Epstein-Barr Virus-Encoded Latent Membrane Protein 2A Downregulates GCNT3 via the TGF- β 1/Smad-mTORC1 Signaling Axis

Juanjuan Liu,^a Yan Zhang,^{a,b} Caixia Yu,^a Jun Li,^a Wen Liu,^a Bing Luo^a

^aDepartment of Pathogenic Biology, School of Basic Medicine, Qingdao University, Qingdao, China

^bDepartment of Clinical Laboratory, Central Hospital of Zibo, Zibo, China

ABSTRACT Increasing evidence shows that Epstein-Barr virus (EBV) infection is closely related to various lymphoid and epithelioid malignancies. However, the underlying mechanisms are unclear. GCNT3 (core 2 β -1,6-acetylglucosaminyltransferase) is a new type of core mucin synthase, and its expression in EBV-associated gastric cancer (EBVaGC) is lower than that in EBV-negative gastric cancer (EBVnGC). EBV-encoded latent membrane protein 2A (LMP2A) is a transmembrane protein with tumorigenic transformation properties. Here, we demonstrated that LMP2A inhibited the transcription of GCNT3 by inhibiting Smad2/3 and Smad4. LMP2A restrained the activation of the mTORC1 pathway by inactivating the transforming growth factor β 1 (TGF- β 1)/Smad pathway and then downregulated GCNT3 expression. The mTORC1-GCNT3 pathway promoted cell proliferation and migration and inhibited G₀/G₁ cell arrest. Related proteins involved in epithelial-mesenchymal transition (EMT) were downstream molecules of the TGF- β 1/Smad-mTORC1-GCNT3 pathway. GCNT3 inhibited autophagy by inducing mTORC1 phosphorylation. These findings indicate that targeting the TGF- β 1/Smad-mTORC1-GCNT3 axis may represent a novel therapeutic target in gastric cancer (GC).

IMPORTANCE Epstein-Barr virus (EBV) is an opportunistic pathogen, and the latent membrane protein 2A (LMP2A) encoded by EBV plays a key role in ensuring the incubation period of EBV. Glycosylation modification is an important marker of cancer cells, and recent studies have reported that it is related to EBV. Our conclusions provide deeper theoretical support for the role of LMP2A and transforming growth factor (TGF)/Smad-mTORC1-GCNT3 in EBV-associated gastric cancer (EBVaGC) and help us to understand glycosylation abnormalities in cancer. Our results may provide novel therapeutic targets for the treatment of gastric cancer against the TGF/Smad-mTORC1-GCNT3 signaling cascade.

KEYWORDS Epstein-Barr virus, TGF/Smad, core 2 β -1,6-acetylglucosaminyltransferase, gastric carcinoma, latent membrane protein 2A, mTORC1 pathway

Epstein-Barr virus (EBV) is an opportunistic pathogen that appears in human diseases after many years of primary infection and is the pathogen of infectious mononucleosis. EBV infection is closely related to various lymphoid and epithelioid malignancies (1). EBV-related cancers include Burkitt's lymphoma, Hodgkin's lymphoma, posttransplant lymphoproliferative disease, non-Hodgkin's lymphoma, nasopharyngeal cancer (NPC), and gastric cancer (GC) (2). In these cells, the EBV genome expresses nine latent proteins encoded by EBV, six of which are expressed in the nucleus (EBV nuclear antigen), three of which are expressed in the membrane (latent membrane protein 1 [LMP1], LMP2A, and LMP2B), and 2 small virus RNAs (1, 3). Increasing evidence suggests that EBV is associated with other epithelioid malignancies, such as gastric cancer (4–6). LMP2A is a transmembrane protein with potential for transformation in epithelial

Citation Liu J, Zhang Y, Yu C, Li J, Liu W, Luo B. 2021. Epstein-Barr virus-encoded latent membrane protein 2A downregulates GCNT3 via the TGF- β 1/Smad-mTORC1 signaling axis. *J Virol* 95:e02481-20. <https://doi.org/10.1128/JVI.02481-20>.

Editor Jae U. Jung, Lerner Research Institute, Cleveland Clinic

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Bing Luo, qdluobing@163.com.

Received 4 January 2021

Accepted 17 February 2021

Accepted manuscript posted online 3 March 2021

Published 26 April 2021

cells and is expressed in several EBV-related malignancies (7). LMP2A plays a key role in ensuring the latent period of EBV and may represent an important risk factor for EBV-related diseases. The emerging importance of LMP2A in epithelial cells has led us to study the effects of LMP2A on signal transduction in gastric cancer.

O-glycan synthase glucosamine (*N*-acetyl) transferase 3 (GCNT3/GNTM/C24GNT/C2GNT2/C2GNTM/C2/4GnT) is a mucin-type synthase responsible for catalyzing core 2 and core 4 O-glycans and forming O-linked glycosylation in protein biosynthesis (8). Previous studies have found that GCNT3 plays an important role in the occurrence and development of colon cancer, pancreatic cancer, and ovarian cancer (9–11). GCNT3 was highly expressed in non-small cell lung cancer (NSCLC) tissues and cell lines, and high expression was significantly associated with advanced tumor lymph node metastasis (TNM) stage, positive lymph node metastasis, and poor overall survival (12). Our previous research found lower GCNT3 expression in EBV-associated gastric cancer (EBVaGC) cells and tissues compared with EBV-negative gastric cancer (EBVnGC), whereas high expression was significantly associated with advanced tumor-lymph node metastasis (13). However, a consensus on the mechanism by which EBV latent genes regulate GCNT3 is lacking.

The mTOR pathway is often activated in cancer, participates in immunosuppression, affects transcription and protein synthesis, regulates cell growth, apoptosis, and autophagy, and affects cancer cell growth and metabolism. Activated mTOR phosphorylates downstream effector eukaryotic initiation factor 4e (eIF4e) binding protein (4E-BP1) and p70 S6 kinase (S6K), which are key components in the pathway that viruses regularly select to ensure their own mRNA translation (14–16). In NPC, the mTOR signaling pathway is a downstream component of the LMP2A-activated PI3-kinase/Akt pathway, which may support the EBV-driven cell expansion process (17). However, there is no clear research on EBVaGC demonstrating that LMP2A regulates mTOR signaling. 4E-BP and eIF4G share the common binding site of eIF4E, leading to impaired assembly of the eIF4F complex (18, 19). mTORC1 phosphorylates 4E-BP, resulting in separation from eIF4E, allowing the formation of eIF4F and the translation process (20, 21).

In recent years, studies have demonstrated that the mTOR pathway is affected by transforming growth factor β (TGF- β) (22, 23). TGF- β is a multifunctional cytokine that plays an important role in regulating cell growth and differentiation (24). Gastric cancer cells express many growth factors, gastrointestinal hormones, and cytokines, which may enhance the growth of these tumor cells through potential autocrine and paracrine pathways (25). For example, elevated levels of TGF- β have been reported in patients with gastric cancer (26, 27). TGF- β plays different roles in a variety of cells. TGF- β induces epithelial-mesenchymal transition (EMT) through the activated form of T β RI or by increasing the expression of Smad2, Smad3, and Smad4 (28, 29). TGF- β promotes the proliferation of mesenchymal cell types, such as the proliferation of renal fibroblasts and smooth muscle cells (30, 31). TGF- β induces or inhibits programmed cell death in different cell types (32). In addition, TGF- β induces cell arrest in almost all nontumor epithelial and endothelial cells, hematopoietic cells, and certain types of mesenchymal cells (33). In recent years, some studies have demonstrated that TGF- β plays a role in mediating cell dormancy (34) and autophagy (35). Cell dormancy and autophagy are biological processes that regulate cell survival and are closely related to tumor progression. In this paper, we present a novel mechanistic insight into the TGF- β 1/Smad-mTORC1-GCNT3 interplay in GC cells.

RESULTS

LMP2A inhibited GCNT3 expression and mTORC1 pathway activation. Based on our previous experiments, we observed lower GCNT3 protein expression in EBVaGC cells and tissues compared with EBVnGC, and high expression is closely related to lymph node metastasis (13). SGC7901 cells with strong GCNT3 expression were selected to transfect LMP2A (SGC7901 cells with pcDNA3.1-LMP2A plasmids [SGC7901-

FIG 1 LMP2A inhibited GCNT3 expression and mTORC1 pathway activation. (A) Microscopic fluorescence showed that the NC and LMP2A plasmids were successfully transfected into the cells. (B) GCNT3 expression in SGC7901-LMP2A cells was downregulated compared to that in SGC7901-NC cells. β -actin was used as a loading control. (C) LMP2A was transiently transfected into SGC7901 cells to study the time-dependent effect of LMP2A on GCNT3 inhibition. (D) After LMP2A was knocked down in GT38, Western blotting showed that GCNT3 expression was upregulated. (E) The mTORC1 signaling pathway was inhibited in EBVaGC cells compared with EBVnGC cells. (F) SGC7901-NC and SGC7901-LMP2A cells were analyzed using antibodies against various proteins involved in mTORC1 signaling. (G) SGC7901 cells were transfected with 3 μ g plasmid for 48 and 72 h and then examined by Western blotting to analyze whether the mTORC1 signaling pathway was inhibited. (H) After knocking down LMP2A in GT38 cells, the mTORC1 signaling pathway was activated. Similar results were obtained in two other independent experiments, and the mean and SD of all three independent experiments were plotted. Error bars indicate SD ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

LMP2A) by Geneticin screening. Microscopic fluorescence showed that the plasmids were successfully transfected into the cells (Fig. 1A). As shown in Fig. 1B, compared to SGC7901 cells with pcDNA3.1 expression vector (SGC7901-NC) (transfection control plasmid), GCNT3 expression in SGC7901-LMP2A cells stably expressing LMP2A was significantly suppressed. LMP2A transiently transfected into SGC7901 cells was used to further study the time-dependent effect of LMP2A on GCNT3 inhibition, and GCNT3 was significantly inhibited at 48 and 72 h after LMP2A transfection (Fig. 1C). GCNT3 was significantly increased after knockdown of LMP2A expression in GT38 EBV-infected cells (Fig. 1D). These findings confirmed the involvement of LMP2A in the inhibition of GCNT3 expression.

The mammalian rapamycin target protein (mTORC1) pathway is the most important energy sensor in the cell and regulates downstream signals to affect cell metabolism, cell cycle progression, and cell growth. Previous reports have confirmed that LMP2A

FIG 2 LMP2A downregulated GCNT3 expression via mTORC1 signaling. (A and B) GT38 cells and SGC7901 cells were treated with MHY1485 or rapamycin for 24 and 36 h and then analyzed by Western blotting for the expression of GCNT3 and mTORC1-signaling components. (C) GT38 cells and SGC7901 cells were treated with MHY1485 or rapamycin for 48 h and then analyzed by Western blotting. (D) GT38 and SGC7901 cells were treated with MHY1485 or rapamycin for 36 h and then analyzed by immunofluorescence for the expression of GCNT3. The relative intensity of GCNT3 fluorescence was measured with ImageJ. (E) SGC7901-LMP2A cells were treated with MHY1485 for 36 h, and the expression of GCNT3 and mTORC1-signaling components was analyzed. (F) GCNT3 protein levels after knockdown of Raptor and mTOR for 48 h (50 nM) in BGC823 and SGC7901 cells. Similar results were obtained in two other independent experiments, and the mean and SD of all three independent experiments were plotted. Error bars indicate SD ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

regulates the mTOR pathway in NPC (17). We investigated the relationship between LMP2A and mTORC1 signaling in gastric cancer. As shown in Fig. 1E, inhibition of mTORC1 signaling was clearly detected in EBVaGC cells rather than EBVnGC as indicated by the decreased phosphorylation of mTOR at Ser²⁴⁴⁸, p-p70s6K (Thr³⁸⁹), and p-4EBP1 (Thr⁷⁰), which are downstream targets of mTORC1. mTORC1 signaling was clearly inhibited in SGC7901 cells stably expressing LMP2A by the decreased phosphorylation of mTOR (Ser²⁴⁴⁸), p70s6K (Thr³⁸⁹), and 4EBP1 (Thr⁷⁰) (Fig. 1F). When LMP2A was transiently transfected into SGC7901 cells, LMP2A also inhibited the phosphorylation of mTOR (Ser²⁴⁴⁸), p70s6K (Thr³⁸⁹), and 4EBP1 (Thr⁷⁰) at 48 and 72 h (Fig. 1G). In EBV-infected epithelial cells, mTORC1 signaling was activated after LMP2A was knocked down with small interfering RNA (siRNA) (Fig. 1H). The above-described results confirmed that LMP2A inhibited mTORC1 pathway activation in GC.

LMP2A downregulated GCNT3 expression via mTORC1 signaling. Experiments were conducted to verify whether the mTOR pathway regulated GCNT3 in GC. Effective concentrations of 2 μM MHY1485 (mTOR activator) and 100 nM rapamycin (mTOR inhibitor) were selected. As shown in Fig. 2A, GCNT3 expression was significantly increased after MHY1485 was added to GT38 cells for 24 and 36 h. Rapamycin effectively inhibited mTORC1 pathway as well as GCNT3 expression in SGC7901 cells (Fig. 2B). When we extended the action time to 48 h, GCNT3 expression was positively correlated with mTORC1 signaling activation (Fig. 2C). Immunofluorescence was used to further verify that the mTORC1 pathway is a key factor in regulating GCNT3 expression (Fig. 2D). At the same exposure time intensity, GT38 with MHY1485 exhibited stronger fluorescence intensities of GCNT3; SGC7901 with rapamycin exhibited weaker fluorescence intensities of GCNT3. The inhibitory effect of LMP2A on GCNT3 expression was reversed after the addition of MHY1485 (Fig. 2E). mTOR is the catalytic subunit of the mTORC1 complex, and Raptor is a protein related to mTOR regulation, which

FIG 3 The 4EBP1-eIF4E complex interacts with GCNT3 to maintain GCNT3 stability. (A) eIF4E protein levels were inhibited in EBVaGC cells compared with EBVnGC cells. (B) Western blot analysis of eIF4E in SGC7901-LMP2A cells and in GT38 cells transfected with LMP2A-siRNA for 48 h. (C) GCNT3 protein levels after knockdown of eIF4E for 48 h (50 nM) in BGC823 and SGC7901 cells. (D) Coimmunoprecipitation in SGC7901 cells followed by Western blotting showed that GCNT3 interacted with eIF4E and 4E-BP1. (E) Coimmunoprecipitation and Western blot analysis showed that LMP2A inhibited the interaction between GCNT3 and eIF4E in SGC7901 cells. (F) Comparison of GCNT3 degradation in SGC7901 cells infected with siNC or siEIF4E and then treated with control (DMSO) or 10 μ g/ml CHX in a time series. (G) Analysis of panel F in triplicate. (H) Comparison of GCNT3 degradation transfected with siNC or siEIF4E in SGC7901 cells and without or with treatment with MG132 (20 μ M). All experiments were performed thrice. Error bars indicate SD ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

combines with the TOR signal motif to promote the recruitment of the mTORC1 substrate (36). Decreased GCNT3 levels were observed following Raptor or mTOR inhibition (Fig. 2F). The above-described results indicated that GCNT3 was a downstream target of the mTORC1 pathway and that LMP2A inhibited GCNT3 expression by inhibiting mTORC1 pathway activation.

The 4EBP1-eIF4E complex interacted with GCNT3 to maintain GCNT3 stability.

GCNT3 was a downstream target of the mTORC1 pathway, and we investigated whether eIF4E regulates GCNT3 in GC. As shown in Fig. 3A, eIF4E expression was reduced in EBVaGC cells compared with EBVnGC cells. LMP2A suppressed eIF4E expression (Fig. 3B). Furthermore, we observed decreased GCNT3 expression following eIF4E inhibition in BGC823 and SGC7901 cells (Fig. 3C). These findings confirmed that LMP2A inhibited GCNT3 expression by inhibiting eIF4E. The SGC7901 cell extract was immunoprecipitated with antibodies against eIF4E, 4EBP1, or GCNT3, and then Western blotting was performed. Coimmunoprecipitation experiments showed that eIF4E, 4EBP1, and GCNT3 formed a complex in the cell (Fig. 3D). LMP2A inhibited the interaction between eIF4E and GCNT3, and complex formation was impaired (Fig. 3E). These data indicate that LMP2A inhibits the formation of the eIF4E-GCNT3 complex and GCNT3 expression by inhibiting eIF4E.

We hypothesized that eIF4E may regulate the protein turnover of GCNT3 in GC. To

FIG 4 LMP2A-inhibited TGF- β 1/Smad signaling was dependent on NF- κ B signaling. (A) Various proteins involved in TGF- β 1/Smad signaling were analyzed by Western blotting in SGC7901-NC and SGC7901-LMP2A cells. (B) The EBV-infected GC cell line GT38 was transfected with siLMP2A and siNC, and the expression of TGF- β 1/Smad components was analyzed. (C) SGC7901-LMP2A cells were treated with BAY11-7082 for 24 h, and cell lysates were examined using antibodies against proteins involved in TGF- β 1/Smad pathways. (D and E) SGC7901 cells were treated with BAY11-7082 for 24 h and analyzed by immunofluorescence for the expression of TGF- β 1/Smad components. The relative intensity was measured by ImageJ. All experiments were performed thrice.

investigate whether eIF4E affects the stability of GCNT3 protein, we detected GCNT3 protein levels in the presence of cycloheximide (CHX), a protein biosynthesis inhibitor. GCNT3 degradation increased with the extension of CHX treatment time; when eIF4E was knocked out, the degradation rate increased (Fig. 3F and G). Further studies showed that in SGC7901 cells treated with the proteasome inhibitor MG132, knock-down of eIF4E could not induce GCNT3 degradation (Fig. 3H). This finding reflected the participation of the ubiquitination degradation pathway. Our results demonstrate that eIF4E binds to GCNT3 and maintains its stability to participate in the degradation of GCNT3, and LMP2A inhibits the above-described effects. In summary, these results provide evidence for LMP2A-eIF4E to regulate GCNT3 protein stability in GC.

LMP2A-inhibited TGF- β 1/Smad signaling was dependent on NF- κ B signaling.

Cross talk of the TGF- β /Smad and mTORC1 signaling pathways has been reported (37). Our previous experiments demonstrated that LMP2A inhibited Smad2 expression via the NF- κ B/miR-155a axis (38). As shown in Fig. 4A, LMP2A not only inhibited the expression of Smad2, Smad3, and Smad4 but also inhibited the expression of p-Smad2 and p-Smad3. In addition, after transfection with LMP2A siRNA, the expression of the above-described genes was upregulated in GT38 EBV-infected epithelial cells (Fig. 4B). These results indicated that LMP2A inhibits the TGF- β 1/Smad pathway activation in GC. LMP2A activates NF- κ B signaling to mediate a variety of malignant phenotypes, thereby promoting tumorigenesis (39, 40). NF- κ B signaling upregulates miR-146a and miR-155a in GC, and miR-146a and miR-155a target Smad4 and Smad2, respectively (38, 41). We hypothesized that LMP2A activated NF- κ B signaling to upregulate microRNAs (miRNAs), thereby inhibiting the expression of Smad family proteins. The

NF- κ B signal inhibitor BAY11-7082 reversed the inhibitory effect of LMP2A on the TGF- β 1/Smad pathway (Fig. 4C). Immunofluorescence showed that the fluorescence intensity of the above-described genes increased in SGC7901 cells after treatment with BAY11-7082 and that the signal in the nucleus was stronger (Fig. 4D and E). Based on the above-described results, we hypothesized that LMP2A activated NF- κ B signaling to mediate its downstream signaling and ultimately inhibited the TGF- β 1/Smad pathway.

The LMP2A/TGF- β 1/Smad axis inhibited the mTORC1 pathway and downregulated GCNT3 transcription. TGF- β 1/Smad and mTORC1 signaling mediates a variety of malignant phenotypes, thereby affecting tumorigenesis. Since LMP2A inhibited the TGF- β 1/Smad and mTORC1 signaling pathways in GC, we hypothesized that a functional interaction exists between these two signaling pathways. Western blots showed that TGF- β 1 activated the mTORC1 signal and upregulated GCNT3 expression (Fig. 5A). SB431542, a specific inhibitor of TGF- β receptor I (TGF- β RI), inhibited Smad2 and Smad3 phosphorylation. As shown in Fig. 5B, SB431542 (10 μ M) completely prevented TGF- β 1-mediated phosphorylation of mTOR, p70S6K, and 4EBP1 and blocked activation of the mTORC1 signal. TGF- β 1 reversed the inhibition of LMP2A on the mTORC1 signaling (Fig. 5C). Next, the specific mechanism by which TGF- β 1/Smad regulates mTORC1 signaling was verified. In SGC7901 and BGC823 cells, siRNA-mediated knockdown of Smad2 and 3 or Smad4 only inhibited the phosphorylation of mTOR and p70S6K induced by TGF- β 1 (Fig. 5D and E). Interestingly, immunofluorescence indicated that knockdown of Smad2/3 or Smad4 in SGC7901 cells inhibited the phosphorylation of mTOR and p70S6K, and the signal in the nucleus was stronger than that in the cytoplasm (Fig. 5F). The expression of Smad2/3 or 4 was increased by transfecting plasmids overexpressing Smad2/3 or 4, which reversed the inhibitory effect of LMP2A on mTOR and p70S6K phosphorylation (Fig. 5G). These results demonstrated that LMP2A inhibited the phosphorylation of mTOR and p70S6K by inhibiting the expression of Smad2/3/4. Coimmunoprecipitation experiments showed that Smad2/3 or Smad4 interacted with mTOR, and LMP2A might inhibit the phosphorylation of mTOR by inhibiting this binding. It is worth noting that LMP2A inhibited the binding of Smad2/3 and Smad4, impairing complex formation and thereby inhibiting the TGF- β 1/Smad pathway (Fig. 5H).

Smad2/3/4 are important molecules in the TGF- β 1/Smad pathway and act as important transcription factors to activate target gene transcription. We assessed whether GCNT3 is the direct target gene of Smad2/3/4. siRNA-mediated knockdown of Smad2/3 or Smad4 inhibited GCNT3 mRNA expression in SGC7901 cells. In addition, LMP2A inhibited GCNT3 mRNA expression; overexpressing Smad2/3 or 4 reversed the inhibitory effect of LMP2A on GCNT3 mRNA level (Fig. 5I). We further used the prediction software JASPAR to detect the promoter region of GCNT3 and found one of the most conserved putative Smad2/3/4 binding sites located at -1719 to -1707 bp in the promoter region of GCNT3 (Fig. 5J). To further confirm the direct binding of Smad2/3/4 to the consensus sequence of the GCNT3 promoter, we performed chromatin immunoprecipitation (ChIP) analysis using antibodies to pull down the components bound by Smad2/3 or Smad4. ChIP analysis revealed a significantly increased binding of Smad2/3 to the putative binding region of the GCNT3 promoter in cells with high expression of Smad2/3 compared with control cells; we obtained the same conclusion using Smad4 antibodies to pull down the Smad4 binding components (Fig. 5K). In summary, GCNT3 was a direct target gene of Smad2/3 or Smad4, and LMP2A downregulated GCNT3 transcription by inhibiting Smad2/3 or Smad4.

GCNT3 mediated autophagy and malignant properties in gastric cancer cells.

To further study the biological function of GCNT3 in GC, we reduced the expression of endogenous GCNT3 in SGC7901 cells by siRNA transfection. The proliferation ability of SGC7901 cells was significantly inhibited when GCNT3 was inhibited, and the inhibitory effect was weakened by MHY1485 (Fig. 6A). The siRNA target sequence to GCNT3 mRNA (siGCNT3) significantly inhibited migration and induced G₀/G₁ cell cycle arrest after transfection in SGC7901 cells, and MHY1485 reversed the reduction in migration and G₀/G₁ cell cycle arrest (Fig. 6B and D and E). CCND1 expression decreased after

FIG 5 The LMP2A/TGF-β1/Smad axis inhibited the mTORC1 pathway and downregulated GCNT3 transcription. For panes A to E, Cells were treated with TGF-β1 (10 ng/ml) for 36 h. (A) GCNT3 and mTORC1 pathway proteins were analyzed by Western blotting after GT38 cells and SGC7901 cells were treated with TGF-β1. (B) SB431542 (10 μM) was applied 3 h before the addition of TGF-β1, and Western blotting for TGF-β1/Smad and mTORC1 components was performed. (C) After SGC7901-LMP2A cells were treated with TGF-β1, cell lysates were examined using antibodies against proteins involved in GCNT3 and mTORC1 signaling. (D and E) SGC7901 and BGC823 cells were transfected for 48 h with a mixture of siSmad2 (50 nM) and siSmad3 (50 nM) or only siSmad4 (50 nM). Proteins involved in TGF-β1/Smad pathways and mTORC1 pathways were assessed by Western blot analysis. (F) SGC7901 cells were transfected with a mixture of siSmad2 (50 nM) and siSmad3 (50 nM) or only siSmad4 (50 nM) and analyzed by immunofluorescence for the expression of p-p70s6K and p-mTOR. (G) SGC7901-LMP2A cells were infected with a mixture of Smad2 and Smad3 or only Smad4 plasmids for 48 h, and cell lysates were analyzed by Western blotting for the expression of Smad2/3/4, p-p70s6K, and p-mTOR. (H) SGC7901 lysates were detected in coimmunoprecipitations using antibodies against Smad2, Smad3, Smad4, and mTOR. IgG antibodies were used as a negative control. (I) GCNT3 mRNA expression was downregulated in SGC7901 cells transfected with a mixture of siSmad2 (50 nM) and siSmad3 (50 nM) or only siSmad4 (50 nM). LMP2A inhibited GCNT3 mRNA expression. GCNT3 mRNA expression was upregulated when SGC7901-LMP2A cells were infected with a mixture of Smad2 and Smad3 or only Smad4 plasmids for 48 h. (J) Motif analysis of Smad2/3/4 binding sites in selected GCNT3 promoter sequences. (K) SGC7901 cells were transfected with a mixture of Smad2 and Smad3 or only Smad4 plasmids for 48 h. Anti-Smad2/3 or anti-Smad4 antibodies were used for ChIP assays. qRT-PCR was performed, and normal rabbit IgG antibody served as the negative control. All experiments were performed thrice. Error bars indicate SD ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

FIG 6 GCNT3-mediated autophagy and malignant properties in gastric cancer cells. SGC7901 cells were transfected with 50 nM scrambled control or siGCNT3 for 48 h. (A) After the indicated period, 10 μ l of CCK-8 solution was added to each well to examine cell proliferation. (B) The cell cycle was (Continued on next page)

siGCNT3 transfection, and MHY1485 reduced the effect of siGCNT3 (Fig. 6C). These results confirmed that knockdown of GCNT3 inhibited cell proliferation and migration and induced G₀/G₁ cell cycle arrest, whereas the mTOR pathway enhanced these effects of GCNT3.

Rapamycin is a known activator of autophagy, and the mTORC1 pathway inhibits the occurrence of autophagy. The MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3) protein exists in two forms. LC3-I can be partially converted to LC3-II by lipidation, which can then be recruited into the precursor of the autophagosome. As shown in Fig. 6F, after transfecting GCNT3 siRNA in SGC7901 cells, the expression of LC3-II was significantly increased, whereas LC3-I had no obvious change. Using immunofluorescence to monitor autophagy in cells, confocal laser scanning microscopy revealed an increased level of punctate LC3B staining after transfection of GCNT3 siRNA (Fig. 6G). In addition, an electron microscope was used to observe the formation of autophagosomes. Compared with control cells, the number of autophagosomes was significantly increased after GCNT3 siRNA transfection (Fig. 6H).

In addition, proteins related to autophagy were tested. After transfection of GCNT3 siRNA, the expression of mTOR, p-mTOR, and p-ULK1 was significantly reduced, and ULK1 expression was significantly increased. ATG7 expression was significantly reduced, whereas the expression of ATG12, ATG16, ATG5, and ATG3 was not significantly different (Fig. 6I and J). This finding suggested that knocking down GCNT3 activated autophagy by inhibiting the mTORC1 pathway and its downstream pathway. Immunoprecipitation showed that GCNT3 interacted with mTOR to form a complex, inducing the expression of mTOR and the activation of mTOR (Fig. 6K). The GCNT3-mTOR complex phosphorylated ULK1, prevented ULK1 from forming complexes with ATG and FIP200, and prevented autophagy in GC cells. In addition, given that EBV is a known activator of autophagy (42), we assessed whether LMP2A affects the formation of autophagosomes in GC. The number of autophagosomes increased in cells stably transfected with LMP2A (Fig. 6L). The above results indicated that knocking down GCNT3 promoted autophagy by downregulating the mTORC1 pathway, and LMP2A might promote autophagy by downregulating GCNT3 and the mTORC1 pathway.

The mTORC1 pathway mediated malignant properties in gastric cancer cells by regulating GCNT3. The mTORC1 pathway is typically activated in cancer and regulates downstream signals to affect cell proliferation, apoptosis, migration and cell cycle progression. The cell proliferation ability was significantly enhanced after MHY1485 treatment and weakened after rapamycin treatment (Fig. 7A). Given that cancer cell metastasis is a key indicator of tumor progression, we found that the migration ability was stronger with MHY1485 treatment and much weaker with rapamycin treatment, and siGCNT3 inhibited the effect of MHY1485 (Fig. 7E and F). After the mTORC1 pathway was inhibited, N-cadherin, vimentin, β -catenin, Snail, and ZEB1 expression were down-regulated, whereas E-cadherin was significantly increased. The opposite result was obtained when the mTORC1 pathway was activated (Fig. 7G). The mTORC1 pathway regulated important proteins involved in EMT to regulate migration. The mTORC1 pathway promoted the proliferation and migration of EBVaGC and EBVnGC cells, whereas these effects were suppressed after transfection with siGCNT3.

Flow cytometry assays were performed to identify the stages of cell cycle progression. Rapamycin resulted in a significant increase in the percentage of G₀/G₁ phase cells

FIG 6 Legend (Continued)

observed using flow cytometry after GCNT3 knockdown. (C) Western blotting was used to analyze the expression of CCND1 after GCNT3 was knocked down. (D) Migration was observed using the Transwell migration assay after knockdown of GCNT3. The number of migrated cells was quantified using ImageJ and expressed as a fold change (bar graph) relative to the appropriate cell line. (E) The apoptosis rate was detected by flow cytometry. (F) SGC7901 cells were transfected with siGCNT3. The lysates were prepared at 48 h following transfection and analyzed by immunoblotting. (G) SGC7901 cells were transfected with siGCNT3 and examined by indirect immunofluorescence using anti-LC3B antibodies. (H) Effect of siGCNT3 transfection on autophagy induction observed by electron microscopy. (I and J) SGC7901 cells were transfected with siGCNT3 and were analyzed by immunoblotting using antibodies against the indicated proteins. (K) Coimmunoprecipitation in SGC7901 cell lysates followed by Western blot analysis showed that GCNT3 interacted with mTOR. (L) Effect of LMP2A on autophagy induction observed by electron microscopy. All experiments were performed thrice. Error bars indicate SD ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

FIG 7 The mTORC1 pathway mediated malignant properties in gastric cancer cells by regulating GCNT3. Cells were treated with MHY1485 (2 μ M) or rapamycin (100 nM) for 36 h. (A) After the indicated period, 10 μ l of CCK-8 (Continued on next page)

and a significant decrease in S phase cells. In addition, rapamycin downregulated CCND1 expression. Opposite results were obtained after treatment with MHY1485, and the percentage of G₀/G₁ phase cells increased after transfection with siGCNT3 (Fig. 7B to D). Under different conditions, the G₂/M phase was not significantly affected (Fig. 7B and C). Treatment with MHY1485 and rapamycin had no significant difference in apoptosis (Fig. 7H). The mTORC1 pathway promoted proliferation and migration and inhibited G₀/G₁ cell cycle arrest in EBVaGC and EBVnGC cells, whereas transfection with siGCNT3 inhibited these effects.

TGF- β 1 activated the mTORC1-GCNT3 pathway to promote cell migration in EBVaGC and EBVnGC cells, and TGF- β 1 promoted apoptosis and G₀/G₁ cell arrest in EBVnGC cells. TGF- β 1 activates a variety of signals to mediate cell proliferation, apoptosis, and migration to regulate tumor progression. For example, TGF- β activates the mTORC1 signaling pathway to induce EMT (23). The effect of TGF- β 1 on the malignant characteristics of GC cells was tested. The migration ability of cells after TGF- β 1 treatment was significantly higher; after adding rapamycin or transfecting siGCNT3 to TGF- β 1-added cells, the cell migration ability was lower than for TGF- β 1-added cells (Fig. 8A and B). TGF- β 1 upregulated the expression of N-cadherin, vimentin, β -catenin, Snail, ZEB1, Twist1, and Slug but downregulated E-cadherin expression (Fig. 8C). This finding indicated that TGF- β 1 promoted cell migration by activating the mTORC1-GCNT3 pathway.

TGF- β 1 promotes apoptosis in GC cells (32), whereas LMP2A resists TGF- β 1-mediated growth inhibition and apoptosis in EBVaGC (43). Consistent with the reported results, TGF- β 1 induced apoptosis in SGC7901 cells, whereas TGF- β 1 did not significantly induce apoptosis in GT38 cells. When LMP2A was knocked down in TGF- β 1-stimulated GT38 cells, apoptosis increased (Fig. 8D and E). This finding suggested that TGF- β 1 inhibited cell growth and promoted apoptosis in EBVnGC, whereas LMP2A inhibited TGF- β 1-mediated growth inhibition and apoptosis in EBVaGC. We demonstrated that TGF- β 1 promoted G₀/G₁ phase cell arrest in SGC7901 cells using flow cytometry (Fig. 8F). TGF- β 1 exhibited different effects on cell proliferation and apoptosis in EBVaGC and EBVnGC, potentially because LMP2A inhibited the formation of Smad2/3/4 complexes and inhibited the activation of the TGF- β 1/Smad pathway. This finding prompted our further assessment of the regulation of EBV on the TGF- β 1 pathway.

DISCUSSION

Glycosylation modification is an important marker of cancer and metabolic diseases, and recent studies have reported that it is related to EBV infections. MAGT1-CDG was described as a disease associated with two different clinical phenotypes caused by defects in glycosylation, and patients with mutations in MAGT1 were characterized by chronic EBV infections (44). When EBV entered epithelial cells, the glycoprotein L (gL) glycosylation mutant N69L/S71V had an epithelial cell-specific hyperfusogenic phenotype (45). LMP2A upregulated the UDP-glucose dehydrogenase gene expression involved in glycosaminoglycan synthesis through the extracellular signal-regulated kinase (ERK) and PI3K/AKT pathways (46). Studies have demonstrated that the abnormal expression of GCNT3 promotes the formation of mucins, thereby enhancing the flexibility of cancer cells and adapting them to adverse tumor microenvironment conditions (10). Abnormal GCNT3 expression affects the occurrence and development of many cancers, including colon cancer, hepatocellular carcinoma, pancreatic cancer, and non-small cell lung cancer (9, 10, 12). We found that GCNT3 was downregulated in

FIG 7 Legend (Continued)

solution was added to each well to examine cell proliferation. (B and C) The cell cycle was observed using flow cytometry in GT38 and SGC7901 cells. (D) Western blotting was used to analyze the expression of CCND1 in GT38 and SGC7901 cells. (E and F) Migration was observed using the Transwell migration assay in GT38 and SGC7901 cells. (G) EMT pathway-associated proteins were detected by Western blot analysis in GT38 and SGC7901 cells. (H) The apoptosis rate was detected by flow cytometry in GT38 cells. All experiments were performed thrice. Error bars indicate SD ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

FIG 8 TGF- β 1 activated the mTORC1-GCNT3 pathway to promote cell migration in EBVaGC and EBVnGC cells, and TGF- β 1 promoted apoptosis and G₀/G₁ cell arrest in EBVnGC cells. (A and B) Migration was observed using the Transwell migration assay in GT38 and SGC7901 cells. (C) EMT pathway-associated proteins were detected by Western blot analysis in GT38 and SGC7901 cells. (D and E) Flow cytometry was used to detect the apoptosis rate in SGC7901 cells (D) and GT38 cells (E). (F) The cell cycle was observed using flow cytometry in SGC7901 cells. (G) Schematic diagram showing the upstream regulators and downstream targets of GCNT3 and their corresponding biological functions in regulating GC aggressiveness. All experiments were performed thrice. Error bars indicate SD ($n \geq 3$). *, $P < 0.05$; **, $P < 0.01$.

EBVaGC cells and tissues and was closely related to lymph node metastasis (13). The mechanism by which EBV regulated GCNT3 still lacks consensus. This research examined the mechanism by which LMP2A regulated GCNT3. A schematic diagram summarizing our findings and suggested signaling events is shown in Fig. 8G. Some studies have demonstrated that patients with EBVaGC have a better prognosis and fewer lymph node metastases compared than EBVnGC patients. In addition, patients with EBVaGC exhibit relatively long survival times because EBVaGC might be more prone to genetic changes associated with better prognosis (47, 48). In this study, LMP2A downregulated GCNT3 expression through the TGF- β 1/Smad-mTORC1 pathway, which might explain the lower metastasis rate and the better prognosis of EBVaGC.

mTOR signaling regulates cell proliferation, growth differentiation, migration, and survival in cancer (49–51). Interestingly, LMP2A activated mTOR signaling to regulate cell growth and proliferation in NPC (17). However, LMP2A inhibited mTORC1 signaling in EBVaGC. We hypothesized that because LMP2A has different regulatory mechanisms in different cancers, the ability of LMP2A to regulate downstream pathways is cell type specific. In addition, we found that LMP2A inhibited mTORC1 signaling activation by suppressing TGF- β 1/Smad signaling. It has been reported that LMP2A inhibits TGF- β stimulation but does not affect the expression of TGF- β or its receptors in tumor cells (43). The EBVaGC cell lines GT38 and GT39 were resistant to TGF- β 1, and there were many mechanisms that lacked TGF- β 1 reactivity, including inactivation or deletion of TGF- β 1 receptor or the Smad protein (52). TGF- β 1 significantly induced PAI-1 and junB expression in HSC-39 cells; however, it induced junB in GT38 cells but rarely induced PAI-1 (52). These results suggested that EBV in GT38 and GT39 cells might be reactivated through the TGF- β 1-induced junB pathway and that different activation patterns of TGF- β 1-mediated signaling molecules resulted in resistance to TGF- β 1. In our study, LMP2A might inhibit TGF- β 1 signaling through various mechanisms that cause cells to lack TGF- β 1 reactivity and inhibit the formation of the Smad complex, thereby inhibiting the activation of mTORC1 signaling. It is worth noting that LMP2A directly downregulated GCNT3 transcription via Smad2/3 and Smad4. In addition, Smad2/3/4 interacted with mTOR to form a complex to phosphorylate mTOR. We discovered for the first time that GCNT3 was a direct target of the mTORC1 pathway. In addition, GCNT3 interacted with mTOR, activated mTOR with feedback, and inhibited autophagy. mTOR might form a multiplex complex with Smad2/3/4 and GCNT3, which is activated by the TGF- β 1/Smad pathway and GCNT3. Moreover, the mTORC1 pathway upregulated GCNT3 to promote GC cell proliferation and migration and inhibit cell cycle arrest. The mTORC1 pathway might target GCNT3 to affect the transfer of glycosyl and thus promote the occurrence and development of GC.

4E-BP1 is a definite target of mTORC1, and eIF4E plays essential roles in translation initiation, nuclear export, and stability control (53). The interaction of 4E-BP and eIF4E excludes the binding of eIF4E and eIF4G, which leads to impaired assembly of the eIF4F complex (19, 20). 4E-BP1 binds tightly to eIF4E and acts as a translation repressor. We found that eIF4E interacted with GCNT3, the target of the mTORC1 pathway, and maintained GCNT3 stability. LMP2A inhibited the formation of eIF4E-GCNT3 complexes to inhibit GCNT3. Interestingly, GCNT3 formed a multiplex complex with eIF4E-4E-BP1. Whether LMP2A affects this complex and affects the inhibition of translation by 4E-BP1 has led to further thought. The interaction between the mTOR-eIF4E pathway and GCNT3 in GC is complex and is closely related to the proliferation, migration, cycle progression, and autophagy. It may be feasible to introduce the regulation and inhibition of mTOR-eIF4E-GCNT3 into the treatment of GC.

TGF- β is a multifunctional cytokine that plays an important role in regulating cell growth and differentiation in many biological systems (24). Increased levels of TGF- β in gastric cancer patients have been reported (25, 26), which prompted us to study the regulation of TGF- β 1 on the LMP2A-mTORC1-GCNT3 pathway. TGF- β plays different roles in different cancers. TGF- β induced EMT by enhancing the activated form of T β RI or by increasing the expression of Smad2, Smad3, and Smad4 (28, 29). TGF- β promoted the proliferation of mesenchymal cell types and inhibited the proliferation of

epithelial cells (30, 31). In most cases, TGF- β induced cell arrest (33). TGF- β induced or inhibited programmed cell death in different cell types (32). In our experiments, TGF- β 1 induced migration by activating the mTORC1-GCNT3 pathway in EBVaGC and EBVnGC; in EBVnGC, TGF- β 1 induced apoptosis and G₀/G₁ cell arrest. These results indicated that TGF- β 1 has a complex function in EBVnGC and EBVaGC. In EBVnGC, TGF- β 1 induced apoptosis, but it promoted migration and the progression of GC by activating the mTORC1-GCNT3 pathway. In EBVaGC, the effect of TGF- β 1 in inducing apoptosis was inhibited by LMP2A.

In the present study, LMP2A inhibited the activation of the mTORC1 pathway by inactivating the TGF- β /Smad pathway and downregulated GCNT3 expression by inactivating the TGF- β /Smad-mTORC1 pathway. In addition, LMP2A inhibited GCNT3 transcription by inhibiting Smad2/3 and Smad4. GCNT3 inhibited autophagy by inducing mTOR phosphorylation. The mTORC1-GCNT3 pathway promoted cell proliferation and migration and inhibited G₀/G₁ cell arrest. The TGF- β 1/Smad-mTORC1-GCNT3 pathway promoted cell migration by regulating genes in its downstream EMT pathway. Our conclusions provide deeper theoretical support for the role of LMP2A and the TGF- β 1/Smad-mTORC1 pathway in EBVaGC and help in understanding glycosylation abnormalities in cancer and its effects on the malignant properties that are enhanced in GC cells. Our results may provide novel therapeutic targets for the treatment of GC against the TGF- β 1/Smad-mTORC1-GCNT3 signaling cascade.

MATERIALS AND METHODS

Cell lines and treatment. A total of six cell lines were used, three EBVaGC cell lines, i.e., GT38, GT39, and SNU719, and three EBV-negative gastric cancer (EBVnGC) cell lines, i.e., AGS, BGC823, and SGC7901. All cell lines (GT38, GT39, SNU719, BGC823, AGS, and SGC7901) were grown in Dulbecco modified Eagle medium (DMEM/F-12; Life Technologies, Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. All cells were maintained at 37°C in a 5% CO₂ incubator. GT38 and GT39 cell lines were sourced from T. Sairenji (Biosignal Division, Department of Biomedical Sciences, Tottori University, Japan).

TGF- β 1 was purchased from Pepro Tech EC Ltd. MHY1485, BAY11-7082, SB431542, MG132, and cycloheximide were obtained from ApexBio (Houston, TX, USA). Rapamycin was purchased from Beyotime Biotechnology Corporation (Shanghai, China). The following primary antibodies were purchased from Abcam (Cambridge, UK): anti-LMP2A, anti-GCNT3, anti-p-4EBP1, anti-p-mTOR, anti-CCND1, and anti- β -actin. The following primary antibodies were purchased from Cell Signaling Technology (Chicago, IL, USA): anti-Smad2, anti-Smad3, anti-Smad4, anti-p-Smad2, anti-p-Smad3, anti-mTOR, anti-p70S6K, anti-4EBP1, anti-p-p70S6K, anti-eIF4E, anti-ULK1, anti-p-ULK1, anti-ATG3, anti-ATG5, anti-ATG7, anti-ATG12, anti-ATG16, anti-N-cadherin, anti-E-cadherin, anti-Vimentin, anti-ZEB1, anti-Snail, anti-Slug, anti- β -Catenin, and anti-Twist1. Anti-rabbit and anti-mouse antibodies conjugated to horseradish peroxidase were purchased from Cell Signaling Technology.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR). Cells were harvested and RNA was extracted using TRIzol reagent (Ambion, USA) according to the manufacturer's instructions. qRT-PCR was performed using the LightCycler 96 system (Roche, Switzerland) to determine GCNT3 mRNA expression. The comparative cycle threshold (C_t) method was used to assess differences in LMP2A transcript levels using GAPDH as an internal loading control. Experiments were performed in triplicate. The following forward and reverse primers were used: GAPDH, 5'-CAAATCCATGGCACCCTCA-3' and 5'-ATCGCCCACTTGATTG-3'; GCNT3, 5'-TGTTACATTGCTGCCACG-3', and 5'-GTGGAGGAGGACACAATCCTT-3'.

Immunofluorescence assay. The cells were placed on slides and fixed with 4% paraformaldehyde for 10 min. Then, slides were washed with phosphate-buffered saline (PBS), blocked, and permeabilized with buffer (1% bovine serum albumin [BSA], 0.15% Triton X-100, 22.52 mg/ml glycine in PBS) at room temperature for 1 h. The slides were incubated with primary antibody overnight at 4°C. The secondary antibody (1:1,000) was incubated for 1 h in the dark at room temperature following three washings with PBS with Tween (PBST), and the cells were then counterstained with Hoechst 33342 (Beyotime, China) for 5 min. The localization of GCNT3 in the cells was observed with a fluorescence microscope (Nikon, Japan).

Western blotting. All cells were lysed using radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor, phenylmethanesulfonyl-fluoride (PMSF), and a phosphatase inhibitor mixture. These samples were transferred to polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany) using SDS-PAGE gel with a 10% mass concentration, blocked with bovine serum albumin (BSA) with a 5% mass concentration at room temperature for 2 h, incubated with the primary antibody overnight at 4°C, cultured with the second antibody the following day, and held at room temperature for 2 h. Following incubation, the membranes were visualized by using an ECL detection kit (Millipore Corporation, Billerica, MA, USA).

Transfection with small interfering RNA (siRNA) and overexpression of LMP2A and Smad2/3/4. Specific small interfering RNAs were designed and synthesized at GenePharma (Shanghai, China). The

sequences of siRNAs were as follows: GCUCAGUGCCGUGAAAUATT for siGNT3-1, GCUACUGCGAG CUGUGUAUATT for siGNT3-2, GAACGAUGAGGAACGUGAAT for siLMP2A, CCUCUUGCUCAGAUGCCUUTT for siRaptor, CCACCGAAUUGGCAGAUUTT for siTOR, GCUAACCCAGAACACUAUATT for sielf4E, GGUUUCGAUAGCAUUAUATT for siSmad2, GCCUGGUCAAGAAACUAATT for siSmad3, GCCAUCGUUGUC CACUGAATT for siSmad4, and UUCUCCGAACGUGUCACGUTT for control siRNA. Cells were transfected with the RNA oligonucleotides using Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Germany) for 48 h at 50 nM per the manufacturer's recommendations. Smad2, Smad3, and Smad4 plasmids were purchased from Genechem (China). LMP2A was cloned into the pcDNA3.1-EF1a-mcs-3flag-CMV-EGFP vector. Three micrograms of LMP2A (pcDNA3.1-LMP2A) or a negative-control vector (pcDNA3.1-vector) plasmid was transfected into SGC7901 cells using Lipofectamine 2000. Cells were harvested for assay at the indicated times. Stable transfection of LMP2A was selected by G418 and observed under a fluorescence microscope.

Immunoprecipitation. Protein complexes were precipitated from whole-cell lysates with anti-elf4E (Santa Cruz Biotechnology), anti-4EBP1 (Santa Cruz Biotechnology), anti-GCNT3 (Santa Cruz Biotechnology), anti-Smad2/3 (Santa Cruz Biotechnology), or anti-Smad4 antibodies (Cell Signaling Technology) and then precipitated with protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Following the instructions, SDS-PAGE and Western blotting were performed after the sample was boiled.

Chromatin immunoprecipitation. A chromatin immunoprecipitation (ChIP) kit was purchased from Cell Signaling Technology, and ChIP was performed according to the manufacturer's recommendations. The antibodies used were normal rabbit IgG against Smad2/3 and Smad4. The primer pairs used for the GCNT3 promoter region were as follows: 5'-GAGAGGATGTGGAGCTCTAAGG-3' (F), 5'-AACTGGTTCTA ATCAGGGTTGC-3' (R).

Cell proliferation assay. Cell proliferation was assessed using cell counting kit-8 (CCK8; Boster Biological Technology, Wuhan, China). For growth curve analysis, cells were seeded in sextuplicate at a density of 8×10^3 cells per well in 96-well plates. After 12 h of incubation, different reagents were added to the experimental wells, and the cells were allowed to grow for another 24, 48, or 72 h. A total of 10 ml of CCK-8 solution was added to each well for 2 h, and viability was measured using a microplate reader at a wavelength of 450 nm.

Cell cycle assay. Cells were seeded into six-well plates at a density of 1×10^6 cells per well and incubated overnight. After treatment for 36 h, the cells were digested by trypsin and washed gently with PBS. The collected cells were fixed in 66% ethanol at 4°C overnight. Then, the cells were incubated with propidium iodide (PI) and RNase A for 25 min in the dark. The cell cycle was analyzed by using flow cytometry (BD Biosciences).

Migration assays. The migration ability of the cells was measured using Transwell plates with 8- μ m pores (Corning, NY, USA). Cells were digested with trypsin and resuspended in serum-free medium. A total of 1×10^5 cells in 200 μ l serum-free medium were seeded into the upper chambers of Transwell plates. Cells attached to the bottom of the chamber membrane were stained with crystal violet after incubation at 37°C for 36 h. Five fields were randomly photographed under each microscope, and the experiments were repeated thrice.

Cell apoptosis detection. Cell apoptosis analyses were detected by flow cytometry analysis using a fluorescein isothiocyanate (FITC)-annexin V apoptosis detection kit I (BD Biosciences, San Jose, CA). Cells were seeded in six-well plates at a density of 1×10^6 cells per well. After treatment for 36 h, the cells were digested and washed twice with cold PBS. Next, 5 μ l FITC-annexin V was added, and the cells were protected from light for 15 min at room temperature. Then, 5 μ l propidium iodide (PI) was added. All experiments were repeated in triplicate.

Electron microscopy. To identify autophagosomes at the ultrastructural level, cells were fixed with 2.5% glutaraldehyde and then dehydrated and embedded. The number of autophagosomes was observed in a randomly selected field of view in each sample.

Statistical analysis. All data are expressed as the means $\pm$ standard error of the mean (SEM). The experimental data were analyzed using a *t* test ($P < 0.05$ was considered significant; ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$). Statistical analyses were conducted using SPSS 19.0 statistical software (Chicago, IL).

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (NSFC 81571995).

J. Liu and B.L. conceived and designed the study. J. Liu, C.Y., and J. Li performed the experiments. J. Liu drafted the manuscript. J. Liu, Y.Z., and W.L. analyzed the experimental data.

We declare no competing financial interests.

REFERENCES

- Young LS, Rickinson AB. 2004. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* 4:757–768. <https://doi.org/10.1038/nrc1452>.
- Delecluse HJ, Feederle R, O'Sullivan B, Taniere P. 2007. Epstein Barr virus-associated tumours: an update for the attention of the working pathologist. *J Clin Pathol* 60:1358–1364. <https://doi.org/10.1136/jcp.2006.044586>.
- Tempera I, Klichinsky M, Lieberman PM. 2011. EBV latency types adopt alternative chromatin conformations. *PLoS Pathog* 7:e1002180. <https://doi.org/10.1371/journal.ppat.1002180>.
- Fukayama M, Hayashi Y, Iwasaki Y, Chong J, Ooba T, Takizawa T, Koike M, Mizutani S, Miyaki M, Hirai K. 1994. Epstein-Barr virus-associated gastric carcinoma and Epstein-Barr virus infection of the stomach. *Lab Invest* 71:73–81.

5. Shibata D, Weiss LM. 1992. Epstein-Barr virus-associated gastric adenocarcinoma. *Am J Pathol* 140:769–774.
6. Sugiura M, Imai S, Tokunaga M, Koizumi S, Uchizawa M, Okamoto K, Osato T. 1996. Transcriptional analysis of Epstein-Barr virus gene expression in EBV-positive gastric carcinoma: unique viral latency in the tumour cells. *Br J Cancer* 74:625–631. <https://doi.org/10.1038/bjc.1996.412>.
7. Scholle F, Bendt KM, Raab-Traub N. 2000. Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. *J Virol* 74:10681–10689. <https://doi.org/10.1128/jvi.74.22.10681-10689.2000>.
8. Yeh JC, Ong E, Fukuda M. 1999. Molecular cloning and expression of a novel beta-1,6-N-acetylglucosaminyltransferase that forms core 2, core 4, and I branches. *J Biol Chem* 274:3215–3221. <https://doi.org/10.1074/jbc.274.5.3215>.
9. Fernandez LP, Sanchez-Martinez R, Vargas T, Herranz J, Martin-Hernandez R, Mendiola M, Hardisson D, Reglero G, Feliu J, Redondo A, Ramirez de Molina A. 2018. The role of glycosyltransferase enzyme GCNT3 in colon and ovarian cancer prognosis and chemoresistance. *Sci Rep* 8:8485. <https://doi.org/10.1038/s41598-018-26468-4>.
10. Rao CV, Janakiram NB, Madka V, Kumar G, Scott EJ, Pathuri G, Bryant T, Kutche H, Zhang Y, Biddick L, Gali H, Zhao YD, Lightfoot S, Mohammed A. 2016. Small-molecule inhibition of GCNT3 disrupts mucin biosynthesis and malignant cellular behaviors in pancreatic cancer. *Cancer Res* 76:1965–1974. <https://doi.org/10.1158/0008-5472.CAN-15-2820>.
11. Gonzalez-Vallinas M, Molina S, Vicente G, Zarza V, Martin-Hernandez R, Garcia-Risco MR, Fornari T, Reglero G, Ramirez de Molina A. 2014. Expression of microRNA-15b and the glycosyltransferase GCNT3 correlates with antitumor efficacy of Rosemary diterpenes in colon and pancreatic cancer. *PLoS One* 9:e98556. <https://doi.org/10.1371/journal.pone.0098556>.
12. Li Q, Ran P, Zhang X, Guo X, Yuan Y, Dong T, Zhu B, Zheng S, Xiao C. 2018. Downregulation of N-acetylglucosaminyltransferase GCNT3 by miR-302b-3p decreases non-small cell lung cancer (NSCLC) cell proliferation, migration and invasion. *Cell Physiol Biochem* 50:987–1004. <https://doi.org/10.1159/000494482>.
13. Liu J, Zhang Y, Liu W, Zhang Q, Xiao H, Song H, Luo B. 2020. MiR-BART1-5p targets core 2 β -1,6-acetylglucosaminyltransferase GCNT3 to inhibit cell proliferation and migration in EBV-associated gastric cancer. *Virology* 541:63–74. <https://doi.org/10.1016/j.virol.2019.12.004>.
14. Kudchodkar SB, Yu Y, Maguire TG, Alwine JC. 2004. Human cytomegalovirus infection induces rapamycin-insensitive phosphorylation of downstream effectors of mTOR kinase. *J Virol* 78:11030–11039. <https://doi.org/10.1128/JVI.78.20.11030-11039.2004>.
15. Schneider RJ, Mohr I. 2003. Translation initiation and viral tricks. *Trends Biochem Sci* 28:130–136. [https://doi.org/10.1016/S0968-0004\(03\)00029-X](https://doi.org/10.1016/S0968-0004(03)00029-X).
16. Walsh D, Mohr I. 2004. Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. *Genes Dev* 18:660–672. <https://doi.org/10.1101/gad.1185304>.
17. Moody CA, Scott RS, Amirghahari N, Nathan CO, Young LS, Dawson CW, Sixbey JW. 2005. Modulation of the cell growth regulator mTOR by Epstein-Barr virus-encoded LMP2A. *J Virol* 79:5499–5506. <https://doi.org/10.1128/JVI.79.9.5499-5506.2005>.
18. Poulin F, Gingras AC, Olsen H, Chevalier S, Sonenberg N. 1998. 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. *J Biol Chem* 273:14002–14007. <https://doi.org/10.1074/jbc.273.22.14002>.
19. Pause A, Belsham GJ, Gingras AC, Donzé O, Lin TA, Lawrence JC, Jr, Sonenberg N. 1994. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* 371:762–767. <https://doi.org/10.1038/371762a0>.
20. Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N. 1998. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev* 12:502–513. <https://doi.org/10.1101/gad.12.4.502>.
21. Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N. 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev* 13:1422–1437. <https://doi.org/10.1101/gad.13.11.1422>.
22. Viel S, Marçais A, Guimaraes FS, Loftus R, Rabilloud J, Grau M, Degouve S, Djebali S, Sanlaville A, Charrier E, Bienvu J, Marie JC, Caux C, Marvel J, Town L, Huntington ND, Bartholin L, Finlay D, Smyth MJ, Walzer T. 2016. TGF-beta inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Sci Signal* 9:ra19. <https://doi.org/10.1126/scisignal.aad1884>.
23. Lamouille S, Derynck R. 2007. Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J Cell Biol* 178:437–451. <https://doi.org/10.1083/jcb.200611146>.
24. Massague J. 1998. TGF-beta signal transduction. *Annu Rev Biochem* 67:753–791. <https://doi.org/10.1146/annurev.biochem.67.1.753>.
25. Tahara E. 1993. Molecular mechanism of stomach carcinogenesis. *J Cancer Res Clin Oncol* 119:265–272. <https://doi.org/10.1007/BF01212724>.
26. Mizoi T, Ohtani H, Miyazono K, Miyazawa M, Matsuno S, Nagura H. 1993. Immunoelectron microscopic localization of transforming growth factor beta 1 and latent transforming growth factor beta 1 binding protein in human gastrointestinal carcinomas: qualitative difference between cancer cells and stromal cells. *Cancer Res* 53:183–190.
27. Naef M, Ishiwata T, Friess H, Buchler MW, Gold LI, Korc M. 1997. Differential localization of transforming growth factor-beta isoforms in human gastric mucosa and overexpression in gastric carcinoma. *Int J Cancer* 71:131–137. [https://doi.org/10.1002/\(SICI\)1097-0215\(19970410\)71:2<131::AID-IJC1>3.0.CO;2-1](https://doi.org/10.1002/(SICI)1097-0215(19970410)71:2<131::AID-IJC1>3.0.CO;2-1).
28. Piek E, Moustakas A, Kurisaki A, Heldin CH, ten Dijke P. 1999. TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J Cell Science* 112:4557–4568.
29. Valcourt U, Kowanetz M, Niimi H, Heldin CH, Moustakas A. 2005. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. *Mol Biol Cell* 16:1987–2002. <https://doi.org/10.1091/mbc.e04-08-0658>.
30. Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R. 1990. TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. *Cell* 63:515–524. [https://doi.org/10.1016/0092-8674\(90\)90448-n](https://doi.org/10.1016/0092-8674(90)90448-n).
31. Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB. 1985. Type beta transforming growth factor: a bifunctional regulator of cellular growth. *Proc Natl Acad Sci U S A* 82:119–123. <https://doi.org/10.1073/pnas.82.1.119>.
32. Schuster N, Kriegstein K. 2002. Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* 307:1–14. <https://doi.org/10.1007/s00441-001-0479-6>.
33. Siegel PM, Massague J. 2003. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 3:807–821. <https://doi.org/10.1038/nrc1208>.
34. Bragado P, Estrada Y, Parikh F, Krause S, Capobianco C, Farina HG, Schewe DM, Aguirre-Ghisso JA. 2013. TGF-beta2 dictates disseminated tumour cell fate in target organs through TGF-beta-R11 and p38alpha/beta signalling. *Nat Cell Biol* 15:1351–1361. <https://doi.org/10.1038/ncb2861>.
35. Ding Y, Kim JK, Kim SI, Na HJ, Jun SY, Lee SJ, Choi ME. 2010. TGF-(beta)1 protects against mesangial cell apoptosis via induction of autophagy. *J Biol Chem* 285:37909–37919. <https://doi.org/10.1074/jbc.M109.093724>.
36. Laplante M, Sabatini DM. 2012. mTOR signaling in growth control and disease. *Cell* 149:274–293. <https://doi.org/10.1016/j.cell.2012.03.017>.
37. Das R, Xu S, Nguyen TT, Quan X, Choi SK, Kim SJ, Lee EY, Cha SK, Park KS. 2015. Transforming growth factor β 1-induced apoptosis in podocytes via the extracellular signal-regulated kinase-mammalian target of rapamycin complex 1-NADPH oxidase 4 axis. *J Biol Chem* 290:30830–30842. <https://doi.org/10.1074/jbc.M115.703116>.
38. Shi Q, Zhang Y, Liu W, Xiao H, Qi Y, Li J, Luo B. 2020. Latent membrane protein 2A inhibits expression level of Smad2 through regulating miR-155-5p in EBV-associated gastric cancer cell lines. *J Med Virol* 92:96–106. <https://doi.org/10.1002/jmv.25579>.
39. Zhang Y, Liu W, Zhang W, Wang W, Song Y, Xiao H, Luo B. 2019. Constitutive activation of the canonical NF- κ B signaling pathway in EBV-associated gastric carcinoma. *Virology* 532:1–10. <https://doi.org/10.1016/j.virol.2019.03.019>.
40. Swanson-Mungerson MA, Caldwell RG, Bultema R, Longnecker R. 2005. Epstein-Barr virus LMP2A alters in vivo and in vitro models of B-cell anergy, but not deletion, in response to autoantigen. *J Virol* 79:7355–7362. <https://doi.org/10.1128/JVI.79.12.7355-7362.2005>.
41. Xiao B, Zhu ED, Li N, Lu DS, Li W, Li BS, Zhao YL, Mao XH, Guo G, Yu PW, Zou QM. 2012. Increased miR-146a in gastric cancer directly targets SMAD4 and is involved in modulating cell proliferation and apoptosis. *Oncol Rep* 27:559–566. <https://doi.org/10.3892/or.2011.1514>.
42. Cavnignac Y, Esclatine A. 2010. Herpesviruses and autophagy: catch me if you can! *Viruses* 2:314–333. <https://doi.org/10.3390/v2010314>.
43. Fukuda M, Longnecker R. 2004. Latent membrane protein 2A inhibits transforming growth factor-beta 1-induced apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. *J Virol* 78:1697–1705. <https://doi.org/10.1128/jvi.78.4.1697-1705.2004>.

44. Blommaert E, Péanne R, Cherepanova NA, Rymen D, Staels F, Jaeken J, Race V, Keldermans L, Souche E, Corveleyn A, Sparkes R, Bhattacharya K, Devalck C, Schrijvers R, Foulquier F, Gilmore R, Matthijs G. 2019. Mutations in MAGT1 lead to a glycosylation disorder with a variable phenotype. *Proc Natl Acad Sci U S A* 116:9865–9870. <https://doi.org/10.1073/pnas.1817815116>.
45. Möhl BS, Chen J, Park SJ, Jardetzky TS, Longnecker R. 2017. Epstein-Barr virus fusion with epithelial cells triggered by gB is restricted by a gL glycosylation site. *J Virol* 91:e01255-17. <https://doi.org/10.1128/JVI.01255-17>.
46. Pan YR, Vatsyayan J, Chang YS, Chang HY. 2008. Epstein-Barr virus latent membrane protein 2A upregulates UDP-glucose dehydrogenase gene expression via ERK and PI3K/Akt pathway. *Cell Microbiol* 10:2447–2460. <https://doi.org/10.1111/j.1462-5822.2008.01221.x>.
47. Matsunou H, Konishi F, Hori H, Ikeda T, Sasaki K, Hirose Y, Yamamichi N. 1996. Characteristics of Epstein-Barr virus-associated gastric carcinoma with lymphoid stroma in Japan. *Cancer* 77:1998–2004. [https://doi.org/10.1002/\(SICI\)1097-0142\(19960515\)77:10<1998::AID-CNCR6>3.0.CO;2-D](https://doi.org/10.1002/(SICI)1097-0142(19960515)77:10<1998::AID-CNCR6>3.0.CO;2-D).
48. Camargo MC, Kim WH, Chiaravalli AM, Kim KM, Corvalan AH, Matsuo K, Yu J, Sung JJ, Herrera-Goepfert R, Meneses-Gonzalez F, Kijima Y, Natsugoe S, Liao LM, Lissowska J, Kim S, Hu N, Gonzalez CA, Yatabe Y, Koriyama C, Hewitt SM, Akiba S, Gulley ML, Taylor PR, Rabkin CS. 2014. Improved survival of gastric cancer with tumour Epstein-Barr virus positivity: an international pooled analysis. *Gut* 63:236–243. <https://doi.org/10.1136/gutjnl-2013-304531>.
49. Martin KA, Blenis J. 2002. Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. *Adv Cancer Res* 86:1–39. [https://doi.org/10.1016/s0065-230x\(02\)86001-8](https://doi.org/10.1016/s0065-230x(02)86001-8).
50. Oldham S, Hafen E. 2003. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol* 13:79–85. [https://doi.org/10.1016/s0962-8924\(02\)00042-9](https://doi.org/10.1016/s0962-8924(02)00042-9).
51. Vogt PK. 2001. PI 3-kinase, mTOR, protein synthesis and cancer. *Trends Mol Med* 7:482–484. [https://doi.org/10.1016/s1471-4914\(01\)02161-x](https://doi.org/10.1016/s1471-4914(01)02161-x).
52. Fukuda M, Ikuta K, Yanagihara K, Tajima M, Kuratsune H, Kurata T, Sairenji T. 2001. Effect of transforming growth factor-beta1 on the cell growth and Epstein-Barr virus reactivation in EBV-infected epithelial cell lines. *Virology* 288:109–118. <https://doi.org/10.1006/viro.2001.1071>.
53. Gosselin P, Martineau Y, Morales J, Czjzek M, Glippa V, Gauffeny I, Morin E, Le Corguillé G, Pyronnet S, Cormier P, Cosson B. 2013. Tracking a refined eIF4E-binding motif reveals Angel1 as a new partner of eIF4E. *Nucleic Acids Res* 41:7783–7792. <https://doi.org/10.1093/nar/gkt569>.