

Autophagy mediates paracrine regulation of vascular endothelial cells

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Gastrin-releasing peptide (GRP) is a proangiogenic ligand secreted by tumors and acts directly upon binding to GRP receptor in endothelial cells. Angiogenesis plays a critical role in the pathology of various diseases, including cancer, as the formation of new blood vessels potentiates the rate of tumor growth and dissemination. GRP increases the migration of endothelial cells, but much is unknown about its role on endothelial cell proliferation and survival, as well as the signaling pathways involved. In the present study, we showed that GRP increases endothelial cell proliferation and tubule formation. There was a time-dependent increase in the levels of phosphorylated AKT, mammalian target of rapamycin (mTOR), and S6R in human umbilical vein endothelial cells treated with GRP. Interestingly, GRP treatment decreased the expression of proautophagic factors, ATG5, BECN1, and LC3 proteins. GRP also attenuated rapamycin-induced formation of autophagosomes. Moreover, overexpression of ATG5 or BECN1 significantly decreased tubule formation induced by exogenous GRP, whereas siRNA against ATG5 or BECN1 resulted in increased tubule formation with GRP treatment. Our results show that GRP inhibits the process of autophagy in vascular endothelial cells, thereby increasing endothelial cell proliferation and tubule formation. Here, we describe a novel role of GRP in the regulation of autophagy of endothelial cells, thereby providing a potential new therapeutic strategy in targeting angiogenesis during cancer progression.

Laboratory Investigation (2013) **93**, 639–645; doi:10.1038/labinvest.2013.57; published online 22 April 2013

KEYWORDS: AKT; autophagy; endothelial cell; GRP; mTOR

Gastrin-releasing peptide (GRP) is both a gut and a neuro-peptide that has been implicated in a number of pathophysiological conditions, including cancer. It has a mitogenic function on various cancer cell types including neuroblastoma in both autocrine and paracrine manner.^{1,2} Moreover, GRP treatment activates intracellular signaling pathways such as the PI3K to stimulate angiogenesis and tumor growth.^{3–5} Although GRP induces endothelial cell migration and enhances angiogenesis in an *in vivo* assay,⁶ much is unknown about its role in the regulation of vascular endothelial cell proliferation. Moreover, downstream signaling pathways mediating the angiogenic effects of GRP are yet to be elucidated.

Cancer progression requires formation of new blood vessels to suffice nutritional requirements of neoplastic cells.⁷ Antiangiogenic therapies targeting tumor cell-derived angiogenic factors have been successfully introduced in clinical trials for metastatic cancer.^{8,9} Tumor cell-derived vascular endothelial growth factor (VEGF) signaling promotes cancer

progression by stimulating endothelial cell proliferation, migration and invasion, increased blood vessel permeability, and by forming a microenvironment for the migration of endothelial cells.¹⁰ In turn, endothelial cells are recruited to tumors to provide ‘angiocrine’ support for the dissemination of tumor cells during cancer progression.¹¹ Moreover, endothelial cell-autonomous VEGF signaling pathway is critical for vascular homeostasis as genetic depletion of VEGF leads to progressive endothelial degeneration.¹² However, the exact action of GRP, which is a known inducer of VEGF signaling, on vascular endothelial cells remains unclear.

Autophagy, an alternative mechanism for cell death, has been identified as a novel therapeutic strategy when there is a failure of the apoptotic machinery in cancer cells. Induction of proautophagic molecules requires a concomitant down-regulation of the AKT/mammalian target of rapamycin (mTOR) signal transduction pathway, as activated mTOR is a negative regulator of autophagy.¹³ Interestingly, GRP

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Received 11 October 2012; revised 1 March 2013; accepted 6 March 2013

treatment enhances AKT signaling in cancer cells,¹⁴ which then phosphorylates mTOR.¹⁵ The role of GRP in the regulation of autophagy via the activation of AKT/mTOR signaling has not been studied yet. Moreover, how the induction of autophagy affects VEGF secretion and tubule formation by endothelial cells is yet to be determined. Targeting GRP/AKT/mTOR signaling axis might provide insights into using autophagy as a new tool to target tumor-associated vascular endothelial cells.

In this study, we demonstrate for the first time that GRP treatment enhances proliferation of human umbilical vein endothelial cells (HUVECs) via upregulation of the PI3K/AKT/mTOR signaling pathway. Moreover, GRP treatment enhanced tubule formation by HUVECs when compared with control. Interestingly, GRP treatment decreased the expression of key autophagic molecules in HUVECs in a time-dependent manner. Furthermore, overexpression or silencing of autophagic molecules ATG5 or BECN1 decreased or increased tubule formation by HUVECs, respectively. Our data indicate a proliferative role for GRP in vascular endothelial cells and identify a novel role for this neuropeptide in the inhibition of autophagy, where autophagy regulates tubule formation by vascular endothelial cells.

MATERIALS AND METHODS

Cell Culture and Reagents

HUVECs obtained from Dr M Freeman (Vanderbilt University Medical Center, Nashville, TN, USA) were cultured in EMM-2 supplemented with growth factors (EGM-2 Single-Quot kit, Lonza, Walkersville, MD, USA) at 37 °C and humidified 5% CO₂. GRP was obtained from TOCRIS Bioscience (Ellisville, MO, USA). Matrigel was purchased from BD Biosciences (Bedford, MA, USA).

Cell Proliferation

HUVECs were seeded in 96-well plate at a density of 5000 cells/well. Next day, GRP (100 nM) was added to each well for up to 24 and 72 h. Then, 20 μ l of the combined MTS/PMS solution was added into each well containing 100 μ l of cells in culture media. The plate was incubated for 1–4 h at 37 °C in a humidified 5% CO₂ atmosphere. Absorbance of the color was measured at a wavelength of 570 nm with Flexstation 3 Molecular Devices (Sunnyvale, CA, USA).

Endothelial Cell Tubule Formation Assay

HUVECs grown to ~70% confluence were trypsinized, counted, and seeded with 48 000 cells per well in 24-well plates coated with 300 μ l of Matrigel (BD Biosciences). These cells were periodically observed by microscope as they differentiated into capillary-like tubule structures. After 6 h, cells were stained with hematoxylin and eosin and photographs were taken by microscope, and representative photographs obtained. Tubules were developed from clear elongated cell bodies that connect to form a polygon network. The average number of tubules was quantified by

randomly selecting three separate \times 200 fields and counting the number of tubules per field.

Co-Culture Assay

Six-well transwell cell culture chamber inserts without collagen and with 0.4 micropores were used to plate HUVECs in the inserts and BE(2)-C cells in the chambers (in duplicate) in a HUVEC/BE(2)-C ratio of 1:10. After 48 h, the insert was removed and stained with hematoxylin and eosin and photographs (\times 200) taken using a microscope.

Immunoblotting

Cells (5×10^5) were collected at various time points, and then washed with ice-cold PBS twice before adding lysis buffer (M-PER Mammalian Protein Extraction reagent, Thermo Scientific, Waltham, MA, USA) and cocktail inhibitor (Sigma, 5 μ g/ml). Equal amounts of protein were loaded onto each well and separated by NuPAGE 4–12% Bis-Tris gel, followed by transfer onto PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% nonfat milk in PBS-T for 1 h at room temperature. The blots were then incubated with antibodies against the human proteins p-mTOR, mTOR, p-AKT, AKT, p-S6R, S6R, ATG5-ATG12, BECN1, β -actin, and LC3I/II for 1 h at 4 °C. Goat anti-rabbit IgG secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated for 45 min at room temperature. Immunoblots were developed by using the chemiluminescence detection system (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol and autoradiography was performed.

Cell Transfection

siRNA ATG5, BECN1, and siRNA control were purchased from Santa Cruz (Santa Cruz Biotechnology). Cells were transfected with 25 nM siRNAs, 3 μ g ATG5 plasmid, or BECN1 plasmid using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). Transfected cells were used for subsequent experiments 48 h later.

Autophagy Assay

HUVECs were transfected with 3 μ g of EGFP-LC3 expression plasmid (a gift from Dr Norboru Mizushima) using Lipofectamine 2000 for 24 h. Cells with punctate GFP signal were counted as autophagic cells based on characteristic lysosomal localization of LC3 protein during autophagy.¹⁶ Punctate GFP cells were quantified by randomly selecting three separate \times 100 fields and counting the number of punctate GFP cells per field. The percent of punctate GFP cells per total GFP transfected cells was calculated and experiments were conducted in triplicate. The fluorescence of EGFP-LC3 was observed using immunofluorescence microscopy.

Statistical Analysis

All results are shown as mean value \pm s.e.m. from at least three independent experiments. Western blot scans are representative of three separate assays. Statistical analysis was performed using Student's *t*-test. The *P*-values of <0.05 were considered to be statistically significant.

RESULTS

GRP Increased HUVEC Proliferation and Tubule Formation

GRP has been reported to induce migration of HUVECs.⁶ To examine whether GRP also induces proliferation of HUVECs, we treated HUVECs with GRP (100 nM) for 24 and 72 h. We found that GRP treatment significantly increased HUVEC proliferation (Figure 1a). We next wanted to determine whether GRP treatment induces tubule formation *in vitro*. HUVECs plated on transwell plates coated with Matrigel were treated either with or without GRP (100 nM) for 6 h. GRP treatment significantly increased tubule formation by HUVECs compared with the control group (Figure 1b). Taken together, these data suggest that exogenous GRP induces endothelial cell proliferation and tubule formation *in vitro*.

GRP Treatment Downregulated Key Autophagic Proteins in HUVECs

Activation of PI3K/AKT pathway is a crucial step in events leading to angiogenesis. GRP also stimulates cancer cell proliferation via activation of the PI3K/AKT pathway.³ The kinase mTOR is a critical negative regulator of autophagy, where activated mTOR suppresses key molecules involved in the induction of autophagy.¹⁷ To validate whether the effect of GRP on HUVEC proliferation and tubule formation is mediated by PI3K/AKT/mTOR pathway, we treated HUVECs with GRP. As expected, we found that GRP treatment increased phosphorylation of AKT, mTOR, and S6R in a time-dependent manner (Figure 2a). As GRP treatment

enhanced phosphorylation of mTOR in HUVECs, we investigated the role of GRP in the regulation of key autophagy proteins. When HUVECs were treated with GRP, the key autophagy proteins ATG5 and BECN1 were significantly downregulated in a time-dependent manner (Figure 2b). Furthermore, expression of LC3I and LC3II was inhibited in a time-dependent manner, indicating decreased formation of autophagosomes on GRP treatment.

To further investigate whether GRP regulates autophagy via the mTOR pathway, we transfected EGFP-LC3 plasmid into HUVECs to visualize the formation of autophagosomes, a hallmark of autophagy. Diffuse cytoplasmic localization of EGFP-LC3 was observed in control cells, whereas rapamycin-treated cells showed significantly increased punctate fluorescence of LC3 proteins at 48 h after transfection. Moreover, GRP dramatically reduced the rapamycin-induced increased punctate fluorescence of EGFP-LC3 proteins (Figure 2c). These data indicate that GRP regulates autophagy of HUVECs via activation of the AKT/mTOR/S6R signaling axis.

Overexpression of ATG5 and BECN1 Decreased HUVEC Tubule Formation

Autophagy is an alternative cell death mechanism that can be activated in tumor cells when other modes of inducing cancer cell death fail. As endothelial cells form new leaky vessels through autocrine and paracrine secretion of proangiogenic molecules during tumor progression, activation of autophagy in these cells might be critical to suppress tumorigenesis. To examine whether autophagy affects the production of tubular structures, HUVECs were transfected with plasmid ATG5 or BECN1 or control vector. A representative image and the average number of tubules counted in three separate ($\times 200$) fields are shown in Figures 3a and b, respectively. Tubules were developed from clear elongated cell bodies that connect to form a polygon network. Treatment of GRP increased the formation of such three-dimensional capillary-like tubule

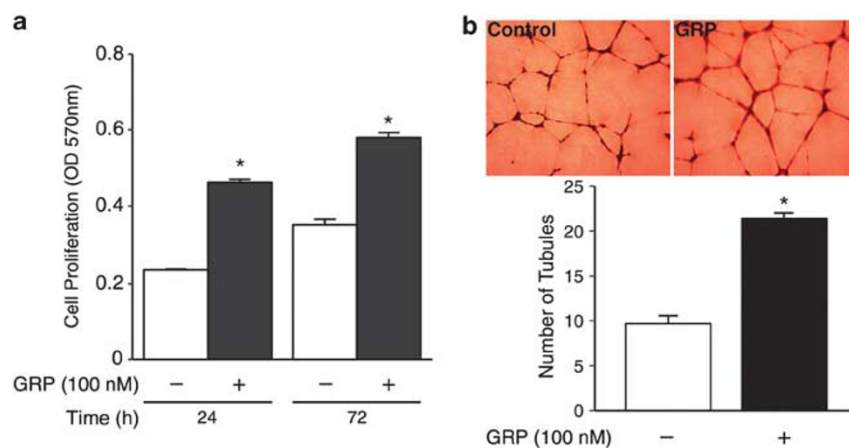


Figure 1 GRP increased HUVEC proliferation and tubule formation. (a) HUVECs treated with GRP (100 nM) resulted in a significant increase in cell proliferation as measured by CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay when compared with control cells. (b) HUVECs were treated with GRP (100 nM) on 24-well plates coated with Matrigel for 6 h. Tubule staining was performed in triplicate (mean \pm s.e.m.; **P* < 0.05 vs control).

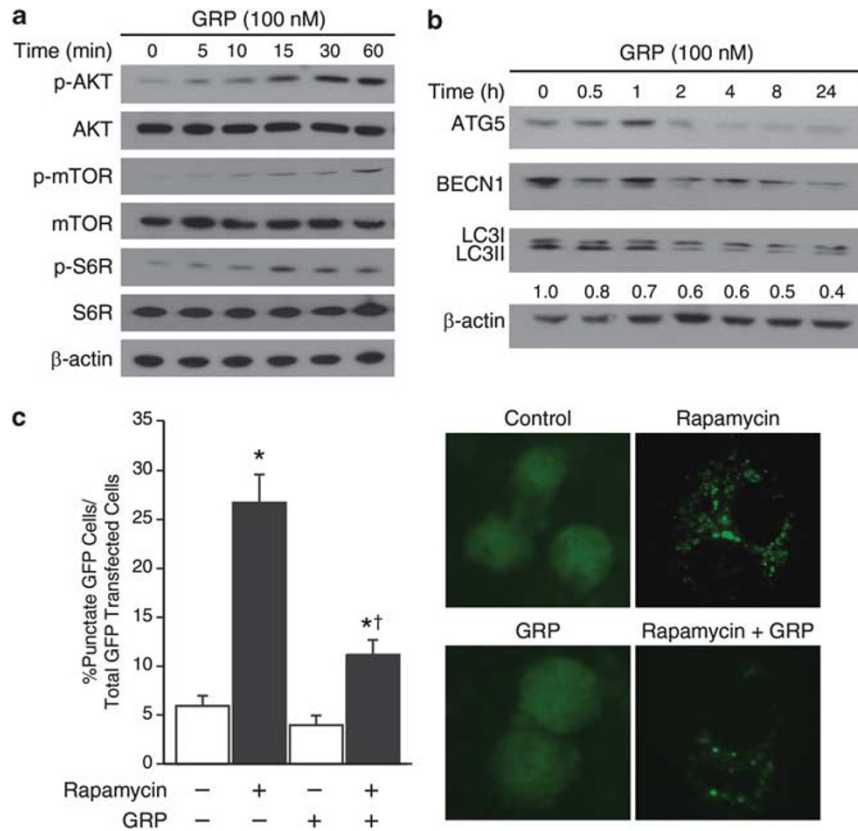


Figure 2 GRP enhanced AKT/mTOR signaling and downregulated ATG5 and BECN1. (a) Phosphorylated and total AKT, mTOR, and S6R protein levels in HUVECs over a time course after GRP treatment (100 nM) for 30 min by western blotting. (b) GRP (100 nM) inhibited expression of ATG5, BECN1, and LC3I/II in HUVECs. Data are representative of three separate experiments. Total proteins (35 μg per lane) were probed with the indicated antibodies in HUVECs. β-Actin was probed to demonstrate equal protein loading. Numbers represent ratio of densitometric values of LC3I to β-actin levels. (c) HUVECs were transfected with EGFP-LC3 plasmid and exposed to 200 nM rapamycin for 24 h and assessed for autophagy after 48 h. Representative fluorescence microscopy photos are shown. Values are mean ± s.e.m. of three separate experiments (**P* < 0.05 vs control, †*P* < 0.05 vs rapamycin alone).

structures when compared with controls (20 ± 2 vs 9 ± 1). Overexpression of either ATG5 or BECN1 formed discontinued tubules and had significantly reduced formation of such polygon networks in spite of GRP treatment when compared with GRP treatment alone (20 ± 2 vs 3.3 ± 0.58 and 20 ± 2 vs 6.7 ± 1, respectively). Immunoblotting was used to confirm overexpression of either ATG5 or BECN1 by stable transfections with or without GRP treatment (Figure 3c). These findings indicate that induction of the autophagy machinery may prevent GRP-induced angiogenesis by HUVECs.

Silencing ATG5 or BECN1 Enhanced HUVEC Tubule Formation

To further characterize the role of autophagy in the regulation of angiogenesis, we downregulated the expression of the two essential autophagy proteins, ATG5 and BECN1. siRNA against either ATG5 or BECN1 increased formation of capillary-like tubule structures with GRP treatment in comparison with GRP treatment alone (27.6 ± 1.5 vs 8 ± 1 and 23 ± 1.5 vs 8 ± 1, respectively; Figure 4a). Moreover, the polygon networks formed upon silencing ATG5 or BECN1

were denser compared with the GRP treatment alone, indicating that targeting proautophagic genes enhanced tubule formation by HUVECs. The average number of tubules counted in three separate (× 200) fields is shown in Figure 4b. Protein levels of ATG5 and BECN1 after siRNA-mediated silencing were confirmed by immunoblotting with or without GRP treatment (Figure 4c). Taken together, these data further confirmed that GRP-mediated increase in angiogenesis by endothelial cells is further enhanced by the inhibition of autophagy.

Inhibition of Autophagy Increased Angiogenesis in Co-Culture of HUVECs with BE(2)-C Cells

To investigate the angiogenic effects via paracrine mechanism, we co-cultured human neuroblastoma BE(2)-C cells and ATG5- or BECN1-silenced HUVECs using transwell inserts without collagen coating and a pore size of 0.4 μm. Interestingly, we found that in the presence of BE(2)-C cells, HUVECs transfected with siRNA against ATG5 or BECN1 exhibited increased formation of net-like structures in comparison with controls (Figure 5; upper panels, arrows). In particular, GRP blocking antibody inhibited the net-like

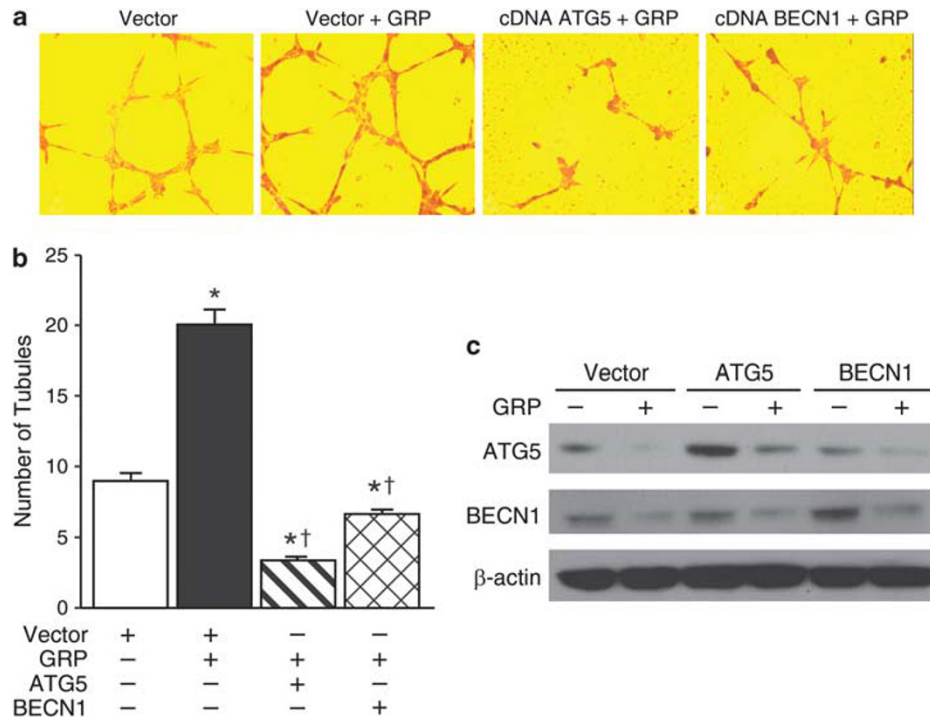


Figure 3 Overexpression of ATG5 or BECN1 decreased HUVEC tubule formation. (a) HUVECs (3×10^5 cells) were transfected with overexpression plasmids encoding ATG5 or BECN1. After 24 h, cells were trypsinized and replated on 24-well plates coated with Matrigel. After 6 h, cells were fixed and stained with hematoxylin and eosin. The slides were examined by microscope ($\times 200$), and representative fields are shown. (b) Stained tubules were based on three separate repeated experiments and shown as the mean \pm s.e.m. (c) Overexpression of ATG5 and BECN1 in HUVECs was confirmed by immunoblotting. β -Actin was probed to demonstrate equal loading ($*P < 0.05$ vs control, $^\dagger P < 0.05$ vs GRP alone).

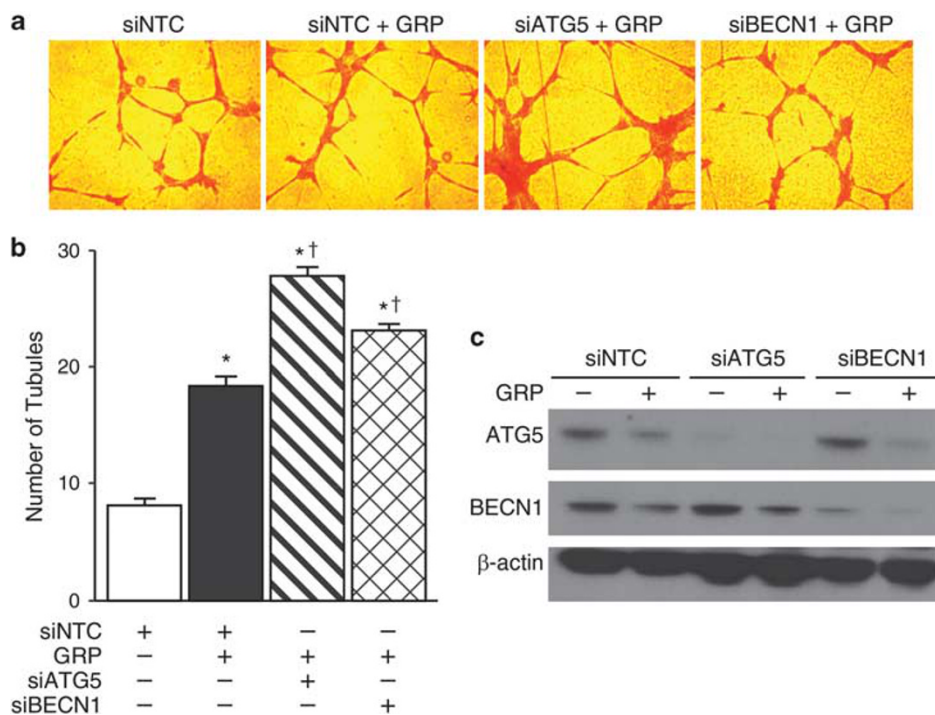


Figure 4 Silencing ATG5 or BECN1 enhanced HUVEC tubule formation. (a) HUVECs (3×10^5 cells) were transfected with 25 nM siRNA against ATG5 or BECN1 for 24 h. After 24 h, cells were trypsinized and treated with GRP on 24-well plates coated with Matrigel. After 6 h, cells were fixed and stained with hematoxylin and eosin. The slides were examined by microscopy ($\times 200$), and representative fields are shown. (b) Stained tubules were based on three separate repeated experiments and shown as the mean \pm s.e.m. (c) Targeted silencing of ATG5 and BECN1 was confirmed by immunoblotting. β -Actin was probed to demonstrate equal loading ($*P < 0.05$ vs control, $^\dagger P < 0.05$ vs GRP alone).

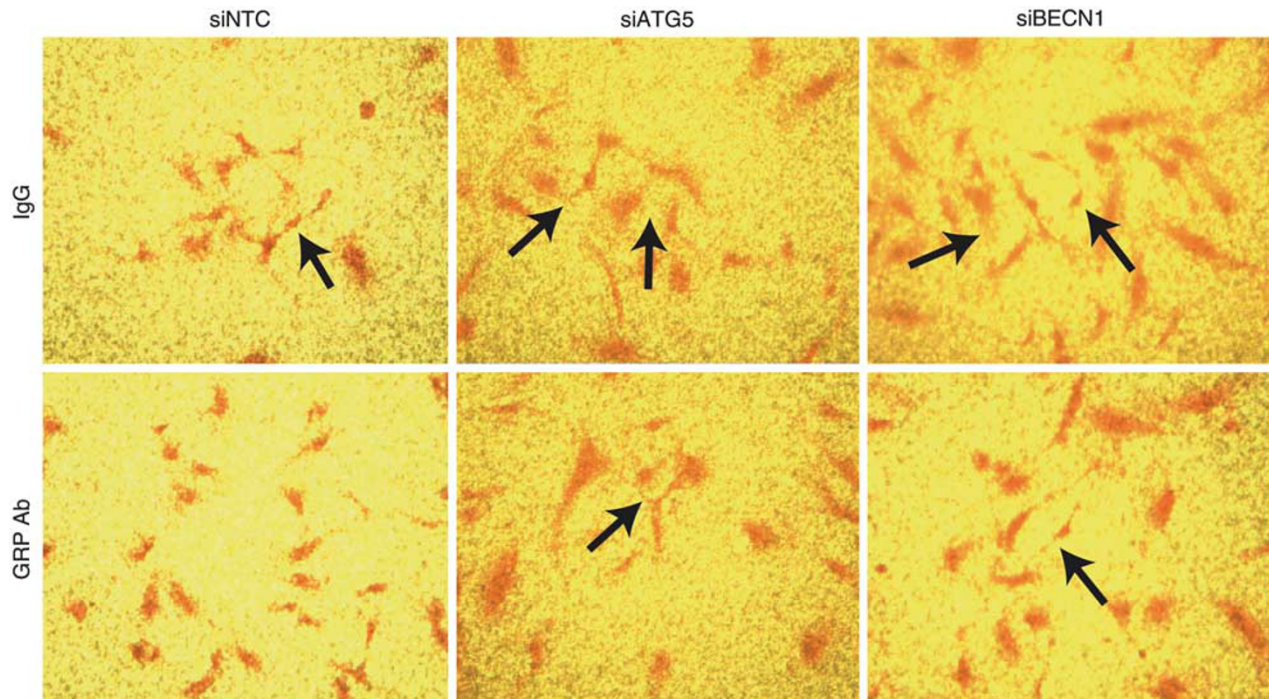


Figure 5 Net-like formation of co-culture with HUVECs transfected with siRNA against ATG5 or BECN1 in BE(2)-C cells. HUVECs were transfected with 25 nM siRNA against ATG5 or BECN1 for 24 h. After 24 h, HUVECs were trypsinized and co-cultured with BE(2)-C cells with or without GRP antibody (GRP Ab; 1 μ g/ml) for 48 h. IgG was used as control. Arrows indicate increased net-like structures (magnification \times 200).

formation observed after silencing the autophagic machinery (Figure 5; lower panels, arrows). Taken together, these data suggest a critical role for GRP-mediated inhibition of autophagy in the paracrine regulation of endothelial cells.

DISCUSSION

GRP is a potent inducer of angiogenesis in various cancer cell types; however, its paracrine regulation of vascular endothelial cell proliferation and tubule formation has not been elucidated. In this report, we describe, for the first time, a novel mechanism of GRP regulation of autophagy in HUVEC proliferation and tubule formation via activation of the PI3K/AKT/mTOR signaling pathway. Furthermore, GRP enhanced HUVEC tubule formation by downregulation of key autophagy proteins such as ATG5 and BECN1.

Angiogenesis, a process essential during normal development, requires an intricate and complex interplay of growth factors and inhibitors, endothelial cells, and the extracellular matrix.¹⁸ Imbalance in any aspect of this critical process contributes to a wide variety of pathological conditions, including cancer. Major breakthroughs have identified signaling pathways and specific proangiogenic growth factors along with their receptors in cancer-derived neoangiogenesis. Conversely, endothelial cells play a more critical role in the process of angiogenesis and cancer progression than acting as mere tubes for the circulation of blood.¹⁹ Cell-autonomous synthesis and secretion of proangiogenic factors are required for the proliferation and survival of endothelial cells, as pharmacological inhibition of

proangiogenic receptors induced endothelial cell apoptosis.²⁰ Moreover, targeting vascular endothelial cells using anticancer drugs have shown to induce apoptosis in these cells and inhibit tubule formation *in vitro*.²¹

GRP treatment has been reported to induce migration of endothelial cells *in vitro* and enhance angiogenesis *in vivo*.⁶ Our data indicate that GRP acts as a mitogen for endothelial cells and induces tubule formation *in vitro*. This observation further confirms the proangiogenic role of GRP during tumorigenesis, as endothelial cell survival, proliferation, and vessel formation are critical steps in neovascularization. GRP and its receptor, GRP-R, utilize the PI3K/AKT signaling pathway as downstream effectors in cancer cells.¹⁴ Interestingly, GRP treatment activated the AKT/mTOR/S6 signaling axis in endothelial cells, demonstrating the critical nature of this pathway downstream of GRP/GRP-R in different kinds of cells. As mTOR negatively regulates autophagy, we speculated a role for GRP in the inhibition of autophagy via the activation of AKT/mTOR pathway. As expected, GRP treatment decreased the expression of proautophagic proteins in a time-dependent manner, and also inhibited rapamycin-induced autophagy. This is a novel finding where a neuropeptide has been shown to suppress the process of autophagy in vascular endothelial cells, providing a better understanding of how induction of autophagy might be a potential antivascular therapy. Interestingly, our results indicate that overexpression of proautophagic proteins inhibited the vasoactive role of GRP and suppressed tubule formation by endothelial cells. Also, silencing autophagic

molecules synergistically enhanced tubule formation by GRP treatment. In particular, we found that co-culture system recapitulated component of the angiogenic phenotype, including formation of net-like structures. Targeting endothelial cells and the perivascular niche using next-generation combinational drug regimens is becoming increasingly important.²⁰ Our co-culture data also highlight the importance of tumor–host interactions during tumor progression. It is plausible that tumor cell-secreted GRP enhances neovascularization in solid tumors by increasing endothelial cell proliferation, survival, and motility. This area of tumor–host interaction becomes increasingly crucial in various cancers such as neuroblastoma, prostate, and small-cell lung cancer, where GRP acts as a critical mitogen and has been implicated in disease progression.

In conclusion, the findings in this study identify a novel role for GRP in vascular endothelial cell proliferation and tubule formation. Our study has further ascertained a plausible mechanism by which tumor cells may enhance tubule formation by endothelial cells by increasing tumor cell-associated GRP secretion and subsequently suppressing endothelial cell autophagy via activation of the PI3K/AKT/mTOR pathway, highlighting the critical nature of host–tumor interactions. Moreover, activation of autophagy by genetic manipulation of the autophagic machinery inhibits the proangiogenic effect of GRP on endothelial cells, providing a potential new tool in targeting angiogenesis during cancer progression.

ACKNOWLEDGEMENTS

We thank Karen Martin for assistance with the manuscript preparation. This work was supported by a grant R01 DK61470 from the National Institutes of Health.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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