Epstein-Barr virus miR-BART3-3p promotes tumorigenesis by regulating senescence pathway in gastric cancer

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\textbf{Running title}
EBV miR-BART3-3p regulates gastric cancer senescence

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Abstract
Epstein–Barr virus-associated gastric cancer (EBVaGC) accounts for about 10% of all gastric cancer cases and has unique pathological and molecular characteristics. EBV encodes a large number of microRNAs, which actively participate in the development of EBV-related tumors. Here we report EBV-miR-BART3-3p (BART3-3p) promotes gastric cancer cell growth in vitro and in vivo. Moreover, BART3-3p inhibits the senescence of gastric cancer cells induced by oncogene (RAS$^{G12V}$) or chemotherapy (Irinotecan). LMP1 and EBNA3C encoded by EBV had also been reported to have anti-senescence effects, however, in EBVaGC specimens, LMP1 expression is very low and EBNA3C is not expressed. BART3-3p inhibits senescence of gastric cancer cells in a nude mouse model and inhibits the infiltration of NK cells and macrophages in tumor by altering senescence associated secretory phenotype (SASP). Mechanistically, BART3-3p directly targeted the tumor suppressor gene TP53 and caused down-regulation of p53’s downstream p21. Analysis from clinical EBVaGC samples also showed a negative correlation between BART3-3p and TP53 expression. It is well known that mutant oncogene (RAS$^{G12V}$) or chemotherapeutic drugs can induce senescence, here we show that both RAS$^{G12V}$ and chemotherapy drug also can induce BART3-3p expression in EBV positive gastric cancer cells, forming a feedback loop, therefore, the EBVaGC senescence remains at a low level. Our results suggest that, although TP53 is seldom mutated in EBVaGC, its expression is fine regulated, in which EBV-encoded BART3-3p may play an important role through inhibiting the senescence of gastric cancer cells.

Introduction
The Epstein–Barr virus (EBV) is a double-stranded DNA virus belonging to the herpes virus family, which is aetiologically linked to lymphoid and epithelial malignancies, such as Burkitt lymphoma, Hodkin’s lymphoma, extranodal NK/T-cell lymphoma, nasopharyngeal carcinoma and gastric carcinoma (GC). EBV infection occurs in 2%–20% of GC, with a worldwide average of 10% (1-3). EBV-encoded small RNAs (EBER) were present in almost all of these tumor cells but not in normal epithelial and stromal cells (4). Elevation of anti-IgG and anti-IgA against viral capsid antigen was found several months before the diagnosis of EBVaGC (5). These findings strongly suggest that EBV plays an important etiological role in the formation and / or development of EBVaGC. Evidence provided by comprehensive molecular analyses proved that EBVaGC is a distinct subset of GC both in terms of its molecular and clinicopathological features (6). Genetic changes that are characteristic of EBVaGC include frequent mutations in PIK3CA and ARID1A, global CpG islands hypermethylation of the promoter region of many cancer related genes. As the most frequently mutated gene in solid tumors, TP53 mutations occurs in about 50% of GC, but is very rare in EBVaGC (6). Another noteworthy feature of EBVaGC is hyper-activation of the PI3K-AKT signaling (6). EBVaGC belongs to latency infection type I or II, in which only EBER, EBNA1 and LMP2A are expressed, but a large number of EBV BART microRNAs are highly expressed (7,8).

p53 is the most important tumor suppressor activated by DNA damage and other stresses (9,10). Activation of p53 pathway leads to temporary or permanent cell cycle arrest, i.e. cell senescence (11). Cell senescence is initiated as a response to cell damage, but its role in tumorigenesis and development is context dependent (12-14). Notably, senescent cells express a vast number of secreted proteins. This phenotype is termed as senescence-associated secretory phenotype (SASP) (15). Some malignant transformed cells undergoing
senescence is due to oncogene activation or loss of tumor suppressor (oncogene-induced senescence, OIS). And this phenotype can be vital in the response to some anticancer treatments, which terms as ‘therapy-induced senescence’ (TIS). Activation of p53/p21<sup>CIP1</sup> and/or p16<sup>INK4A</sup> tumor suppressor pathway is essential for both OIS and TIS. Partial loss of <i>PTEN</i> leads to moderate activation of the PI3K-AKT pathways, which interrupts OIS (16).

The tumorigenesis role of EBV latent infection in host cells is accomplished by manipulating a series of host genes, such as genes related to cellular stress responses, senescence, proliferation, etc. The fine regulation of host genes is considered of great importance for EBV pathogenesis. LMP1, as the most famous latent protein of EBV, suppresses the expression of p16<sup>INK4a</sup>, commonly believed to be a key regulator of replicative senescence. LMP1 also prevents RAS-induced premature senescence (17,18). <i>In vitro</i>, EBV infection of B cells induces a continuous proliferation of B cell, resulting in the immortal lymphoblastoid cell lines (LCLs). It has been reported that EBNA3C acts as a host DDR (DNA damage response) inhibitor to prevent B proliferation from stagnation and promote its immortalization (19). Although LMP1 is present in nasopharyngeal carcinoma, but only a few EBVaGC specimens expressed LMP1. This raises the question of whether EBV affects the senescence process through other molecules in EBVaGC.

EBV was the first virus to be found to encode microRNAs. It encodes approximately 25 microRNA precursors and 44 mature microRNAs, which are divided into two major clusters: BART and BHRF-1(20). The BHRF-1 cluster expresses only in lytically infected cells or cells with latency type III infections (21). EBVaGC specimens express a large number of miR-BARTs, whereas almost all EBV oncoproteins are seldom expressed (8). Up to date, the genes targeted by EBV miR-BARTs are associated with oncogenesis, apoptosis, epithelial-mesenchymal transition (EMT) and signal transductions, many of them are involved in tumor progression or have an impact on tumor immune response (22-25). We speculate that <i>TP53</i>, although not mutated, is also closely regulated in EBVaGC, and EBV-encoded miR-BARTs may contribute to p53 modulation and further affect the senescence of GC cells.

In the present study, we show that <i>EBV-miR-BART3-3p</i> (BART3-3p), as a relative high expressed microRNA in EBVaGC, can promote the proliferation and inhibit the senescence of GC cells by directly targeting the CDS region of <i>TP53</i> and inhibiting PTEN. By fine tuning the two key molecules in the senescence pathway, BART3-3p promotes the development of EBVaGC.

Results

<i>EBV-miR-BART3-3p targets tumor suppressor TP53 in GC</i>

In order to find the EBV BART microRNAs that may regulate p53, we searched all the BART microRNAs seed sequences, and found that EBV BART3-3p has several binding sites can interact with <i>TP53</i>. To investigate whether BART3-3p was one of the possible mechanisms for p53 inactivation or down-regulation in EBVaGC, BART3-3p mimics were transfected into two EBV negative gastric cancer cell lines, SGC7901 and AGS. p53 and its downstream target p21 were significantly suppressed at protein levels by BART3-3p mimics (Figure 1A). Considering that the binding sites of BART3-3p and <i>TP53</i> mRNA predicted by bioinformatics were located in the <i>TP53</i> CDS region, we co-transfected BART3-3p mimics and <i>TP53</i> expression-vectors GFP-p53 which lacked <i>TP53</i> 3'UTR and 5'UTR into SGC7901 and AGS. p53 and its downstream target p21 were significantly suppressed at protein levels by BART3-3p mimics (Figure 1B). We also co-transfected GFP-p53 and BART3-3p mimics into <i>TP53</i>-deficient cell
line KATOIII, and the results further confirmed that exogenous GFP-p53 was suppressed by BART3-3p (Figure 1C). BART3-3p mimics also significantly decreased the mRNA levels of TP53 and its target genes, CDKN1A and BAX (Figure 1D). Further study showed that BART3-3p reduced the p53 transcriptional activity (Figure 1E). To clarify whether TP53 is a direct cellular target gene for BART3-3p, luciferase reporter assays were performed by co-transfection of a BART3-3p mimics with a full length of TP53 3’UTR or CDS-containing luciferase reporter vector into HEK293 cells, respectively. It is the CDS but not the 3’UTR luciferase activity was significantly reduced by BART3-3p mimics (nc) (Figure 1F), suggesting that the CDS region of TP53 mRNA may contain the target sites directly targeted by BART3-3p. An online tool for microRNA target prediction RNAhybrid showed two possible binding sites existed in the CDS of TP53 mRNA (from the nucleotide position 511 and 647, respectively) by the seed sequence of BART3-3p (Figure 1G). The luciferase activity of the WT TP53-CDS but not the mutant CDS was significantly reduced by BART3-3p but not by negative control mimics (Figure 1H). These results strongly suggest that BART3-3p directly binds to TP53 CDS region and inhibits its transcription. microRNAs bind to their target genes and carry them to RNA-induced silencing complex (RISC), in which Argonaute 2 (Ago2) functions as a platform. SGC7901 cells were transfected with BART3-3p mimics, and then RNA immunoprecipitation was performed by anti-Ago2 antibody. BART3-3p mimics significantly increased the TP53 mRNA level that binds to Ago2 comparing to NC mimics (Figure 1I). Taken together, BART3-3p targets TP53 through binding to its CDS region.

The expression of EBV-miR-BART3-3p and TP53 was correlated in EBVaGC specimens. We collected 20 cases of EBVaGC and 20 cases of non-EBVaGC tissue samples for analyzing the expression levels of TP53 and BART3-3p. The clinic pathological parameters of GC specimens were listed in Supplementary Table 3. The diagnostic standard of EBVaGC was in situ hybridization positive EBER staining (Figure 2A). BART3-3p can only be detected in the EBVaGC specimens (although its expression levels varied in the specimens), but not in the non-EBVaGC specimens (Figure 2B). The average TP53 expression levels are lower in EBVaGC specimens comparing to that of non-EBVaGC specimens, but the difference has not reached the significant threshold ($P = 0.1785$, Figure 2C). We used chi-square test to analyze the correlation between the expression levels of BART3-3p and TP53 in EBVaGC specimens. We divided the 20 cases of EBVaGC into two groups BART3-3p high and BART3-3p low; as well as TP53 high and TP53 low groups (see Methods and Materials for detail). Chi-square test showed that BART3-3p in EBVaGC was negatively correlated with TP53 ($P < 0.01$, Figure 2D). The above clinical specimens data suggests a possibility that BART3-3p is involved in the regulation of TP53 expression in EBVaGC.

**EBV-miR-BART3-3p promotes GC growth in vitro and in vivo**

We next investigated the function of BART3-3p under the text of tumor biology in GC cells. BART3-3p mimics were transfected into SGC7901 and AGS cell lines. Colony formation assay (Figure 3A) and CCK8 assay (Figure 3B) demonstrated that expression of BART3-3p can increase the proliferation ability of GC cells compared with negative control. In order to study whether BART3-3p promotes gastric cancer growth in vivo, we established a xenograft mouse model. SGC7901 cells were transfected with b3-3p agomir or negative control (nc) agomir for 12h, then transplanted into the liver capsule of the nude mice. Injection
of b3-3p agomir or nc agomir to corresponding groups of nude mice through their tail veins on day 7 and day 14. All the mice were sacrificed in day 28 (Figure 3C). Both groups have one or several transplanted tumors in the liver, the average tumor volumes of the b3-3p agomir group is larger than that of the nc group (Figure 3D). To verify the results, we expressed BART3-3p in SGC7901 and AGS cells by lentivirus-mediated transduction and named the cells as SGC7901 LV-b3-3p and AGS LV-b3-3p (Figure S1A). Compared with their respective controls, the p53 of these two cell lines were decreased (Figure S1B). BART3-3p inhibitor increased the expression of p53 and its downstream p21 protein in both of the two cell lines (Figure S1C). Consistent with the above results, BART3-3p inhibitor can reverse the cell proliferation induced by BART3-3p in SGC7901 LV-b3-3p and AGS LV-b3-3p cell lines (Figure 3E). CCK8 assay also showed the same function of BART3-3p inhibitor on cell proliferation in SGC7901 LV-b3-3p and AGS LV-b3-3p cells (Figure 3F). These results suggest that BART3-3p promotes the proliferation of GC cells both in vitro and in vivo.

**EBV-miR-BART3-3p inhibits cell senescence in vitro**

p53 and PTEN are the two masters in the senescence pathway. In addition to directly targeting TP53, we also observed that PTEN was inhibited by BART3-3p both at the protein and mRNA levels, but not through a direct targeting relationship (Figure S2). We proposed that BART3-3p may take part in the regulation of cells senescence. To evaluated this hypothesis, we used Irinotecan (a chemotherapeutic drug) and RAS\textsuperscript{G12V} plasmid to induce either TIS (treatment induced senescence) (26) or OIS (oncogene induced senescence) (27) in SGC7901 LV-b3-3p/SGC7901 LV-nc cells. Both Irinotecan and RAS\textsuperscript{G12V} significantly induced senescence in SGC7901 LV-nc cells, but their effects were compromised in the SGC7901 LV-b3-3p cells, as assessed by senescence-associated-beta-galactosidase (SA-\β-gal) activity (Figure 4A, 4B). EBV encoded oncoprotein LMP1 has been reported to inhibit senescence (18), so we transfected LMP1 into SGC7901 cell line, and it turned out LMP1’s effect on senescence is similar to that of BART3-3p (Figure 4C,4D). Irinotecan or RAS\textsuperscript{G12V} up-regulated the protein levels of p53, whereas BART3-3p reversed this effect (Figure 4E). We next examined the SASP (senescence-associated secretory phenotype) cytokines by qRT-PCR. Cytokines (IL-6 and IL-8), as well as MMP1 and CDK4 were repressed by BART3-3p (Figure 4F). The IL-6 protein secreted by GC cells is also inhibited by BART3-3p (Figure 4G). Luciferase reporter assay determined that BART3-3p can inhibit the transcription activity of NF-κB but not STAT3, and NF-κB is the master transcriptional factor that promoting the secretory activity of senescent cells (Figure 4H). BART3-3p can inhibit the RAS\textsuperscript{G12V} induced senescence in human embryonic lung fibroblast cell line MRC-5 (Figure S3). In addition to LMP1, EBV-encoded EBNA3C also can inhibit B cell senescence (19). However, EBNA3C are not expressed in EBVaGC, and LMP1 expression has been negative in most of the EBVaGC specimens (7,28). TCGA Data from 295 GC specimens further confirms this result (6). Here, we also detected the expression levels of LMP1 and EBNA3C in 20 cases of EBVaGC and 20 cases of non-EBVaGC tissue samples, and found that only 3 cases of EBVaGC had LMP1 expression, while the remaining samples were unable to detect LMP1 and EBNA3C expression (Figure 4I). These results imply the importance of BART3-3p on senescence in EBVaGC, since there is very limited expression of LMP1 and EBNA3C in those cancers.
EBV-miR-BART3-3p inhibits cell senescence in vivo

The in vitro study showed a solid effect of BART3-3p on cell senescence; we then examined the in vivo effect of this discovery by xenografting SGC7901 cells into nude mice (under their liver capsule). Mice were randomly divided into two groups, one was treated with b3-3p agomir, the another were treated with nc agomir. All mice were sacrificed in 4 weeks, and the tumors were stripped from the liver. SA-β-gal staining showed that the number of senescent cells in the b3-3p agomir group was less than that in the nc agomir group (Figure 5A). The expression levels of TP53, CDKN1A, CDK4 and SASP cytokines (IL-1A, IL-1B, IL-6, IL-8) were decreased in b3-3p agomir group compared with nc group (Figure 5B). The NK cells and macrophages infiltrated in the tumor tissues of b3-3p agomir group were reduced comparing to nc group by means of flow cytometry (Figure 5C), but the number of macrophages infiltrated in the livers has no differences between the two groups (Figure S4). These results suggest that BART3-3p can inhibit senescence in vivo and possibly reduce the recruitment of immune cells by down-regulating important SASP molecules, thus inhibiting the anti-tumor immune response.

RASG12V and chemotherapy induce BART3-3p expression in EBV positive GC cells, which constitutes a feedback loop to keep cell senescence in a low level

It has been reported that activation of NF-κB can increase the expression of a variety of EBV microRNAs(29). Since RASG12V and chemotherapy can activate the NF-κB pathway (26), it triggered a question whether RASG12V and chemotherapy induce BART3-3p expression in EBV positive GC cells. It turned out that either irinotecan or RASG12V can significantly increase BART3-3p expression, while adding NF-κB inhibitor BAY11-7028, the increase of BART3-3p was reversed (Figure 6A). We noticed that both RASG12V and irinotecan can activate NF-κB signaling, and the activation was compromised by the addition of BAY11-7028 (Figure 6B). These results suggest that RASG12V and chemotherapy can increase the expression of BART3-3p, most probably by activating the NF-κB signaling. In the AGS-EBV cells, when b3-3p inhibitors were introduced, the RASG12V-induced senescence can be significantly enhanced (Figure 6C). This result suggests that although RASG12V can induce cancer cell senescence, meanwhile, they also can induce BART3-3p expression, and BART3-3p can inhibit senescence, therefore, the cell senescence is kept in a low level in the EBVaGC cells, which normally have wild type TP53.

Discussion

EBVaGC has recently attracted a lot of attention. This type of gastric cancer has its unique biological characteristics, such as hypermethylation and rare TP53 mutation. The role of EBV and its encoded RNAs in the tumorigenesis and progression of EBVaGC still needs intensive study. EBV is a very low-profile virus, with very few molecules expressed during latent infection, but BART microRNAs are the exceptions, with high abundance during latent infection. The high expression of low-immunogenicity microRNAs and limited expression of high-immunogenicity viral proteins will benefit the virus to survive from the anti-virus immunity system of the host cells, as a result of long-term evolution process. More attention should be paid to the function and clinical significance of the EBV microRNAs. In this study, we reported that BART3-3p is a potent inhibitor of senescence, which is the first time that EBV has been reported to inhibit GC cell senescence through miR-BARTs.

EBV BART3-3p promoted GC cells proliferation and inhibited senescence through
targeting TP53, and therefore played a key role in promoting tumor progression in EBVaGC. Senescence is a state of permanent cell cycle arrest when cells confront different damaging stimuli. Due to the activation of an irreversible proliferation arrest, cellular senescence is seen as a strong safeguard against tumorigenesis (30). Loss of p53 activity or loss of p16INK4A, a frequent strategy employed by tumor cells, leads to elimination of the senescence response (31). Partial loss of PTEN leads to moderate activation of PI3K/AKT pathway, which disrupts OIS (16). Multiple microRNAs had been reported to play a regulatory role in cell senescence. For example, miR-30 inhibits both p16INK4A and p53, two key senescence effectors, leading to efficient senescence disruption (32). miR-137 targets KDM4A mRNA during Ras-induced senescence and activates both p53 and Rb pathways (33). miRNA-34a induces esophageal squamous cancer cell senescence-like changes via the down-regulation of SIRT1 and up-regulation of p53/p21 (34). In this study, p53 and its downstream p21 were negatively regulated by BART3-3p, but p16 did not appear to be affected (results not shown).

Senescent cells are not dormant cells but are metabolically active (35). They express a large number of secreted proteins which is known as SASP (senescence-associated-secretory phenotype) (15). SASPs are observed in tumors cell senescence, which involves a large number of inflammatory cytokines (most notably IL-1a, IL-1b, IL-6 and IL-8) and chemokines and provide a strong link between senescence and anti-tumor immunity. Here, we found that several SASP molecules were significantly reduced by BART3-3p, both in vivo and in vitro (Figure 4F, 5B). It is noteworthy that SASP differs in different tissues and in different stimuli, but inflammatory cytokines, such as IL-6 and IL-8, are highly conserved parts of SASP and play a major role in maintaining SASP responses in the senescent cell itself and in the affected tissues. Given that NF-κB and STAT3 are the major driver of the SASP, we tested whether BART3-3p affects them. We found that BART3-3p inhibited the luciferase reporter activity of NF-κB but not STAT3, suggesting that the effect of BART3-3p on SASP was achieved by restraining NF-κB activity (Figure 4F). SASP modulates the infiltration of immune cells in tumor tissues, and we found that the number of NK and macrophages in the tumor microenvironment is decreased in the BART3-3p agomir group comparing the nc group, while the number of macrophages in liver tissues was no different between the two groups (Figure 5C, Figure S4). This result suggests that the reduction of immune infiltrating cells may be one of the reasons for the greater tumor size in BART3-3p agomir group. Recent studies seem to have different perspectives on the relationship between SASP and tumorigenesis. Milanovic M et al. reported that post-senescent cells with ‘hijacked’ SASP exerted their detrimental potential at relapse by driving a much more aggressive growth phenotype using p53-regulatable models of acute lymphoblastic leukemia and acute myeloid leukemia (36). Menendez JA et al. suggested that in neoplastic background, cell senescence seems to be a beneficial response to tumor progression by controlling tumor growth (37).

Some studies have addressed the relationship between EBV and cell senescence. EBV infection in vitro drives primary human B cells into indefinitely proliferating lymphoblastoid cells. This process of growth transformation depends on a subset of viral latent oncoproteins and non-coding RNAs. The viral latent oncoproteins EBNA3C was required to attenuate the EBV-induced DNA damage response to prevent proliferation arrest (19). EBV onco-protein LMP1 can inhibit RAS mediated induction of p16INK4a and p21WAF to
promote senescence bypass; LMP1 also prevents RAS-induced senescence in fibroblasts IMR90 and REF52 (18). However, *EBNA3C* is not expressed, and *LMP1* is seldom expressed in EBVaGC specimens. There are very limited expression of *LMP1* and almost no expression of *EBNA3C* in the 20 EBVaGC specimens (Figure 4), which suggests that these EBV-encoded oncoproteins are unlikely to play an anti-senescence role in EBVaGC. Here we report that EBV-encoded BART3-3p regulates cellular senescence pathway in gastric cancer. BART3-3p plays a negative role in the regulation of p53/p21 in EBVaGC, and also inhibits the expression of PTEN protein, thus preventing GC cells from senescence and promoting EBVaGC progression. We speculate that strict control of cell senescence is essential for the survival of EBV, whether in newly infected cells or in EBV-associated tumors. BART3-3p, as a relatively high expressed non-coding RNA in EBVaGC, its regulation of senescence has important significance for both virus survival and GC process.

We recently reported another BART microRNA—BART5-3p, which can directly target *TP53* by combining with *TP53* 3’UTR, thereby promoting the progression of EBV-positive epithelial tumors (38). Unlike BART5-3p, BART3-3p binds to the *TP53* CDS region but not 3’UTR (Figure 1F). Much is known about how miRNAs regulate gene expression comes from studies that miRNA binding sites located in the 3’UTR of mRNA. The work of Hauser *J et al.* pointed out that target sites located in the CDS are most potent in inhibiting translation, while sites located in the 3’UTR are more efficient in triggering mRNA degradation (39). Both BART3-3p and BART5-3p have similar effects on cell biological behavior, but BART3-3p has a more obvious senescence-inhibition phenotype of gastric cancer cells. This may be because BART3-3p not only directly targets p53/p21, but also represses PTEN pathway. Li X’s group had revealed that both BART7-3p and BART1 can inhibit PTEN signaling (23,24). So, one conclusion can be made that the master tumor suppressors are under the attack of EBV microRNAs.

There are a few of target genes has been reported to be regulated by BART3-3p. Lei T *et al.* reported BART3-3p overcame the growth suppressive activity of DICE1 and stimulated cell proliferation (40). Kang D *et al.* identified CASZ1a as a target gene of BART3-3p and accordingly demonstrated that it is one of the EBV microRNAs with anti-apoptosis effect (41). According to them, BART3-3p can be combined with the 3’UTR area of both DICE1 and CASZ1a. Because BART3-3p targets several important tumor suppressor genes, it has oncogene characteristics in the development of EBV-associated epithelial tumors. Our study revealed a novel function of BART3-3p for inhibition of oncogene or chemotherapy-induced senescence through suppressing p53 and PTEN, which has significance to keep the senescence in a quiet low level in EBVaGC.

It has been reported that BARTs is upregulated by NF-κB, and LMP1 induces BART miRNA expression through NF-κB signaling (29). Here we report that both OIS and TIS can up-regulate the expression of BART3-3p, which constitutes a negative feedback mechanism to keep the senescence in a low level of the GC cells. When EBV-positive GC cells undergo OIS or TIS, they can activate NF-κB signal, thus can up-regulate the expression of BART3-3p. Increased BART3-3p can negatively regulate the senescence by targeting p53/p21 signal and partly inhibiting PTEN (Figure 7). According to a series of reported BART target genes, BART molecule may represent an important strategy for EBV to regulate host cell proliferation, death, senescence and anti-infection immune response.
EBV encoding microRNAs should be given enough attention, since they do not directly produce viral proteins, thus do not form traditional viral antigens to be recognized by the host immune system.

**Experimental Procedures**

**Cell culture and reagents**

SGC7901 (EBV-negative GC cell line), KATOIII (GC cell line) cells were maintained in RPMI-1640 (Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum; AGS (EBV-negative GC cell line) cells were maintained in Ham’s F-12 medium (Hyclone) supplemented with 10% fetal bovine serum; AGS-EBV (EBV-positive GC cell line) and EBV-positive AKATA cells (47,48) also received 400 μg of G418/ml. HEK293 cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum. All cells were maintained at 37°C with 5% CO₂.

miRNA mimics, inhibitors were purchased from GenePharma Inc (Shanghai, China, the sequence information are listed in the Supplementary Table 1). EBV-miR BART3-3p and negative control agomirs were purchased from Ribobio (Guangzhou, China). The cells were transfected with RNAs and/or plasmids using Lipofectamine 3000 (Invitrogen).

**Clinical gastric cancer specimens**

EBVaGC was defined as positive EBER using in situ hybridization (ISH), as described later in the text. Formalin-fixed paraffin-embedded (FFPE) surgical specimens, including adequate cases of EBVaGC and non-EBVaGC, were obtained from the Xiangya Hospital (Hunan, China) selected by the senior pathologist. 20 EBVaGC and 20 non-EBVaGC specimens were collected for the analysis of BART3-3p, TP53, LMP1 and EBNA3C expression. Written informed consent was obtained from all study participants. Collections and using of tissue samples were approved by the ethical review committees of the Xiangya Hospital of Central South University and were in accordance with the Declaration of Helsinki.

**TaqMan microRNA assay**

Total RNAs from surgical specimens were extracted using the RecoverAll total nucleic acid isolation kit for FFPE according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). The miRNA (mature BART3-3p or U6)-specific RT primers, and miRNA-specific TaqMan probes were purchased from Thermo (Waltham, MA, USA), and the miRNAs expression were detected by TaqMan microRNA assays according to the manufacturer’s protocol. The results were normalized to U6.

**Lentivirus transduction**

Lentivirus packaging was made by GenePharma Inc. A lentiviral vector plasmids LV3 (H1/GFP&Puro) was used in this study to construct the stable cell lines. The randomized flanking sequence control (mock) and EBV-miRBART3-3p were purchased from GenePharma Inc and transduced into cells following the manufacturer’s instructions.

**Western blot**

Protein extracts were resolved by SDS–polyacrylamide gel, transferred to PVDF membranes, and probed with antibodies against p53 (DO-1) (Santa Cruz; 1:500), p21 (Cell Signaling Technology; 1:500), GAPDH (Sangon Biotech; 1:5000). HRP-conjugated secondary antibody (Cell Signaling Technology) was used as the secondary antibody. The antigen-antibody reaction was visualized by enhanced Chemi-luminescence assay.

**Tumor xenografts procedures in nude mice.**

All nude mice (4–5 weeks old, male) were purchased from the Central Animal Facility of Central South University. To assess tumor growth and infiltration of immune cells, 16 nude
mice were received a 0.5-cm cut on their abdominal sections, and their liver were pushed part out of the abdominal cavity, then, 50 μl of SGC7901 cells (1×10^6) transfected with b3-3p agomir or nc agomir (2nmol) for 24 hours with Matrigel (BD, #354234) were injected into their liver capsules, finally, their liver were carefully pushed back to their abdominal cavity after pressing the pinhole with alcohol cotton balls for 2 min. After 7 days, the 16 mice were randomly divided into two groups. One group was injected with 10 nmol BART3-3p(b3-3p) agomir into the tail vein and another group was injected with 10 nmol nc-agomir. Repeated injection of 10 nmol BART3-3p agomir or nc agomir on the 14th day in the corresponding group. All mice were sacrificed in 4 weeks. The tumors were stripped from the livers and the tumor volumes were measured regularly and calculated using the formula: (A×B^2)/2, where A is the largest diameter and B is the perpendicular diameter. Part of the tumor tissues were fixed in 4% paraformaldehyde for 24 h and transferred to gradient ethanol. Tumors were embedded in paraffin, sectioned with LeiCa RM2245 and processed for histological examinations. Frozen sections were also prepared directly from tumor tissues. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The protocol was approved by the Animal Ethics Committee of Central South University.

**Cell preparation and FACS analysis**

Resected xenograft tumors were washed in PBS. Tumor tissues were cut into small pieces by scissors in RPMI1640 medium, collagenase D and DNase I, and incubated for 6 minutes at 37°C, then passing through a 70-µm cell strainer (BD Falcon, NJ, USA). The resulting cells are purified using Percoll (GE healthcare, Waukesha, WI, USA) density gradient centrifugation. For analysis of macrophages and NK cells, the cells were stained with F4/80-APC (clone RM8), anti-CD49b-PE (clone DX5), anti-CD11b-APC/Cy7 (clone M1/70), which were purchased from Biolegend (San Diego, CA, USA). Samples were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, MA, USA), and the data were analyzed with FlowJo sofware (TreeStar, Olten, Switzerland).

**Plasmids and Dual-Luciferase Reporter Assay**

The full length of TP53 3′-UTR or CDS were amplified and subcloned into the pmirGLO dual-luciferase miRNA target expression vector. TP53 CDS mutant plasmids were synthesized and constructed by GeneCreate Biotech (Wuhan, China). The pp53-TA-luc luciferase reporter plasmid was purchased from Beyotime (Jiangsu, China) to monitor the transcriptional activity of p53. For miRNA target gene verification, HEK293 cells were seeded in 48-well plates and co-transfected with 200 ng of the luciferase reporter plasmid (TP53 3′-UTR, CDS, wild type or mutant) along with 20 pmol of BART3-3p or negative control (NC) mimics; For p53 transcriptional activity detection, SGC7901 cells were cotransfected with 200 ng of pp53-TA-luc luciferase reporter plasmid and 40 ng of Renilla plasmid along with 20 pmol of BART3-3p or NC mimics. Firefly and Renilla luciferase activities were measured via a Dual-GLO luciferase assay system (Promega, Madison, WI, USA). The data were expressed as the relative firefly luciferase activity normalized to the value of Renilla luciferase.

**Analysis of mRNA levels**

Total RNAs were extracted using TRIzol reagent (Invitrogen). For mRNA reverse transcription, 2 μg of RNA was used to synthesize cDNA using a RevertAid first strand cDNA synthesis kit (Thermo) according to the
manufacturer’s protocol. For miRNA reverse transcription, 1 μg of RNA was used to synthesize cDNA using a Mir-X miRNA First-Strand synthesis kit (Takara, Otsu, Japan) according to the manufacturer’s protocol. The levels of gene transcripts were detected by qRT-PCR using specific primers and a SYBR premix Ex TaqII kit (Takara). The expression levels of miRNA and mRNA were quantified by measuring cycle threshold (Ct) values and normalized to U6 and Actin, respectively. The data were further normalized to the negative control, unless otherwise indicated. The primers used for qRT-PCR are listed in Supplementary Table 2.

**ELISA**

ELISA was performed as previously described (49). An ELISA kit for IL-6 was obtained from R&D Systems (Minneapolis, MN, USA).

**Colony formation assay**

Cells with different treatment were planted into 6-wells plates (1000 cells per well) and incubated for 12 days. Plates were washed with PBS and stained with crystal violet. The number of colonies with more than 50 cells was counted.

**SA-β-gal staining of histological sections and cultured cells**

For xenografts tissues samples, SA-β-gal staining was performed in frozen sections using Senescence β-Galactosidase Staining Kit (Beyotime, #C0602). Briefly, 12-μm tissue frozen sections were fixed at room temperature for 15 min with fix solution, washed three times with PBS, and incubated 48 h at 37°C with the staining solution containing X-gal, sections were then counterstained with eosin. Cells were stained for SA-β-gal activity according to the instruction manual. Briefly, SGC7901 and MRC5 cells were fixed with fix solution for 15 min at room temperature. Fixed cells then were washed with PBS and stained at 37°C for 24 h.

**In situ hybridization**

EBER ISH was performed on the FFPE tissue samples slides by EBER1 probe ISH kit (Zsbio, Beijing, China). Only those with a universal and unequivocal nuclear staining within almost all tumor cells were interpreted as EBV positivity.

**Statistical Analysis**

Statistical analysis was determined by independent t test or ANOVA using SPSS17.0 and GraphPad Prism 5. Significance parameters were set at P vales of < 0.05.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.
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Figures

Figure 1. EBV BART3-3p inhibits p53 expression by targeting its CDS region. (A) Western blotting analysis of p53 and p21 protein expression in SGC7901 and AGS cells transfected with negative control mimics (nc) or EBV BART3-3p mimics (b3-3p) for 48h. (B) Co-transfected with GFP-p53 and mimics (b3-3p or nc) to SGC7901 and AGS cells for 48h, exogenous and endogenous p53 were analyzed by western blot. (C) KATOIII cells were co-transfected with GFP-p53 and mimics (b3-3p or nc) for 48h. Western blotting shows the p53. (D) Gene expression levels of TP53, BAX and CDKN1A in SGC7901 cell transfected with mimics (b3-3p or nc) for 48h were examined by RT-qPCR(n=3). Expression levels were normalized to nc. (E) BART3-3p regulates p53 transcriptional activity. SGC7901 cells were co-transfected with 200 ng of p53-responsive reporter pp53-TA-Luc plasmid and 50 ng Renilla plasmid and 20 pmol mimics (b3-3p or nc) (n=3). Luciferase activity was measured 48h later, and the data were showed as the relative Firefly luciferase activity normalized to the value of Renilla luciferase. (F) HEK293 cells were co-transfected with luciferase reporters carrying TP53 3’UTR (or CDS) and mimics (b3-3p or nc) (n=4). 48h later, luciferase activity was measured. The data were showed as the relative Firefly luciferase activity normalized to the value of Renilla luciferase. (G) BART3-3p and its putative binding sequences in the CDS region of TP53. Mutants of the CDS of TP53 gene were generated in the complementary sites that bind to the seed regions of BART3-3p. (H) HEK293 cells were co-transfected with luciferase reporters carrying either predicted miRNA target site in TP53 CDS (WT) or its corresponding mutants (Mut1 and Mut2) and mimics (b3-3p or nc). Luciferase activity was measured 48h later. The data were showed as the relative Firefly luciferase activity normalized to the value of Renilla luciferase. (I) SGC7901 cells were transfected with mimics (b3-3p or nc) for 48h, and cells lysates were immunoprecipitated using either anti-Ago2 antibody or a negative control IgG. The mRNA levels of TP53 binding to AGO2...
were detected by RT-qPCR (n=4). n represents biologically independent samples and data are shown as the mean ±95% CI limit. Statistical significance relative to control was assessed by the unpaired two-tailed Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control group (nc). n.s, not significant. GAPDH were used for proteins loading control. Actin was used for normalizing the expression of mRNAs.

Figure 2. Expression of BART3-3p is negatively correlated with TP53 in EBVaGC specimens.

(A) Representative images of EBER expression in GC specimens using in situ hybridization (ISH). Left indicates a negative EBER staining, and right indicates a positive staining.

(B-C) Expression levels of BART3-3p (B) and TP53 (C) in 20 cases of EBVaGC (T1 - T20) and 20 cases of non-EBVaGC (T21 - T40) specimens were detected by stem-loop RT-qPCR (for non-coding RNAs) or RT-qPCR (for genes). The expression values of case T1 are set as 1 (left panel). The right panel shows the individual values of each sample of BART3-3p or TP53 in two groups. Data are shown as the mean ±95% CI limit. Statistical significance between two groups was assessed by the unpaired two-tailed Student’s t test. ****P < 0.0001.

(D) Correlation analysis of the expression levels of BART3-3p and TP53 in EBVaGC specimens. The median expression values of BART3-3p and TP53 in 20 EBVaGC samples were calculated as a criterion for differentiating ‘high’ and ‘low’ groups. The differences of TP53 expression between BART3-3p (high) and BART3-3p (low) groups were analyzed by χ² test.
**Figure 3. BART3-3p promotes tumor growth in vitro and in vivo.** (A) A total 1000 gastric cancer cells transfected with b3-3p mimics (or nc mimics) were seeded in six-well plates. Colony formation assays were performed and the representative images are shown on the left and quantification is shown on the right (n=3). (B) Proliferation of gastric cancer cells transfected with b3-3p mimics (or nc mimics) was analyzed with the CCK8 assay (n=3). (C) Schematic representation of the nude mice SGC7901 xenograft tumor experimental protocol. (D) Representative tumor photos of SGC7901 xenografts treated with b3-3p agomir or nc agomir in nude mice. Tumor volume was measured after sacrifice (n=8, each group). (E) SGC7901 LV-b3-3p cells and AGS LV-b3-3p cells were transfected with either nc or BART3-3p inhibitors for 6 h, then 1000 cells were reseed. Colony formation assays were performed and the representative images are shown on the left and quantification is shown on the right (n=3). (F) Proliferation of SGC7901 LV-b3-3p cells and AGS LV-b3-3p cells transfected with nc or b3-3p inhibitor was analyzed with the CCK8 assay. n represents biologically independent samples and data are shown as the mean ± 95% CI limit. Statistical significance relative to control was assessed by the unpaired two-tailed Student’s t test. *P < 0.05, ** P <0.01.
Figure 4. BART3-3p inhibits cellular senescence in vitro. (A-B) SGC7901 LV-b3-3p or SGC7901 LV-nc cells were treated with Irinotecan (A) for 48h or transfected with 2μg RAS<sup>G12V</sup> plasmid (B) for 48 h, and stained for SA-β-gal (n=3). (C) SGC7901 cells were transfected with 2μg LMP1 plasmid for 24h, then treated with Irinotecan for another 48h, and stained for SA-β-gal (n=3). (D) SGC7901 cells were co-transfected with 1μg LMP1 (or negative control) plasmid and 1μg RAS<sup>G12V</sup> plasmid for 48h, and stained for SA-β-gal. Representative images are shown on the left and quantification is shown on the right (n=3). (E) Western blotting analysis of p53 protein expression in SGC7901 LV-b3-3p or SGC7901 LV-nc cells treated with Irinotecan or transfected with RAS<sup>G12V</sup> plasmid for 48 h. (F) mRNA levels of the indicated genes in SGC7901 cells transfected with nc or BART3-3p mimics (n=3). Actin was used for normalizing the expression of mRNA. (G) Protein levels of the IL-6 was in the cell-culture supernatants of SGC7901 cells transfected with mimics (nc or BART3-3p) were analyzed by ELISA (n=3). (H) Luciferase reporter plasmid PGL3-NF-κB or plasmid STAT3-LUC was co-transfected with mimics (nc or b3-3p) (n=5) in SGC7901 cells for 24h. The data were showed as the relative Firefly luciferase activity normalized to the value of Renilla luciferase. (I) Expression levels of LMP1 and EBNA3C in GC specimens were detected by RT-qPCR. The expression values of case T2 LMP1 are set as 1. n represents biologically independent samples and data are shown as the mean ± 95% CI limit. Statistical significance relative to control was assessed by the unpaired two-tailed Student’s t test. *P < 0.05, **P < 0.01, ***P <0.001.
Figure 5. BART3-3p inhibits cellular senescence in vivo. (A) SA-β-gal staining of the tumor tissues from the xenograft tumor mouse models (see Figure 3C for nude mice experiment procedure). Representative images are shown on the left and quantification is shown on the right (n=6). (B) mRNA levels of the indicated genes in the xenograft tumor tissues (n=4), assayed by RT-qPCR. (C) Frequencies of macrophages and NK cells in xenograft tumor tissues were determined by flow cytometry (n=6). Cells were gated on CD11b+F4/80+ and CD49b+ populations, respectively. Representative flow cytometric figures were shown. n represents biologically independent samples. The data are shown as the mean ± 95% CI limit. Statistical significance relative to control was assessed by the unpaired two-tailed Student’s t test. *P < 0.05; **P < 0.01, *** P <0.001 comparing with control group.
Figure 6. RAS^{G12V} and chemotherapy can induce BART3-3p expression in EBV positive GC cells, which constitutes a feedback loop to keep cell senescence in a low level. AGS-EBV cells were treated with Irinotecan (2.5 μg/ml) alone or together with BAY 11-7028 (2.5μM) for 12h; or transfected with RASG12V alone for 24h or after 12 hours of transfection, BAY 11-7028 (2.5μM) was added for another 12h. Then, BART3-3p expression were detected by qRT-PCR. U6 was used for normalizing the expression of BART3-3p. (A) BART3-3p expression levels were calculated from 3 independent experiments and data are shown as the mean ± 95% CI limit. **P < 0.01, ***P <0.001. (B) Expression levels of phos-p65 (Ser536) were detected by Western blotting. (C) AGS-EBV cells were transfected with 2μg RAS^{G12V} plasmid and b3-3p inhibitor (or nc inhibitor as negative control) for 48 h, and stained for SA-β-gal (n=3). Representative images are shown on the left and quantification is shown on the right.
Figure 7. A proposed model demonstrating the role of BART3-3p in EBVaGC.

Senescence-inducing triggers, such as mutant oncogenes (RAS<sup>G12V</sup>), or chemotherapeutic drugs can induce GC cells senescence, meanwhile, the senescent GC cells secret SASP factors which can recruit immune cells to inhibit GC progression. In EBVaGC, the same triggers also induce BART3-3p expression, and BART3-3p can inhibit senescence through inhibiting p53/p21/PTEN, therefore, the cell senescence is kept in a low level in the EBVaGC cells, which normally have wild type TP53. Lower levels of SASP secretion implies lower levels of immune attack. Together, BART3-3p promotes tumor progression in EBVaGC through inhibiting senescence pathway.
Epstein-Barr virus miR-BART3-3p promotes tumorigenesis by regulating senescence pathway in gastric cancer

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