be used for pilot drug studies looking at pharmacokinetics of specific Pgp and BCRP substrates and their disposition in the brain.

Cdh5-Cre as a Model of Endothelial Cell Deletion of Pgp and BCRP

The Cdh5-Cre model had the most convincing evidence for Cre localization by the tdTomato reporter, where it is clear that Cre is localized to endothelial cells only. Cre/tdTomato expression was consistent and strong without variation between animals. To our surprise the cKO animals did not recapitulate the tdTomato phenotype in targeting the endothelial cells. BCRP was lost in only some of the brain endothelial cells while deletion of Pgp was weak in the BBB cKOs. The promoter seems robust and specific enough to drive Cre expression in endothelial cells. The only inadequacy could be that the promoter is not targeting all endothelial cells, but upon evaluating the tdTomato characterization it seems all or at least a great majority of the endothelial cells are expressing the Cre enzyme. It is unclear why loss of Pgp and BCRP in the endothelial cells is not occurring. Characterizing additional animals will aide in confirming the cKO phenotype for these mice.

LPV-Cre as a Model of Choroid Plexus Deletion of Pgp and BCRP

The LPV-Cre model had the weakest expression of tdTomato (Cre expression). This raised the question of whether this weak expression would result in enough Cre to excise the ABC transporter floxed alleles. After staining the cKO tissues for BCRP it became clear that there is not enough Cre for excision. While the Pgp-CP KO does not seem useful since mice do not express Pgp in the CP, we initially thought that mice would express Pgp since other mammals do (i.e. humans). In light of the incapability of
\textit{LPV}-Cre to knockout proteins in the CP, the \textit{Ptgds}-Cre model may serve for this compartment. We could then simply compare a BBB KO to an AB/CP KO where they would represent the comparison between the BBB versus the BCSFB, respectively.

\section*{Significance of Using Conditional Knockouts of Pgp and BCRP in the Brain}

Global Pgp and BCRP knockout mice have shown the importance of these drug transporters to drug movement, specifically at the BBB. However, due to the unique localization and expression level of Pgp and BCRP between the AB, BBB, and CP, global deletion of transporters cannot tease apart each barriers influence on drug movement. Since many therapeutic agents are or could be substrates from Pgp and BCRP, it is crucial for the development of novel drugs to estimate the role of each transporter within each barrier (AB, BBB, and CP). These cKO models have the potential to answer these questions. As a growing number of drugs are given IT, an understanding of how drug transporters at the AB and CP modulate or prevent drugs from blood to CSF will open the door to new strategies to improve drug delivery to the CSF. IT delivery of drugs is common to patients that experience metastasis of cancer cells to the CSF. The CSF harbors and protects these cells from chemotherapeutics given IV with help from drugs transporters at the BBB. With the aide of cKO models, drug penetration at the BBB versus the BCSFB can be studied as well as the comparison of the contribution of Pgp and BCRP at each barrier. Blood to CSF drug penetration is no longer only determined by the BBB and CP, but also influenced by the AB. Adding this level of complexity also supports the use of cKOs to determine the regional influence of drug transporters at the three barrier sites.
LIST OF REFERENCES


VITA

Rachel Laurene Scheib, the daughter of Robin and Gary Scheib, was born in 1987 in Grand Rapids, Michigan. Upon completing her B.S. in Biotechnology at Ferris State University located in Big Rapids, Michigan, she attended graduate school at the University of Tennessee Health Science Center in Memphis, Tennessee. During this time Rachel was involved in many student and professional organizations while serving in several leadership roles within them; Women in Medicine and Science, Graduate Student Executive Committee, The Imhotep Society, and St. Jude Women’s Club. Upon approval of this manuscript, she will receive a Masters of Science degree in Biomedical Sciences with a concentration in Cancer and Developmental Biology in May 2014. She is currently training as a histotechnologist and pursuing HTL and QIHC certifications.