LRIG1 inhibits hypoxia-induced vasculogenic mimicry formation via suppression of the EGFR/PI3K/AKT pathway and epithelial-to-mesenchymal transition in human glioma SHG-44 cells

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Abstract Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) is a pan-negative regulator of the epidermal growth factor receptor (EGFR) signaling pathway. The aim of this study was to investigate the underlying mechanism of LRIG1 in the regulation of vasculogenic mimicry (VM) formation in glioma cells. We constructed an enhanced green fluorescent protein plasmid (pEGFP) system, pEGFP-C1-LRIG1, for overexpression of LRIG1, and transfected it into human glioma cell line SHG-44. Under hypoxic conditions induced by CoCl₂, we investigated the effects of LRIG1 overexpression on VM formation and VM-dependent malignant behaviors including migration, invasion, and proliferation. Additionally, we explored the effects of LRIG1 on the expression levels of major components of the EGFR/PI3K/AKT pathway as well as E-cadherin and vimentin. We found that LRIG1 overexpression is able to inhibit hypoxia-induced VM formation, migration, invasion, and proliferation. Furthermore, LRIG1 overexpression counteracts hypoxia-induced increase in the expression of phosphorylated EGFR (pEGFR), PI3K (pPI3K), and AKT (pAKT) and reverts hypoxia-induced alteration in E-cadherin and vimentin expression levels. In LRIG1 knockdown SHG-44 cells,

however, hypoxia-induced VM formation and alteration in E-cadherin and vimentin expression levels were exacerbated. These results suggest that the inhibitory effects of LRIG1 are most likely mediated by suppression of the EGFR/PI3K/AKT pathway and epithelial-mesenchymal transition (EMT) process. Our findings provide compelling evidence implicating LRIG1 in glioma pathophysiology, suggesting that gene therapy using LRIG1 may serve as a treatment for this disease.

Keywords LRIG1 · Vasculogenic mimicry · Epithelial-mesenchymal transition · EGFR/AKT/PI3K pathway

Introduction

Glioma is the most common form of malignant brain tumor in adults (Omuro and DeAngelis 2013). High-grade gliomas have a tendency to infiltrate surrounding tissues and give rise to metastases (Van Meir et al. 2010). In recent years, numerous studies have revealed that during development and progression, the microenvironment of a glioma can become increasingly hypoxic and the tumor cells undergo adaptive changes, which allow them to survive and even progress in this hypoxic environment (Persano et al. 2013). Hypoxia is a potent stimulus for neovascularization, which is essential for the malignant behaviors of glioma to provide increasing amounts of blood flow for nutrient and oxygen supply (Mongiardi 2012). Angiogenesis is likely the predominant process of neovascularization, but in addition to angiogenesis, vasculogenic mimicry (VM) has been proven to be a new and important neovascularization process (Seftor et al. 2012). VM is the formation of a fluid-conducting network characterized by vascular channels that are exclusively lined by tumor cells mimicking endothelial cells (Misra et al. 2012). This process

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is different from classic vasculogenesis by endothelial cells because of the lack of an endothelial monolayer, but it has a function in sustaining tumor microcirculation (Paulis et al. 2010). Previous studies have found that VM formation is closely associated with development of malignant gliomas (Wang et al. 2013; Wang et al. 2012), and yet, the mechanisms involved in the regulation of VM process in glioma cells still remain elusive.

Growth factors and their receptor tyrosine kinases (RTKs) are major regulators of the cellular processes of tumors (Kumar et al. 2012). Deregulation of growth factor signaling pathways has been widely accepted as a hallmark of glioma formation (Van Meir et al. 2010; Verhaak et al. 2010); malignant gliomas often occur as a result of growth factor receptors being constitutively activated. As one of the best characterized oncogenes, the gene for epidermal growth factor receptor (EGFR) is amplified in 30–50 % of malignant gliomas (Van Meir et al. 2010), while components of its downstream signaling cascade have been associated with key steps in the development of this disease (Kumar et al. 2012).

Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) is a pan-negative regulator of the EGFR signaling pathway and acts as a tumor suppressor protein in tumorigenesis (Gotoh 2009). Cellular expression and distribution of LRIG1 correlate with the prognosis of glioma patients (Guo et al. 2006; Holmlund et al. 2009). Downregulation of LRIG1 expression in glioma cells by RNA interference can promote aggressive malignant properties through activation of EGFR and protein kinase B (AKT) (Xie et al. 2013). Based on these findings and the current understanding of the interaction between LRIG1 and EGFR, we hypothesized that LRIG1 inhibits VM formation of glioma cells through suppression of the EGFR/AKT-related pathways. To test our hypothesis, we transfected LRIG1 into SHG-44 cells (a highly metastatic human glioma cells line) and found that LRIG1 inhibits hypoxia-induced VM formation and VM-dependent malignant behaviors including migration, invasion, and proliferation. We further demonstrate that suppression of the EGFR/PI3K/AKT signaling pathway and epithelial-mesenchymal transition (EMT) process is critical for the inhibitory effects of LRIG1.

Materials and methods

Reagents and cell line

The pGEM-T easy cloning vector kit was purchased from Promega (Madison, WI, USA). The eukaryotic expression vector pEGFP-C1 was purchased from Clontech (Palo Alto, CA, USA). The SHG-44 human glioma cell line was obtained from the Central Laboratory of Medical School of Xi'an Jiaotong University (Xi'an, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10 % (v/v) fetal bovine serum (FBS) and maintained at 37 °C in humidified air containing 5 % CO₂. All other biochemical reagents and chemicals were purchased from Amresco (Solon, OH, USA) and Clontech.

Plasmid construction and transfection

The full-length complementary DNA (cDNA) of LRIG1 was amplified from total RNA extracted from human peripheral blood cells by RT-PCR using the following primer pairs: forward primer 5'-CGCTCGAGATGGCGCGGCCGGTCCGGGGAGGG-3'; reverse primer 5'-CCATCGATTAGCTTTTGTGGTGCCAAACAGCAG-3'. The LRIG1 cDNA was then cloned into pGEM-T easy vector, and correct cloning was confirmed by sequencing. In order to evaluate transfection efficiency, a plasmid expressing enhanced green fluorescent protein (EGFP)-fused LRIG1 was constructed by inserting the full-length LRIG1 cDNA encoding open reading frame (ORF) into pEGFP-C1 vector at *XhoI/ClaI* sites. The insertion of LRIG1 was verified by double digestion of the plasmid with *HindIII* and *XbaIII*. The constructed pEGFP-C1-LRIG1 plasmid was then purified and transfected into SHG-44 cells using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). The empty pEGFP-C1 vector served as a negative control. Twenty-four hours after transfection, the transfection efficiency was determined by counting the percentage of EGFP-positive cells under a fluorescence microscope (BX51, Olympus, Tokyo, Japan). Western blot was also performed to detect LRIG1 expression in transfected SHG-44 cells.

VM formation assay

Hypoxic conditions stimulated by cobalt chloride (CoCl₂) induce VM formation in SHG-44 cells (Xu et al. 2013a). Therefore, in this study, we created hypoxic conditions using CoCl₂ to investigate the effect of LRIG1 on hypoxia-induced VM formation of SHG-44 cells. Briefly, a 24-well plate was coated with 20 µl per well of the mixture of Matrigel® (BD Biosciences, San Jose, CA, USA) and FBS (v/v=1:1) for 20 min at room temperature (RT). Then, 1 × 10⁴ SHG-44 cells were seeded into each well. They were either untransfected (the normoxia and mock groups) or transfected with either the empty pEGFP-C1 vector (the control group) or the pEGFP-C1-LRIG1 plasmid (the transfected group). Afterward, all groups of cells were incubated at 37 °C in a 5 % CO₂ atmosphere. Then, cells were treated either with 100 µM of CoCl₂ (the mock, control, and transfected groups) or with phosphate-buffered saline (PBS) vehicle (the normoxia group) for 24 h. Ten nonoverlapping fields were selected from each well and photographed at ×200 magnification using an Olympus BX51 microscope. VM was evaluated by counting the number of tube-like structures per field using ImageJ software (version 1.44, <http://imagej.nih.gov/ij/>).

Cell migration and invasion assay

Transwell migration and Matrigel® invasion assays were performed to evaluate the effect of LRIG1 on migration and invasion of SHG-44 cells under hypoxic condition. In brief, 2×10^4 SHG-44 cells (untransfected or transfected with the pEGFP-C1-LRIG1 plasmid or empty pEGFP-C1 vector) were suspended in 200 μ l medium containing 1 % FBS and then seeded on the upper chamber of a Transwell filter (8- μ m pore size, Costar, Cambridge, MA, USA). Cells were grouped in the same way as in VM formation assay. For cells in the mock, control, and transfected groups, CoCl_2 was added to a final concentration of 100 μ M, while an equivalent quantity of PBS vehicle was added to cells in the normoxia group. Six hundred microliters of medium supplemented with 10 % FBS was used as a chemoattractant in the bottom chamber. All groups of cells were incubated at 37 °C, 5 % CO_2 for 12 h. The migrated cells on the lower membrane were stained with crystal violet and counted under an inverted microscope (Olympus BX51, Olympus). For the Matrigel® invasion assay, 100 μ l of a mixture of Matrigel® and FBS ($v/v=1:1$) was added to the upper chamber of a Transwell filter and incubated for 20 min at RT to allow the mixture to solidify. Then, 8×10^4 SHG-44 cells (untransfected or transfected with the pEGFP-C1-LRIG1 plasmid or empty pEGFP-C1 vector) were seeded, grouped, and treated in the same way as in the migration assay. All experimental groups were incubated at 37 °C, 5 % CO_2 for 24 h. The invasive cells were stained and counted.

Cell proliferation assay

The effect of LRIG1 on the proliferation of SHG-44 cells under hypoxic condition was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 5×10^3 SHG-44 cells exposed to various transfection treatments were cultured in each well of 96-well plates. Then, the cells were grouped and subjected to hypoxic conditions as described in the VM formation assay. After incubation for 24 h, MTT (5 mg/ml; Sigma, St. Louis, MO, USA) was added, and the 96-well plates were incubated at 37 °C for an additional 4 h. Then, the supernatant was removed and 150 μ l dimethyl sulfoxide (DMSO) was added to each well. After shaking for 15 min, the optical density (OD) values were read at 490 nm with a reference wavelength of 620 nm using a plate reader (Bio-Rad, Hercules, CA, USA).

Western blot

SHG-44 cells subjected to different transfection treatments were seeded into six-well plates at a density of 1×10^6 cells/well for Western blot experiments. Cells were grouped and then exposed to the same hypoxic conditions as used in the VM formation assay. After 24-h incubation, total lysates from

SHG-44 cells were extracted and analyzed by Western blot according to standard procedures. The following antibodies were used: LRIG1 (Abcam, Cambridge, MA, USA, Catalog No. 36707), EGFR (Catalog No. 2232), phospho-EGFR (p-EGFR, Tyr 1068, Catalog No. 2220), AKT (Catalog No. 9272), phospho-AKT (p-AKT, Ser 473, Catalog No. 9271), PI3K (Catalog No. 4292), phospho-PI3K (p-PI3K, Tyr 458, Catalog No. 4228) (Cell Signaling, Danvers, MA, USA), E-cadherin (Abcam, Catalog No. 15148), and vimentin (Santa Cruz Biotech, Santa Cruz, CA, USA, Catalog No. 7558). Proteins were detected using the enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). The bands were quantified using ImageJ software. Beta-actin served as an internal control.

Silencing RNA targeting LRIG1

Transfection of small interfering RNA (siRNA) targeting LRIG1 was performed in SHG-44 cells using ON-TARGET plus SMART pool (human LRIG1 siRNA product # L-013940-00-0020, Dharmacon, Lafayette, CO, USA). Briefly, SHG-44 cells were plated in 24-well culture plates at a density of 1×10^5 cells per well. Twenty-four hours after plating, cells were transfected with 100 nmol/L LRIG1 siRNA for an additional 24 h. ON-TARGET plus nontargeting pool (product # D-001810-10-20, Dharmacon) was used as a control. Then, Western blot was performed to detect LRIG1 expression in transfected SHG-44 cells. Afterward, the transfected cells (the LRIG1 siRNA and siRNA control groups) and untransfected cells (the normoxia and mock groups) were subjected to VM formation assay and E-cadherin and vimentin expression analysis as described above.

Statistical analysis

The data are presented as means $\pm$ standard deviation (SD) or as means $\pm$ standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison tests. A *P* value less than 0.05 was considered statistically significant.

Results

Overexpression of LRIG1 in transfected SHG-44 cells

Twenty-four hours after the transfection with pEGFP-C1-LRIG1, LRIG1 expression in the transfected SHG-44 cells was detected by fluorescence microscopy and Western blot. The transfection efficiency was

Fig. 1 LRIG1 expression in SHG-44 cells transfected with pEGFP-C1-LRIG1. **a** LRIG1 expression in the transfected cells detected by fluorescence microscopy 24 h after transfection. **b** Western blot analysis of LRIG1 expression. The results were normalized to the untransfected group and presented as means $\pm$ SD ($N=4$). *** $P<0.001$, ANOVA followed by Tukey's post hoc test. Bar=25 μ m

approximately 60–70 % (Fig. 1a). Western blot analysis showed that the level of LRIG1 in the transfected cells increased over 3-fold when compared with untransfected cells or cells transfected with empty pEGFP-C1 vector ($P<0.001$) (Fig. 1b).

LRIG1 inhibits hypoxia-induced VM formation

In the next step, we sought to determine whether LRIG1 transfection could inhibit hypoxia-induced VM formation in SHG-44 cells. As shown in Fig. 2, SHG-44 cells in the normoxia

Fig. 2 LRIG1 inhibits hypoxia-induced VM formation after 24-h treatment with CoCl_2 . For quantitative analysis, ten nonoverlapping fields were selected from each culture well and the experiment was performed in quadruplicate. Data are presented as means $\pm$ SEM. *** $P<0.001$ vs. the normoxia group, ### $P<0.001$ vs. the mock group, ANOVA followed by Tukey's post hoc test. Bar=20 μ m

group showed poor formation of tube-like structures, while cells in the mock group exhibited significantly extensive tubular network upon treatment with CoCl_2 for 24 h. Quantitative analysis showed that there was a 12.5-fold increase in the mean number of tube-like structures per field in the mock group compared with the normoxia group (56.8 ± 12.2 vs. 4.2 ± 2.6 , $P < 0.001$). When SHG-44 cells were transfected with pEGFP-C1-LRIG1, the VM formation induced by CoCl_2 treatment was significantly decreased, as evidenced by an 82 % reduction in the average number of tube-like structures per field in the transfected group as compared with the mock group (10.2 ± 3.0 vs. 56.8 ± 12.2 , $P < 0.001$). This was not the case for the cells transfected with empty pEGFP-C1 vector. Taken together, these data demonstrate that LRIG1 overexpression is able to suppress hypoxia-induced VM formation in SHG-44 cells.

LRIG1 inhibits hypoxia-induced migration and invasion

To gain insights into the inhibitory role of LRIG1 in hypoxia-induced vasculogenesis, we performed Transwell migration and Matrigel® invasion assays to evaluate the effect of LRIG1 on migration and invasion of SHG-44 cells under hypoxic condition. As shown in Figs. 3 and 4, treatment with CoCl_2 could significantly increase migration and invasion of

SHG-44 cells when compared to normoxic condition. The mean number of migrated and invaded cells in the mock group was 2.1-fold and 4.0-fold higher than those in the normoxia group ($P < 0.001$). However, this effect of CoCl_2 was counteracted by transfection with pEGFP-C1-LRIG1 in SHG-44 cells. The transfected group showed an 80 and 42 % decrease in the mean number of migrated and invaded cells, respectively, in comparison with the normoxia group ($P < 0.001$). Collectively, these results reveal that LRIG1 transfection successfully inhibits hypoxia-induced migration and invasion.

LRIG1 inhibits hypoxia-induced proliferation of SHG-44 cells

Next, we used MTT assay to examine the effect of LRIG1 overexpression on proliferation of SHG-44 cells under hypoxic stimulus. As shown in Fig. 5, CoCl_2 incubation considerably increased the proliferation of SHG-44 cells, which was manifested by a 50 % increase in absorbance at 490 nm in the mock group when compared with the normoxia group. However, this proliferative effect was attenuated by transfection with pEGFP-C1-LRIG1, as evidenced by a significant lower mean OD (490 nm) value in the transfected group than in both the mock and control groups ($P < 0.001$), suggesting

Fig. 3 LRIG1 inhibits hypoxia-induced migration after 12-h treatment with CoCl_2 . For quantitative analysis, ten nonoverlapping fields were selected from each culture well and the experiment was performed in quadruplicate. Data are presented as means $\pm$ SEM. *** $P < 0.001$ vs. the normoxia group, #### $P < 0.001$ vs. the mock group, ANOVA followed by Tukey's post hoc test. Bar = 10 μm

Fig. 4 LRIG1 inhibits hypoxia-induced invasion after 12-h treatment with CoCl_2 . For quantitative analysis, ten nonoverlapping fields were selected from each culture well and the experiment was performed in quadruplicate. Data are presented as means $\pm$ SEM. *** $P < 0.001$ vs. the normoxia group, ### $P < 0.001$ vs. the mock group, ANOVA followed by Tukey's post hoc test. Bar = 10 μm

Fig. 5 LRIG1 inhibits hypoxia-induced proliferation after 24-h treatment with CoCl_2 , as measured by MTT assay. Data are expressed as means $\pm$ SEM for three independent determinations. *** $P < 0.001$ vs. the normoxia group, ### $P < 0.001$ vs. the mock group, ANOVA followed by Tukey's post hoc test

that LRIG1 transfection is able to suppress hypoxia-induced proliferation in SHG-44 cells.

LRIG1 suppresses hypoxia-induced EGFR/PI3K/AKT pathway activation and epithelial-to-mesenchymal transition

To explore the molecular mechanisms underlying the inhibitory effects of LRIG1 on hypoxia-induced VM formation, migration and invasion, and proliferation, we examined the expression of both LRIG1 and the key components of the EGFR/PI3K/AKT signaling pathway in SHG-44 cells under normoxic and hypoxic conditions. The results are presented in Fig. 6. We found that hypoxia could considerably reduce LRIG1 level in all groups of SHG-44 cells (Fig. 6a). Additionally, as compared to the normoxia group, cells in the mock group showed significantly increased protein levels of pEGFR, pPI3K, and pAKT upon exposure to CoCl_2 treatment, indicating that hypoxic stimulus by CoCl_2 caused activation of the EGFR/PI3K/AKT pathway. Under the same hypoxic condition, however, the expression levels of pEGFR, pPI3K, and pATK in the

transfected group were considerably decreased by transfection with pEGFP-C1-LRIG1, demonstrating that overexpression of LRIG1 in SHG-44 cells prevents hypoxia-induced activation of the EGFR/PI3K/AKT pathway (Fig. 6b). In addition, as detected by Western blot, CoCl₂ exposure could significantly reduce expression of epithelial protein E-cadherin and increase expression of cytoskeleton protein vimentin (Fig. 7). Considering that E-cadherin and vimentin are important molecular markers of EMT, these results imply that hypoxic exposure in SHG-44 cells promoted EMT. Following transfection with pEGFP-C1-LRIG1, the expression of E-cadherin in transfected cells was dramatically increased while expression of vimentin was decreased (Fig. 7). These findings reveal that LRIG1 overexpression suppresses hypoxia-induced EMT in SHG-44 cells, indicating that LRIG1 acts as a negative regulator of EMT process.

Silencing RNA targeting LRIG1

In order to obtain further evidence supporting the inhibitory effects of LRIG1 on hypoxia-induced VM formation and EMT, we employed siRNA to knockdown the expression of LRIG1 in SHG-44 cells. As shown in Fig. 8a, after transfection with LRIG1 siRNA for 24 h, Western blot analysis showed that the expression of LRIG1 in the transfected cells was significantly reduced as compared to untransfected cells or cells transfected with siRNA control ($P<0.001$). These results indicate successful knockdown of LRIG1 by siRNA. Next, we performed VM formation assay and Western blot analysis to investigate whether LRIG1 knockdown could aggravate the effects of hypoxia on VM formation and EMT in SHG-44 cells. After treatment with CoCl₂ for 24 h, we found that SHG-44 cells transfected with LRIG1 siRNA displayed better formation of tube-like

Fig. 6 Hypoxia reduces LRIG1 expression in SHG-44 cells (a) and LRIG1 counteracts hypoxia-induced increases in the expression of pEGFR, pPI3K, and pAKT (b), as detected by Western blot. For quantitative analysis, data are shown as means $\pm$ SD ($N=4$). * $P<0.05$ and ** $P<0.01$ vs. the normoxia group, # $P<0.05$ and ## $P<0.01$ vs. the mock group, ANOVA followed by Tukey's post hoc test

Fig. 7 LRIG1 reverts hypoxia-induced alteration in E-cadherin and vimentin expression levels, as detected by Western blot. For quantitative analysis, data are shown as means $\pm$ SD ($N=4$). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs. the normoxia group, # $P<0.05$ and ### $P<0.001$ vs. the mock group, ANOVA followed by Tukey's post hoc test

structures than those in the mock and siRNA control groups (Fig. 8b). Quantitative analysis showed that there was a significant increase in the mean number of tube-like structures per field in the LRIG1 siRNA group compared with the mock group (55.4 ± 8.2 vs. $45.3\pm$

6.7 , $P<0.05$). In addition, after transfection with LRIG1 siRNA, SHG-44 cells showed a lower expression of E-cadherin and a significantly higher expression of vimentin than those in the mock and siRNA control groups. Collectively, these data further demonstrate the

Fig. 8 Knockdown of LRIG1 by siRNA aggravates the effects of hypoxia on VM formation and EMT. **a** Western blot analysis of LRIG1 expression in LRIG1 knockdown SHG-44 cells. The results were normalized to the untransfected group and presented as means $\pm$ SD ($N=4$). *** $P<0.001$. **b** Knockdown of LRIG1 exacerbates hypoxia-induced VM formation after 24-h treatment with CoCl_2 . For quantitative analysis, ten nonoverlapping fields were selected from each culture well and the experiment was performed in quadruplicate. Data are presented as means $\pm$ SEM. *** $P<0.001$ vs. the normoxia group, # $P<0.05$ vs. the mock group. $\text{Bar}=20\ \mu\text{m}$. **c** Knockdown of LRIG1 aggravates hypoxia-induced alteration in E-cadherin and vimentin expression levels, as detected by Western blot. For quantitative analysis, data are shown as means $\pm$ SD ($N=4$). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs. the normoxia group, ### $P<0.01$ vs. the mock group. All statistical analyses were performed using ANOVA followed by Tukey's post hoc test

inhibitory effects of LRIG1 on hypoxia-induced VM formation and EMT.

Discussion

In the current study, we report for the first time that overexpression of LRIG1 in pEGFP-C1-LRIG1 vector-transfected SHG-44 cells can inhibit hypoxia-induced VM formation, migration and invasion, and cell proliferation. Additionally, LRIG1 overexpression is able not only to counteract hypoxia-induced increase in the expression of pEGFR, pPI3K, and pAKT, but also to revert hypoxia-induced alterations in E-cadherin and vimentin expression levels. Furthermore, knockdown of LRIG1 by siRNA can aggravate the effects of hypoxia on VM formation and EMT. These findings suggest that the inhibitory effects of LRIG1 on hypoxia-induced VM formation are most likely mediated by suppression of the EGFR/PI3K/AKT pathway and EMT process.

VM refers to a process in which tumor cells replace endothelial cells and form a vessel with a lumen (Seftor et al. 2012). Since it was first described in aggressive human uveal melanomas in 1999 (Maniotis et al. 1999), VM has been reported in various types of cancers, including highly invasive primary and metastatic ovarian cancer, hepatocellular carcinoma, and glioma (Zhang et al. 2007). Although there is still debate as to whether VM is an active process of tumor cells or a consequence of vessel regression (Seftor et al. 2012), numerous studies have demonstrated that VM formation is essential for growth, invasion, and metastasis of human malignant tumors (Fan and Sun 2010). For gliomas, there are sparse studies regarding the association between VM and prognosis of the disease, but researchers have found that VM formation in glioma tissues correlates with increasing malignancy and higher aggressiveness and that patients with VM formation had much poorer clinical outcomes than those without VM formation (Wang et al. 2013; Wang et al. 2012), suggesting a functional advantage imparted by VM related to the survival of the aggressive tumor cell phenotype (Plate et al. 2012). There are two important features of aggressive glioma cells capable of VM formation: first, these cells exhibit a remarkable degree of plasticity, and second, these cells lost expression of some tumor cell markers but have a multipotent phenotype usually associated with embryonic stem cells and endothelial progenitor cells (Seftor et al. 2012). The functional plasticity and multipotent phenotype associated with VM are supported by a complex combination of signaling pathways, of which the hypoxia-related signaling cascade plays a key regulatory role (Paulis et al. 2010). Hypoxic culturing is able to induce VM formation in several highly aggressive human glioma cell lines (Huang et al. 2014). Consistent with these reports, using SHG-44 cells (another highly aggressive glioma

cell line), we found that treatment with hypoxia-mimicking agent CoCl_2 could lead to significantly increased VM formation as well as cell migration, invasion, and proliferation. However, these hypoxia-induced alterations were suppressed in LRIG1-overexpressing SHG-44 cells. In addition, in LRIG1 knockdown cells, we found that the hypoxia-induced VM formation was exacerbated. These results indicate that LRIG1 is a negative regulator for VM formation and other behaviors of glioma cells.

LRIG1 has been proven to be a suppressor of tumor growth by counteracting the signaling of EGFR family members (Gotoh 2009). Inhibition of EGFR signaling by LRIG1 results from the physical interaction between both proteins, which subsequently induces the recruitment of E3 ubiquitin ligases to promote lysosomal degradation of the EGFR-LRIG1 complex (Johansson et al. 2013). In glioma cells, EGFR is usually overexpressed and its downstream signaling cascades are frequently activated during the development of malignancy (Ye et al. 2010). The activation of EGFR downstream cascades mainly depends on autophosphorylation of EGFR, which can be enhanced by hypoxia (Taylor et al. 2012). Upon autophosphorylation, the phosphorylated tyrosine residues in the cytoplasmic tail serve as docking sites for various downstream effectors that contain Src homology-2 (SH2) and phosphotyrosine-binding (PB) domains, subsequently stimulating their associated signaling pathways and mediating transcription of genes required for the growth and progression of tumor (Ye et al. 2010). PI3K and AKT are important downstream effectors of EGFR, and the EGFR/PI3K/AKT pathway plays a major oncogenic role in glioma (Xu et al. 2013b). In the current study, we found that increased expression of pEGFR, pPI3K, and pAKT induced by hypoxic stress was suppressed in pEGFP-C1-LRIG1-transfected cells, suggesting that the negative effects of LRIG1 on VM formation and other

Fig. 9 Hypothetical scheme of the proposed mechanism for the inhibitory effects of LRIG1

cell behaviors may be mediated by the EGFR/PI3K/AKT pathway. In addition, the EGFR/PI3K/AKT pathway can crosstalk with the Notch pathway and contribute to the function of Notch signaling (Xu et al. 2013b). Because Notch signaling has been implicated to be intimately involved in the process of VM, we therefore speculate that LRIG1 overexpression may also have an impact on the Notch signaling cascade. Further studies are required to examine this possibility.

Furthermore, we found that LRIG1 overexpression could counteract hypoxia-induced alterations in E-cadherin and vimentin expression levels, but knockdown of LRIG1 showed the opposite effect. These findings suggest a negative role for LRIG1 in regulating the EMT process of glioma cells. EMT contributes to VM formation in various tumor cells (Wang et al. 2014; Du et al. 2014); meanwhile, EGFR regulates EMT process through activation of the downstream effectors such as PI3K and AKT (Ye et al. 2010). There exists precedent for modulating EMT through the suppression of EGFR. Zhou et al. (2014) showed that suppression of EGFR by miRNA-7 is able to inactivate the ERK-1/2/ATK signaling cascade, thus reversing EMT and attenuating metastasis in epithelial ovarian cancer cells. Zuo et al. (2011) found that EGFR activation can induce EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin in HNSCC SCC10A cells through the ERK-1/2 and PI3K signaling pathways. Based on these findings, the negative effects of LRIG1 on EMT are most likely attributed to inactivation of the EGFR/PI3K/AKT pathway triggered by the antagonistic interaction between EGFR and LRIG1, consequently suppressing VM formation and VM-dependent malignant behaviors in glioma cells (the hypothetical scheme is shown in Fig. 9).

Conclusion

In summary, the current study shows that overexpression of LRIG1 is able to suppress hypoxia-induced VM formation and VM-dependent malignant behaviors including migration, invasion, and proliferation in SHG-44 cells. Moreover, the knockdown of LRIG1 can exacerbate the effects of hypoxia on VM formation and EMT. Our data suggest that the effect of LRIG1 is mediated by suppression of the EGFR/PI3K/AKT pathway and EMT process. These findings provide compelling evidence implicating a role for LRIG1 in glioma pathophysiology and suggesting that therapy upregulating LRIG1 expression or a gene-therapy-based treatment introducing LRIG1 may prove to be successful in the treatment of this disease.

Conflict of interest The authors report no conflicts of interest.

References

- Du J, Sun B, Zhao X et al (2014) Hypoxia promotes vasculogenic mimicry formation by inducing epithelial-mesenchymal transition in ovarian carcinoma. *Gynecol Oncol* 133(3):575–583
- Fan YZ, Sun W (2010) Molecular regulation of vasculogenic mimicry in tumors and potential tumor-target therapy. *World J Gastrointest Surg* 2(4):117–127
- Gotoh N (2009) Feedback inhibitors of the epidermal growth factor receptor signaling pathways. *Int J Biochem Cell Biol* 41(3):511–515
- Guo D, Nilsson J, Haapasalo H et al (2006) Perinuclear leucine-rich repeats and immunoglobulin-like domain proteins (LRIG1-3) as prognostic indicators in astrocytic tumors. *Acta Neuropathol* 111(3):238–246
- Holmlund C, Haapasalo H, Yi W et al (2009) Cytoplasmic LRIG2 expression is associated with poor oligodendroglioma patient survival. *Neuropathology* 29(3):242–247
- Huang M, Ke Y, Sun X et al (2014) Mammalian target of rapamycin signaling is involved in the vasculogenic mimicry of glioma via hypoxia-inducible factor-1alpha. *Oncol Rep* 32(5):1973–1980
- Johansson M, Oudin A, Tiemann K et al (2013) The soluble form of the tumor suppressor Lrig1 potently inhibits in vivo glioma growth irrespective of EGF receptor status. *Neuro Oncol* 15(9):1200–1211
- Kumar M, Nagpal R, Hemalatha R et al (2012) Targeted cancer therapies: the future of cancer treatment. *Acta Biomed* 83(3):220–233
- Maniotis AJ, Folberg R, Hess A et al (1999) Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* 155(3):739–752
- Misra RM, Bajaj MS, Kale VP (2012) Vasculogenic mimicry of HT1080 tumour cells in vivo: critical role of HIF-1alpha-neuropilin-1 axis. *PLoS One* 7(11):e50153
- Mongiardi MP (2012) Angiogenesis and hypoxia in glioblastoma: a focus on cancer stem cells. *CNS Neurol Disord Drug Targets* 11(7):878–883
- Omuro A, DeAngelis LM (2013) Glioblastoma and other malignant gliomas: a clinical review. *JAMA* 310(17):1842–1850
- Paulis YW, Soetekouw PM, Verheul HM, Tjan-Heijnen VC, Griffioen AW (2010) Signalling pathways in vasculogenic mimicry. *Biochim Biophys Acta* 1806(1):18–28
- Persano L, Rampazzo E, Basso G, Viola G (2013) Glioblastoma cancer stem cells: role of the microenvironment and therapeutic targeting. *Biochem Pharmacol* 85(5):612–622
- Plate KH, Scholz A, Dumont DJ (2012) Tumor angiogenesis and anti-angiogenic therapy in malignant gliomas revisited. *Acta Neuropathol* 124(6):763–775
- Seftor RE, Hess AR, Seftor EA et al (2012) Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. *Am J Pathol* 181(4):1115–1125
- Taylor TE, Furnari FB, Cavenee WK (2012) Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance. *Curr Cancer Drug Targets* 12(3):197–209
- Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ (2010) Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA Cancer J Clin* 60(3):166–193
- Verhaak RG, Hoadley KA, Purdom E et al (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17(1):98–110
- Wang SY, Yu L, Ling GQ et al (2012) Vasculogenic mimicry and its clinical significance in medulloblastoma. *Cancer Biol Ther* 13(5):341–348
- Wang SY, Ke YQ, Lu GH et al (2013) Vasculogenic mimicry is a prognostic factor for postoperative survival in patients with glioblastoma. *J Neurooncol* 112(3):339–345

- Wang W, Lin P, Sun B et al (2014) Epithelial-mesenchymal transition regulated by EphA2 contributes to vasculogenic mimicry formation of head and neck squamous cell carcinoma. *Biomed Res Int* 2014: 803914
- Xie R, Yang H, Xiao Q et al (2013) Downregulation of LRIG1 expression by RNA interference promotes the aggressive properties of glioma cells via EGFR/Akt/c-Myc activation. *Oncol Rep* 29(1):177–184
- Xu G, Bai X, Wang M, Xie W, Li R, Li C (2013a) Characterization of glycolytic phenotype of SHG44 human glioma cells under hypoxic conditions and its association with cell proliferation and apoptosis. *Nan Fang Yi Ke Da Xue Xue Bao* 33(3):406–411
- Xu P, Zhang A, Jiang R et al (2013b) The different role of Notch1 and Notch2 in astrocytic gliomas. *PLoS One* 8(1):e53654
- Ye F, Gao Q, Cai MJ (2010) Therapeutic targeting of EGFR in malignant gliomas. *Expert Opin Ther Targets* 14(3):303–316
- Zhang S, Zhang D, Sun B (2007) Vasculogenic mimicry: current status and future prospects. *Cancer Lett* 254(2):157–164
- Zhou X, Hu Y, Dai L et al (2014) MicroRNA-7 inhibits tumor metastasis and reverses epithelial-mesenchymal transition through AKT/ERK1/2 inactivation by targeting EGFR in epithelial ovarian cancer. *PLoS One* 9(5):e96718
- Zuo JH, Zhu W, Li MY et al (2011) Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin. *J Cell Biochem* 112(9):2508–2517