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The Functional Interrelationship between Gap Junctions and Fenestrae in Endothelial Cells of the Liver Organoid

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Abstract Functional intact liver organoid can be reconstructed in a radial-flow bioreactor when human hepatocellular carcinoma (FLC-5), mouse immortalized sinusoidal endothelial M1 (SEC) and A7 (HSC) hepatic stellate cell lines are cocultured. The structural and functional characteristics of the reconstructed organoid closely resemble the *in vivo* liver situation. Previous liver organoid studies indicated that cell-to-cell communications might be an important factor for the functional and structural integrity of the reconstructed organoid, including the expression of fenestrae. Therefore, we examined the possible relationship between functional intact gap junctional intercellular communication (GJIC) and fenestrae dynamics in M1-SEC cells. The fine morphology of liver organoid was studied in the presence of (1) irsogladine maleate (IM), (2) oleamide and (3) oleamide followed by IM treatment. Fine ultrastructural changes were studied by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and compared with control liver organoid data. TEM revealed that oleamide affected the integrity of cell-to-cell contacts predominantly in FLC-5 hepatocytes. SEM observation showed the presence of fenestrae on M1-SEC cells; however, oleamide inhibited fenestrae expression on the surface of endothelial cells. Interestingly, fenestrae reappeared when IM was added after initial oleamide exposure. GJIC mediates the number of fenestrae in endothelial cells of the liver organoid.

Keywords Electron Microscopy · Defenestration · Fenestra · Fenestrae formation · Liver sieve · Pore · Porosity · Sinusoidal endothelial cell · Transendothelial channel · Transendothelial transport

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Introduction

Previously, we reported that functional liver organoid was able to reassemble when human hepatocellular carcinoma cells (FLC-5), mouse immortalized sinusoidal endothelial cells (M1-SEC) and mouse immortalized hepatic stellate cells (A7 HSC) were cocultured in a radial-flow bioreactor (RFB). The intricate structural arrangement of the reconstructed liver organoid was proven to be successful as urea production in function of time could be measured as a biochemical functional marker in the supernatant. Furthermore, fenestrae were clearly detected as a structural indicator on the surface of M1-SEC cells (Saito et al.,

2006). We postulated at that time that cell-to-cell communications were an important factor in the successful reconstruction of a functional liver organoid in the RFB.

Gap junctional intercellular communication (GJIC) is one of the family members of junctional complex systems that allow transport of low-molecular weight molecules such as ions and second messengers. GJIC complexes also have an important role in regulating cell growth and tissue homeostasis (Evans & Martin, 2002). Several studies on vascular endothelial cells have shown that gap junctions between the cellular layers of arteries have important roles for altering the phenotype of endothelial cells and regulating the vascular diameter (de Wit, Hoepfl & Wolfle, 2006; Rummery & Hill, 2004; van Veen, van Rijen & Jongsma, 2006). However, to date, there is no evidence available describing the relation between SEC fenestrae and gap junctions.

Liver sinusoidal endothelial fenestrae have been shown to be responsive under numerous physiological (Wisse et al., 1985) and pathophysiological (Fraser, Dobbs & Rogers, 1995) conditions, including various pharmaceutical compounds (Braet & Wisse, 2002). The function of these peculiar membrane-bound holes which lack any diaphragm has been demonstrated in several relevant medical conditions such as cirrhosis, lipid transport and blood flow regulation (Oda, Han & Nakamura, 2000). In brief, these open pores act as bidirectional guardians for regulating the transendothelial transport of solutes, particles and food substances between the liver sinusoidal blood and the hepatocytes (Wisse, 1970).

In recent times, special attention has been paid to learning more about the origin (fenestrae formation) and loss (defenestration) of liver sinusoidal endothelial fenestrae (Braet, 2004). More recently, similar mechanisms in the dynamics and formation of diaphragmed fenestrae have been described in an endothelioma cell line (Ioannidou et al., 2006); and in the liver sinusoidal field, it was demonstrated that endothelial fenestrae could be induced in M1-SEC cells (Saito et al., 2004) and primary cultured SEC cells (Braet et al., 1998, 2007) using anti-actin agents. At present, the majority of fenestral studies are focused on finding ways to increase the liver sieve's porosity by pharmaceutical means (Braet et al., 2004; Yokomori et al., 2004). By doing so, one may hope to restore the normal liver sieving function in humans suffering from one or another fenestrae-related disorder (Braet, 2004).

In the present study, we examined the effects of the pharmaceutical compounds irsogladine maleate (IM) and oleamide on the ultrastructure and numerical dynamics of endothelial fenestrae of M1-SEC cells cultured in the RFB. IM and its antagonist oleamide have been reported to influence gap junctional function in epithelial and endothelial cells (Uchida et al., 2005; Nakashima et al., 2000; Inoguchi et al., 1995). Furthermore, there is mounting preliminary evidence illustrating the interrelation between functional gap-junctional complexes and various transendothelially mediated processes (Zahler et al., 2003; Feng et al., 1997).

The aims of our study were therefore to explore (1) the possible functional relationship between GJIC and hepatic fenestrae and (2) whether functional GJIC can induce fenestrae in endothelial cells of the liver organoid.

Materials and Methods

Cell Culture

In this study, we used a functional human hepatocellular carcinoma cell line (FLC-5) known to express drug metabolism enzymes (e.g., human-type carboxyl esterase or cytochrome) and liver-specific proteins such as albumin. *In vitro*, this cell line retains its capability for three-dimensional reorganization and possesses distinct microvilli on the cell surface. As a nonparenchymal endothelial cell line we used the immortalized M1-SEC line (Matsuura et al., 1998). The immortalized HSC line A7 (Matsuura et al., 1999) was established by isolating nonparenchymal cells from an H-2Kb-tsA58 transgenic mouse liver transfected with the simian virus 40 large T antigen gene (Jat et al., 1991). Cocultures of FLC-5, M1 and A7 cells were grown in ASF104 medium enriched with 2% fetal bovine serum.

Drug Compounds

IM [2,4-diamino-6-(2, 5-dichlorophenyl)-striazine maleate], an antiulcer drug which increases the activity of GJIC complexes via the cyclic adenosine monophosphate (cAMP) pathway, was obtained from Nippon Shinyaku (Kyoto, Japan). Oleamide is a sleep-inducing drug for animal purposes that inhibits GJIC function and was purchased from Sigma (St. Louis, MO).

Electron Microscopy

Fine structural changes were observed by means of transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

For TEM, cultured cells were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (PB) for 1 h and post-fixed with 1% OsO_4 in 0.1 M PB for 1 h at 4°C. Specimens were dehydrated in ethanol and subsequently embedded in a mixture of Epon-araldite. Thin sections (60 nm) were cut with a diamond knife mounted on an LKB (Bromma, Sweden) ultratome and stained with aqueous uranyl ace-

tate. Specimens were examined with a JEOL (Tokyo, Japan) 1200EX electron microscope at 80 k*V*.

For SEM, cultured cells were fixed with 1.2% glutaraldehyde in 0.1 M PB at pH 7.4 and postfixed with 1% OsO_4 in 0.1 M PB. The fixed cells were rinsed twice with phosphate-buffered saline, subsequently dehydrated in ascending concentrations of ethanol, critical point-dried using carbon dioxide and coated by vacuum-evaporated carbon and ion-sputtered gold. Specimens were observed using a JSM-35 (JEOL) at an accelerated voltage of 10 k*V*.

Monolayer Culture Experiments of M1 Cells

Confluent dishes of M1 cells were cultured in ASF 104 medium with or without 10 μM IM for 3 days. IM was replaced every 24 h. Fine morphology of fenestrae of M1 cells was observed by SEM. For immunofluorescence studies, a rabbit polyclonal antibody directed against a 23-amino acid C-terminal peptide sequence within the cytoplasmic domain of mouse Cx43 (Chemicon, Temecula, CA; AB1728) was used at a 1:500 dilution in 0.1 M PB. Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR; A-11008) was used as secondary antibody and diluted 1:1,000 in 0.1 M PB containing 1:200 goat serum. Next, dishes were washed three times in PB for 5 min. Samples were examined with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

RFB Cocultures

FLC-5 cells (1×10^7) were seeded in the RFB. After 5 days, 1×10^7 A7 cells were added in a similar manner, followed by another addition of 5×10^6 M1 cells 5 days later. Next, the cell line cocultures were allowed to culture for an additional 3 days to permit liver organoid reconstruction in the RFB as described (Saito et al., 2006). The RFB system used in this study is made of a 5-ml radial-flow chamber, a mass flow controller and a closed circuit reservoir (RA-5; ABLE, Tokyo, Japan). Culture medium was changed manually to maintain optimal glucose, lactate and pH levels (Saito et al., 2006).

IM and Oleamide Drug Treatment

The fine morphology of liver organoid was studied in the presence of (1) IM, (2) oleamide and (3) oleamide followed by IM treatment. More specifically, 10 μM IM was added every 24 h from day 14 until day 16. Oleamide (100 μM) was added every 24 h from day 13 until day 14. For the combined treatment, oleamide was added in a similar manner as outlined above, followed by another addition of 10 μM IM for 2 days.

Subsequently, samples were prepared for electron microscopy as outlined above, and fine ultrastructural changes were studied by TEM and SEM. Treated samples were compared with control monolayers and/or liver organoid data.

Results

In monolayers of M1 cells, the presence of connexin 43 was confirmed by confocal microscopy. A distinct positive staining for connexin 43 in the form of dots could be observed at the cell-cell contact sites (Fig. 1). SEM investigation of M1-treated cells showed an increased number of small pores resembling coated pits when treated with IM (Fig. 2).

TEM observation of the reconstructed liver organoid showed that cultured cells form multiple layers of cells mimicking liver tissue organization. The cells are closely interconnected with the neighboring cells in both control (Fig. 3a) and IM (Fig. 3b) conditions. Intercellular junctions are clearly present and keep the cells together as in the liver tissue context.

When the liver organoid was exposed to oleamide, the number of cell-to-cell contacts was overall significantly reduced,but loss was more pronounced in the areas where FLC-5 hepatocytes reside (Fig. 3c). Additionally, an overall loss in tissue architecture of the liver organoid could be observed and, instead, large clefts between the individual cells became apparent.

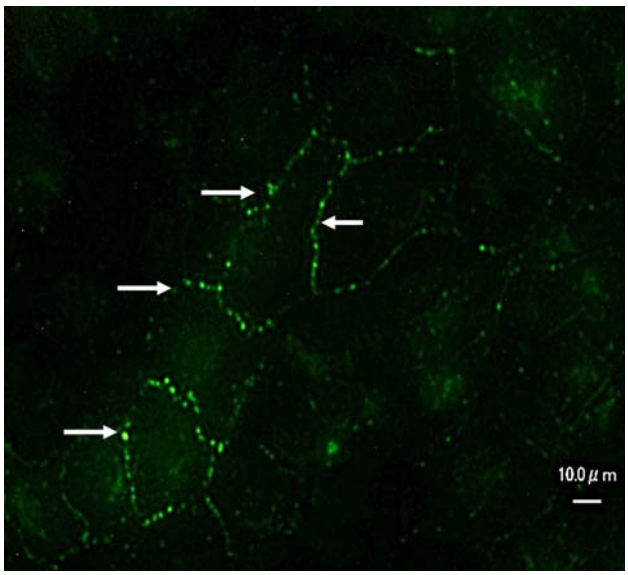

Fig. 1 Confocal laser microscopic image of M1 monolayer cell cultures stained for connexin 43. *Arrows* denote positive connexin 43 staining at the cell-to-cell border of cultured M1 cells. Scale bar = 10 μm

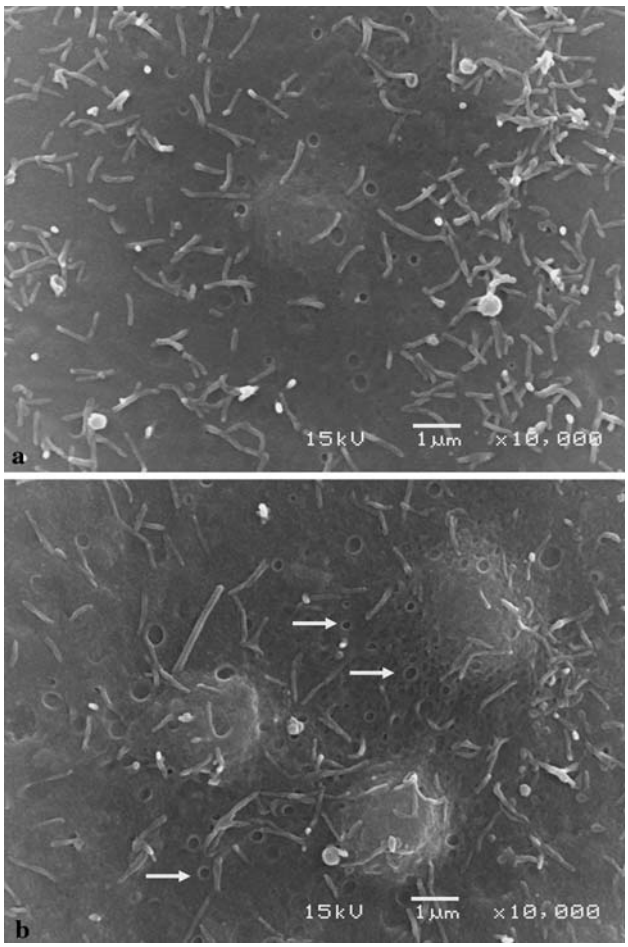

Fig. 2 SEM images of M1 cells in monolayer cell culture conditions. (**a**) Only a few small pores could be observed on the surface of control M1 cells. (**b**) An increased number of small pores (*arrows*) resembling coated pits could be observed under IM conditions. Scale bar = 1 μm

Under the combined oleamide/IM treatment conditions, cell-to-cell contact recovered to a large extent (Fig. 3d) and organoid organization was comparable to control conditions (Fig. 3a).

SEM observation of M1 control cells showed small pores (<100 nm) on the surface (Fig. 4a). In IM conditions, besides the presence of small pores, large membrane-bound openings with a diameter size of 200 nm could be routinely detected, indicative of fenestrae (Fig. 4b). On the contrary, oleamide exposure resulted in an almost nonporous surface in M1 cells (Fig. 4c), whereas oleamide administration followed by IM treatment restored the porous aspect of M1 cells as seen after IM treatment alone, i.e., small pores and fenestrae (Fig. 4d).

Discussion

Liver transendothelial transport seems to be a very complex system regulated by numerous structural and molecular pathways which use a variety of signaling molecules, receptors and (cytoskeletal) proteins. Coated pits, vesiculovacuolar organelles, caveolae, micropinocytotic vesicles and fenestrae are all subcellular structural components of the endothelium which play a key role in handling, processing and delivering transendothelial transport cargo from the liver sinusoidal vascular bed to the hepatocytes (Braet & Wisse, 2002). The model of how transport across the liver sinusoidal endothelial barrier is regulated becomes surprisingly even more complicated from this study as we demonstrated that functional GJIC from SEC and/or hepatocytes is able to upregulate or at least to sustain the porosity (area of fenestrae/area of endothelial surface) of M1 SEC cells from a distance (Fig. 4).

This study did not enlighten us about the exact mechanisms by which GJIC regulates fenestral dynamics. However, we hypothesize the following pathway based on our previous and present studies, including the existing literature. In the RFB system, culture medium flows continually, and share stress might influence the structure and function of cells. It is known that share stress changes the cytoskeleton and gene expression and induces cell stretching via connexin 43-mediated autocrine vascular endothelial growth factor (VEGF) secretion (Pimentel et al., 2002; Yamada et al., 2005). Furthermore, it is reported that cAMP levels are increased by IM, which concomitantly enhances GJIC function (Kawasaki et al., 2002). cAMP also induces VEGF gene expression. In line with this, based on the data presented here and preliminary studies, we collected evidence that (1) FLC-5 cells express mRNA of connexins 26, 32 and 43 and connexin 26 is located in the cytoplasm and connexin 32 at the cell rims and (2) M1 cells express mRNA for connexin 43 and are immunoidentified at the cell boundaries. Furthermore, we confirmed the functional integrity of GJIC by the Lucifer yellow assay (*data not shown*). We therefore hypothesize that VEGF secretion is enhanced in FLC-5 cells and acts directly on M1 cells. In addition, it has been reported that VEGF increases fenestral permeability in hepatic sinusoidal endothelial cells (Yokomori et al., 2003; DeLeve et al., 2004) and other sources of vascular endothelium (Roberts & Palade, 1995; Feng et al., 1999; Chen et al., 2002) via paracrine and autocrine pathways. We propose therefore the VEGF-mediated mechanism as one of the plausible mechanisms for the increased vascular permeability observed in the reconstructed liver organoid (Fig. 5).

In conclusion, functional and structural intact GJIC of SECs and/or hepatocytes is able to increase the number of fenestrae in M1-SECs of the liver organoid grown in the RFB coculture system. IM is a promising pharmaceutical compound to be tested first for its beneficial effects on the liver sieve's porosity *in vivo*. This opens up an entire new

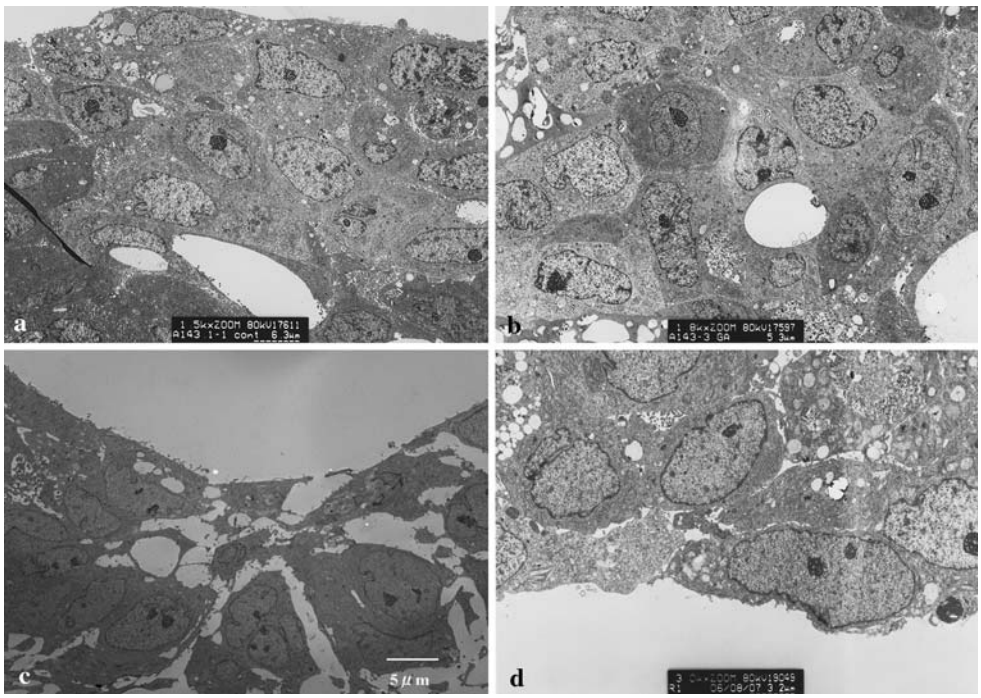

Fig. 3 Low-magnification TEM images of RFB cocultures. (**a**) TEM observation of control liver organoid shows that cells are organized in multiple layers and interconnected with the neighboring cells, closely resembling liver tissue. Scale bar = 6 μm. (**b**) IM-exposed RFB cocultures show features similar to those observed under control conditions (*see* **a** for comparison). Scale bar = 5 μm. (**c**) Oleamide exposure resulted in a significant decrease in the number of cell-to-cell contacts and, instead, large clefts between the individual cells can be observed. Scale bar = 5 μm. (**d**) Oleamide followed by IM treatment resulted in restoration of the liver oganoid tissue context. Scale bar = 3 μm

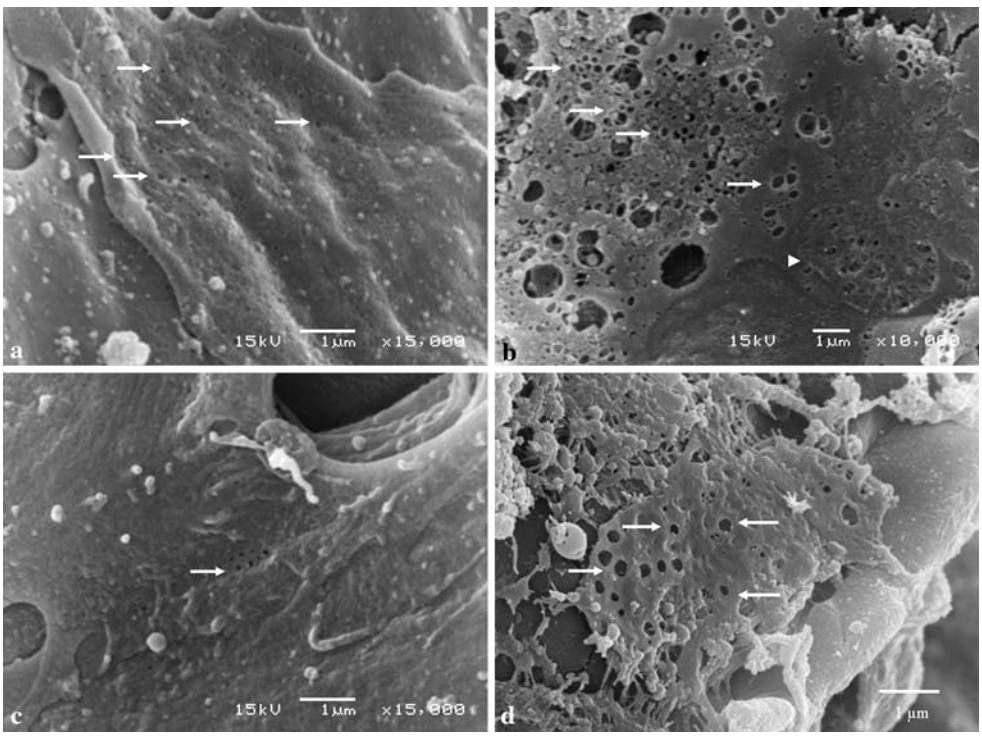

Fig. 4 SEM images of the surface of M1 cells cultured in the RFB system. (**a**) Small pores (*arrows*) can be observed on the surface of control M1 cells. (**b**) After IM exposure, small pores (*arrows*) and large openings with a diameter size of 200 nm (fenestrae) and organized in plates could be routinely detected (*arrowhead*). (**c**) Oleamide treatment resulted in a nonporous appearance of M1 cells. Small pores (*arrow*) could occasionally be observed on the surface of M1 cells. (**d**) Combined oleamide/IM treatment resulted in the reappearance of the porous surface as observed after IM treatment only (for comparison, *see* **b**). Scale bars = 1 μm

field in fenestral research in which gap junctions will be targeted, modulated and manipulated in order to restore the liver sieve's porosity or its ability to sieve. The relevant alcohol- or CCl_4-induced defenestration model of cirrhosis in rats in combination with IM and/or oleamide drug treatment might serve as a first step to bridge the gap between the exciting *in vitro* RFB data presented here and the potential future *in vivo* applications.

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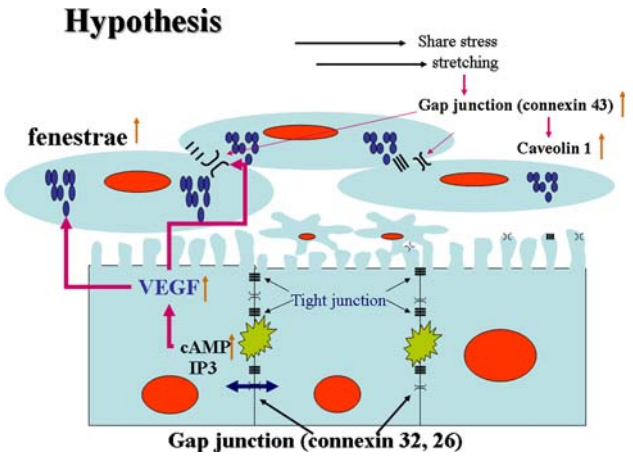

Fig. 5 Schematic presentation of the postulated mechanism by which GJIC might regulate fenestral number in M1 endothelial cells under RFB coculture conditions. As outlined in detail in the Discussion section, the molecules connexin, cAMP and VEGF play a central role in this process. *IP3* inositol 1,4,5-trisphosphate

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