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# Kaposi Sarcoma–Associated Herpesvirus G Protein–Coupled Receptor Enhances Endothelial Cell Survival in Part by Upregulation of Bcl-2

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## ABSTRACT

**Background:** Kaposi sarcoma–associated herpesvirus (KSHV) encoded G protein–coupled receptor (vGPCR) is a constitutively active lytic phase protein with significant homology to the human interleukin-8 receptor. vGPCR is necessary and sufficient to induce angiogenesis as well as the spindle cell proliferation characteristic of Kaposi sarcoma (KS) lesions. We previously demonstrated that Bcl-2, an antiapoptotic protein, is upregulated in KS lesions. The aim of this study was to determine if vGPCR enhances endothelial cell survival through upregulation of Bcl-2 expression and to elucidate the signaling pathways involved.

**Methods:** Primary human umbilical vein endothelial cells were transduced with a recombinant retrovirus expressing vGPCR and then subjected to serum starvation. Cell viability and apoptosis were analyzed by fluorescence-activated cell sorting. Bcl-2 expression was determined by real-time quantitative reverse transcription polymerase chain reaction and immunoblotting. Specific pharmacological inhibitors of phosphatidylinositol 3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) were employed to elucidate the signaling pathways involved. Bcl-2 expression was knocked down using small interfering RNA (siRNA).

**Results:** Endothelial cells expressing vGPCR showed increased survival after serum starvation and upregulation of Bcl-2 messenger RNA (mRNA) and protein. The vGPCR-induced increases in both Bcl-2 mRNA and protein levels were dependent on PI3K signaling but not on mTOR. Moreover, siRNA inhibition of Bcl-2 resulted in significant abrogation of the observed vGPCR-mediated cell survival advantage.

**Conclusions:** Taken together, the results demonstrate that Bcl-2 is a mediator of vGPCR-induced endothelial cell survival and is a downstream effector of Akt in this process.

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## INTRODUCTION

Kaposi sarcoma–associated herpesvirus (KSHV) is the etiologic agent of Kaposi sarcoma (KS), an endothelial cell sarcoma that is highly comorbid with human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS) development.[1] KS is a multifocal tumor that affects the skin, gastrointestinal tract, lung, and lymph nodes. The KS lesions are characterized by proliferating spindle cells, prominent angiogenesis, and leukocyte infiltration. Four clinical forms of KS have been described.[1,2] *Classic* KS is an indolent tumor of elderly men of Mediterranean origin. *Endemic* KS is prevalent in sub-Saharan Africa, where it is one of the most frequently occurring tumors. *Iatrogenic* KS has been identified in transplant recipients undergoing immunosuppressive therapy. *Epidemic* AIDS-KS is the most common neoplastic manifestation of AIDS in the United States and Europe. KSHV (also known as human herpesvi-

rus 8) has been implicated in the development of all epidemiological forms of KS, primary effusion lymphoma,[3] and multicentric Castleman disease.[4] The DNA of KSHV is found in the spindle cells and endothelial cells of the KS lesion, suggesting that the presence of KSHV has a critical role in the induction and maintenance of the tumor.[5] This critical role is further supported by the fact that the KSHV genome contains several genes capable of inhibiting apoptosis, causing oncogenic transformation, and modulating the immune response.[2]

The KSHV virally encoded G protein–coupled receptor (vGPCR) is a constitutively active lytic phase protein with significant homology to the human interleukin-8 receptor. vGPCR has been demonstrated to promote cell proliferation, enhance cell survival, modulate cell migration, stimulate angiogenesis, and recruit inflammatory cells, both in expressing cells and in neighboring (bystander) cells.[6] vGPCR has angiogenic and tumorigenic properties[7] and has been shown to be sufficient to induce an angiogenic phenotype and KS lesions.[8-10] It has been proposed that vGPCR induction of the angiogenic phenotype in KS lesions ultimately leads to tumorigenesis, and is, therefore, essential to the proliferation of the disease.[11-13]

For cells to become angiogenic and then tumorigenic, they must first obtain a survival advantage so they may become proliferative under nutrient-deficient conditions. vGPCR stimulates a network of intracellular signaling cascades, including the prosurvival phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway that appears to play a central role in sarcomagenesis induced by KSHV.[14-16] Akt is a serine/threonine protein kinase that is involved in mediating numerous cellular phenotypes associated with cancer, including cell proliferation, survival, angiogenesis, and tissue invasion.[17] However, the downstream effectors of Akt signaling in KS are not fully understood. Bcl-2, a protooncogene known to prolong cellular viability and to antagonize apoptosis, is a downstream effector of Akt signaling.[18] We previously showed that Bcl-2 is upregulated in spindle cells within KS lesions and its expression increases as the pathological stage of KS advances.[19]

The aim of this study was to assess whether vGPCR conferred a survival advantage to primary endothelial cells and whether this advantage could be attributed to upregulation of Bcl-2 expression. We further sought to elucidate the signaling pathways involved.

## METHODS

### Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Allendale, NJ) and maintained in M-199 (Gibco/Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 1% penicillin/streptomycin (Gibco/Invitrogen), and 50 μg/mL endothelial cell growth supplement (ECGS; BD Biosciences, San Jose, CA).

### Retroviral Infections

Recombinant retroviral vectors carrying either enhanced green fluorescent protein (eGFP) alone (BABE) or eGFP and vGPCR (BABE-vGPCR) cassette(s)[20] were used to infect endothelial cells. HUVECs at passage 6 were used for all experiments. Cells were transduced at 40%-60% confluency by adding a volume of virus equal to one-fifth of the culture media to complete media supplemented with 8 μg/mL hexadimethrine bromide (Polybrene; Sigma-Aldrich, St. Louis, MO). Cells were incubated overnight at 37°C in 5% $CO_2$, and the media was changed the following day. Fluorescence from the eGFP was used to approximate transduction efficiency between 48-72 hours postinfection; transduction efficiency was usually in the range of 70%-80%. Approximately 2-3 days postinfection, cells were subjected to treatment.

### Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells transduced with either BABE or BABE-vGPCR were allowed to grow to near confluency (90%). Once cells were confluent, apoptotic events were analyzed using the Annexin-V-APC Kit (Molecular Probes; Invitrogen, Grand Island, NY) according to the manufacturer's protocol following serum starvation in media supplemented with 0.5% FBS. Briefly, cells were washed in phosphate-buffered saline (PBS), trypsinized, and collected by centrifugation (1,500 rpm, 5 minutes). The supernatant was aspirated, and cells were rinsed with PBS (0.5 mL). Following a centrifugation step as described above, supernatant was aspirated and cells were resuspended in 250 μL of Annexin Binding Buffer (Invitrogen). Cells were stained with 5 μL Annexin-V-APC (1:5 dilution of stock) and incubated at room temperature for 15 minutes. Propidium iodide (1 μL) was added to the cells, and they were analyzed immediately after staining.

### Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Cells transduced with BABE or BABE-vGPCR were allowed to grow to near confluency (90%). Total RNA was harvested from the cells using the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions following serum starvation in media supplemented with 0.5% FBS. Briefly, cells were lysed

on the tissue culture dish with RLT lysis buffer (supplemented with beta-mercaptoethanol) and collected from the plate by homogenization with a 25-gauge needle and syringe. RNA was precipitated with ethanol, bound to the column, washed, and eluted in 30 μL RNase-free water. RNA was DNase-treated with TURBO DNA-free Kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA was precipitated overnight at −20°C with absolute ethanol and 3 M sodium acetate and resuspended in 30 μL of RNase-free water the following day. Absorbance readings were taken for each sample on a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE).

The iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA) was used to synthesize cDNA according to the manufacturer's instructions, using 250-500 ng RNA per 20 μL reaction. The cDNA product (1 μL) was used in a subsequent real-time qRT-PCR reaction with BIO-RAD SYBR Green in a BIO-RAD iCycler. The sequences of the PCR primers (IDT, Inc, Coralville, IA) were Bcl-2 Forward, 5′-tcc gat cag gaa ggc tag agt t-3′; Bcl-2 Reverse, 5′-tcg gtc tcc taa aag cag gc-3′; Human 36B4 (h36B4) Forward, 5′-tgg aga cgg att aca cct tc-3′; h36B4 Reverse, 5′-ctt cct tgg cct ca acct tag-3′. Prior to performing real-time qRT-PCR on experimental samples, primer concentrations were optimized to provide equal priming efficiency (~100%) for each primer pair. Negative controls, including cDNA reactions without reverse transcriptase or RNA and PCR mixtures lacking cDNA, were included in each PCR. Following amplification, specificity of the reaction was confirmed by melt curve analysis. Relative quantitation was determined using the comparative $C_T$ method with data normalized to h36B4 and calibrated to the average $\Delta C_T$ of the indicated control sample.

## Western Blotting

Cells transduced with BABE or BABE-vGPCR were allowed to grow to near confluency (90%). Protein was extracted from the cells using RIPA buffer (25 mM TrisHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Sigma-Aldrich) supplemented with protease (Roche Chemicals, Indianapolis, IN) and protein phosphatase (Sigma-Aldrich) inhibitor cocktails following serum starvation in media supplemented with 0.5% FBS. Lysates were cleared by centrifugation (15,000 rpm, 4°C, 15 minutes) and transferred to a new tube. Protein concentration was assayed by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Proteins (20 μg total) were separated on a NuPAGE 4%-12% SDS-PAGE gel (Invitrogen) using MOPS running buffer under reducing conditions. Proteins were separated for 2 hours at 120 volts and then transferred to a PVDF Invitrolon membrane (Invitrogen) for 1.5 hours at 30 volts. Once the blot transfer was complete, it was blocked in 5% nonfat dry milk in 0.1% Tris-buffered saline with Tween (TBST) for 1 hour at room temperature. Blots were incubated in primary antibody overnight at 4°C on a rocker. Following the overnight incubation, blots were washed 3 times quickly with 0.1% TBST, once for 15 minutes and twice for 5 minutes (all on an orbital shaker). The blots were then incubated with HRP-conjugated secondary antibodies to the appropriate species (Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature and then washed as described above. A second wash with TBS for 15 minutes was added to the end of the washing steps to remove the Tween 20 from the blot. Bound antibody-protein complexes were detected with ECL Plus (Amersham Biosciences) according to the manufacturer's instructions. Primary antibodies used were anti-human Bcl-2 (Dako USA, Carpinteria, CA), anti-phospho-Akt (Ser473) and anti-Akt (Cell Signaling Technology, Beverly, MA), and anti-β-actin (abcam, Cambridge, MA). β-actin expression was used as a loading control.

## Small Interfering (siRNA) Transfection

HUVECs were transduced with either BABE or BABE-vGPCR at approximately 40%-60% confluency and incubated at 37°C until they were 90% confluent. Cells were then collected and transfected with siRNA (SMARTpool; Dharmacon, Lafayette, CO) targeting Bcl-2 messenger RNA (mRNA) (or scrambled RNA as a control) using the Neon Transfection System (Invitrogen) according to the manufacturer's instructions. Cells were plated in 2 mL media immediately following transfection. Twenty-four hours later, the media was changed and cells were serum starved for 24 hours. Cells were then collected and stained with Annexin-V-APC and propidium iodide and analyzed by FACS for apoptotic events and cell survival as described above.

## Inhibition Assays

HUVECs were transduced with either BABE or BABE-vGPCR at approximately 40%-60% confluency and incubated at 37°C until they were 90% confluent. Cells were serum starved during which time they were also treated with inhibitors LY294002 (50 μM; Cell Signaling Technology) or rapamycin (50 nM; Calbiochem, San Diego, CA) prepared in dimethyl sulfoxide (DMSO). Control cells were treated with an equal amount of DMSO. Following the serum starvation, cells were harvested for protein and/or mRNA analysis.

## Statistical Analysis

Statistical significance was determined by Student *t* test or 1-way analysis of variance followed by

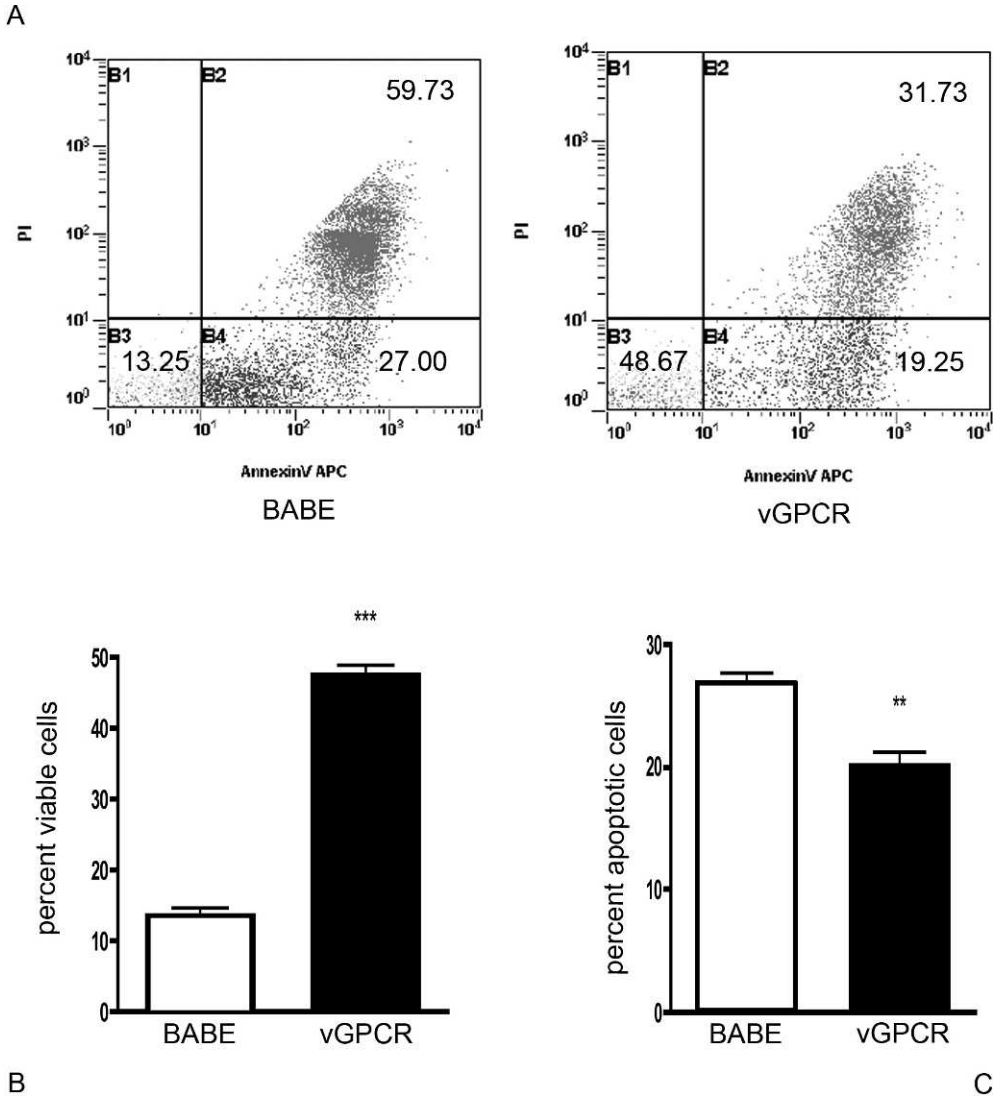


**Figure 1. vGPCR enhances cell survival in HUVECs. BABE and BABE-vGPCR transduced HUVECs were serum starved for 24 hours and then harvested and stained with Annexin-V-APC and propidium iodide for FACS analysis. (A) Representative histograms from BABE and BABE-vGPCR transduced HUVECs. (B) The percent of viable cells was determined by the number of cells negative for propidium iodide staining. (C) The percent of apoptotic cells was measured by immunostaining for Annexin-V on the cell surface. Data are presented as the mean of 3 independent experiments ± SEM (n=3) (**$P<0.001$, ***$P<0.0001$).** FACS, fluorescence-activated cell sorting; HUVECs, human umbilical vein endothelial cells; SEM, standard error of the mean; vGPCR, virally encoded G protein–coupled receptor.

the Tukey post hoc *t* test using GraphPad Prism v.4 software (GraphPad Software, La Jolla, CA). Data are presented as the mean ± standard error of the mean.

## RESULTS

### vGPCR Promotes Survival and Reduces Apoptosis in Endothelial Cells

KSHV-infected cells have been shown to be resistant to growth factor deprivation.[16] To determine whether expression of vGPCR alone conferred a survival advantage to endothelial cells during a period of serum starvation, primary HUVECs were transduced with a retrovirus expressing vGPCR and eGFP (BABE-vGPCR) or eGFP alone (BABE) and 2-3 days later were serum starved for 24 hours. Cell viability and apoptosis were assessed by FACS after propidium iodide staining that is excluded from viable cells and by immunostaining with Annexin-V-APC that is exposed on the surface of the cell membrane in apoptotic cells.

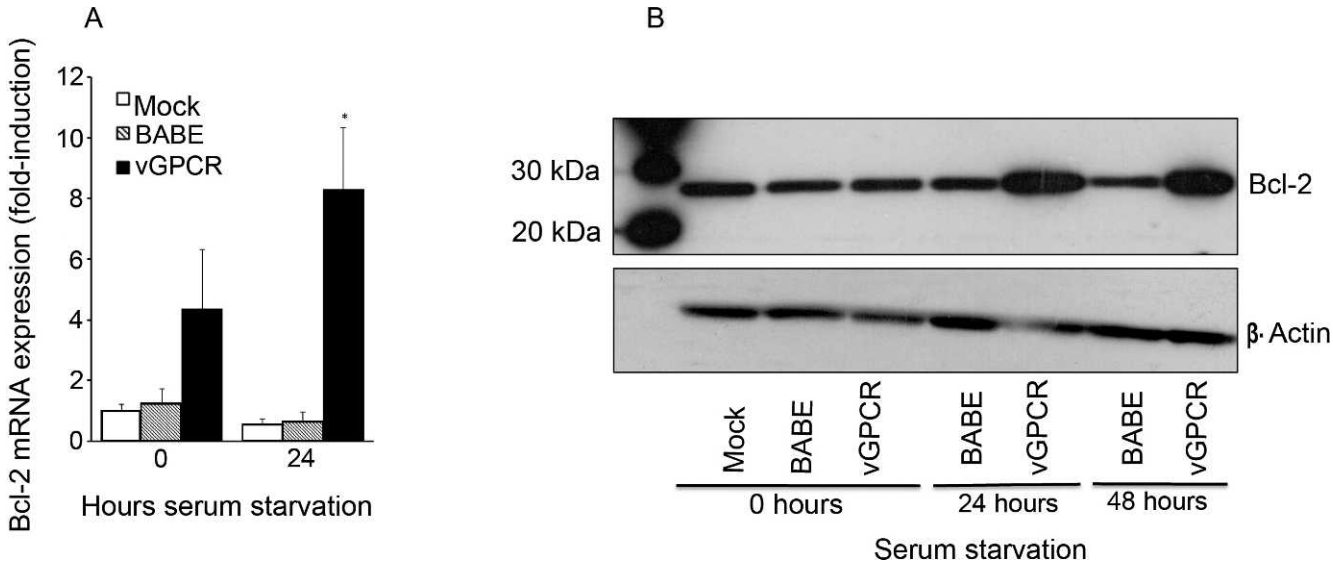


**Figure 2. vGPCR upregulates Bcl-2 mRNA and protein levels in endothelial cells. Mock, BABE, and BABE-vGPCR transduced HUVECs were serum starved for the indicated times. (A) Bcl-2 mRNA levels were measured by real-time qRT-PCR. Each sample was analyzed in triplicate and normalized to the level of 36B4 mRNA. The data are presented as fold change (mean ± SEM) relative to the mock-infected sample at 0 hours (*$P<0.05$). (B) Cell lysates were prepared and analyzed by Western blot using antibodies to Bcl-2 and β-actin as a loading control.** HUVECs, human umbilical vein endothelial cells; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SEM, standard error of the mean; vGPCR, virally encoded G protein–coupled receptor.

Significantly more viable cells were among the cells transduced with BABE-vGPCR (Figure 1B). Expression of vGPCR also correlated with a significant decrease in apoptosis (Figure 1C). These data demonstrate that expression of vGPCR alone confers a survival advantage to serum-starved endothelial cells.

## vGPCR Upregulates Bcl-2 mRNA and Protein Levels in Endothelial Cells

Bcl-2 is a key regulator of programmed cell death. Our next investigation was whether Bcl-2 levels were affected by vGPCR expression in endothelial cells. HUVECs were transduced with BABE-vGPCR or BABE and serum starved for 24 hours. Total cellular RNA and protein were collected for analysis of Bcl-2 mRNA by real-time qRT-PCR and protein levels by Western blot analyses, respectively. vGPCR-transduced cells showed significant upregulation of both Bcl-2 mRNA (Figure 2A) and protein (Figure 2B) compared to controls. These data clearly show that Bcl-2 expression is upregulated in endothelial cells expressing vGPCR.

## vGPCR-Induced Upregulation of Bcl-2 mRNA and Protein Levels in Endothelial Cells Depends on PI3K/Akt Signaling

Previous studies have shown that vGPCR induces the activation of the prosurvival PI3K/Akt pathway.[16] To determine whether vGPCR-induced Bcl-2 upregulation was dependent on PI3K/Akt signaling, vGPCR-expressing HUVECs were treated with the PI3K-specific inhibitor LY294002.[21] As demonstrated in Figure 3A, LY294002 completely blocked phosphorylation and thus activation of Akt in vGPCR-expressing cells. Furthermore, treatment with LY294002 decreased accumulation of Bcl-2 protein in vGPCR expressing cells (Figure 3B). This decrease coincided with an approximately 7-fold decrease in Bcl-2 mRNA levels in vGPCR-expressing cells treated with LY294002 (Figure 3C). These results suggest that PI3K/Akt signaling plays an important role in vGPCR-induced regulation of Bcl-2 and places Bcl-2 downstream of Akt in the PI3K signal transduction cascade.

Previous work demonstrated that vGPCR promotes activation of mammalian target of rapamycin (mTOR), and treatment with rapamycin prevents vGPCR-induced sarcomagenesis.[22] The authors showed that the observed activation of the mTOR pathway was a result of constitutive signaling of Akt in vGPCR-expressing cells.[22] Our results indicate that Akt is an important factor in vGPCR-induced regulation of Bcl-2. To assess whether vGPCR-mediated expression of Bcl-2 was also dependent on mTOR signaling, HUVECs that had been transduced with BABE-vGPCR were serum starved and treated with rapamycin for 24 hours. Bcl-2 mRNA levels were then measured by real-time qRT-PCR. The levels of Bcl-2

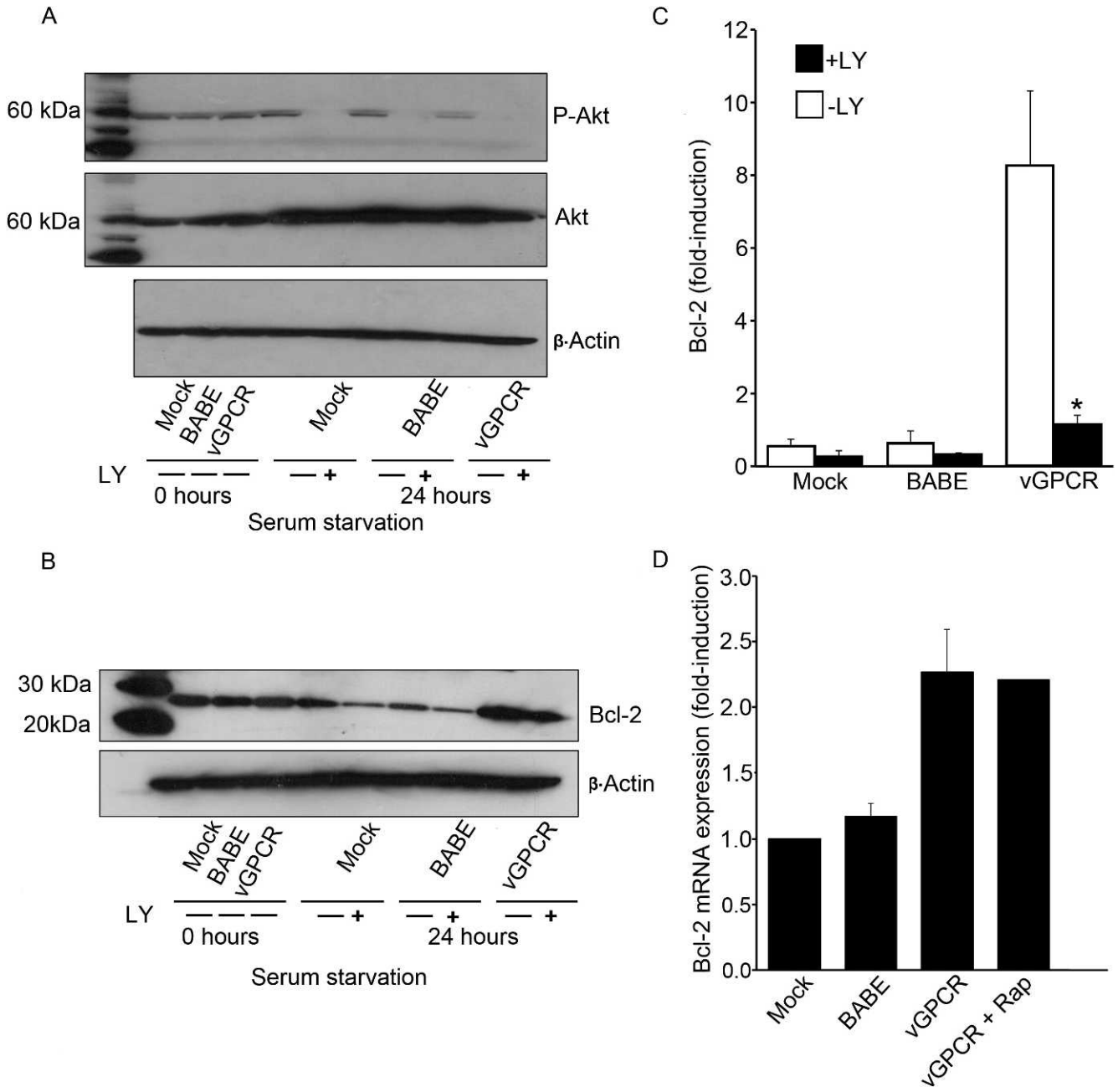


**Figure 3. vGPCR-induced upregulation of Bcl-2 mRNA and protein levels is dependent on PI3K/Akt in HUVECs. (A) Mock, BABE, and BABE-vGPCR transduced HUVECs were serum starved for 24 hours while being treated with PI3K/Akt inhibitor (+LY, 50 μM LY294002) or vehicle (-LY). Cell lysates were harvested at 0 and 24 hours following treatment and analyzed by Western blot for activation of Akt using the antibody to phospho-Akt (Ser473) (p-Akt). Blots were stripped and reprobed with antibodies to total Akt and β-actin was used as a loading control. (B) The same cell lysates were analyzed by Western blot for Bcl-2 expression and β-actin as loading control. (C) Mock, BABE, and BABE-vGPCR transduced HUVECs were serum starved for 24 hours while being treated with PI3K/Akt inhibitor (+LY, 50 μM LY294002) or vehicle (-LY). Total RNA was extracted and Bcl-2 mRNA levels were measured by real-time qRT-PCR following treatment. Each sample was analyzed in triplicate and normalized to the level of 36B4 mRNA. The data are presented as fold change (mean ± SEM) relative to the mock-infected sample without inhibitor. (*$P<0.05$). (D) Mock, BABE, and BABE-vGPCR transduced HUVECs were treated with 50 nM rapamycin (Rap) or vehicle under serum-starvation conditions for 24 hours. Total RNA was extracted and Bcl-2 mRNA levels were measured by real-time qRT-PCR following treatment. Each sample was analyzed in triplicate and normalized to the level of 36B4 mRNA. The data are presented as fold change (mean ± SEM) relative to the mock-infected sample without inhibitor.** HUVECs, human umbilical vein endothelial cells; mRNA, messenger RNA; PI3K, phosphatidylinositol 3-kinase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SEM, standard error of the mean; vGPCR, virally encoded G protein–coupled receptor.

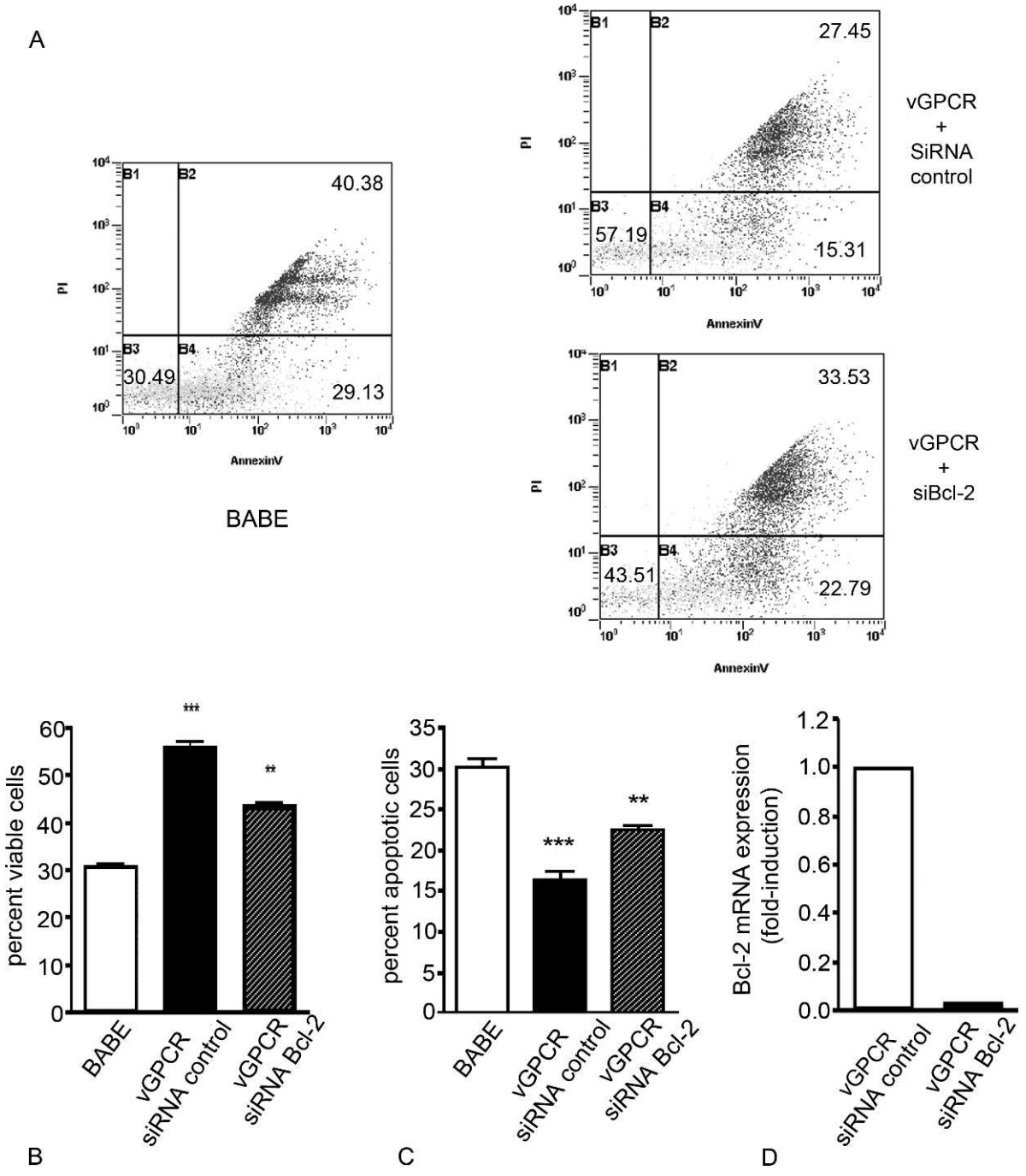


**Figure 4. vGPCR-enhanced cell survival in HUVECs is dependent, in part, on Bcl-2. BABE-vGPCR transduced HUVECs were transfected with siRNA targeting Bcl-2 or a scrambled siRNA as control and then serum starved for 24 hours. Cells were then harvested and stained with Annexin-V-APC and propidium iodide for FACS analysis. (A) Representative histograms from HUVECs treated with BABE, BABE-vGPCR + control siRNA, and BABE-vGPCR + Bcl-2–targeted siRNA. (B) The percent of viable cells was determined by the number of cells negative for propidium iodide staining. (C) The percent of apoptotic cells was measured by immunostaining with Anexin-V on the cell surface. (D) Knockdown of Bcl-2 mRNA in BABE-vGPCR transduced HUVECs transfected with Bcl-2–targeted siRNA was confirmed by real-time qRT-PCR. Each sample was analyzed in triplicate and normalized to the level of 36B4 mRNA. The data are presented as fold change (mean ± SEM) relative to cells transfected with control scrambled siRNA. Data are presented as the mean of 3 independent experiments ± SEM (n=3) (**$P<0.001$, ***$P<0.0001$).** HUVECs, human umbilical vein endothelial cells; mRNA, messenger RNA; PI3K, phosphatidylinositol 3-kinase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SEM, standard error of the mean; siRNA, small interfering RNA; vGPCR, virally encoded G protein–coupled receptor.

mRNA did not change with rapamycin treatment (Figure 3D). These data indicate that vGPCR-induction of Bcl-2 expression is independent of the mTOR pathway, albeit still downstream of Akt.

### vGPCR-Induced Enhanced Survival of Endothelial Cells Is Partially Dependent on Bcl-2

To determine the role of Bcl-2 in vGPCR-mediated survival advantage, RNA interference was used to knock down Bcl-2 in endothelial cells. HUVECs were transduced with BABE-vGPCR retroviral vector or vector alone and then subsequently transfected with siRNA targeting Bcl-2 mRNA. Twenty-four hours after transfection with siRNA, cells were serum starved for 24 hours. Cells were then harvested, and cell viability and apoptosis were analyzed by FACS. There was a statistically significant decrease in vGPCR-mediated survival in cells transfected with siRNA to Bcl-2 (Figures 4A and 4B). Consistent with these results, knockdown of Bcl-2 resulted in a significant increase in apoptosis (Figures 4A and 4C). RNA interference-mediated knockdown of Bcl-2 mRNA was confirmed by real-time qRT-PCR (Figure 4D). These data implicate Bcl-2 as a mediator of the vGPCR-induced survival advantage; however, they also suggest that other prosurvival mechanisms are involved in the process since complete abrogation of the survival advantage was not achieved with the knockdown of Bcl-2 alone.

## DISCUSSION

KSHV has been identified as the etiological agent of KS; however, the molecular pathogenesis of this virus remains poorly understood. Evidence suggests that expression of vGPCR is necessary and sufficient to induce endothelial cell transformation and the angiogenic phenotype characteristic of KS.[7,12] Cell survival is an essential component of the angiogenic and tumorigenic process. Results from this study clearly show that vGPCR protects endothelial cells from serum starvation–induced apoptosis, thus providing them with a survival advantage. We also observed a significant upregulation of Bcl-2 expression in endothelial cells expressing vGPCR that was dependent on PI3K/Akt signaling. Inhibition of Bcl-2 expression by RNA interference significantly abrogated the survival advantage mediated by vGPCR, suggesting an important role for Bcl-2 in sarcomagenesis induced by KSHV.

The Bcl-2 gene has been implicated in a number of cancers, including B- and T-cell lymphomas and cervical, lung, breast, prostate, and colorectal cancers.[23-28] The Bcl-2 gene is also thought to be involved in resistance to conventional cancer treatment.[29,30] We previously reported that Bcl-2 is expressed within KS lesions and its expression increases as the pathological stage of KS advances.[19] In agreement, Long et al reported that the levels of Bcl-2 were higher in visceral KS lesions compared to levels found in cutaneous and mucosal KS.[31] Bcl-2 suppresses the intrinsic pathway of apoptosis by binding proapoptotic Bcl-2 family members.[32] Therefore increased expression of Bcl-2 provides tumor cells with a survival advantage under conditions that would otherwise lead to cell death. Currently, small molecule inhibitors of Bcl-2, such as ABT,[33] are under development for the treatment of malignancies dependent on Bcl-2.[34,35]

Recent evidence suggests that activation of the PI3K/Akt pathway can lead to upregulation of Bcl-2 expression via activation of the transcription factors NF-κB[36] (nuclear factor kappa B) and CREBP (cAMP responsive element binding protein).[37] The PI3K/Akt pathway is strongly activated in endothelial cells expressing vGPCR (Figure 3A), promotes the protection of endothelial cells from apoptosis, and represents one of the key mechanisms in vGPCR oncogenesis.[15,22] Akt activation is a molecular hallmark of KS, further supporting a role for this pathway in sarcomagenesis induced by KSHV.[15] Here we show that vGPCR upregulation of Bcl-2 expression is dependent on PI3K/Akt signaling. The precise mechanism by which this pathway upregulates Bcl-2 remains to be determined. In preliminary studies, inhibition of NF-κB using the specific inhibitor BAY completely blocked vGPCR induction of Bcl-2 expression (data not shown). A recent study by Gao et al[38] suggests another mechanism by which Bcl-2 may be upregulated in KSHV-infected cells. The replication and transcription activator encoded by KSHV ORF50, a critical regulator for the shift of the KSHV replication cycle from latency to lytic activation, directly transactivates the cellular Bcl-2 promoter.[38] Thus, upregulation of Bcl-2 within KS lesions may enhance viral replication by prolonging the life of lytically infected endothelial cells and render them more susceptible to proliferative effects of inflammatory cytokines, another molecular hallmark of KS.

Emerging evidence suggests that mTOR is a key effector of Akt signaling in KS development.[22] Akt promotes activation of mTOR through inactivation of the tuberous sclerosis complex.[39] Rapamycin, a potent inhibitor of mTOR, has proven to be a very effective treatment of iatrogenic KS and has previously been reported to affect the levels of proteins of the Bcl-2 family in different tumor cells.[40] In this study, rapamycin treatment of vGPCR-expressing cells failed to block upregulation of Bcl-2 expression (Figure 3D). Therefore we postulate that vGPCR activation of Akt

can lead to endothelial cell survival through multiple mechanisms. Indeed, knockdown of Bcl-2 by RNA interference only partially abrogated the survival advantage imparted by vGPCR expression. Employing the Human Apoptosis $RT^2$ Profiler PCR Array (SABiosciences, Frederick, MD), we identified two additional antiapoptotic genes, Bcl-2A1 and BIRC3, that are significantly upregulated in vGPCR expressing cells (data not shown). More work is required to determine the contribution of these factors to vGPCR-enhanced cell survival and oncogenesis.

## CONCLUSIONS

We have identified Bcl-2 as a downstream effector of PI3K/Akt signaling leading to enhanced cell survival in endothelial cells expressing vGPCR. These results suggest a mechanism by which vGPCR promotes oncogenesis and increases our understanding of the molecular pathogenesis of KSHV. Moreover, these results could lead to new treatment strategies for treatment of KS.

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