MiR-34a regulates blood–tumor barrier function by targeting protein kinase Cε

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ABSTRACT **MicroRNA-34a (miR-34a) functions to regulate protein expression at the post-transcriptional level by binding the 3′ UTR of target genes and regulates functions of vascular endothelial cells. However, the role of miR-34a in regulating blood–tumor barrier (BTB) permeability remains unknown. In this study, we show that miR-34a overexpression leads to significantly increased permeability of BTB, whereas miR-34a silencing reduces the permeability of the BTB. In addition, miR-34a overexpression significantly down-regulates the expression and distribution of tight junction–related proteins in glioma endothelial cells (GECs), paralleled by protein kinase Cε (PKCε) reduction. Moreover, luciferase reporter gene analysis shows that PKCε is the target gene of miR-34a. We also show that cotransfection of miR-34a and PKCε inversely coregulates BTB permeability and protein expression levels of tight junction–related proteins. Pretreatment of ψεRACK, a PKCε-specific activator, decreases BTB permeability in miR-34a–overexpressed GECs and up-regulates expression levels of tight junction proteins. In contrast, pretreatment of εV1-2, a specific PKCε inhibitor, gives opposite results. Collectively, our findings indicate that miR-34a regulates BTB function by targeting PKCε; after phosphorylation, PKCε is activated and contributes to regulation of the expression of tight junction–related proteins, ultimately altering BTB permeability.**

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INTRODUCTION

Glioma is a common primary malignant tumor originating from adult brain glial cells and is characterized by aggressive growth, poor prognosis, and short survival period. Except for surgical treatment, chemotherapy is the main therapeutic approach to glioma. However, the presence of the blood–tumor barrier (BTB) restricts the use of anticancer drugs in the CNS, which affects the efficacy of chemotherapy (Fukaya *et al.*, 2010; Ji *et al.*, 2010; Kuijlen *et al.*, 2010). To transport drugs selectively to the tumor tissue, investigations on selective increase of BTB permeability and ways to inhibit malignant glioma progression at the molecular level will provide a novel avenue of therapeutic strategies and further improve efficacy.

Human microRNA-34 (miR-34) family members are involved in the regulation of cell cycle, senescence, and apoptosis and play a crucial role in tumor development and progression. MiR-34a, member of the miR-34 family (miR-34a, b, and c), is mainly located in the brain. MiR-34a consists of 22 nucleotides distributed at 1p36.23 and inhibits target gene translation at the posttranscriptional level by binding the 3′ or 5′ untranslated region (UTR; Wang *et al.*, 2011). Previous studies showed loss of function or low expression of miR-34a in a variety of tumors, such as leukemia, multiple myeloma, breast cancer, choriocarcinoma, and glioma, and miR-34a contributed to regulation of cell proliferation, invasion, migration, apoptosis, and autophagy via different target genes (Li *et al.*, 2012; Pang *et al.*, 2013). In addition, miR-34a down-regulated the expression of c-Met, Notch-1, Notch-2, and CDK6 and thus inhibited the proliferation and invasion of tumor cells, promoted apoptosis, and intercepted cell fate, preventing the occurrence of brain tumors (Li *et al.*, 2011; Tivnan *et al.*, 2011; Silber *et al.*, 2012). Besides its role against tumor cells, miR-34a also regulates the functions of endothelial cells

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Abbreviations used: BTB, blood–tumor barrier; GEC, glioma endothelial cell; HRP, horseradish peroxidase; NC, negative control; PKC, protein kinase C; TEER, transendothelial electric resistance.

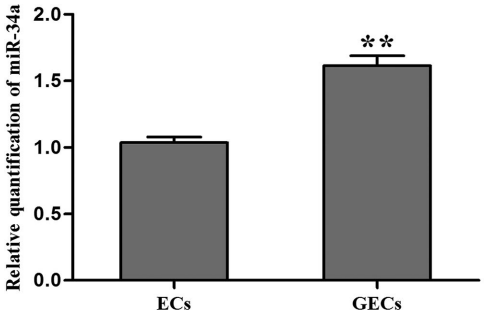

FIGURE 1: Relative expression of miR-34a in ECs and GECs. The expression of miR-34a was detected by quantitative real-time PCR. Relative gene expression was normalized to U6, and analysis was performed by the $2^{-\Delta\Delta Ct}$ method. Data represent means ± SD ($n = 6$). $^{**}p < 0.01$ vs. EC group.

and plays an important role in the growth of tumor blood vessels in angiogenesis. Up-regulation of miR-34a expression level in endothelial progenitor cells is also associated with the inhibition of silent information regulator 1 (SIRT1) expression and the induction of endothelial cells senescence (Ito *et al.*, 2010). However, whether miR-34a regulates BTB permeability has not been fully investigated.

Protein kinase C (PKC) is a phospholipid-dependent, calcium-activated protein serine/threonine kinase consisting of single multichains and is the main mediator of a variety of cell signaling pathways. PKC can mediate oncogene signals and regulate cell cycle, apoptosis, and growth, which are involved in tumor development and progression (Prévostel *et al.*, 1995). Among PKC subtypes, PKCε is most abundant in the CNS and is an important intracellular signaling molecule responsible for various cell physiological functions. Increasing evidence has highlighted the contribution of PKCε to the cell apoptosis signaling pathway and tumor occurrence, invasion, and metastasis in a variety of cells. The degree of invasion and metastasis of oncogenes was consistent with the level of PKCε in human glial cells (Besson *et al.*, 2002; Hiwasa *et al.*, 2002). PKCε is an important signaling molecule associated with various intracellular proteins and is capable of regulating the expression and phosphorylation levels of these proteins (Lu *et al.*, 2006, 2007). However, knowledge of the expression and function of PKCε in human cerebral microvascular endothelial cells (hCMEC/D3; ECs) is limited.

The purpose of this study is to investigate whether miR-34a has an effect on BTB permeability through incomplete complementary binding to the 3′ UTR of PKCε. The expression and distribution of tight junction proteins were determined to further clarify the molecular mechanism of miR-34a regulation of BTB permeability by targeting PKCε.

RESULTS

MiR-34a was expressed in normal ECs and increased significantly in glioma endothelial cells

The endogenous expression levels of miR-34a in ECs and glioma endothelial cells (GECs) were detected by quantitative real-time PCR. As shown in Figure 1, miR-34a was expressed both in ECs and GECs, but the expression level in GECs was higher than that in ECs.

Overexpression of miR-34a significantly impaired BTB permeability

To explore the effects of miR-34a on the functions of BTB, overexpression or silenced plasmids of miR-34a were constructed and transfected into ECs, respectively, and the transfected efficacy of overexpression or silencing was evaluated by quantitative real time-PCR.

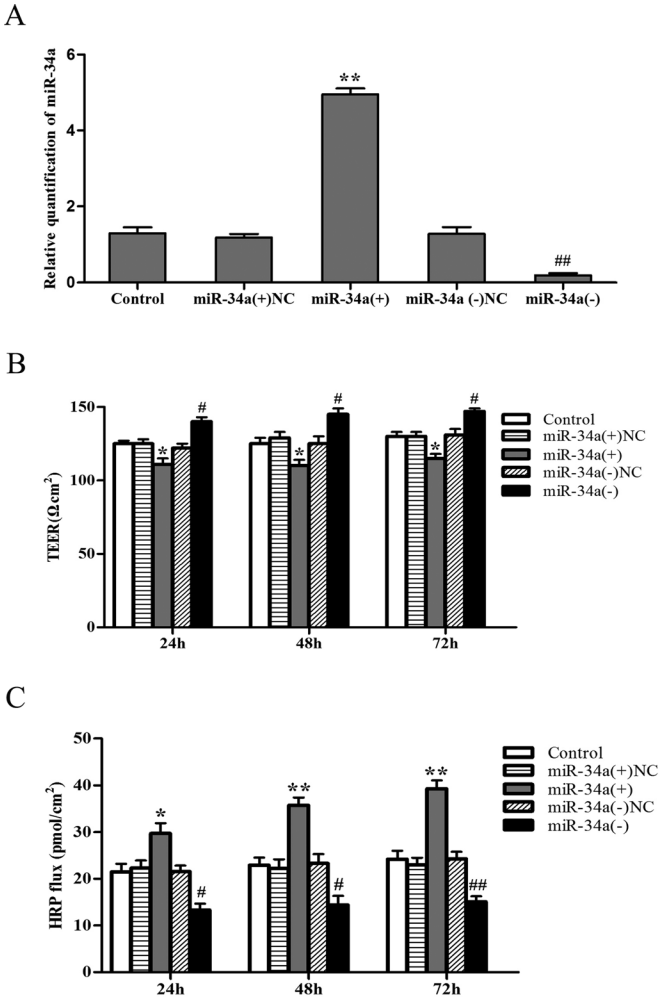

FIGURE 2: The permeability of BTB was altered after overexpression or silencing of miR-34a. (A) Relative expression of miR-34a after transfection with overexpression and silencing plasmids. The endogenous expression of miR-34a was detected by quantitative real-time PCR. The relative gene expression was normalized to U6, and analysis was performed by the $2^{-\Delta\Delta Ct}$ method. Data represent means ± SD ($n = 6$). (B) TEER values of GECs expressed as $\Omega \cdot cm^2$. Data represent means ± SD (n = 5). (C) HRP flux was calculated as $pmol/cm^2$. Data represent means ± SD ($n = 5$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. miR-34a (+) NC group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. miR-34a (–) NC group.

As shown in Figure 2A, miR-34a expression was increased significantly in miR-34a–overexpressed (miR-34a (+)) cells compared with miR-34a (+) negative control (NC) cells, whereas miR-34a expression was decreased significantly in miR-34a–silenced (miR-34a (–)) cells compared with miR-34a (–) NC cells. The expression level of miR-34a in the miR-34a (+) NC and miR-34a (–) NC groups showed no significant difference compared with the respective control groups.

Then miR-34a–overexpressed or –silenced ECs were cocultured with U87 glioma cells to establish an in vitro BTB model. Transendothelial electric resistance (TEER) and horseradish peroxidase (HRP) flux assays were carried out to evaluate whether the overexpression or silencing of miR-34a affected the permeability of the BTB. As shown in Figure 2B, the TEER values of GECs decreased in the miR-34a (+) group compared with the miR-34a (+) NC group, whereas they increased in the miR-34a (–) group compared with the miR-34a (–) NC group. The HRP flux in the miR-34a (+) group was higher than

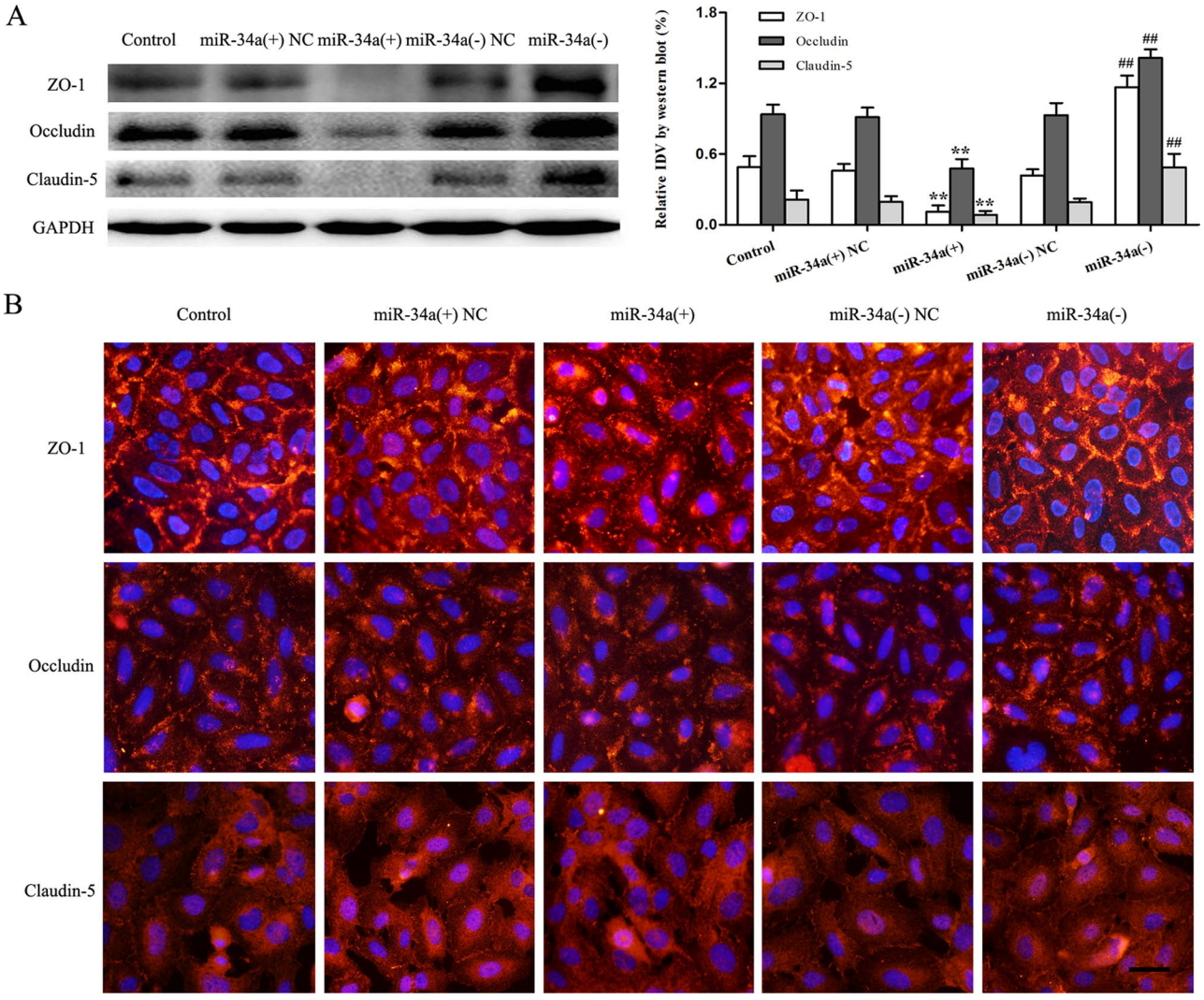

FIGURE 3: The protein expression of tight junction–related proteins was altered in GECs after overexpression or silencing of miR-34a. (A) Protein expression levels of ZO-1, occludin, and claudin-5 as assessed by Western blot. The relative IDVs of ZO-1, occludin, and claudin-5 are shown using GAPDH as an endogenous control. Values represent the means ± SD ($n = 5$, each group). $^{**}p < 0.01$ vs. miR-34a (+) NC group; $^{\#\#}p < 0.01$ vs. miR-34a (–) NC group. (B) The distribution and expression of tight junction-related proteins as detected by immunofluorescence assay. ZO-1, occludin, and claudin-5 were respectively labeled with fluorescent secondary antibody, and nuclei (blue) were labeled with DAPI. Scale bars, 20 μm.

in the miR-34a (+) NC group, whereas it was lower in the miR-34a (–) group than in the miR-34a (–) NC group (Figure 2C). The TEER value and HRP flux in the miR-34a (+) NC and miR-34a (–) NC groups were not obviously changed compared with the respective control groups.

Overexpression of miR-34a reduced the protein expression levels of tight junction–related proteins in GECs

To clarify the potential mechanisms in the changes in BTB permeability, we determined protein expression levels of ZO-1, occludin, and claudin-5 by Western blot assay. As shown in Figure 3A, protein expression levels of ZO-1 and occludin were down-regulated significantly in miR-34a (+) compared with miR-34a (+) NC cells, whereas they were up-regulated significantly in miR-34a (–) compared with miR-34a (–) NC cells. The expression levels of ZO-1, occludin, and claudin-5 in miR-34a (+) NC and miR-34a (–) NC cells showed no significant difference compared with respective control cells. Moreover, immunofluorescence analysis of ZO-1, occludin, and claudin-5 showed that ZO-1 and occludin were localized on the cell–cell boundaries and showed a continuous distribution in miR-34a (+) NC, miR-34a (–) NC, and miR-34a (–) cells, but claudin-5 was mainly expressed in the cytoplasm. However, a discontinuous distribution of ZO-1 and occludin was observed in miR-34a (+) cells, whereas the expression of ZO-1 and occludin increased in miR-34a (–) cells (Figure 3B).

The expression levels of p-PKCε and PKCε in ECs and GECs

To determine whether PKCε play a role in the process of miR-34a regulation of BTB permeability, the mRNA and we analyzed protein expression levels of PKCε in ECs and GECs by reverse transcription PCR (RT-PCR) and Western blot assay, respectively. As shown in Figure 4A, PKCε mRNA was expressed in both ECs and GECs, but there was no significant difference of PKCε mRNA expression levels in ECs compared with GECs. However, the protein expression levels of p-PKCε and PKCε decreased significantly in GECs compared with ECs (Figure 4B).

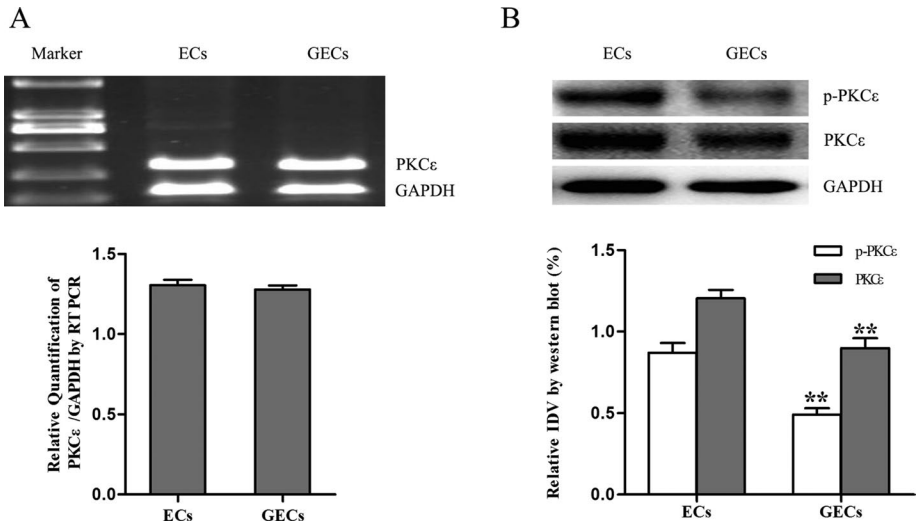

FIGURE 4: The relative expression of PKCε in ECs and GECs. (A) mRNA expression of PKCε as detected by RT-PCR. (B) Protein expression of p-PKCε or PKCε as detected by Western blot assay. GAPDH was used as inner control. Data represent means ± SD ($n = 5$). $^{**}p < 0.01$ vs. EC group.

Overexpression or silencing of miR-34a significantly affected the protein expression of p-PKCε and PKCε

To clarify whether overexpression and silencing of miR-34a affect expression of PKCε, we evaluated mRNA and protein expression levels of PKCε in overexpression or inhibition of miR-34a cells by RT-PCR and Western blot assays. As shown in Figure 5A, there was no significant difference of PKCε mRNA expression level between miR-34a (+) and miR-34a (+) NC cells or between miR-34a (–) and miR-34a (–) NC cells. However, Western blot assay showed that protein expression levels of p-PKCε and PKCε decreased in the miR-34a (+) group compared with the miR-34a (+) NC group, whereas that of the miR-34a (–) group increased compared with the miR-34a (–) NC group (Figure 5, B and D).

MiR-34a targeted the 3′ UTR of PKCε

To elucidate the molecular mechanisms responsible for the miR-34–induced down-regulation of PKCε expression, we conducted a luciferase reporter assay. Using TargetScan Human Release 6.2, PKCε was predicted to harbor putative miR-34a–binding sites in the 3′ UTR, which shared the same seed region with miR-34a (Figure 6A). The seed sequence for miR-34a to PKCε 3′ UTR was indicated. To verify that PKCε was a functional target of miR-34a, we cloned the reporter plasmids containing the wild-type 3′ UTR of PKCε (PKCε wt) and a mutant-type 3′ UTR of PKCε (PKCε mut). As shown in Figure 6B, there was no significant difference of luciferase activity in PKCε mut + miR-34a (+) cells compared with cells transfected with PKCε mut and miR-34a (+) NC. However, the luciferase activity significantly decreased in cells cotransfected with PKCε wt and miR-34a (+) plasmids compared with miR-34a (+) NC and PKCε wt plasmid–cotransfected cells. The result suggested that miR-34a targeted the 3′ UTR of PKCε.

The BTB permeability was inversely coregulated by cotransfection of miR-34a and PKCε

To clarify whether PKCε was involved in the miR-34a–mediated regulation of BTB function, we further investigated combined transfection in GECs. We evaluated the BTB permeability of cotransfected cells using TEER and HRP flux assays. As shown in Figure 7A, the TEER value in the miR-34a (+) + PKCε–silenced (PKCε (–)) group decreased compared with the miR-34a (+) + PKCε–overexpressed (PKCε (+)) group, and the TEER value in the miR-34a (–) +PKCε (–) group decreased compared with the miR-34a (–) + PKCε (+) group. There was no significant difference of TEER value in the miR-34a (+) + PKCε (+) group compared with the miR-34a (+) NC + PKCε (+) NC group and in the miR-34a (–) + PKCε (–) group compared with the miR-34a (–) NC + PKCε (–) NC group at 48 and 72 h. However, the TEER value in the miR-34a (–) + PKCε (–) group slightly increased compared with the miR-34a (–) NC+PKCε (–) NC group at 24 h. Meanwhile, the HRP flux in the miR-34a (+) + PKCε (–) group increased compared with the miR-34a (+) + PKCε (+) group, and the HRP flux in the miR-34a (–) + PKCε (–) group increased compared with the miR-34a (–) + PKCε (+) group. The HRP flux decreased in the miR-34a (+) + PKCε (+)and miR-34a (–) + PKCε (–) groups compared with the miR-34a (+) NC + PKCε (+) NC and miR-34a (–) NC + PKCε (–) NC groups, respectively (Figure 7B). In addition, the TEER value increased in the miR-34a (+) + PKCε (+) group compared with the miR-34a (+) group, whereas the TEER value in the miR-34a (–) +PKCε (–) group decreased compared with the miR-34a (–) group (Supplemental Figure S1). In the miR-34a (+) + PKCε (+) group, the overexpression of PKCε could reverse the down-regulation of PKCε expression caused by miR-34a overexpression. Conversely, in the miR-34a (+) + PKCε (–) group, miR-34a overexpression and silencing of PKCε contribute to maximizing the opening of the BTB. The results indicate that miR-34a has negative regulatory effects on PKCε after cotransfection of miR-34a and PKCε in GECs.

The expression levels of ZO-1, occludin, and claudin-5 were inversely coregulated by cotransfection of miR-34a and PKCε

To investigate further the relationship of miR-34a with PKCε, we examined the protein expression levels of ZO-1, occludin, and claudin-5 in cotransfected GECs by Western blot assay. As shown in Figure 8, the protein expression levels of ZO-1, occludin, and claudin-5 in the miR-34a (+) + PKCε (–) group were down-regulated compared with the miR-34a (+) + PKCε (+) group. Furthermore, the protein expression levels of ZO-1, occludin, and claudin-5 in the miR-34a (–) + PKCε (–) group were down-regulated compared with the miR-34a (–) + PKCε (+) group. In the miR-34a (+) + PKCε (+) group, ZO-1 and occludin expression levels were down-regulated, whereas claudin-5 was up-regulated compared with the miR-34a (+) NC + PKCε (+) NC group. Further, the expression levels of ZO-1, occludin, and claudin-5 in the miR-34a (–) + PKCε (–) group were down-regulated compared with the miR-34a (–) NC + PKCε (–) NC group. The results suggested that miR-34a and PKCε displayed inverse regulatory effects on the expression levels of ZO-1, occludin, and claudin-5 in GECs.

BTB permeability and expression of tight junction–related proteins were affected by PKCε-specific activator ψεRACK or inhibitor εV1-2 in miR-34a–overexpressed or –silenced cells

To determine the role of PKCε activity in miR-34a–overexpressed or –silenced cells, we evaluated the effect of the PKCε specific

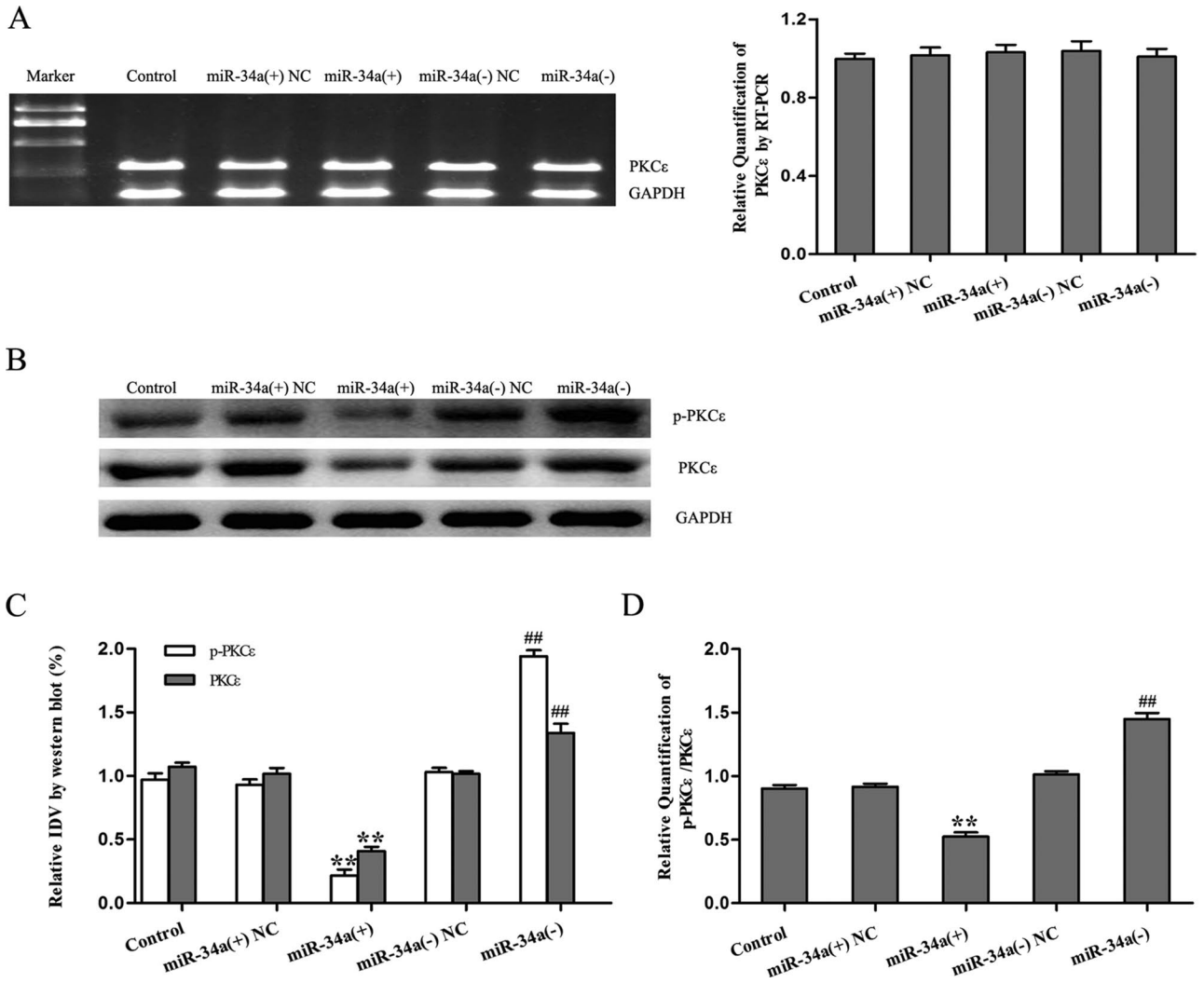

FIGURE 5: Overexpression or silencing of miR-34a significantly affected the protein expression of p-PKCε and PKCε. (A) PKCε mRNA expression levels as assessed by RT-PCR. IDVs are shown using GAPDH as endogenous control. (B) p-PKCε and PKCε protein levels as assessed by Western blot. (C) Relative IDVs of p-PKCε/GAPDH and PKCε/GAPDH by Western blot. (D) Relative IDV of p-PKCε/PKCε by Western blot. IDVs are shown with GAPDH as endogenous control. Values represent the means ± SD ($n = 5$). $^{**}p < 0.01$ vs. miR-34a (+) NC group; $^{\#\#}p < 0.01$ vs. miR-34a (–) NC group.

activator ψεRACK (PKCεA) and inhibitor εV1-2 (PKCεI) on BTB permeability and expression of tight junction–related proteins. As shown in Figure 9, the TEER value increased and HRP flux decreased significantly in the PKCεA and miR-34a (+) + PKCεA groups compared with the respective control and miR-34a (+) groups. In contrast, the TEER value decreased and HRP flux increased significantly in the PKCεI and miR-34a (–) + PKCεI groups compared with the respective control and miR-34a (–) groups.

Moreover, the protein expressions of p-PKCε, PKCε, p-PKCε/PKCε, ZO-1, and occludin, as well as of claudin-5, were up-regulated significantly in PKCεA and miR-34a (+) + PKCεA groups compared with the respective control and miR-34a (+) groups. Conversely, expression of these proteins was down-regulated in the PKCεI and miR-34a (–) + PKCεI groups compared with the respective control and miR-34a (–) groups (Figure 10). These results show that miR-34a can alter the activity of PKCε by p-PKCε and further regulate BTB permeability.

DISCUSSION

MicroRNAs are a group of small, noncoding RNAs, ~20–22 base pairs in length, which bind to the 3′ UTR of a target gene, degrade target gene mRNA, or inhibit gene translation, thus playing an important role in the regulation of gene expression at the posttranscriptional level (Zhang *et al.*, 2007). MiR-34a, a member of the miR-34 family, contributes to regulation of the cell cycle, differentiation, senescence, and apoptosis (Tarasov *et al.*, 2007; Aranha *et al.*, 2011; Iqbal *et al.*, 2014). The present study shows that miR-34a expression in GECs is significantly higher than in ECs. Previous work showed that miR-34a is highly expressed in aging endothelial cells, and up-regulated miR-34a expression levels inhibits SIRT1 expression and induces aging of endothelial cells (Lee *et al.*, 2010; Zhao *et al.*, 2010). Growing evidence has shown that microRNAs are involved in angiogenesis and regulation of blood-brain barrier (BBB) permeability (Suárez and Sessa, 2009; Kalani *et al.*, 2014; Lopez-Ramirez *et al.*, 2014), but the effect of miR-34a in GECs and on BTB permeability has not been elucidated.

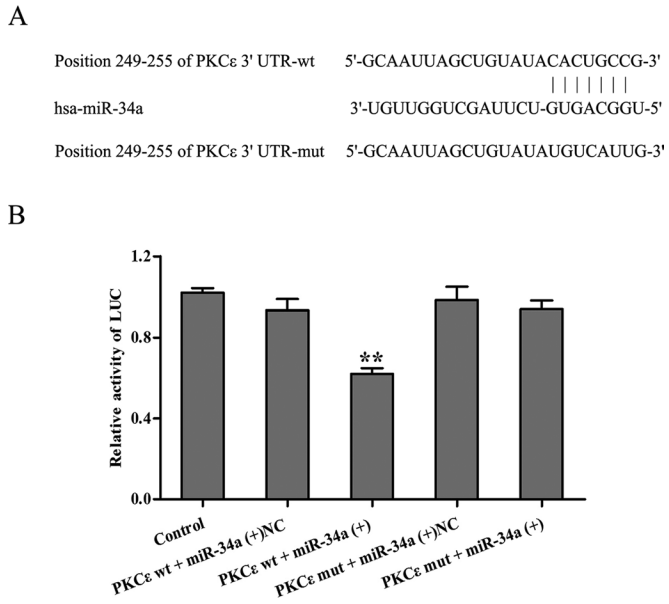

FIGURE 6: PKCε is a direct target of miR-34a. (A) The putative binding site of PKCε 3′UTR matching with the seed region of miR-34a was predicted with the help of TargetScan. (B) Relative activity of LUC was expressed as firefly/renilla luciferase activity. Values are means ± SD ($n = 6$). $^{**}p < 0.01$ vs. PKCε wt + miR-34a (+) NC group.

In the present study, we showed that the permeability of the BTB was increased compared to that of the BBB at 72 and 96 h when cocultured (as shown in Supplemental Figure S2), and overexpression of miR-34a significantly increased the permeability of the BTB compared to the BBB (as shown in Supplemental Figure S3), which implied that miR-34a selectively opened the BTB rather than the BBB. MiR-34a overexpression in GECs led to an increased BTB permeability, whereas miR-34a silencing reduced BTB permeability, leaving the molecular mechanism unclear. Vascular endothelial cells are continuously distributed along with blood vessels, forming a selective permeation barrier between vascular wall and blood (Cines *et al.*, 1998; Pober and Sessa, 2007). Tight junctions among the adjacent endothelial cells are the important structures to maintain the integrity of both the BBB and the BTB and play a key role in regulating endothelial barrier functions. Once tight junctions are destroyed, the barrier permeability increases, and endothelial dysfunction occurs (Bazzoni and Dejana, 2004; Dejana, 2004). In this study, the increased BTB permeability after miR-34a overexpression was the result of the opening of tight junctions in GECs. ZO-1, occludin, and claudin-5 are the key mediators for tight junctions between endothelial cells (Taddei *et al.*, 2008; Ronaldson *et al.*, 2009). ZO-1 is a member of the guanylate kinase–like family and is located at the cytoplasmic membrane surface; occludin and claudin-5 are responsible for the integrity of the endothelial cell membrane and form tight junctions between adjacent cells and then connect to cytoskeletal proteins via ZO-1, thus regulating tight junctions and

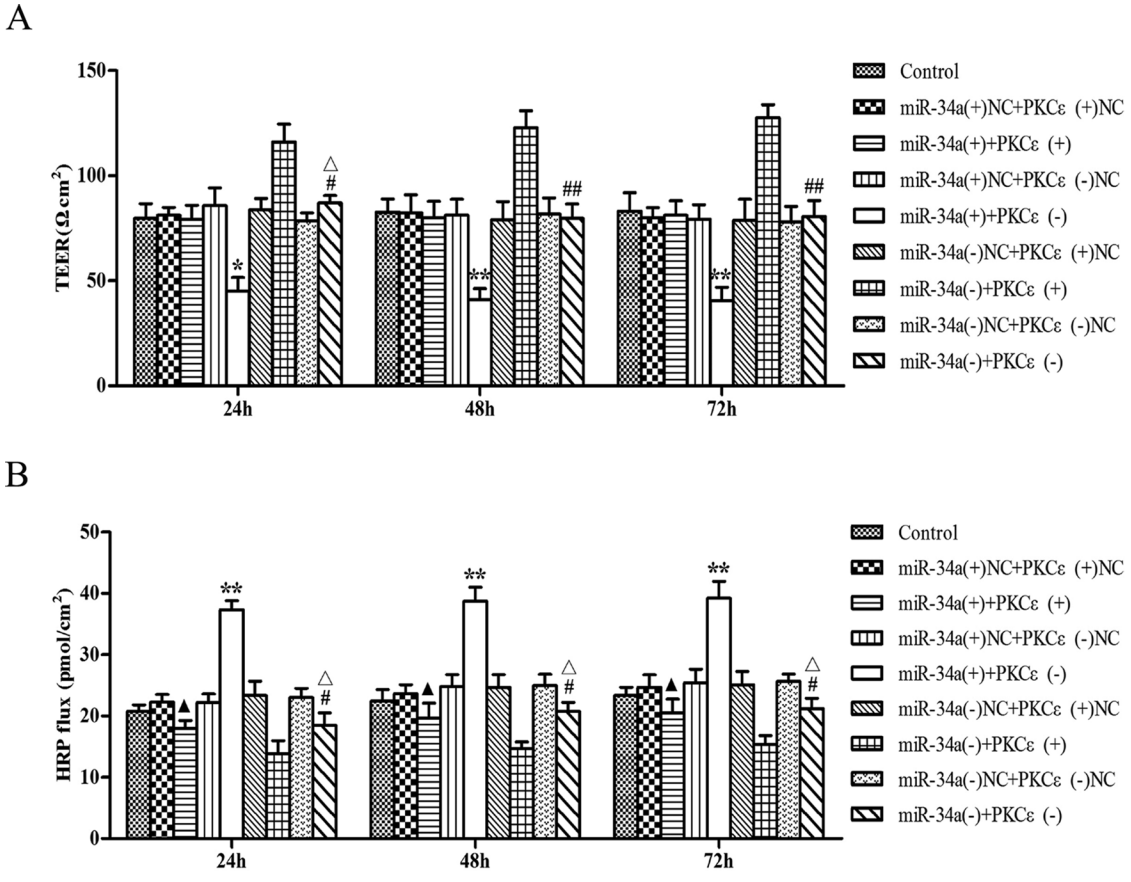

FIGURE 7: The permeability of BTB was inversely coregulated by miR-34a and PKCε in GECs. (A) TEER values of GECs were expressed as Ω·cm^2. (B) HRP flux was calculated as pmol/cm^2. Data represent means ± SD ($n = 5$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. miR-34a (+) + PKCε (+) group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. miR-34a (–) + PKCε (+) group; $^{\blacktriangle}p < 0.01$ vs. miR-34a (+) NC + PKCε (+) NC group; $^{\triangle}p < 0.05$ vs. miR-34a (–) NC + PKCε (–) NC group.

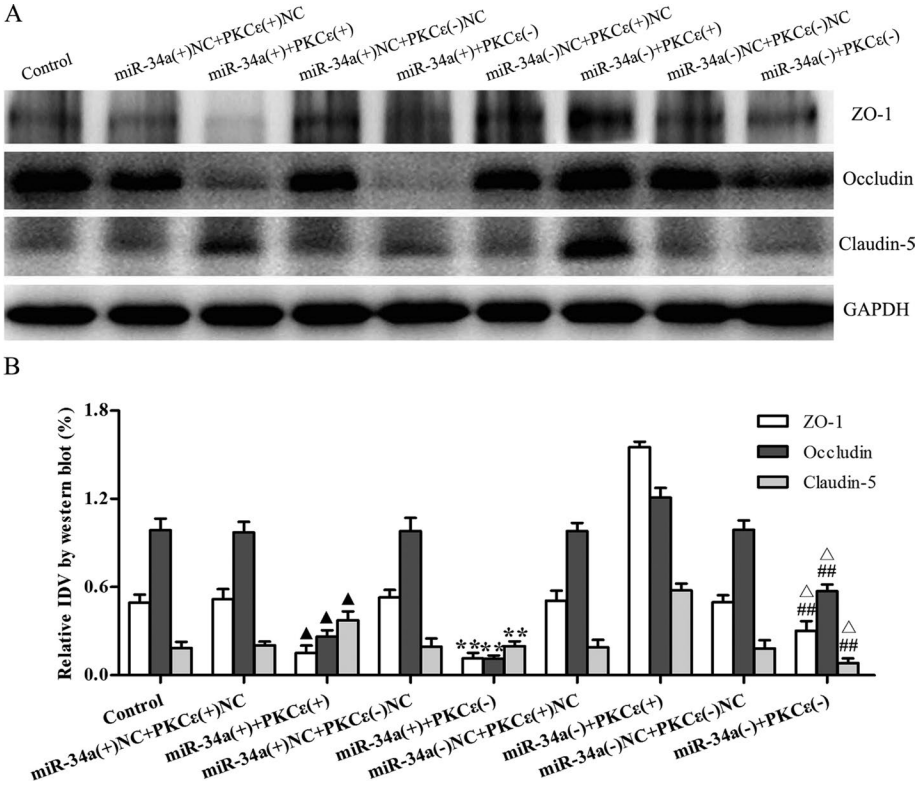

FIGURE 8: The expression levels of tight junction–related proteins were inversely coregulated by miR-34a and PKCε in GECs. (A, B) Protein expression levels of ZO-1, occludin, and claudin-5 as determined by Western blot. IDVs are shown with GAPDH as endogenous control. Data represent the means ± SD ($n = 5$). $^{**}p < 0.01$ vs. miR-34a (+) + PKCε (+) group; $^{\#\#}p < 0.01$ vs. miR-34a (–) + PKCε (+) group; $^{\blacktriangle}p < 0.01$ vs. miR-34a (+) NC + PKCε (+) NC group; $^{\triangle}p < 0.05$ vs. miR-34a (–) NC + PKCε (-) NC group.

endothelial cell permeability (Niessen, 2007; Luissint *et al.*, 2012). The results of this study show that overexpression of miR-34a down-regulates ZO-1, occludin, and claudin-5 expression in GECs, whereas miR-34a silencing gives opposite results. Of importance, immunofluorescence analysis showed that ZO-1 and occludin are continuously distributed at the edge of the GECs; after miR-34a overexpression, immunological reaction of ZO-1 and occludin was attenuated and the distribution was discontinuous. These results strongly imply that overexpression of miR-34a increased BTB permeability through a paracellular pathway, that is, by opening tight junctions. Studies showed that papaverine reduced occludin, claudin-5, and F-actin expression and increased BTB permeability through protein kinase A and HSP70 expression (Wang *et al.*, 2010). In addition, EMAP-II and bradykinin down-regulated claudin-5, occludin, and ZO-1 expression, thus opening tight junctions in GECs and enhancing BTB permeability (Liu *et al.*, 2008; Xie *et al.*, 2010, 2012). Our observations that overexpression of miR-34a impaired BTB permeability and down-regulation of ZO-1, occludin, and claudin-5 expression in GECs are highly consistent with these findings.

MiR-34a may guide ribosome binding to the 3′ UTR in the target gene and posttranscriptionally inhibit the translation of the target gene (Li *et al.*, 2009; Craig *et al.*, 2011; Wang *et al.*, 2011). Therefore the functions of miR-34a depend on the target molecules. As the common signaling molecule responsible for tight junctions, PKC can adjust the permeability of the pathway adjacent to ECs (Fleegal *et al.*, 2005; Willis *et al.*, 2010). PKCε, a member of the PKC family, plays an important role in a variety of physiological functions and signal transmissions (Basu and Sivaprasad, 2007; Jain and Basu, 2014). The results of the present study show that PKCε is expressed in both ECs and GECs, but the expression is significantly lower in GECs. This indicates that PKCε inhibited the functions of the BTB. In addition, we further demonstrated that miR-34a overexpression significantly reduced the protein expression of PKCε and p-PKCε, and miR-34a silencing resulted in the increase of PKCε and p-PKCε protein expression, but mRNA expression levels did not change. These results show that the regulation of miR-34a by PKCε is at the posttranscriptional level. Dual luciferase reporter assays further confirmed that miR-34a directly binds to the 3′ UTR of the PKCε but not ZO-1, occludin, or claudin-5 (Supplemental Figure S4). Thus we speculate that miR-34a can regulate PKCε activity and influence BTB permeability in vitro.

To investigate further the correlation between miR-34a and target gene PKCε, we investigated cotransfections in GECs. The results showed that miR-34a and PKCε displayed inverse regulatory effects on the permeability of BTB and the expression levels of ZO-1, occludin, and claudin-5 after cotransfection of miR-34a and PKCε in GECs. This indicated that miR-34a and PKCε play a synergistic effect on the inverse regulation of BTB permeability and the expression levels of ZO-1, occludin, and claudin-5. Previous studies showed that PLCγ activated PKCε, reduced the EGF-induced redistribution of ZO-1 and occludin, and increased tight junctions between Caco-2 cells, thereby reducing the permeability of Caco-2 cells (Suzuki *et al.*, 2008). Interleukin-25 also protected BBB function through activation of PKCε and up-regulation of ZO-1, occludin, and claudin-5 expression (Sonobe *et al.*, 2009). Thus our findings confirmed that miR-34a regulated BTB permeability by targeting PKCε, which regulated the expression of the tight junction–related proteins of ZO-1, occludin, and claudin-5.

Previous reports showed that inactive PKC is mainly present in the cytoplasm of endothelial cells and may transfer to the cell membrane after activation; the activated PKC then functions to regulate endothelial cells function and signal transduction pathways (Newton, 1997; Ron and Kazanietz, 1999). Addition of PKCε agonists reduced ZO-1 and occludin redistribution in Caco-2 cells and increased TEER values between cells, thereby reducing permeability between cells (Suzuki *et al.*, 2008). Given that miR-34a could inversely regulate the protein expression of PKCε and p-PKCε in this study, we used pretreatment with the PKCε-specific activator ψεRACK and inhibitor εV1-2 in miR-34a–overexpressing or miR-34a–silenced GECs, respectively, to verify whether miR-34a regulated BTB permeability by altering PKCε activity. The results demonstrated that the PKCε-specific activator ψεRACK decreased BTB permeability and increased the expression of tight junction proteins in miR-34a-overexpressed GECs, whereas pretreatment with the inhibitor εV1-2 achieved opposite results. These findings implied that PKCε is activated after phosphorylation, and the activated PKCε regulates the expression of ZO-1, occludin, and claudin-5, leading to the opening or

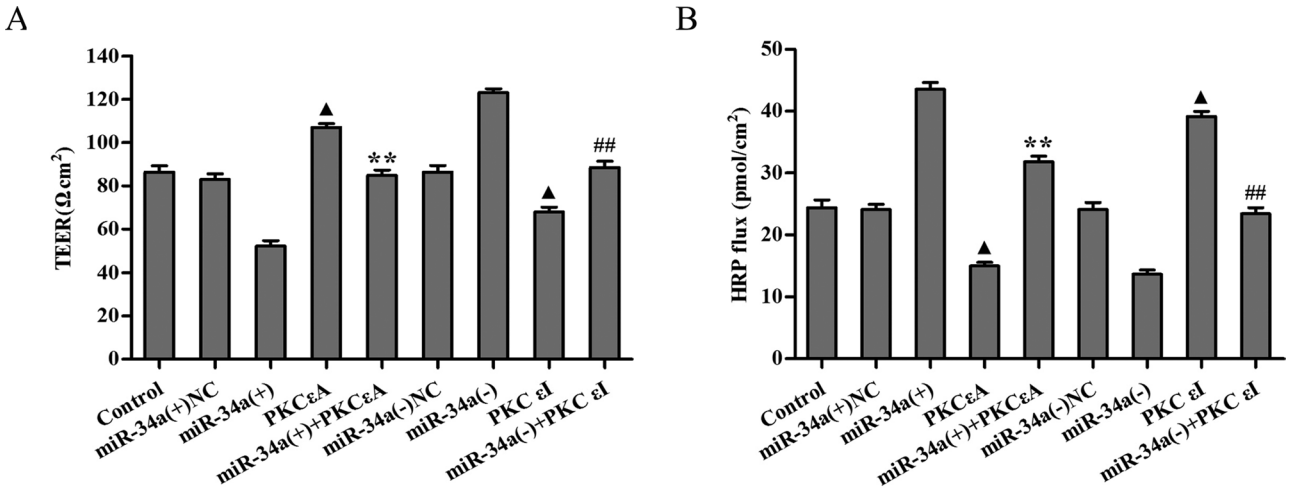

FIGURE 9. The permeability of BTB was regulated by PKCε activator ψεRACK and inhibitor εV1-2 peptides (100 nM). (A) TEER values of GECs expressed as Ω·cm². (B) HRP flux calculated as pmol/cm². Data represent means ± SD (n = 5). ▲$p < 0.01$ vs. control group; **$p < 0.01$ vs. miR-34a (+) group; ##$p < 0.01$ vs. miR-34a (–) group.

closing of tight junctions between endothelial cells, ultimately regulating BTB permeability.

In summary, the present study shows for the first time that miR-34a in GECs can regulate PKCε activity, alter the expression and distribution of tight junction proteins ZO-1, occludin, and claudin-5, and thus regulate BTB permeability. These findings provide a new insight into miRNA-34a regulation of BTB function.

MATERIALS AND METHODS

Cell lines and cultures

ECs (passages 30–40) were kindly provided by P. O. Couraud (Institute Cochin, Paris, France), and they maintained stable growth and endothelial marker characteristics (Weksler et al., 2005, 2013). Cells were cultured as described by Weksler *et al*. (2005) in endothelial basal medium (EBM-2; Lonza, Walkersville, MD) supplemented

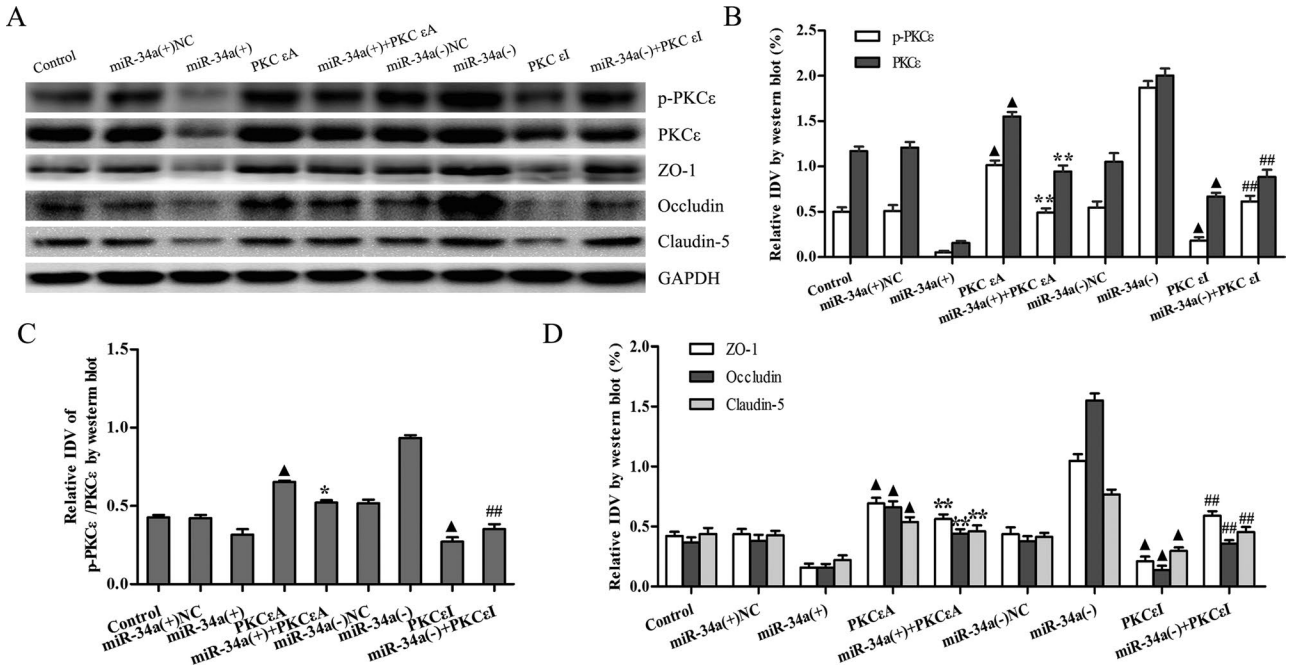

FIGURE 10: The expression of p-PKCε and PKCε, as well as of tight junction–related proteins, in GECs after pretreatment with PKCε activator ψεRACK or inhibitor εV1-2 (100 nM). (A) p-PKCε, PKCε, ZO-1, occludin, and claudin-5 protein levels as assessed by Western blot. (B) Relative IDVs of p-PKCε/GAPDH and PKCε/GAPDH as detected by Western blot. (C) Relative IDV of p-PKCε/PKCε as assessed by Western blot. (D) Relative IDVs of ZO-1 and occludin as determined by Western blot. IDVs are shown with GAPDH as endogenous control. Values represent the means ± SD ($n = 5$). ▲$p < 0.01$ vs. control group; *$p < 0.05$ and **$p < 0.01$ vs. miR-34a (+) group; ##$p < 0.01$ vs. miR-34a (–) group.

with 5% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria), 1% chemically defined lipid concentrate (Life Technologies, Paisley, United Kingdom), 1.4 μmol/l hydrocortisone (Sigma-Aldrich, St. Louis, MO), 5 μg/ml ascorbic acid (Sigma-Aldrich), 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (PAA Laboratories), 1% penicillin-streptomycin (Life Technologies), and 1 ng/ml human basic fibroblast growth factor (Sigma-Aldrich). Human glioblastoma U87 cell line and human embryonic kidney 293T cell line (passages 20–30) were purchased from the Shanghai Institutes for Biological Sciences Cell Resource Center (Shanghai, China) and maintained in high-glucose DMEM and supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). All cells were maintained at 37°C with 5% CO_2 in a humidified atmosphere, and the medium was renewed every 2–3 d.

Establishment of an in vitro BTB model

The in vitro BTB model was established by coculturing ECs and U87 cells in a Transwell system (0.4-μm pore size; Corning, Corning, NY) as described previously (Ma *et al.*, 2014). U87 cells were seeded at a density of 2×10^4/well in six-well plates and cultured for 48 h. ECs were subsequently seeded at a density of 2×10^5/well on the upper side of inserts pretreated with 150 μg/ml Cultrex Rat Collagen I. The culture medium was then renewed every 2 d. After 4 d of culture, GECs from the BTB models were obtained and used for further analysis.

Transfection and grouping

ECs were seeded on 24-well plates before transfection and incubated until 80% confluency was reached. For overexpression of miR-34a, cells were transfected with the DNA plasmid vector miR-34a-pPG/miR/EGFP/blasticidin (GenePharma, Shanghai, China) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After 48 h of transfection, cells were subjected to blasticidin selection for 3–4 wk. Resistant cells were sorted to obtain only green fluorescent protein (GFP)–expressing cells and maintained in medium supplemented with 0.4 mg/ml blasticidin. miR-34a antagomir was synthesized (GenePharma) and transfected into ECs using Lipofectamine 2000 reagent according to the manufacturer's instructions. Plasmid carrying a nontargeting sequence was used as NC. The transfection efficacy of miR-34a–overexpressed plasmid and antagomir was evaluated by quantitative real-time PCR. The high transfection efficacy of antagomir was sustained for 8 d (Supplemental Figure S5). Transfected cells were divided into five groups: control, untransfected; miR-34a (+) NC, negative control of miR-34a–overexpressed; miR-34a (+), miR-34a overexpressed; miR-34a (–) NC, negative control of miR-34a–silenced; and miR-34a (–), miR-34a– silenced.

For overexpression or silencing of PKCε, DNA plasmid vector PKCε-pGCMV/MCS/T2A/EGFP/Neo or PKCε-pGPU6/GFP/Neo (GenePharma) was transfected respectively into ECs using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h of transfection, the cells were subjected to G418 (Sigma-Aldrich) selection for 3–4 wk. GFP-expressing cells were maintained in medium supplemented with 0.4 mg/ml G418. The transfected efficacy of PKCε plasmids was assessed by RT-PCR. To achieve cotransfection, DNA plasmid vectors of miR-34a–overexpressed plasmid, miR-34a antagomir, and their respective NCs were transfected to the aforementioned PKCε-stable transfected cells using Lipofectamine 2000 according to the manufacturer's instructions. Cotransfected cells were obtained after 48 h of culture and divided into nine groups (Table 1).

In some experiments, the stable transfected ECs were pretreated with PKCεA (500 nM; AnaSpec, Fremont, CA; Bright *et al.*, 2008) or PKCεI (500 nM; AnaSpec; Brandman *et al.*, 2007) after 4 d of coculture with U87. Cells were divided into nine groups: control; miR-34a (+) NC; miR-34a (+); PKCεA, PKCε-selective activator–pretreated; miR-34a (+) + PKCεA; miR-34a (–) NC; miR-34a (–); PKCεI, PKCε-selective inhibitor pretreated; miR-34a (–) + PKCεI.

RT-PCR and quantitative real-time PCR

Total RNA was extracted from the cells with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The RNA concentration and quality were determined for each sample by the 260/280-nm ratio using a NanoDrop Spectrophotometer (ND-100; NanoDrop, Wilmington, DE). The cDNA was generated using the RNA PCR Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Primer sets specific for PKCε were as follows: PKCε forward, AGC CTC GTT CAC GGT TCT A, and PKCε inverse, CTG TCC AGC CAT CAT CTC G. Then PCR was performed with the following parameters: 30 cycles of 94°C for 5 min, 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min. The amplified products were separated by electrophoresis on a 1% polyacrylamide gel and visualized under an automated electrophoresis gel imaging analyzer (Chemi Imager 5500 V2.03; Alpha Innotech, San Leandro, CA), and integrated density values (IDVs) were assayed by Fluor Chem 2.0. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control, and the value of PKCε was expressed as intensity relative to the level of GAPDH.

Group	Stable transfected cell	Cotransfection
Control	Untransfected	None
miR-34a (+) NC + PKCε(+) NC	PKCε (+) NC	miR-34a (+) NC
miR-34a (+) + PKCε (+)	PKCε (+)	miR-34a (+)
miR-34a (+) NC + PKCε (–) NC	PKCε (–) NC	miR-34a (+) NC
miR-34a (+) + PKCε (–)	PKCε (–)	miR-34a (+)
miR-34a (–) NC + PKCε (+) NC	PKCε (+) NC	miR-34a antagomir NC
miR-34a (–) + PKCε (+)	PKCε (+)	miR-34a antagomir
miR-34a (–) NC + PKCε (–) NC	PKCε (–) NC	miR-34a antagomir NC
miR-34a (–) + PKCε (–)	PKCε (–)	miR-34a antagomir

TABLE 1: Grouping of cotransfected cells.

For quantitative real-time PCR, RNA samples were inversely transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) with the following parameters: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and then maintained at 4°C. Real-time PCR was performed for 40 cycles using TaqMan Universal Master Mix II (Applied Biosystems) with the TaqMan MicroRNA Assay of miR-34a and U6 (Applied Biosystems): 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. All quantitative RT-PCR analyses were conducted by means of a 7500 Fast Real-Time PCR System (Applied Biosystems). The relative gene expression was normalized to U6, and analysis of relative expression values for mRNA was performed by the $2^{-\Delta\Delta Ct}$ method.

TEER and HRP flux assays

After the BTB cell model was constructed, TEER measurement was performed at different time points using a Millicell-ERS instrument (Millipore, Billerica, MA). Briefly, U87 cells were seeded at 2×10^4/well in a six-well plate for 2 d. ECs were subsequently seeded at a density of 2×10^5/well on the upper side of inserts pretreated with 150 µg/ml Cultrex Rat Collagen I. On the fourth day of coculture, TEER was measured. Background electrical resistances were subtracted before final resistances (ohms·centimeter^2) were calculated. To further determine the permeability of the in vitro BTB, HRP (44 kDa) permeability was detected. After the BTB cell model was constructed, 1 ml of culture fluid containing 10 µg/ml HRP was added to the upper side of inserts and 2 ml of culture medium was added to the well. Then 5 µl of culture medium was collected from each well at different time points, and the collected samples were measured using TMB colorimetry with a spectrophotometer at 370 nm. HRP permeability was calculated from the standard curve and expressed as picomoles per square centimeter.

Western blot assay

Cells were lysed, and the supernatant extracts were quantified for protein using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Hangzhou, Jiangsu, China). Equal amounts of protein (40 µg) were loaded onto SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 for 2 h and subsequently incubated overnight with primary antibodies against PKCε (1:500; Proteintech, Chicago, IL), p-PKCε (Ser-729, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), ZO-1 (1:500; Life Technologies), occludin (1:250; Life Technologies), claudin-5 (1:500; Life Technologies), and GAPDH (1:10,000; Proteintech), respectively. Then the membranes were incubated with specific antibodies in combination with HRP-conjugated anti-mouse immunoglobulin G (IgG) or HRP-conjugated anti-rabbit IgG antibodies for 2 h at room temperature. The blots were visualized using enhanced chemiluminescence (ECL Kit; Santa Cruz Biotechnology) and scanned using Chemi Imager 5500 V2.03 software. Then the IDVs of bands for PKCε, p-PKCε, ZO-1, occludin, and claudin-5 were calculated by Fluor Chen 2.0 software and expressed as ratio of intensities relative to the level of GAPDH.

Reporter vectors constructs and luciferase assays

Potential binding site between the 3′ UTR of PKCε mRNA and the seed region of miR-34a was predicted by TargetScan Human Release 6.2 (www.targetscan.org). The sequence of PKCε mRNA-3′ UTR was amplified by PCR and cloned into Luc expression vector pmirGLO; then the recombinant vector of pmirGLO-PKCε-3′ UTRs was inserted into the vector of pcDNA3.3. The resulting pcDNA3.3-miR-34a constructs were verified by sequencing (GenePharma). MiR-34a–overexpressed and miRNA NC plasmids were transfected together with PKCε wt 3′ UTR or PKCε mutatant (Mut) 3′ UTR reporter plasmid, respectively, into HEK293T cells using Lipofectamine LTX and Plus (Life Technologies) according to the manufacturer's instructions. After 48 h of transfection, cells were harvested and analyzed by luciferase assay using a Dual-Luciferase Reporter Assay System (Promega, Beijing, China). Luciferase expression was given as relative light units (firefly/*Renilla* luciferase) to determine whether PKCε was the target of miR-34a in vitro.

Immunofluorescence assays

Immunofluorescence assays were performed to detect the expression and distribution of tight junction proteins in GECs. Briefly, cells were cultured on insert filters and fixed in 4% paraformaldehyde for 20 min. Then the cells were blocked in 5% BSA for 2 h at room temperature and incubated with primary antibodies for ZO-1, occludin, and claudin-5 (1:50; Life Technologies) overnight. After three washes with PBS, cells were incubated with Alexa Fluor 555–labeled goat anti-mouse IgG or anti-rabbit IgG secondary antibody (1:500; Beyotime Institute of Biotechnology, Hangzhou, Jiangsu, China) for 2 h. The nuclei were then stained with 0.5 µg/ml 4′,6-diamidino-2-phenylindole (DAPI; Beyotime Institute of Biotechnology) for 8 min. The fluorescence was visualized under an immunofluorescence microscope (Olympus, Tokyo, Japan), and images were collected by the Chemi Imager 5500 V2.03 software.

Statistical analysis

SPSS 19.0 software (SPSS, Chicago, IL) was used for statistical analysis. Data are expressed as mean ± SD. Differences between two groups were examined for significance with the Student's *t* test. One-way analysis of variance and Dunnett's posttest were used to determine significance among multiple groups. $p < 0.05$ was considered to be statistically significant.

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