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New approaches to *in vitro* models of blood–brain barrier drug transport

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The pharmaceutical industry has been searching for an *in vitro* blood-brain barrier (BBB) model that preserves *in vivo* transporter functions in CNS drug discovery and development. The application of conditionally immortalized cell lines derived from transgenic animals harboring temperature-sensitive SV40 large T-antigen gene, is a rational and promising approach to such a workable *in vitro* BBB model. The established brain capillary endothelial cell lines retain the *in vivo* transport rate of several compounds and various forms of gene expression. Furthermore, this new approach has enabled the development of stable and reproducible co-culture models with a pericyte cell line and/or an astrocyte cell line.

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▼ More than 98% of candidate CNS-targeting drugs have been halted mid-development because of poor permeability across the blood-brain barrier (BBB), presenting a major problem to the pharmaceutical industry [1,2]. Over the past decade, many scientists have been searching for a workable *in vitro* model to assess *in vivo* BBB drug permeability. Several *in vitro* culture systems have been proposed, however, the major BBB transporter, the hexose transport system, was found to be down-regulated in these models, compared with that found *in vivo*. Therefore, Caco-2 cells have been used for *in vitro* screening to identify BBB-permeable drugs, but this has limitations because it is a human colon cancer cell line and not a BBB cell line; no 'workable' *in vitro* BBB model has yet been produced.

P-Glycoprotein (P-gp) is the most important BBB transporter, from a pharmaceutical viewpoint, because it prevents accumulation of drugs in the brain through its role in the brain-to-blood efflux transport of drugs [3–5]. Therefore, the design of drugs that are not P-gp substrates is considered to be a possible strategy for obtaining higher BBB permeability [6]. The function of P-gp at the BBB has been analyzed thoroughly, using knockout mice. In humans, we know that P-gp is expressed at the BBB [7], but its contribution to BBB efflux transport requires further study. However, P-gp is not the only transporter that mediates the efflux system at the BBB. We also know of efflux transport systems for neurotransmitter metabolites [8] and uremic toxins [9]. In addition, Cooray *et al.* have reported that ABCG2/BCRP, which belongs to the same transporter family as P-gp (the ATP binding cassette (ABC) transporter superfamily), is localized at the luminal membrane of human brain capillary endothelial cells (BCEC) [10]. Despite the recent progress of BBB research [11–13], the mechanisms and functions of the BBB remain to be fully clarified. Thus, a reasonable approach would be to develop *in vitro* BBB models that accurately reflect the BBB properties *in vivo*, and to employ them in the prediction of *in vivo* BBB drug permeability.

Advantages and limitations of *in vitro* BBB models

To date, many culture cell systems have been established as *in vitro* BBB and blood-cerebrospinal fluid barrier (BCSFB) models, as summarized in Tables 1 and 2. Such models can be used to estimate permeability, using small amounts of candidates, and to elucidate the molecular mechanisms of BBB and BCSFB functions. Preparation of samples for quantitative analysis is also relatively easy. Studies using primary cultured bovine BCEC [3] and immortalized mouse BCEC [14] have revealed that P-gp functions as an efflux pump at the BBB. These studies have demonstrated that the efflux transport system at the BBB actively prevents the entry of hydrophobic drugs.

Despite their advantages, cultured cell systems are not ideal models for analyzing BBB

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Table 1. *In vitro* blood-brain barrier models

Cell type	Species	Name of cell line	Source	Nature of transformation	Refs
Conditionally immortalized	Mouse	TM-BBB	Transgenic animal	#SV40 tsA58 T antigen	[22]
	Rat	TR-BBB	Transgenic animal	#SV40 tsA58 T antigen	[23]
	Rat	CR3	Primary cells	SV40 tsA58 T antigen	[47]
Immortalized	Mouse	MBEC4	Primary cells	SV40 T antigen	[14]
	Mouse	ME-2	Primary cells	Without gene transfection	[48]
	Rat	RBEC1	Primary cells	SV40 T antigen	[49]
	Rat	rBCEC4	Primary cells	Polyoma virus T antigen	[50]
	Rat	GPNT	Cell line (GP8)	SV40 T antigen	[51]
	Rat	RBE4	Primary cells	Adenovirus E1A gene	[52]
	Rat	RCE-T1	Primary cells	Rous sarcoma virus	[53]
	Bovine	t-BBEC-117	Primary cells	SV40 T antigen	[54]
	Bovine	SV-BEC	Primary cells	SV40 T antigen	[55]
	Porcine	PBMEC	Primary cells	SV40 T antigen	[56]
	Human	SV-HCEC	Primary cells	SV40 T antigen	[57]
	Human	HBEC-51	Primary cells	SV40 T antigen	[58]
	Human	BB19	Primary cells	Human papilloma E6E7 gene	[59]
Primary	Mouse				[60]
	Rat				[61]
	Bovine				[62]
	Bovine				[63]

#, transform before cell isolation

permeability, the main reason being that severe down-regulation of transport functions occurs in cultured cells [15,16]. In bovine primary cultured BCEC, the expression of glucose transporter 1 (GLUT1) is suppressed 150-fold in comparison to the *in vivo* level [17]. In addition, the function of primary cultured BCEC varies from batch to batch. Although such variations are small in immortalized BCEC, the BBB function remains suppressed. The BBB *in vivo* expresses the multidrug resistance protein 1a (mdr1a) gene, encoding P-gp, whereas an immortalized mouse BCEC line, MBEC4, does not express this but expresses another subtype [18], mdr1b, which is not expressed at the BBB *in vivo*. Therefore, it can be concluded that *in vivo* BBB permeability cannot be predicted from the results obtained with primary cultured or immortalized BCEC.

Conditionally immortalized cell lines established from transgenic animals

Immortalized BCEC lines have been established by introducing genes, such as SV40 large T-antigen gene, into primary cultured cells. By contrast, conditionally immortalized BCEC have been established by using transgenic mice [19] and rats [20] harboring the temperature-sensitive SV40 large T-antigen gene (tsA58 T-antigen gene). Use of these transgenic animals has advantages in that it is possible to establish cell lines from tiny amounts of tissues and the established cell lines show better maintenance of *in vivo* functions.

The temperature-sensitive SV40 large T-antigen gene product is activated at 33°C, but is inactive in the mice and rats at 37°C. The transgenic rats grow almost normally [20], whereas the mice develop choroid plexus tumors and die within five months [19]. To establish immortalized cell lines, cells isolated from the tissues are cultured at 33°C (Figure 1), at which point some cells acquire immortalized characteristics. Temperature-sensitive SV40 large T-antigen is inactivated by shifting the culture temperature to 37°C, under which condition cell growth is repressed or arrested. Therefore, these immortalized cells are conditionally immortal.

Table 2. *In vitro* blood-cerebrospinal fluid barrier models

Cell type	Species	Name of cell line	Source	Nature of transformation	Refs
Conditionally immortalized	Rat	TR-CSFB	Transgenic animal	#SV40 tsA58 T antigen	[27]
Immortalized	Rat	Z310	Primary cells	SV40 T antigen	[64]
Primary	Rat				[65]
	Rabbit				[66]
	Porcine				[67]

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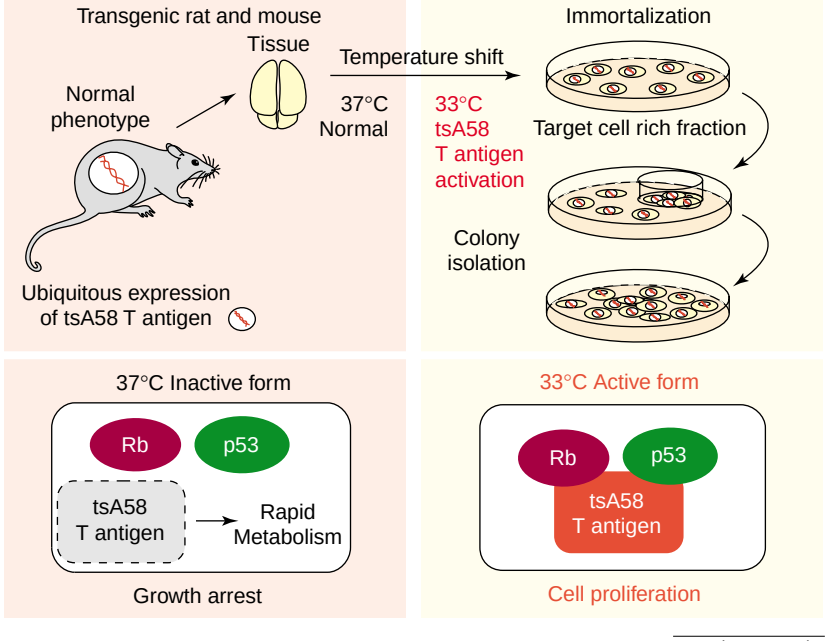

Figure 1. Establishing conditionally immortalized cell lines from transgenic animals harboring temperature-sensitive SV40 large T-antigen gene (tsA58 T-antigen gene). The strategy for establishing conditionally immortalized cell lines from transgenic animals is shown here. The tsA58 T-antigen gene product is stably expressed in all tissues in transgenic animals and is inactive at 37°C. When isolated tissue cells are cultured at 33°C, the gene product turns into the activated form and some cells acquire immortalized characteristics. The cloned cell lines are obtained by colony isolation for the rapidly growing cells.

By using transgenic mice and rats, conditionally immortalized cell lines were established from various tissues, including liver, kidney and gastric tissue [19,21]. In most cases, the conditionally immortalized cell lines retain contact inhibition, indicating that they are not tumor cells. They also show better retention of *in vivo* functions compared with traditionally immortalized cells [19]. This is probably because the temperature-sensitive SV40 large T-antigen gene was introduced into the cells before the start of *in vitro* culture, and the transgene does not disrupt the expression of other essential genes, because transgenic animals exhibiting normal phenotype were selected and used for the study [20].

The following conditionally immortalized cell lines relating to the barrier organs have been established (Figure 2)

- BBB-related cells, TM-BBB and TR-BBB from mouse and rat BCEC, respectively [22,23], TR-AST from rat astrocytes [24] and TR-PCT from rat pericytes [25,26];
- BCSFB-related cells, TR-CSFB from rat choroid plexus epithelial cells [27];
- Inner blood-retinal barrier-related cells, TR-iBRB from rat retinal capillary endothelial cells [28], TR-MUL from rat retinal Müller cells [29] and TR-rPCT from rat retinal pericytes [30];
- Blood-placental barrier-related cells, TR-TBT from rat syncytiotrophoblast cells [31].

Conditionally immortalized endothelial cells differentiated from bone marrow cells (TR-BME) have also been established [32] and, by using these cell lines, it has become possible to compare function and gene expression between cell lines. It would be difficult to establish immortalized cell lines of very small barrier organs, such as inner blood-retinal barrier-related cells, by using traditional methods of gene transfection. The afore-mentioned conditionally immortalized cell lines have identical genetic backgrounds and are derived from the same animal at the same age, thus, sets of these BBB-related cells in co-culture systems should be useful tools for analyzing cell-to-cell interaction.

Characteristics of conditionally immortalized BBB- and BCSFB-related cell lines

Tables 3 and 4 summarize the expressions of transporters in conditionally immortalized cell lines, TM-BBB, TR-BBB and TR-CSFB. These *in vitro*-cultured cell models express most of those transporters that are expressed *in vivo*. A brief description of the characteristics of the newly established conditionally immortalized cell lines follows.

Characteristics of TR-BBB cells

TR-BBB cells express BBB markers, such as von Willebrand factor, acetylated LDL uptake and γ-glutamyl transpeptidase, and show spindle fiber-shaped morphology, similar to that of primary cultured BCEC [23]. In addition, TR-BBB cells express BBB transporters, including mdr1a, large neutral amino acid transporter 1 (LAT1), GLUT1 and monocarboxylic acid transporter 1 (MCT1). TM-BBB cells also possess these characteristics [22]. Regarding transport function, transport activity for 3-O-methyl-D-glucose (3-OMG), a specific substrate for GLUT1, in TR-BBB cells is estimated to be 20% of that of *in vivo* BBB measured by means of the Brain Uptake Index and/or Brain Efflux Index methods. This indicates that TR-BBB cells retain 20% of *in vivo* glucose transport function at the BBB. Primary cultured bovine BCEC have 0.7% to 2% of *in vivo* BBB D-glucose transport

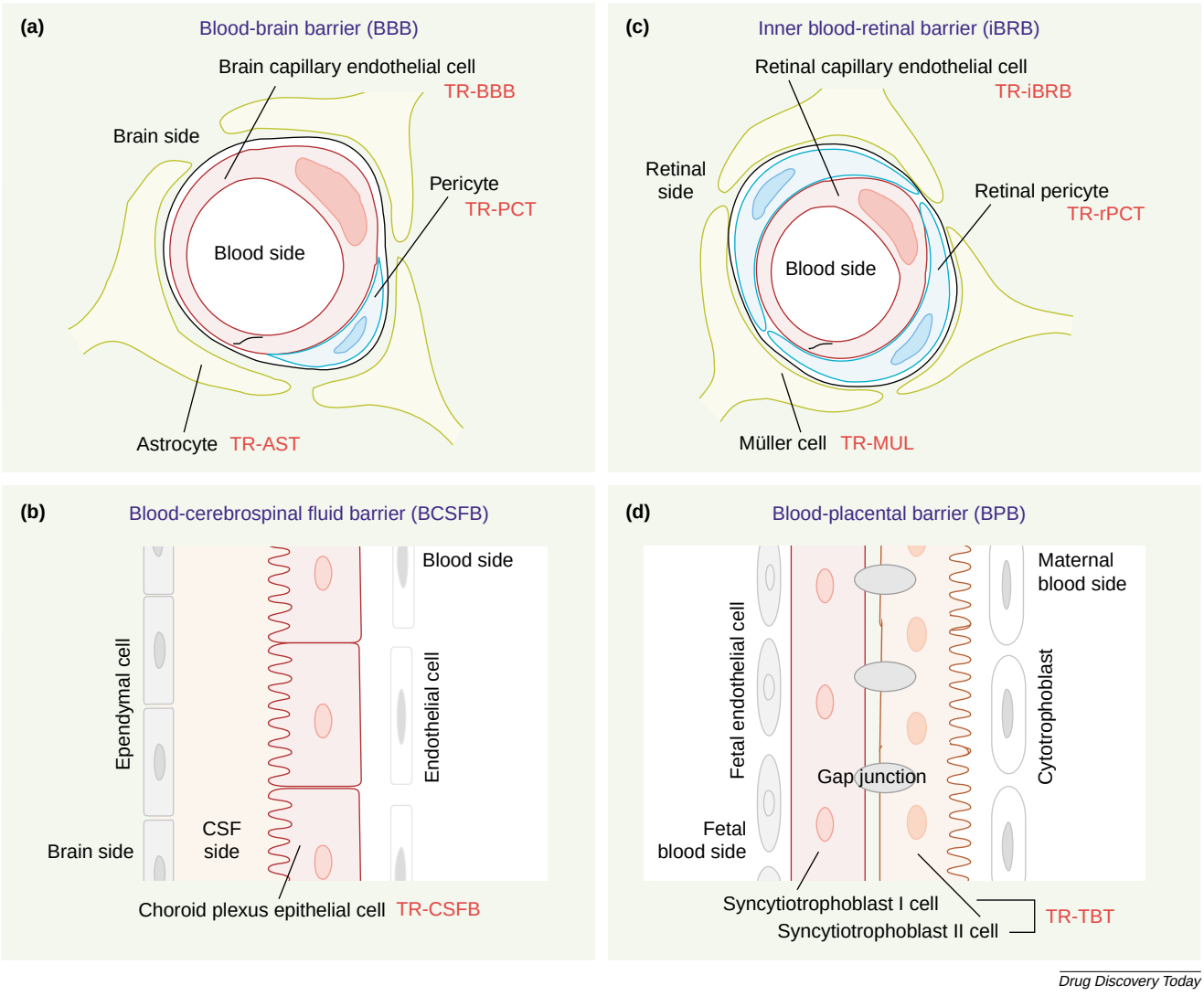

Figure 2. Conditionally immortalized cell lines of rat blood-organ barriers. Conditionally immortalized cell lines were established from the cells forming the barrier system, including blood-brain barrier (BBB) **(a)**, blood-cerebrospinal fluid barrier (BCSFB) **(b)**, inner blood-retinal barrier (iBRB) **(c)** and blood-placental barrier (BPB) **(d)**. Brain capillary endothelial cells, choroid plexus epithelial cells, retinal capillary endothelial cells and syncytiotrophoblast cells form the barrier in the BBB, BCSFB, iBRB and BPB, respectively. The cells surrounding capillary endothelial cells, such as astrocytes, pericytes and Müller cells, regulate the barrier functions of endothelial cells. The name of each cell line is indicated by red text.

function [17], therefore, TR-BBB cells exhibit at least ten-fold greater functional activity than primary cultured cells.

In vivo/*in vitro* comparison is an important issue when using immortalized cell lines as the BBB model. As shown in Table 3, TR-BBB and TM-BBB cells exhibited expression of numerous transporters that are also expressed in the *in vivo* BBB. TM-BBB cells exhibited expression and transport activity of creatine transporter (CRT) and organic anion transporting polypeptide 2 (oatp2) [33,34]. At the *in vivo* BBB, CRT was found to be localized in BCEC and was found to supply creatine [33]. Oatp2 plays a role in the brain-to-blood efflux transport of sulfate-conjugated steroids at the BBB [34] and Gao *et al.* (1999) have reported that oatp2 is localized at the BCEC [35]. TR-BBB cells express organic anion transporter 3 (OAT3) [9], and OAT3 is localized at the abluminal membrane of the BCEC, and mediates the brain-to-blood transport of neurotransmitter metabolites [8]. Therefore, TR-BBB and TM-BBB cells can be used to search for and analyze unidentified transport systems at the BBB. In addition to CRT, oatp2 and OAT3, the BBB expression and function of various transporters, such as GABA transporter 2 (GAT2/BGT-1) [36], system A transporter (ATA2) [37] and taurine transporter (TAUT) [38], have been clarified using these *in vitro* models.

Another important *in vivo*/*in vitro* comparison for consideration is that between *in vivo* BBB permeability and *in vitro*

Table 3. Transporters localized at the blood-brain barrier and expression of them in conditionally immortalized brain capillary endothelial cell lines

Transporters and transport system	(Substrates)	Expression	
		In vivo	*In vitro*
Energy transporters			
GLUT1	(D-Glucose, Dehydroascorbic acid)	+	+ (TM) (TR)
MCT1	(L-Lactate/monocarboxylates)	+	+ (TR)
CRT	(Creatine)	+	+ (TM)
Amino acid transporters			
LAT1/4F2hc[system L]	(Large neutral amino acids)	+	+ (TR)
CAT1 [system y^+]	(Cationic amino acids)	+	ND
EAAT1, 2, 3	(Anionic amino acids)	+	ND
ATA2 [system A]	(Small neutral amino acids)	+	+ (TR)
TAUT	(Taurine)	+	+ (TR)
System β	(β–Amino acids)	+	ND
ASCT2 [system ASC]	(L-Ala and others)	+	+ (TM) (TR)
System T	(Thyroid hormones)	+	ND
Neurotransmitter transporters			
GAT2/ BGT1	(GABA)	+	+ (TM)
SERT	(Serotonin)	+	+ (TM)
NET	(Norepinephrine)	+	+ (TM)
Organic anion and cation transporters			
OAT3	(PAH, HVA, Indoxyl sulfate)	+	+ (TR)
oatp2	(Digoxin, Organic anions)	+	+ (TM)
OCTN2	(Carnitine)	+	ND
Others			
CNT2	(Nucleosides)	+	ND
ABC transporters			
ABCB1	(Vincristine, Cyclosporin A)	+	+ (TM) (TR)
ABCC1	(Leukotriene C_4 and others)	+	+ (TR)
ABCG2	(Mitoxantrone, Topotecan)	+	+ (TR)

Abbreviations: GABA, γ-aminobutylic acid; HVA, homovanillic acid; PAH, *p*-aminohippuric acid ND, not determined; TM, TM-BBB (mouse); TR, TR-BBB (rat)

transport activity. As illustrated in Figure 3, the BBB permeability clearances estimated by TR-BBB and TM-BBB cells were sufficiently similar to those measured *in vivo*. This finding supports the idea that TR-BBB and TM-BBB cells can be used to estimate the BBB permeability of drugs. One limitation of these cell lines is that the expression level of mdr1a is lower than that in the *in vivo* BBB. However, it is possible to estimate the contribution of the efflux permeability clearance by mdr1a by using its stable expressing cells. Therefore, the combination of TR-BBB cells, TM-BBB cells and mdr1a expressing cells might provide a reliable *in vitro* assay system, including high-throughput screening, to discover BBB-permeable drugs.

Application of TR-BBB cells [39]

Cultured cells are useful to investigate regulation of BBB functions, especially transporter functions and tight-junction properties, and can help us to understand drug distribution under pathophysiological conditions, such as brain edema and inflammation. When TR-BBB cells were cultured under hypertonic conditions, mRNA expressions of TAUT [38] and ATA2 [37] were significantly induced. These inductions are likely to enhance the ability of BCEC to control cellular volume and to regulate osmolality in the brain through efflux of substrates and coupled ions as osmolytes.

Large T-antigen interacts with many kinds of protein, including Rb protein and p53, and these interactions often affect cell functions. Therefore, the effect of large T-antigen has to be considered when using cells into which the large T-antigen gene has been introduced. The conditionally immortalized cells can be analyzed under which conditions T-antigen activity is reduced or absent because temperature-sensitive large T-antigen is degraded rapidly at 37°C [40,41].

Co-culture system using TR-BBB, TR-AST and TR-PCT cells

The BBB is considered to be regulated by the astrocytes and

Table 4. Transporters localized at the blood-cerebrospinal fluid barrier and expression of them in conditionally immortalized choroid plexus epithelial cell lines

Transporters	(Substrates)	Expression	
		In vivo	*In vitro*
Organic anion and cation transporters			
OAT1		+	ND
OAT3		+	ND
oatp1		-[a]	-
oatp2		+	ND
oatp3		+	+
OCT2	(Choline, TEA)	+	ND
OCT3	(Guanidine, TEA)	+	ND
ABC transporters			
ABCB1		+	+
ABCC1		+	+
ABCG2		+	+

Abbreviation: ND, not determined
[a]mRNA expression of oatp1 was not detected in isolated rat choroid plexus by RT–PCR [44].

pericytes that exist around the brain capillaries. However, the mechanisms of this regulation are still unknown. Clarifying the interactions among BCEC, astrocytes and pericytes would provide important information for the construction of an *in vitro* BBB model that reflects *in vivo* function. For this purpose, conditionally immortalized cell lines of these three types of cells have been established (Figure 4). Table 5 summarizes the BBB co-culture model that has been developed previously. Conditionally immortalized rat astrocytes, TR-AST cells, exhibit the characteristics of type 2 astrocytes, that is, the expression of GFAP, A2B5, glutamine synthetase, GLT-1, system x_c^- and GLAST [24]. Conditionally immortalized rat pericytes, TR-PCT cells, also exhibit the *in vivo* characteristics of pericytes (i.e. the expression of platelet-derived growth factor receptor β, angiopoietin-1, osteopontin and intercellular adhesion molecule 1) [25,26]. Furthermore, transforming growth factor-β1 induces the expression of α-smooth muscle actin in TR-PCT cells; this induction is suppressed by subsequent treatment with basic fibroblast growth factor.

When TR-BBB cells were cultured with conditioned medium of TR-AST cells, the expression of mdr1a was significantly induced, whereas that of mdr1b was not [42]. Therefore, studies on mdr1a expression using this culture system could enable the identification of key factors that regulate an important BBB function, that is, the brain-to-blood efflux transport system for drugs.

The physiological role of pericytes is poorly understood. The conditioned medium of TR-PCT cells induced the expression of the tight-junction protein, occludin, but not that of mdr1a, suggesting that pericytes are involved, at least partly, in maintaining the tight-junctions of the BBB. Furthermore, results suggest that angiopoietin-1 that is secreted from TR-PCT cells is involved in regulating occludin expression at the BBB [43].

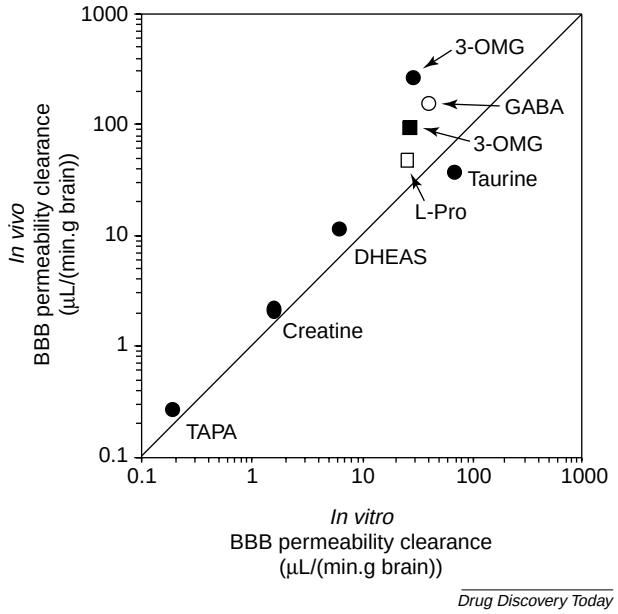

Figure 3. Comparison of the BBB permeability clearance between *in vivo* and *in vitro* experiments. Closed symbols and open symbols indicate the blood to brain influx and the brain to blood efflux in *in vivo* BBB permeability clearance, respectively (reported previously [33,34,37,77-80]). The BBB permeability clearance was estimated from transport activity measured by TR-BBB (square) or TM-BBB (circle) cells [22,23,33,34,36-38,80]. The estimations were performed with the following surface area parameters: TM-BBB, 18 cm^2/mg protein; TR-BBB, 22 cm^2/mg protein; *in vivo* brain capillary, 100 cm^2/g brain. For the clearance estimation of DHEAS, 34.4 μM of the affinity constant and 37.5 pmol/(min·mg protein) of the maximum transport rate were used, instead of the published value (37.5 nmol/(min×mg protein)), because the unit was mistyped in the published data. Abbreviations: 3-OMG, 3-O-methyl-D-glucose; DHEAS, dehydroepiandrosterone sulfate; GABA, γ-aminobutylic acid; L-Pro, L-proline; TAPA, H-Tyr-D-Arg-Phe-β-Ala-OH (Opioid Peptide).

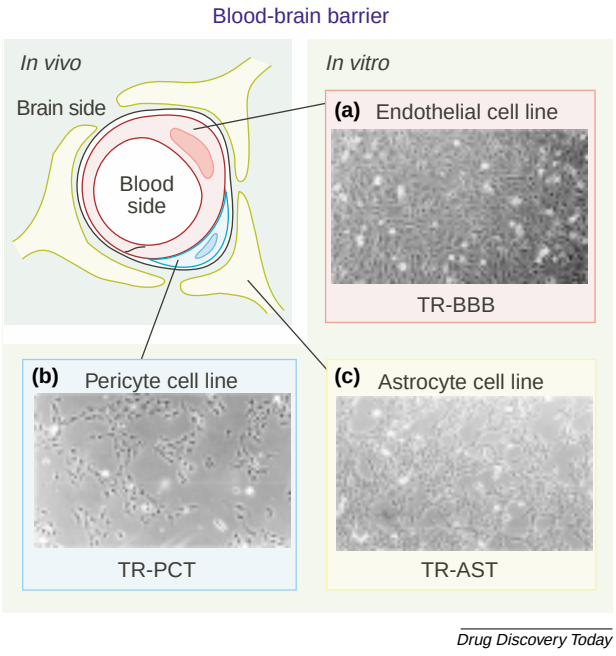

Figure 4. Phase contrast microscopic images of conditionally immortalized cells forming the blood-brain barrier. Conditionally immortalized rat brain capillary endothelial cell line, TR-BBB **(a)**; astrocyte cell line, TR-AST **(b)**; and pericyte cell line, TR-PCT **(c)**. Magnification X100.

Figure 5 shows four types of co-culture system; a non-contact co-culture model (a), a conditioned medium culture model (b), a contact co-culture model (c), and a mixed culture model (d). In the mixed culture model, three types of cell lines are cultured in the same culture plate and TR-BBB cells can be isolated by means of a cell sorter with anti-Tie2 antibody labelling. Using this mixed culture, the direct contact effect of these cells could be analyzed.

TR-CSFB cells

Choroid plexus epithelial cells form the BCSFB, which plays an important role in exchanging substances between the cerebrospinal fluid and the circulating blood. As summarized in Table 2, the isolated choroid plexus and primary cultured cells have been used mainly to investigate BCSFB transport of drugs. However, choroid plexus is an extremely small tissue and many animals are needed in studies to investigate BCSFB transport. To resolve this problem, a conditionally immortalized rat choroid plexus epithelial cell line, TR-CSFB [27] has been established. TR-CSFB cells also retain several *in vivo* BCSFB functions. TR-CSFB cells possess system A transport activity at the apical membrane, in accordance with the fact that system A exists at the brush-border membrane of *in vivo* choroid plexus epithelial cells. In addition, Na^+/K^+ ATPase is localized

Table 5. *In vitro* models for studying paracrine interactions between the blood-brain barrier-composing cells

Endothelial cells			Other cells			Systems	Refs
Cell type	**Species**	**Name of cell line**	**Cell type**	**Species**	**Name of cell line**		
Conditionally immortalized	Rat	TR-BBB	Conditionally immortalized	Rat	TR-AST	Conditioned medium,	[42, 43]
			Conditionally immortalized	Rat	TR-PCT	contact/non-contact culture and mix culture	
Immortalized	Bovine	t-BBEC-117	Primary	Rat	Astrocytes (embryo)	Conditioned medium	[54]
Immortalized	Human	ECV304 (Umbilical vein endothelial cell)	Immortalized Primary	Rat Rat	C6 glioma Astrocytes (newborn)	Contact culture and conditioned medium	[68]
Primary	Bovine		Primary	Rat	Astrocytes (newborn)	Contact culture	[69]
						Conditioned medium	[70]
Immortalized	Rat	RBE4	Primary	Rat	Astrocytes (newborn)	Conditioned medium	[52]
			Immortalized	Rat	C6 glioma	Plasma membranes and non-contact culture	[71]
Primary	Bovine		Primary	Rat	Astrocytes (newborn)	Conditioned medium	[72]
Immortalized	Mouse	Me-ly (derived from ME-2)	Primary Immortalized	Mouse Rat	Astrocytes (newborn) C6 glioma	Conditioned medium	[73]
			Primary	Mouse	Cerebrovascular smooth muscle cells		
Primary	Rat		Primary	Rat	Astrocytes (newborn)	Conditioned medium	[74]
Immortalized	Mouse	ME-2 and Me-ly	Immortalized	Rat	C6 glioma	Contact culture	[75]
Immortalized	Mouse	ME-2	Immortalized	Rat	C6 glioma	Contact culture	[76]

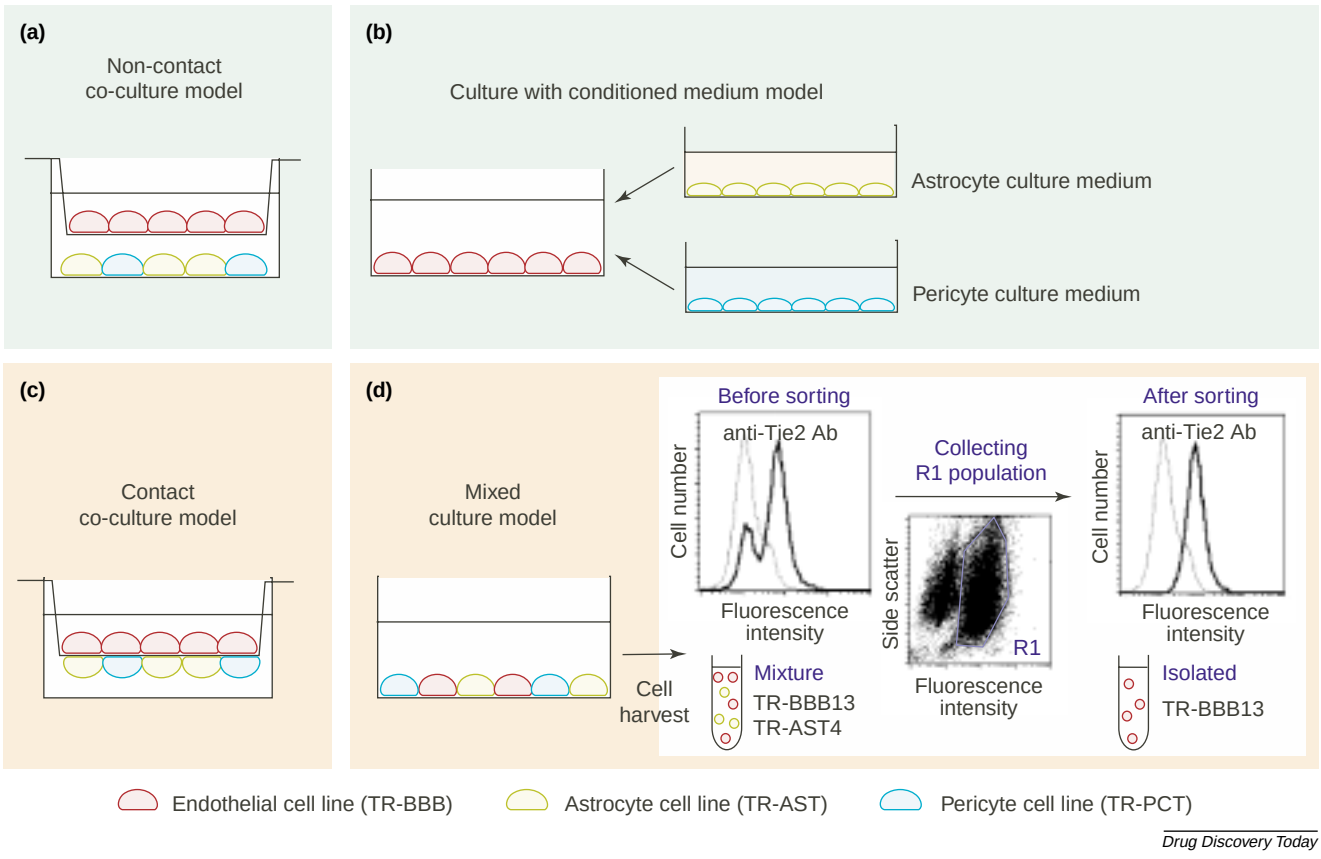

Figure 5. *In vitro* blood-brain barrier models constructed with conditionally immortalized cell lines**. (a)** Non-contact co-culture model: endothelial cells are cultured with astrocytes and pericytes using cell culture inserts without contact. Endothelial cells are cultured on the upper side of the inserts. Astrocytes and pericytes are cultured on plates. The proteins secreted from astrocytes or pericytes interacts with endothelial cells through the pores on the inserts. **(b)** Culture with conditioned medium model: conditioned medium is prepared from a single culture of either astrocytes or pericytes. The conditioned medium contains proteins secreted from astrocytes or pericytes. Then, endothelial cells are cultured with conditioned medium of astrocytes or pericytes. **(c)** Contact co-culture model: endothelial cells are cultured with astrocytes and pericytes using a cell culture insert with contact. Endothelial cells are cultured on the upper side of the insert. Astrocytes and pericytes are cultured on the opposite side of the insert. In addition to the soluble factor effect, this system produces a partial contact effect. **(d)** Mixed culture model: endothelial cells, astrocytes and pericytes are cultured in the same culture plate. Right side panel, TR-BBB cells are isolated from a mixture of TR-BBB and TR-AST cells by means of a cell sorter with anti-Tie2 (an endothelial cell-specific marker) antibody labelling. Red, yellow and blue cells indicate endothelial cells, astrocytes and pericytes, respectively.

at the apical membrane of TR-CSFB cells and ABCC1 mRNA is expressed in TR-CSFB cells (Table 4), as it is in *in vivo* BCSFB. Estrone-3-sulfate undergoes CSF-to-blood efflux transport, and TR-CSFB cells exhibit uptake activity for estrone-3-sulfate. Organic anion transporting polypeptide 3 (oatp3) has been found to be involved in this efflux transport using TR-CSFB cells (Table 4) [44]. Before this report, it was believed that oatp1 is expressed at the brush-border membrane of choroid plexus epithelial cells [35]. However, the amino acid and nucleotide sequences of oatp1 and oatp3 are very similar, and this study revealed that oatp3, not oatp1, is expressed at the brush-border membrane. This was discovered using careful RT-PCR and immunohistochemical analyses using oatp3-specific primers and antibodies, respectively [44]. Although further study is necessary, it is clear that TR-CSFB cells maintain several *in vivo* functions, as described previously, and this cell line represents a useful tool for the analysis of BCSFB functions and the regulation of BCSFB transporters.

Prospects and limitations

The conditionally immortalized cell lines of the blood-organ barriers possess many *in vivo* functions, as described here, but nevertheless, these cell lines fail to retain some of the *in vivo* properties. TR-BBB and TM-BBB cells do not form rigid tight-junctions, as seen in the *in vivo* BBB [22,23]. Although TR-BBB cells express tight-junction proteins, such as claudin-5, occludin and junctional adhesion molecule [23], the expression of these proteins might be down-regulated. Cells without tight-junctions have been

reported to make tight-junctions after introduction of the tight-junction protein, claudin-1, gene [45,46], therefore, the possibility that TR-BBB and TM-BBB cells could recover tight-junctions through expression of the claudin-5 gene in BCEC is of great interest. Tight-junctions might function as an insulator between the apical and basolateral surfaces of polarized cells, and thus play an important role in constructing cell polarity. Therefore, reconstructing tight-junctions might improve the polarity of TR-BBB and TR-CSFB cells and enable us to obtain more reliable data from transcellular transport studies using Transwell™.

We are now able to prepare stable co-culture systems using conditionally immortalized cell lines (Figures 4 and 5), which should function better as *in vitro* models than single-cultures. Furthermore, comparison of the gene expression patterns of TR-BBB, TR-AST and TR-PCT cells in co-culture with that of each cell type in single-culture would reveal the genes involved in the regulation mechanisms of BBB functions. Subtraction cloning of the genes that are selectively expressed in freshly isolated BCEC versus those in the conditionally immortalized BCEC would also reveal those genes that are down-regulated by conditional immortalization. The products of these genes could be key molecules in regulating the *in vivo* BBB properties. In this case, it might be possible to fully restore the *in vivo* BBB functions of TR-BBB cells by introducing 'BBB onset genes', affording a superior *in vitro* BBB model [2].

Conclusion

At present, conditionally immortalized cell lines have many advantages over primary cultured cells and immortalized cells as *in vitro* BBB models, even in single-culture. In developing new drugs to treat CNS and eye diseases, it is essential to be able to predict drug permeability across the barriers in the brain and retina. For this purpose, reliable model systems are essential. *In vitro* BBB models using conditionally immortalized cell lines could be a powerful tool for CNS drug delivery and discovery research, and could be applicable to high-throughput screening systems to estimate drug permeability across the BBB.

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