A transgenic zebrafish model for the in vivo study of the blood and choroid plexus brain barriers using claudin 5

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ABSTRACT

The central nervous system (CNS) has specific barriers that protect the brain from potential threats and tightly regulate molecular transport. Despite the critical functions of the CNS barriers, the mechanisms underlying their development and function are not well understood, and there are very limited experimental models for their study. Claudin 5 is a tight junction protein required for blood brain barrier (BBB) and, probably, choroid plexus (CP) structure and function in vertebrates. Here, we show that the gene claudin 5a is the zebrafish orthologue with high fidelity expression, in the BBB and CP barriers, that demonstrates the conservation of the BBB and CP between humans and zebrafish. Expression of claudin 5a correlates with developmental tightening of the BBB and is restricted to a subset of the brain vasculature clearly delineating the BBB. We show that claudin 5a-expressing cells of the CP are ciliated ependymal cells that drive fluid flow in the brain ventricles. Finally, we find that CP development precedes BBB development and that claudin 5a expression occurs simultaneously with angiogenesis. Thus, our novel transgenic zebrafish represents an ideal model to study CNS barrier development and function, critical in understanding the mechanisms underlying CNS barrier function in health and disease.

KEY WORDS

Claudin 5, Tight junction, Zebrafish, Blood brain barrier, Choroid plexus, Transgene
INTRODUCTION

The central nervous system (CNS) is protected by three specialized barriers that shield the vulnerable brain tissue from potential threats and actively regulate exchange of ions and nutrients. The blood brain barrier (BBB) is formed by endothelial cells between blood and brain interstitial fluid and has extensive control over the immediate microenvironment of the CNS (Abbott et al., 2010, 2006). Less studied are the blood-cerebrospinal fluid (CSF) barrier, formed by the epithelial cell layer of the choroid plexus (CP) between blood and ventricular CSF, and the epithelial cell layer of the meningeal arachnoid between blood and subarachnoid CSF (Abbott et al., 2010, 2006; Obermeier et al., 2013).

The BBB and blood-CSF barrier tissues have tight junctions (TJs), consisting of protein complexes that seal adjacent cells and actively regulate barrier integrity (Greene and Campbell, 2016). TJs are protein complexes containing occludins and claudins that provide a physical barrier to block free paracellular diffusion of solutes and macromolecules (Abbott et al., 2010). More than 20 different claudin isoforms are known, of which at least four, Claudin 1, 3, 5 and 12, are involved in establishing and regulating TJs in mammalian brain endothelial cells (Abdelilah-Seyfried, 2010; Greene and Campbell, 2016). Of these, claudin 5 is the most strongly expressed in mammalian brain microvessels (Zhang et al., 2012). Although this protein was shown to be important for barrier integrity in mice, the expression of claudin 5 is not conserved in the murine CP and is not a definitive marker of the BBB (Nitta et al., 2003).

Links with BBB breakdown or dysfunction have been shown for neurodegenerative processes, inflammation and infection. These include, but are not limited to, Alzheimer's disease (Schenk and de Vries, 2016; Zenaro et al., 2016), multiple sclerosis (Macrez et al., 2016; Schenk and de Vries, 2016), amyotrophic lateral sclerosis (Garbuzova-Davis et al., 2012; Garbuzova-Davis and Sanberg, 2014), vascular dementia (Ueno et al., 2016), autoimmune encephalitis (Platt et al., 2017) and infectious meningoencephalitis (Coureuil et al., 2017; Gibson and Johnston, 2015; Swanson and McGavern, 2015). Targeting the breakdown or dysfunction of the BBB in CNS disease has significant potential as a treatment target, but is severely hampered by a lack of experimental models and current treatment is limited to broad spectrum immunosuppression/anti-inflammatory treatment with, for example, glucocorticosteroids (Obermeier et al., 2013). Furthermore, to improve drug delivery in CNS disease, specific modulation of therapeutic delivery to the brain with limited neurotoxicity is critical (Greene and Campbell, 2016), but there are very limited experimental models that allow the required noninvasive imaging and mechanistic studies (Greene and Campbell, 2016; O’Keeffe and Campbell, 2016; Zhang et al., 2010).

In vitro experimental models exist to study different aspects of the BBB, but none of them can completely mimic the complex interplay between BBB and other cells, such as...
immune cells or pathogens. In addition, in vivo systems are often restricted by the use of a single method, such as single molecular tracer injections or immunohistochemistry. Moreover, real-time in vivo imaging of both the BBB and CP is not possible in current models (Blanchette and Daneman, 2015). Therefore, we set out to develop a new model and demonstrate its potential in understanding the mechanistic biology of the BBB and CP.

The zebrafish (*Danio rerio*) model has proven to be highly accessible for real-time imaging especially in combination with fluorescently labeled tissues, and is therefore extensively used to study development and many aspects of human disease (Holtzman et al., 2016). Recently, two transgenic zebrafish lines were developed to visualize CNS angiogenesis and BBB development in vivo, demonstrating that these events occur simultaneously (Umans et al., 2017). The zebrafish BBB and CP are similar to higher vertebrates, and Claudin 5a has been suggested to play an essential role in establishment and maintenance of these barriers between systemic circulation and CNS (Henson et al., 2014; Jeong et al., 2008; Xie et al., 2010). Early in development, at 14 hours post fertilization (hpf), *claudin 5a* is expressed in the entire developing zebrafish CNS (Zhang et al., 2010). However, soon after, labeling is confined to the CP and brain vasculature (from 20 hpf and 48 hpf onwards, respectively) (Xie et al., 2010; Zhang et al., 2010). Functional studies have shown size-dependent exclusion of fluorescent tracers injected in the circulation from 2 days post fertilization (dpf) onwards, indicative of the functional maturation of the BBB shortly after TJ formation (Fleming et al., 2013; Jeong et al., 2008; van Leeuwen et al., 2014; Xie et al., 2010). Furthermore, Claudin 5a has been suggested to be involved in the establishment of the neuroepithelial ventricular barrier, which is essential for brain ventricle expansion and subsequent brain development (Zhang et al., 2012, 2010). With the use of several enhancer trap lines the presence of a diencephalic and myelencephalic CP (dCP and mCP, respectively) has been suggested to be present in zebrafish larvae early in development (Bill and Korzh, 2014; Bill et al., 2008; García-Lecea et al., 2008; Henson et al., 2014). Therefore, we considered Claudin 5a to be an excellent candidate as the basis for our new in vivo model for the BBB and CP, which adds specificity to the currently existing model systems.

In this study, we have identified *claudin 5a* as the zebrafish gene equivalent to human claudin 5. We have generated a *claudin 5a* reporter transgenic with high fidelity expression in both the BBB and CP, making time-lapse imaging of early development of these structures possible. Using our new transgenic we demonstrate that the CP forms prior to expression of *claudin 5a* in brain blood vessels and validate our model by showing that once the BBB is established *claudin 5a* expression coincides with new BBB vessel formation. In addition, *claudin 5a* expressing cells in the CP are ciliated ependymal cells that drive fluid flow early in development.
RESULTS

Zebrafish \textit{claudin 5a} is the human claudin 5 orthologue

In order to identify the zebrafish (Dr) orthologue of human (Hs) protein claudin 5 (CLDN5 gene) we used a BLASTP search of the zebrafish genome (GRCz10) using the protein sequence of Hs claudin 5. Two zebrafish proteins, Claudin 5a and Claudin 5b, were identified as being most similar by protein sequence (56.9% and 54.8% identical, respectively; Figure 1A). Alignment of the zebrafish protein sequences to the Hs sequence could not identify which of these two proteins is the orthologue. Examination of the genomic region and closer gene relatives of \textit{claudin 5a} and \textit{5b} clearly showed that \textit{claudin 5a} shared synteny with Hs CLDN5 and that \textit{claudin 5b} was only present in ray-finned fish (Figure 1B, C; data not shown). In addition, examination of zebrafish expression patterns (Thisse et al., 2004) showed that \textit{claudin 5a} was expressed in the CNS ventricle region while \textit{claudin 5b} had a cardiovascular patternning. Together, we took this as sufficient evidence that claudin 5a was the correct target gene. Using BAC recombineering (Abe et al., 2011) we inserted enhanced green fluorescent protein (EGFP) at the translation start site of the \textit{claudin 5a} gene with ~200 Kb of flanking sequence to maximize fidelity of EGFP expression to endogenous \textit{claudin 5a} (Supplementary Figure S1).

\textit{Claudin 5a} is expressed in the CP of zebrafish at 1 dpf

To study the developmental expression of \textit{claudin 5a} in \textit{TgBAC(cldn5a:EGFP)} larvae and correlate this to previous performed immunohistochemical analysis (Xie et al., 2010; Zhang et al., 2012), we performed noninvasive imaging of the brain region of larvae daily between 1 and 9 dpf and imaging of adult zebrafish at 1.5 years (Figure 2). As early as 24 hpf, GFP expression was observed in the area of the mCP and dCP (Figure 2B, arrows). The mCP consisted of a large sheet of cells covering the roof of the hindbrain ventricle early in development (Figure 2B,C) that developed into a compact cluster located in the midline of the larval head at 3 dpf (Figure 2D). In addition to expression in both CPs, labeling in brain parenchyma, presumably co-localizing with vasculature, and spinal cord was observed from 3 dpf onwards (Figure 2D, arrow). Between 3 and 5 dpf, \textit{claudin 5a} expression rapidly expanded in the entire parenchyma (Figure 2F). Interestingly, strong labeling in the midline of the larval head was observed (Figure 2B, open arrow). This labeling appeared at the same time in both CPs, connected these structures, and was sustained through development (Figure 2J, J'). In addition to \textit{Claudin 5a}:GFP expression during development and maturation of the BBB and CP, expression was maintained in the BBB of adult fish at 1.5 years (Figure 2K-M). Unfortunately, both CPs were difficult to access in whole adult brain, thus CP labeling in adults could not be confirmed. Although Claudin 5a specifically labels CNS barriers in zebrafish, transient expression was observed in the caudal hematopoietic tissue (CHT), the tip of the tail and the heart region (Supple
Figure 1. Zebrafish Claudin 5a is the homologue of human claudin 5.

[A] Protein sequence alignment of human (Hs; Homo sapiens) claudin 5, zebrafish (Dr; Danio rerio) Claudin 5a and Claudin 5b using Clustal Omega. [B,C] Syntenic analysis of claudin 5a (B) and claudin 5b (C) genes. (B) Green: Syntenic genes in the claudin 5a locus across Latimeria chalumnae (Lc), Oryzias latipes (Ol), Danio rerio (Dr), Xenopus laevis (Xl) and Homo sapiens (Hs). Purple: Syntenic genes in the claudin 5a locus across Osteichthyes (Lc, Ol, Dr). Blue: Unique gene present in the claudin 5a locus of Xenopus laevis. Open bar, 250 Kb. (C) Claudin 5b is only present in Actinopterygii (ray-finned fish), here represented by Oryzias latipes (Ol) and Danio rerio (Dr), which show conserved synteny. Syntenic genes in the claudin 5b locus across Actinopterygii.
Figure 2. Developmental expression of claudin 5a.

[A] Lateral view of a Casper zebrafish larva at 1 dpf and 7 dpf. Boxed areas represent the brain regions of which confocal images are shown in B-J. [B-J] Z-stacks of dorsal view of larval head to visualize development of GFP expression from 1 to 9 dpf. GFP expression can be found in the dCP and mCP from 1 dpf onwards (B, closed arrow). In addition, labeling is observed in the midline connecting the dCP and mCP (B, open arrow, J').
This expression was only present during early development and disappeared in later larval stages (data not shown). Collectively, our TgBAC(cldn5a:EGFP)\textsuperscript{vum1} larvae and adult zebrafish showed specific expression in brain vasculature and, in larvae, in both CPs labeling the BBB and blood-CSF barrier, respectively.

**Claudin 5a expression in brain vasculature rapidly expands between 3 dpf and 4 dpf**

To study if Claudin 5a can be found in tight junctions of brain vasculature and therefore represents the BBB, we injected our construct in the vascular specific reporter line Tg(kdrl:mCherry)\textsuperscript{is5} (Jin et al., 2005) to generate a double transgenic line, Tg(kdrl:mCherry)\textsuperscript{is5};TgBAC(cldn5a:EGFP)\textsuperscript{vum2}. Using the previously described detailed anatomical description of vasculature development (Isogai et al., 2001), we observed that expression of claudin 5a first appeared in the mesencephalic vein (MsV) and middle cerebral vein (MCeV) at 3 dpf (Figure 3A, B). Subsequent expansion of claudin 5a expression between 3 and 4 dpf occurred in large vessels first (Figure 3C, D). At 5 dpf, nearly all vessels, veins and arteries, show green fluorescence indicating that claudin 5a is expressed in virtually all the vessels in the zebrafish brain (Figure E, F). Intriguingly, a certain number of specific areas never showed claudin 5a expression (15 of 16 larvae, three biological independent experiments, Figure 3G): the primordial midbrain channels (PMBC), choroidal vascular plexus (CVP) (Figure 3G, arrows), anterior cerebral vein frontally located (ACeV) (Figure 3I-K), and at the location of the midbrain the dorsal midline junction (DMJ) and dorsal longitudinal vein (DLV) (Figure 3L-N). In addition, the strong labeling in the midline of the larval head did not co-localize with blood vessels (Figure 3H, open arrow). The Claudin 5a-deficient regions were sustained through development until at least 9 dpf and were present independent of zebrafish background [WT, Casper or Tg(kdrl:mCherry)].

**Claudin 5a expression occurs prior to new BBB vessel formation**

As stated above, we had identified that Claudin 5a was first present in the larger vessels. Development and expansion of brain vasculature continues after 4 dpf, when claudin 5a expression is established. The timing of tight junction protein expression in the BBB has been unstudied for long due to the absence of a suitable in vivo model (Haddad-Tóvolli et al., 2017). Recently, two transgenic zebrafish lines were developed that showed that tight junction protein expression occur together with CNS angiogenesis (Umans et al., 2017). Therefore, we aimed to test and validate our model in this respect. Using

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**Figure 2. Developmental expression of claudin 5a. (continued)**

From 3 dpf onwards, labeling in brain parenchyma is observed (D, open arrow). [K] Diffuse GFP expression in brain region of adult zebrafish, 1.5 years, with corresponding brightfield image. The boxed area in K and its inset is enlarged in M and L, respectively. [L] Brightfield image of blood vessel in adult brain, co-localizing with [M] Claudin 5a:GFP expression. Scale bars: 100 μm in B-J; 1 mm in K; 200 μm in L,M.
long time-lapse imaging over 12 h, starting at 96 hpf (3 dpf), in our Tg(kdrl:mCherry)\textsuperscript{is5};TgBAC(cldn5a:EGFP)\textsuperscript{vum2} double transgenic, we were able to identify sprouting vessels and follow their growth and correlation with claudin 5a expression. Careful analysis of sprouting vessels revealed that, in every case, claudin 5a expression was observed simultaneously with the initiation of a new vessel (Figure 4; Movies S1 and S2). This indicates that components of tight junctions were expressed from the initiation of BBB angiogenesis and demonstrates the essential nature of early expression of these junctions in BBB development.

**Zebrash fish larvae possess two separate blood-CP barriers that exhibit collective cell migration**

To determine the position of the claudin 5a-expressing cells being a major component of the blood-CP barrier, its localization in respect to the vasculature was analyzed in
more detail in the double transgenic line $Tg(kdrl:mCherry)^{is5};TgBAC(cldn5a:EGFP)^{vum2}$. Three-dimensional confocal analysis revealed that the ACeV and prosencephalic artery (PrA) formed a vascular circuit early in development, which overlapped with claudin 5a expression at the location of the dCP (Figure 5A, B). In the mCP in the roof of the hindbrain ventricle a similar pattern was seen: the DLV and both posterior cerebral veins (PCEv) formed a vascular circuit closely related to the cells expressing claudin 5a (Figure 5C, D). This co-localization did not change during the course of days (Figure 5A-D, com-

Figure 4. Initiation of claudin 5a expression coincides with blood vessel formation. [A,B] Still images of two time lapses of blood vessel development in $Tg(kdrl:mCherry)^{is5};TgBAC(cldn5a:EGFP)^{vum2}$ larva at 96 hpf. Images were taken every 2.5 h for a period of 12.5 h on blood vessels in the optic tectum of the midbrain. Images of single channels show blood vessel development ($kdrl:mCherryCAAX$) on the top row, coinciding with development of claudin 5a in the middle row. Merged images are shown in the bottom row. Scale bars: 20 μm. See corresponding movies of time lapses (Movies S1 and S2).
Figure 5. Blood-CP barrier.

[A] Dorsal view of dCP of Tg(kdrl:mCherry);TgBAC(cldn5a: EGFP)um2 larva at 4 dpf, showing the close correlation between the vasculature (red) and claudin 5a-expressing cells (green). The AceV and PrA form a vascular circuit surrounding the dCP. Transversal view with 3D model (A′) and single Z-slice at the dotted line in A (A′′) visualizes the close correlation between these structures. [B] Dorsal view of dCP in a larva at 9 dpf, with transversal view in a 3D model (B′) and single Z-slice at dotted line (B′′). [C] At 9 dpf, the
blood-dCP barrier is formed by the PrA. At the level of the mCP, the PCEV and DLV form a vascular circuit. Transversal view in C′ with a 3D model and C′′ with a single Z-slice at the level of the dotted line in C show the close correlation between the mCP and vasculature. [D] Dorsal view of mCP in a larva at 9 dpf, with transversal view in a 3D model (D′) and single Z-slice at dotted line (D′′). AceV, anterior cerebral vein; DLV, dorsal longitudinal vein; PCEV, posterior cerebral vein; PrA, prosencephalic artery. [E] First and last images of time lapse of dCP cell migration at 48-51 dpf. Time lapse is presented in Movie S3. [F] First and last images of time lapse of mCP cell migration at 48-51 dpf. Time lapse is presented in Movie S4. [G-I] Dorsal view of head of TgBAC(cldn5a:EGFP)vum1 larvae between 1 dpf and 3 dpf, the timeframe in which the major morphological transformation is observed. Transversal section is shown for every time point in G′,H′,I′ and G′′,H′′,I′′, corresponding to the dotted lines depicted in G-I. This visualizes the superficial localization of the GFP-expressing cells of the mCP and dCP. Scale bars: 50 μm in A-F; 100 μm in G-I.

Claudin 5a expression delineates the structured epithelial sheet of the CP

The CP is a contiguous epithelial sheet with tight junctions (Lun et al., 2015). To further validate our transgenic, and demonstrate its utility in studying the fine structure of the CP, we labeled endogenous protein via immunohistochemistry with a monoclonal antibody to mammalian claudin 5 for comparison. Antibody labeling identified a tight network of epithelial cells with claudin 5 localized to the cell margins in both the mCP and dCP structures (Figure 6A-E). This correlated with cldn5a:EGFP expression in the TgBAC(cldn5a:EGFP)vum1 and light-sheet imaging was able to resolve the same network of cells and cell junctions, even though the subcellular localizations are not exactly the same (Figure 6F-J). Differences in localization of EGFP expression were found, due to accumulation of expressed protein in the cytoplasm of cells in the TgBAC(cldn5a:EGFP)vum1 transgene and localization of antibody labeling at the cell membranes.

The cldn5a:EGFP-expressing sheet contains ciliated ependymal cells that drive cerebral spinal fluid flow

The cells of the CP are a specialized type of ependymal cells, which line the brain ventricles. To confirm the identity of our cldn5a:EGFP cells we stained for glutamylated tubulin to label cilia. We could image single cilia from cldn5a:EGFP cells in the mCP and
dCP as early as 2 dpf (Figure 7A-D). Only monociliated cells were found at all stages examined in the fore and hindbrain (Figure 7A-P). We could determine the polarity of the cldn5a:EGFP cells on the basis of the abundant labeling of glutamylated tubulin in the skin, which revealed that cilia project into the brain ventricles (Figure 7B,D,F,H,J,L,N,P). CSF is under constant flow that is thought to result from a combination of secretion of CSF from cells of the CP and the beating of the cilia lining the brain ventricles (Kramer-Zucker et al., 2005; Sawamoto et al., 2006). Using injection of fluorescently labeled beads
we were able to observe vigorous fluid flow in the CSF in both the fore- and hindbrain ventricles (Movies S5 and S6).

Claudin 5 as a prominent TJ protein is a consistent feature between the BBB and blood-CSF barrier (Bill and Korzh, 2014). Here we have used this feature to create an in vivo model for real-time analysis of the development, structure and function of the BBB and CP by generating a transgenic zebrafish line that expresses EGFP under the claudin 5a promoter. The high homology and synteny with human, the conservation along the teleost lineage and the previous characterization of Claudin 5a in zebrafish makes cldn5a a logical candidate (Abdelilah-Seyfried, 2010; Xie et al., 2010; Zhang et al., 2012).

We show that developmental expression of cldn5a:EGFP is restricted to, and starts in both CPs and the midline at 1 dpf, thereby narrowing down the previously shown
whole-mount in situ hybridizations (Zhang et al., 2010). The presence of Claudin 5a at the CPs at 1 dpf coincides with the inflation of the ventricles (Zhang et al., 2012, 2010) and corroborates its role in this process. Claudin 5a is crucial for tightening the neuro-epithelial paracellular barrier, and probably also important for proper formation of the CP, allowing the production of cerebral spinal fluid (CSF) and inflation of both ventricles. These ventricles are connected and form a system through which continues flow of CSF is ensured (Turner et al., 2012). Expression of claudin 5a possibly outlines the entire ventricular system, which can be an explanation of the midline staining we observe. Expression in the brain vasculature is only found at 3 dpf.

Within the functional highly diverse CNS, the microvasculature is expected to consist of a heterogeneous population of brain microvascular endothelial cells (BMECs) (Wilhelm et al., 2016). A considerable majority of CNS microvasculature comprise capillaries, of which the BMECs preferentially express genes related to transport of ions and nutrients (Macdonald et al., 2010). BMECs of venules instead show higher expression of genes involved in inflammatory-related processes and were shown to have a looser organization of tight junctions as compared to capillaries. This suggests a vessel-specific unique role in physiology and pathophysiology (Macdonald et al., 2010). It is likely that the majority of expression of tight junction-related genes cover all vessel types to sustain the protective function of the BBB. Therefore, the observation made in this study that some blood vessel segments lack claudin 5a expression was highly surprising. In mice, similar heterogeneous expression of claudin 5 has been observed in the spinal cord, with highest expression in capillaries and small venules and less expression in larger venules (Paul et al., 2013). Induction of experimental autoimmune encephalitis (EAE) led to loss of claudin 5 expression specifically in venules, suggesting an important vessel specific role for claudin 5 in this condition (Paul et al., 2013). Another plausible explanation for the variation in Claudin 5a presence in our model is the anatomical localization of the blood vessels in respect to brain tissue. Blood vessel segments lacking claudin 5a expression were all located at the borders of the brain and in close proximity to meninges. Therefore, it is likely that these vessels are located outside the parenchyma and do not possess a BBB.

Development of the CNS vascular network involves complex changes in endothelium and surrounding tissue and the timing of BBB formation in this process is difficult to pinpoint (Malinovskaya et al., 2016). Elaborate studies in rodents and zebrafish have shown that CNS vascularization during development mainly occurs through angiogenesis derived from the perineural vascular plexus driven by VEGF and CNS-specific Wnt/beta-catenin signaling (Blanchette and Daneman, 2015; Hagan and Ben-Zvi, 2015; Obermeier et al., 2013; Umans et al., 2017). Within a few days after initiation of vessel formation, restricted properties have been demonstrated by exclusion of fluorescent dyes from the CNS. Remarkably, this seem to happen before astrocyte generation and ensheathment...
of vessels occur, while these events have always been considered to be essential for BBB establishment (Blanchette and Daneman, 2015). Recently, within a transgenic zebrafish model for BBB development, it was observed for the first time that barriergenesis occurs simultaneously with CNS angiogenesis (Umans et al., 2017). Moreover, expression of TJ proteins is present at the initiation of angiogenesis in the CNS of mice with subsequent increase of TJ functionality during embryogenesis (Daneman et al., 2010, 2009). In the opossum, it has been demonstrated that newly formed blood vessels possess functional properties from their initiation (Ek et al., 2006). Our study offers a possible mechanism for this, whereby new vessels express *claudin 5a* immediately to form TJs, reflecting developmental steps observed in other models and demonstrating how our transgenic will enable determination of TJ and BBB specification and functionality.

The timespan between initial TJ expression and a functionally intact BBB was for long believed to be the main reason for differences in BBB permeability at different ages. However, considering that these events coincide, an alternative explanation is a prolonged permeability of the barrier between blood and CP (Ek et al., 2006; Saunders et al., 2012). The last decade it has become clear that the blood-CP barrier has more functions than solely CSF production and makes significant contributions to brain homeostasis. Junctional, enzymatic and transporter proteins have been identified and the CP may serve as entry route for immune cells, compounds and even pathogens (Lun et al., 2015). The blood-CP barrier is formed by a monolayer of cuboidal epithelial cells, i.e. ependymal cells, which surround stromal tissue and capillaries, and are joined together by tight junctions (Lun et al., 2015). Studies with enhancer trap lines were the first to describe the two CPs in zebrafish and suggested that at least four different cell lineages develop into stromal, epithelial, endothelial and astroglial components (Bill and Korzh, 2014). The previously described enhancer trap line shows a broad expression profile, of an unidentified gene, in the region of the CP (Bill et al., 2008; García-Lecea et al., 2008). In precision, our transgenic line corroborates and extends those findings and shows cells originating from the roofplate express *claudin 5a* and develop into ciliated ependymal cells.

The treatment of CNS disease is severely impeded by an inability to modulate the entry and exit of therapeutic compounds. Therefore, an improved understanding of the biology of the BBB and blood-CP barrier is of essential importance to improve treatment. Claudin 5 is a tempting target for manipulation, since many hydrophilic drugs prefer to cross the BBB via the paracellular pathway (Greene and Campbell, 2016; Saunders et al., 2013). However, to reduce potential dangerous consequences extensive studies in in vivo models are needed before this therapy can be applied in the clinical setting (O’Keeffe and Campbell, 2016). Promising results were achieved in a mice model for cerebral edema, a major cause for morbidity and mortality in a wide variety of conditions, such as severe traumatic brain injury, neurological cancers and brain infections. Strategies to prevent or treat this condition are limited, but transient modulation of claudin-5
with RNAi led to reduced brain swelling and a better outcome (Campbell et al., 2012). In zebrafish, a mutated fragment of clostridium perfringens enterotoxin has been shown to specifically target and modulate claudin 5, inducing transient paracellular permeability of the BBB (Liao et al., 2016). As zebrafish have also proven to be an outstanding model for new compound screens (North et al., 2007; Robertson et al., 2014), analysis of pharmacodynamics of the compound entry into the CNS is now eminently feasible.

**MATERIALS AND METHOD**

**Identification of zebrafish Claudin 5 and BAC recombineering**

Human (genome build GRCh38.p7) CLDN5 protein sequence (NP_003268.2) was used in a BLAST search of the zebrafish protein database (genome build GRCz10). Zebrafish Claudin 5a and Claudin 5b proteins were identified and aligned with human claudin 5 using Clustal Omega. Using synteny and expression pattern zebrafish claudin 5a was confirmed as the homologue of human CLDN5. A search of zebrafish genome BACs identified BAC 187M8 from the CHORI211 library (Robert Geisler and Pieter de Jong, Children’s Hospital Oakland Research Institute) as suitable for generation of a fluorescent reporter line due to significant flanking sequence up- and downstream of the claudin 5a gene. Primers were designed with a forward primer with 50 bp upstream and including the ATG codon of claudin 5a and 24 bp of the targeting vector containing EGFP and a Kanamycin resistance cassette (Dee et al., 2016). The reverse primer contained the reverse complement sequence of the 50 bp downstream of the ATG codon of claudin 5a and the reverse complement of the end of the cassette sequence. Forward primer: AACTTCTAAACTCCCTTTTAGTACCATCAGGAGTGGGAAAAAGAAACGATGGTGA-CAAGGGCGAGGAGCTGTTC; reverse primer: GTCCGCGACGCACAGGATCAGACCCAG- GAGCTCAAAAGCCGGAGGGATCTGCAAGAATTCGCCCATTGA.

Tol2 homology arms (Supplementary Figure S1) were added as described previously (Gray et al., 2011). Two nanolitres of recombineered BAC DNA at a concentration of 50 ng/μl combined withitol2 mRNA (Abe et al., 2011) at 30 ng/μl were injected to perform transgenesis.

**Zebrafish**

Maintenance of adult zebrafish took place at 26°C in aerated 5 liter tanks, in a 10:14 h light:dark cycle. Eggs were collected within the first hour post fertilization and injected at the 1-4 cellular stage. Injection was performed as described previously (Benard et al., 2012). Initial transgenesis was performed on: (1) WT zebrafish (van der Sar et al., 2004); (2) casper zebrafish, transparent because these zebrafish lack pigment (White et al., 2008); and (3) Tg(kdrl:mCherry), with red fluorescent endothelial cells (Jin et al., 2005). All
procedures involving zebrafish embryos and larvae and adult were performed in compliance with local animal welfare laws under Dier Ethische Commissie (DEC) protocol (MMI 12-01).

**Transgenesis**

At 4 dpf, larvae injected with the construct were analyzed for transgenic expression with a Leica MZ16FA fluorescence microscope. F0-embryos expressing EGFP in the brain region were selected and grown until reproducing age. Subsequent selection took place and F1 larvae with good expression were used for egg production. F2 larvae were used for further analysis and experiments described here. Stable germline transgenics \(\text{TgBAC(cldn5a:eGFP)}^{\text{vum1}}\) and \(\text{Tg(kdrl:mCherry)}^{\text{is5};\text{TgBAC(cldn5a:eGFP)}}^{\text{vum2}}\) were generated and used for the experiments.

**Whole-mount zebrafish larval staining**

Visualization of Claudin 5 expression in the BBB of zebrafish larvae was done by performing whole-mount immunohistochemical staining on fixed larvae. For this, larvae were euthanized at indicated time points with tricaine (E10521, Sigma-Aldrich) and fixed in 4% (V/V) paraformaldehyde/PBS (100122, Electron Microscopy Sciences, Hatfield, USA) at 4°C overnight or at room temperature (RT) for 4 h in microfuge tubes. Fixed larvae were dehydrated and stored in 100% methanol at −20°C until anti-claudin 5 staining was performed. In short, larvae were rehydrated, rinsed with 1% PBTx (PBS+1% Triton X-100), permeated in 0.24% trypsin in PBS and blocked for 3 h in block buffer [10% normal goat serum (NGS) in 1% PBTx (V/V)] Incubation with the primary antibody was performed overnight at RT [mouse anti-Claudin 5 (4C3C2), 187364, Invitrogen; 1:500 dilution] in antibody buffer [PBTx containing 1% (V/V) NGS and 1% (W/V) BSA]. After washing again with PBTx and incubation for 1 h in block buffer, embryos were incubated in the secondary antibody (goat anti-mouse Alexa-647, A21070, Invitrogen; 1:400 dilution), overnight at 4°C. Embryos were then washed with PBTx 5 times, 10 min each. For staining with anti-glutamylated tubulin, washing with PBST (PBS +0.1% Tween) was applied after fixation. Samples were transferred to 1% Triton X-100 in PBST and incubated at RT for 1-5 days to permeabilize. Larvae were placed in the blocking buffer [0.5% (V/V) Triton, 2% (V/V) normal goat serum in PBST] at room temperature for 2 h. Subsequently, the blocking buffer was removed and replaced with blocking buffer containing the primary antibody [anti-glutamylated tubulin (GT335) mouse IgG, AdipoGen Life Sciences, Liestal, Switzerland; 1:650 dilution]. Specimens were incubated in this solution at 4°C overnight. Larvae were then washed in PBST three times, 30 min each, on a rotator and incubated in secondary antibody (goat anti-mouse Alexa-568, Invitrogen; 1:500 dilution) in the blocking buffer for 4 h at room temperature. Embryos were washed with PBST three times, 10 min each.
Microscopy

For imaging, embryos were mounted in a drop of 1.5% low melting agarose placed on the surface of 1% agarose gel layer in a 35-mm petri dish [A9414, Sigma-Aldrich (now Merck, Darmstadt, Germany)] or embedded in 1% low melting-point agarose [12841221-01, Boehringer Mannheim (Roche Diagnostics), Basel, Switzerland] dissolved in egg water (60 μg/ml instant ocean see salts) in an eight-well microscopy μ-slide (http://www.ibidi.com). Analysis was performed with a confocal laser scanning microscope (confocal, Leica TCS SP8 X; microscope, Leica DMI 6000). LAS software and ImageJ software were used to generate 3D models, adjust brightness and contrast and create overlays. Adult zebrafish were euthanized with tricaine (E10521, Sigma-Aldrich) and directly embedded in 2% low melting-point agarose (12841221-01, Boehringer Mannheim) dissolved in egg water (60 μg/ml instant ocean see salts) with the dorsal side up. The cranial roof was removed to expose the brain. Analysis was performed with a Leica MZ16FA Fluorescence Stereo Microscope. Brightfield and fluorescence images were generated with a Leica DFC420C camera using LAS software.

Time-lapse imaging

Time-lapse imaging was performed with light-sheet fluorescence microscopy on a Zeiss Z1 with Z.1 detection optics 20×1.0 NA water immersion objective lens. Zebrafish larvae were mounted in 0.8% low melting point agarose (A9414, Sigma-Aldrich) in E3 containing 0.168 mg/ml tricaine (E10521, Sigma-Aldrich). Z-stacks were captured every 30 min over 12.5 h or 16 h. Maximum intensity projections were generated in Zeiss Zen software. Image processing (cropping, generation of merged images, and linear adjustment of pixel levels) was performed in Fiji ImageJ 2.0.0. Tracking was performed using the Manual Tracking plugin included in Fiji.

Bead flow assay

Nacre fish were injected with 4×103 1.75 mm beads (fluoresbrite carboxylate; Polysciences Inc., Warrenton, USA) into the hindbrain ventricle 2 dpf. Bead flow was imaged 24 hours post infection in the hind- and forebrain ventricles with a Nikon Ti-E with a CFI Plan Apochromat λ 20×, 0.75NA objective lens, and using Intensilight fluorescent illumination with ET/sputtered series fluorescent filters (Chroma, Bellows Falls, VT, USA). Images were captured with Neo sCMOS, 2560×2160 Format, 16.6 mm×14.0 mm Sensor Size, 6.5 μm pixel size camera (Andor, Belfast, UK) and NIS-Elements (Nikon, Richmond, UK). Images were processed (cropping, contrast enhancement) using NIS-Elements.
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SUPPLEMENTARY INFORMATION

Supplementary information contains two figures and six movies

**Supplemental Figure S1. BAC recombineering**
Illustration of BAC recombineering strategy. CHORI211 library BAC 187M8 was targeted with the insertion of eGFP following the ATG of the claudin 5a gene. Tol2 recombination sites were then added to increase likelihood of stable genome integration.

**Supplemental Figure S2. Extra-neuronal regions with transient expression of Claudin 5a**
Sagittal images of Tg(cldn5a:GFP) larvae at different time points showing transient expression at other locations than the central nervous system. Expression was observed in the caudal hematopoietic tissue (CHT) between 2-4 dpf, in the tip of the tail and in the heart region at 9 dpf.
Supplemental Movie S1. Angiogenesis 1
Blood vessel development in Tg(kdrl:mCherry)\textsuperscript{iS};TgBAC(cldn5a:EGFP)\textsuperscript{num2} larva at 96 hpi. Orientation: dorsal view, blood vessels in the optic tectum of the midbrain. Time lapse made over 12.5 hours. Corresponding still images can be found in Figure 4A.

Supplemental Movie S2. Angiogenesis 2
Blood vessel development in Tg(kdrl:mCherry)\textsuperscript{iS};TgBAC(cldn5a:EGFP)\textsuperscript{num2} larva at 96 hpi. Orientation: dorsal view, blood vessels in the optic tectum of the midbrain. Time lapse made over 12.5 hours. Corresponding still images can be found in Figure 4B.

Supplemental Movie S3. Cell tracking dCP development
Cell tracking of Cldn5a:EGFP positive cells in the forebrain ventricle demonstrating that this structure formed via collective cell migration. Z-stacks were captured every 30 minutes over 16 hours between 2 and 3 dpf. Corresponding still images are presented in Figure 5E.

Supplemental Movie S4. Cell tracking mCP development
Cell tracking of Cldn5a:EGFP positive cells in the forebrain ventricle demonstrating that this structure formed via collective cell migration. Z-stacks were captured every 30 minutes over 16 hours between 2 and 3 dpf. Corresponding still images are presented in Figure 5F.

Supplemental Movie S5. ventricular circulation, beads, dCP
CSF flow in the forebrain ventricle visualized by time lapse imaging of fluorescent beads. Dorsal view with the anterior of the larvae to the left, posterior to the right. Images were captured every 200 ms and presented at 50 frames per second.

Supplemental Movie S6. ventricular circulation, beads, mCP
CSF flow in the hindbrain ventricle visualized by time lapse imaging of fluorescent beads. Dorsal view with the anterior of the larvae to the left, posterior to the right. Images were captured every 200 ms and presented at 50 frames per second.

REFERENCES


Chapter 6 addendum

Claudin 5a expression during mycobacterial CNS invasion

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INTRODUCTION

Blood-brain barrier (BBB) dysfunction or break-down has been linked to fungal, bacterial and viral meningoencephalitis (Coureuil et al., 2017; Gibson and Johnston, 2015; Swanson and McGavern, 2015). In the case of tuberculous meningitis, evidence for BBB disruption are increased levels of vascular endothelial growth factor in the cerebrospinal fluid (CSF) (van der Flier et al., 2004; Visser et al., 2015). However, very little experimental models exist for the mechanistic studies needed to support BBB dysfunction during TBM. The model presented in chapter 5 is one of the first transgenic zebrafish lines allowing for real time in vivo imaging of infectious pathophysiology at the level of the BBB and blood-CSF barrier formed by the Choroid Plexus (CP) (van Leeuwen et al., 2018).

In this proof-of-principal study, we examined the effect of the presence of Claudin 5a, one of the functional components of tight junctions in the BBB and blood-choroid plexus (CP) barrier, on mycobacterial invasion of the central nervous system (CNS). For this, we used our double transgenic Tg(kdrl:mCherry)is5;TgBAC(cldn5a:EGFP)vum2 zebrafish larvae described in chapter 6, with labeling of the vasculature, BBB and CP. As shown in Figure 3 of chapter 6, claudin 5a expression rapidly expands after 2 days post fertilization (dpf). In addition, functional studies have shown size-dependent exclusion of fluorescent tracers from the CNS from 2 dpf onwards, which indicates maturation of the BBB starting at 2 dpf and coincides with the claudin 5a expression (Fleming et al., 2013; Jeong et al., 2008; van Leeuwen et al., 2014; Xie et al., 2010). Therefore, to study mycobacterial migration across a functionally intact BBB, injection of crimson-fluorescent bacteria was performed at 4 dpf.

SYSTEMIC INFECTION

Systemic infection of zebrafish larvae (Figure 1A) resulted in the formation of early granulomas and spread of single bacteria in the entire CNS of all zebrafish larvae at 5 dpi (23 granulomas in 8 larvae, data not shown). Interestingly, bacterial clusters often formed in close proximity to blood vessels that lacked claudin 5a expression, in particular in the diencephalic CP (dCP) in the forebrain ventricle (FBV) and the myelencephalic CP (mCP) in the hindbrain ventricle (HBV) (5 resp. 6 granulomas of total 23 granuloma in 8 larvae, data not shown; Fig 1B, arrows). The dCP is formed by the anterior cerebral vein (ACeV) and prosencephalic artery (PrA) and because bacterial cluster formed next to these vessels we hypothesized that bacteria traverse the blood vessels that form the CP to leave the circulation (Figure 1 C-F). Additionally, two early granulomas were formed in close proximity to the midbrain junction (Figure 1 G-I), and four were formed near the choroidal vascular plexus and the eye (data not shown). These blood vessels are
Figure 1. Systemic dissemination of *M. marinum* and invasion of the CNS

[A] Bright field image of casper Tg(kdrl:mCherry)\textsuperscript{105};TgBAC(cldn5a:EGFP)<\textsuperscript{vum2} zebrafish larvae at 9 dpf. Red arrow indicates the caudal vein as injection spot at 4 dpf to induce systemic infection, boxed area represents area of interest enlarged in B and G. [B] Dorsal view of the head of a double transgenic larvae with red fluorescent blood vessels and green fluorescent expression representing claudin 5a, 5 days after infection with crimson *M. marinum* E11 (pictured as cyan to aid visualization). Image shows infectious foci localized near both CPs (arrows). Boxed area is enlarged in C-F and shows the cluster in close proximity to the dCP. [C] Blood vessels in red, with the prosencephalic artery (PrA) procephalic and anterior cerebral vein (ACeV) as part of the blood-CP barrier, [D] Claudin 5a expression in blood vessels and ependymal cells of the CP, [E] *M. marinum* in blood vessels and surrounding tissue, [F] merged image of C-E. [G] Dorsal view of the head of a double transgenic larvae with multiple infectious foci in the CNS, boxed areas are enlarged in H and J, with I and K resp. as corresponding single green channel to show claudin 5a expression specifically. [H, I] clustering of bacteria in close proximity to a blood vessel without claudin 5a expression and bacteria co-localizing with the blood vessel wall, suggesting that mycobacterial traversal...
also devoid of claudin 5a expression and it is highly likely that bacteria that established these early granulomas originated from these vessels lacking claudin 5a expression. While single mycobacteria could be found in virtually all blood vessel of the CNS, few bacterial clusters were formed near blood vessels expressing claudin 5a, in respect to the total number of early granulomas (6 out of 23 granulomas, Figure 1 G, J, K). This difference suggests that expression of claudin 5a indicates that functional characteristics of a BBB are present and form an obstacle but do not completely block the traversal of *M. marinum* into the CNS as is shown in chapter 5 (van Leeuwen et al., 2018).

**LOCAL INFECTION INTO THE VENTRICULAR SYSTEM**

To examine the possibility of the CPs as passage for mycobacteria to enter the brain tissues, *M. marinum* was injected directly into the hindbrain ventricle of our double transgenic Tg(kdrl:mCherry)\textsuperscript{is5};TgBAC(cldn5a:EGFP)\textsuperscript{vum2} larvae at 4 dpf and infection was examined at 3 dpi (n=2) and 5 dpi (n=1) (Figure 2A, B). According to the observed vigorous fluid flow in the CSF (chapter 5, supplemental movies S5, S6), bacteria were found to spread across the entire ventricular system and were predominantly found in the HBV and FBV. Intriguingly, at 3 and 5 dpi single bacteria were found to co-localize with ependymal cells of the dCP and the mCP (Figure 2 C-F). These bacteria resided within cells (Figure 2G, arrow) or co-localized with claudin 5a expression, suggestive for an extracellular or perhaps paracellular localization (Figure 2H, arrows).

**CONCLUDING REMARKS**

Taken together, although the numbers are small, this proof-of-principle study illustrates the value of our Tg(kdrl:mCherry)\textsuperscript{is5};TgBAC(cldn5a:EGFP)\textsuperscript{vum2} in vivo reporter line for studying the pathogenesis of infectious meningoencephalitis. Systemic infection resulted in mycobacterial CNS invasion and formation of early granulomas predominantly near vessels lacking claudin 5a expression (17 out of 23 granulomas). This indicates that functional components of the BBB play an important role in restricting mycobacterial migration into the CNS and that infected phagocytic cells tend to use the route with least resistance (van Leeuwen et al., 2018). However, the lack of labeling of additional structures or tight junctions in this study, does not allow for a complete description of the localization and behavior of bacteria within the brain.
of the pathophysiological steps. One can also speculate that these blood vessels are located outside the brain parenchyma, possibly near meninges, and therefore have a different functional organization.

The interesting observation that after both systemic and ventricular infection a large proportion of early granulomas and bacteria were found in the dCP or mCP, raises the questions whether and why these particular spots are more attractive than others for bacteria to migrate from the blood into tissues. In addition, it brings into question if bacterial transport is possible in both directions, from blood to CP and visa versa. The presence of various immune cells in the CP, including CP macrophages and dendritic cells, and the hypothesis that the CP provides immune cell passage into the CNS might explain why this is an attractive location (Lun et al., 2015; Shechter et al., 2013). Further-
more, the CP might function as a space where infected macrophages reside to either recruit additional immune cells or to activate the adaptive immune system. Thereby the CP might serve as an entry route for mycobacteria in the ventricle (back) into the CNS, regardless if their origin is the circulation or the CSF, in order to further spread infection.

In conclusion, here we show the use of our \textit{Tg(kdrl:mCherry)^{is5};TgBAC(cldn5a:EGFP)^{vum2}} zebrafish line in studying mycobacterial CNS invasion. Larger experiments, additional labeling of immune cells and tight junction proteins and functional studies with fluorescent tracers, might facilitate in answering the above-mentioned questions.
REFERENCES


