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ORIGINAL ARTICLE

Nanoparticles (NPs)-mediated lncBCMA silencing to promote eEF1A1 ubiquitination and suppress breast cancer growth and metastasis

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Abstract Long non-coding RNAs (lncRNAs) play an important role in cancer metastasis. Exploring metastasis-associated lncRNAs and developing effective strategy for targeted regulation of lncRNA function *in vivo* are of utmost importance for the treatment of metastatic cancer, which however remains a big challenge. Herein, we identified a new functional lncRNA (denoted lncBCMA), which could stabilize the expression of eukaryotic translation elongation factor 1A1 (eEF1A1) *via* antagonizing its ubiquitination to promote triple-negative breast cancer (TNBC) growth and metastasis. Based on this regulatory mechanism, an endosomal pH-responsive nanoparticle (NP) platform was engineered for systemic lncBCMA siRNA (siBCMA) delivery. This NPs-mediated siBCMA delivery could effectively silence lncBCMA expression and promote eEF1A1 ubiquitination, thereby leading to a significant inhibition of TNBC tumor growth and metastasis. These findings show that lncBCMA could be used as a potential biomarker to

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predict the prognosis of TNBC patients and NPs-mediated lncBCMA silencing could be an effective strategy for metastatic TNBC treatment.

1. Introduction

With the rapid development of medical technology, great achievement has been gained in cancer diagnosis and treatment over the years. However, metastasis still occurs in many cancer patients and accounts for ∼90% of cancer-associated deaths[1−3]. For example, it is estimated that metastasis occurs in ∼40% of breast cancer patients with an overall 5-year survival rate less than 30%[4−6]. In the past decade, although the survival rate of cancer patients has been significantly improved due to the rapid development of early diagnosis, limited achievement has been made in the treatment of metastatic cancer. At present, chemotherapy and targeted therapy are still the first-line treatment modalities for metastatic cancer patients[6−8]. However, drug resistance and severe toxicity significantly restrict the therapeutic outcomes. Thus, acquainting the biological process of cancer metastasis and uncovering the key regulators contributing to cancer metastasis are of utmost importance for the development of effective therapeutic strategy.

Cancer metastasis is a complex process that involves multiple sequential steps, mainly including the detachment of tumor cells from the primary sites, migration and invasion, traveling to different sites, and final adhesion and proliferation[9−12]. In the past decade, much effort has been paid to explore the intrinsic reasons for cancer metastasis, in which long non-coding RNAs (lncRNAs) have been recognized as a type of important regulators[13−15]. LncRNAs are one subtype of RNA transcripts with a length more than 200 nucleotides (nt) that can transcriptionally and/or post transcriptionally regulate targeted gene function[16−18]. Currently, many functional lncRNAs such as metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) have been identified and their critical roles in cancer development and progression have been also extensively investigated[19−23]. Nevertheless, compared to the large number of lncRNAs that accounts for more than 50% of human genome transcripts[24−26], these uncovered lncRNAs are still the tip of the iceberg. Therefore, much effort is still required to explore more functional lncRNAs and elucidate their biological functions in regulating cancer development and progression. More importantly, although the importance and regulatory mechanisms of currently discovered lncRNAs have been extensively explored, few have been successfully translated into clinical use due to the absence of effective strategy to regulate lncRNA function *in vivo*.

To address these issues, we herein analyzed the lncRNA expression profile of metastatic tumors of breast cancer patients and identified a completely new lncRNA (denoted breast cancer metastasis-associated RNA, lncBCMA). Molecular mechanism study reveals that high lncBCMA expression could enhance the ability of breast cancer cells to proliferate, migrate, and invade *via* antagonizing the ubiquitination of eukaryotic translation elongation factor 1A1 (eEF1A1), an important GTP-binding protein that is highly expressed in many cancer types (*e.g.*, breast, liver, colon, and pancreatic cancer)[27−31]. Based on this regulatory mechanism and considering that there are no available inhibitors for lncBCMA, we further employed RNA interfering (RNAi) technology to *in vivo* regulate lncBCMA function as this technology could silence any target genes, especially the "undruggable" portions of human genome[32−34]. However, the endosomal entrapment is still a major challenge to restrict the therapeutic outcomes of RNAi technology[32−36]. To address this issue, we further constructed an endosomal pH-responsive nanoparticle (NP) platform for lncBCMA siRNA (siBCMA) delivery and *in vivo* lncBCMA silencing. Our results demonstrate that the NPs-mediated siBCMA delivery could efficiently silence lncBCMA expression in the tumor tissue and dramatically inhibit TNBC tumor growth and metastasis.

2. Materials and methods

2.1. Materials

Alkyl-modified polyamidoamine (PAMAM) dendrimer (G0-C14) and methoxyl-polyethylene glycol-*b*-poly (2-(diisopropylamino) ethylmethacrylate) (Meo-PEG-*b*-PDPA) polymer were prepared using the previously reported methods[37−39]. The siRNA targeting lncBCMA were provided by IGE Bio (Guangzhou, China). and the detailed information is as follows: siBCMA-1, 5′-GUG UGU UAC UAG AGA AGU UdTdT-3′ (sense sequence) and 5′-AAC UUC UCU AGU AAC ACA CdTdT-3′ (antisense sequence); siBCMA-2, 5′-GAC AGA GAC UAG CUC GUA AdTdT-3′ (sense sequence) and 5′-UUA CGA GCU AGU CUC UGU CdTdT-3′ (antisense sequence). Cy5-labled siBCMA-1 was purchased from IGE Bio. and Cy5 was labeled at the 5′-end of both sense and antisense strands. Lipofectamine 3000 (Lipo3K), fetal bovine serum (FBS), penicillin-streptomycin, Dulbecco's modified Eagle medium (DMEM), and, trypsin were obtained from Invitrogen Corp (California, USA). All other reagents and solvents are of analytical grade and used directly.

2.2. Patients and tissue samples

Tumor samples of 129 female TNBC patients and three hormone receptor (HR)-positive breast cancer patients between January 2010 and September 2018 were collected from the Breast Tumor Center, Sun Yat-sen Memorial Hospital, Sun Yat-sen University. Among the 129 TNBC patients, 42 patients had recurrence and metastasis. The tumor samples included 130 surgically resected tumors and two matched tumor tissues collected from two TNBC patients before (surgically resected tumors) and after post-operative lung metastasis (core needle biopsies). Pathological diagnosis, Ki67, and other biomarkers were independently determined by two pathologists. All the samples were collected with informed consents from the patients according to the International Ethical Guidelines for Biomedical Research Involving Human

Subjects (CIOMS). The study was approved by the Institutional Review Board (IRB) of Sun Yat-sen Memorial Hospital.

2.3. Cell culture

Triple-negative breast cancer (TNBC) cells (MDA-MB-231, Luc-expressing MDA-MB-231, and MDA-MB-468), hormone receptor (HR)-positive breast cancer cells (MCF-7 and T47D), human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells (SKBR3 and BT474), and breast epithelial cells (MCF-10A) were cultured in medium with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were tested every 3 months to ensure no *Mycoplasma* contamination using Mycoplasma Detection Kit (Solarbio, China) and used for no longer than 10 generations.

2.4. Preparation and characterizations of siRNA-loaded NPs

Nanoprecipitation method was used to prepare the siRNA-loaded NPs. In brief, 10 mg of Meo-PEG-*b*-PDPA was first dissolved in 1 mL of dimethylformamide (DMF). In parallel, siRNA (10 μL, 0.1 nmol/μL in deionized water) and G0-C14 (50 μL, 5 mg/mL in DMF) were prepared and mixed with the above Meo-PEG-*b*-PDPA solution. The mixture was added dropwise to RNase-free water (5 mL) with continuous stirring (Eppendorf, 1000 rpm). The obtained NP suspension was then transferred to a dialysis tube (MWCO 100K, MilliporeSigma, USA) for purification *via* centrifugation (Eppendorf, 2800 rpm × 10 min). After three times of PBS washing (3 × 5 mL), the obtained siRNA-loaded NPs denoted NPs(siBCMA-1) were suspended in 1 mL of PBS solution. Dynamic light scattering (DLS, Malvern, USA) was used to examine the NP size and zeta potential. The NP morphology was visualized on a transmission electron microscope (TEM, Tecnai G^2 Spirit BioTWIN). To determine the encapsulation efficiency (EE%) of siRNA, the NPs loading with Cy5-siBCMA-1 were prepared using the method described above and the EE% of siBCMA-1 was determined *via* examining the fluorescence intensity of Cy5-siBCMA-1 in the NPs.

2.5. LncBCMA silencing and over-expression

MDA-MB-231 and MDA-MB-468 cells in 6-well plates (50,000 cells per well) were incubated with the NPs(siBCMA-1), Lipo3k/siBCMA-1, or Lipo3k/siBCMA-2 complexes at a siRNA dose of 30 nmol/L, respectively. Twenty-four hours later, the culture medium was changed with 2 mL of fresh medium with 10% FBS and the cells were further incubated for 48 h. Thereafter, the cells were trypsinized and collected for reverse transcription quantitative polymerase chain reaction (qRT-PCR) and Western blot analysis. For the lncBCMA over-expression, MCF-7 cells in 6-well plates (50,000 cells per well) were incubated with Lipo3k/lncBCMA-expressing plasmid complexes at a plasmid dose of 1 μg/mL. Twenty-four hours later, the culture medium was changed with fresh medium and the cells were further incubated for 48 h. Subsequently, the cells were trypsinized and collected for qRT-PCR and Western blot analysis.

2.6. Proliferation and clone formation

MDA-MB-231 and MDA-MB-468 cells in 6-well plates (20,000 cells per well) were incubated with the NPs(siBCMA-1), Lipo3k/siBCMA-1, or Lipo3k/siBCMA-2 complexes at a siRNA dose of 30 nmol/L, respectively. Twenty-four hours later, the culture medium was removed and the cells were further incubated in 2 mL of fresh medium with 10% FBS. At different time points, the cell viability was determined using AlamarBlue assay (ThermoFisher, USA). To examine the proliferation of cells with lncBCMA over-expression, MCF-7 cells in 6-well plates (20,000 cells per well) were incubated with Lipo3k/lncBCMA-expressing plasmid complexes at a plasmid dose of 1 μg/mL. Twenty-four hours later, the culture medium was replaced with 2 mL of fresh medium with 10% FBS and the cell viability was also examined using AlamarBlue assay. For the clone formation study, the cells in 6-well plates (20,000 cells per well) were treated with the formulas described above for 24 h. After refreshing the medium, the cells were further incubated for one week and the cell clones were observed under an optical microscope (Olympus, Japan).

2.7. Migration and invasion

The lncBCMA was silenced in MDA-MB-231 and MDA-MB-468 cells using the NPs(siBCMA-1), Lipo3k/siBCMA-1, or Lipo3k/siBCMA-2 complexes while lncBCMA was over-expressed in MCF-7 cells using the Lipo3k/lncBCMA-expressing plasmid complexes according to the method described above. Thereafter, the cells were trypsinized and then added to the upper chamber of 24-well Boyden chamber (Corning, USA) with 8M-insert at a density of 2000 cells/well. Twenty-four hours later, the cells attached to the bottom of the upper chamber were imaged and counted to assess the cell migration ability. For the invasion assay, the upper chamber of 24-well Boyden chamber was first coated with 20% Matrigel (Corning, USA) and then the trypsinized cells described above were also added. The cells attached to the bottom of the upper chamber were imaged and counted after 24-h incubation.

2.8. Animals

Healthy BALB/c (normal and nude) mice and NSG (NOD/SCID/IL2Rγ^{null}) mice (female, 4−5 weeks old) were purchased from the Sun Yat-sen University Experimental Animal Center (Guangzhou, China). All *in vivo* studies were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University.

2.9. Evaluation of in vivo metastasis

Luciferase (Luc)-expressing MDA-MB-231 cells with lentivirus-mediated stable lncBCMA silencing by shRNA were established according to the manufacturer's protocols. Subsequently, healthy nude mice ($n = 5$) were received the intravenous injection of the Luc-expressing cells (5×10^6 cells per mouse). At different time points, D-luciferin substrate was intraperitoneally injected to mice (150 mg/kg) and Perkin-Elmer IVIS Lumina III (USA) imaging system was used to observe the tumor progression. The tumor growth was constantly monitored until all the mice died and the lungs were collected for histological analysis.

2.10. Establishment of highly metastatic MDA-MB-231 cells

Healthy nude mice ($n = 3$) received the injection of Luc-expressing MDA-MB-231 cells into foot pad and the cells migrated to the lymph nodes of mouse limbs were monitored

using the above Perki-Elmer imaging system. After the formation of metastatic tumors, the lymph nodes were collected and the tumor cells were isolated for further incubation and proliferation in the medium with 10% FBS.

2.11. *Orthotopic and patient-derived xenograft (PDX) tumor-bearing mouse*

Orthotopic tumor-bearing mouse was constructed by subcutaneous injection with the mixture of DMEM medium containing highly metastatic MDA-MB-231 cells and Matrigel in 1/1 volume ratio (200 μL with a cell density of 1×10^7 cells/mL) into the second pair of mammary fat pads of healthy female nude mice. The tumor-bearing mice were used for the following *in vivo* experiments when the tumor size reached ~100 mm^3. For the PDX model, fresh tumor tissues of TNBC patients were cut into tiny pieces and then subcutaneously transplanted into back region of healthy NSG mice. The tumor-bearing mice were also used for the following *in vivo* experiments when the tumor size reached ~100 mm^3.

2.12. *Inhibition of orthotopic tumor growth and metastasis*

Orthotopic tumor-bearing mice were randomly divided into four groups ($n = 5$) and received intravenous injection of either (i) PBS; (ii) naked siBCMA-1 (1 nmol per mouse); (iii) NPs(siNC) (1 nmol per mouse) or (iv) NPs(siBCMA-1) (1 nmol per mouse). After all administration, mice were continuously monitored for weight loss and tumor growth. Measurement of tumor was taken by measuring perpendicular diameters using a caliper and tumor volume was calculated according to Eq. (1):

$$V = W^2 \times L/2 \quad (1)$$

where W and L are the shortest and longest diameters, respectively. The tumor growth was constantly monitored until all the mice died. The tumors and lungs of all dead mice were harvested and sectioned for histological analysis.

2.13. *Inhibition of PDX tumor growth*

PDX tumor-bearing mice were randomly divided into four groups ($n = 5$) and given an intravenous injection of either (i) PBS; (ii) naked siBCMA-1 (1 nmol per mouse); (iii) NPs(siNC) (1 nmol per mouse) or (iv) NPs(siBCMA-1) (1 nmol per mouse). After all administration, mice were continuously monitored for weight loss and tumor growth. Measurement of tumor was taken by measuring perpendicular diameters using a caliper and tumor volume was calculated according to Eq. (1). At the end of observation, the tumor tissues were harvested and sectioned for histological analysis.

2.14. *Statistical analysis*

The *in vitro* data were presented as mean ± S.D. of three independent experiments. Statistical significance was determined by a two-tailed Student's t test assuming equal variance. A P value < 0.05 is considered statistically significant.

3. Results

3.1. *High lncBCMA expression is correlated with metastasis and poor prognosis of TNBC patients*

To explore the functional lncRNAs that may regulate TNBC metastasis, the tumor samples of breast cancer patients with low metastasis characteristic (HR-positive, $n = 3$) and high metastasis characteristic (TNBC, $n = 3$) (Supporting Information Table S1)[40–42] were collected, and their lncRNA expression profiles were analyzed (Fig. 1A). Additionally, the primary tumors and postoperative metastatic tumors in the lungs of two matched TNBC patients (Supporting Information Table S2) were also collected for lncRNA expression analysis (Fig. 1B). It can be found that five lncRNAs are over-expressed in the primary tumors of TNBC patients and their expression is further up-regulated in the metastatic tumors (Fig. 1C). With this information, we next examined the expression of these five lncRNAs in TNBC cell line (MDA-MB-231) and HR-positive breast cancer cell line (MCF-7). As displayed in Fig. 1D, all five lncRNAs show higher expression in MDA-MB-231 cells. Among them, a new lncRNA is particularly noted as its expression is around 45-fold higher in MDA-MB-231 cells than in MCF-7 cells. The full length of this lncRNA is 637 nt (chr17:19112000−19112636) that refers to ENST00000573866.2 (UCSC database) or ENSG00000262202 (GeneCards). The biological function of this lncRNA in cancer development and progression has not been explored. Therefore, we herein denoted this lncRNA as breast cancer metastasis-associated RNA (lncBCMA) and further examined its expression in other types of breast cancer cells. From the results of Fig. 1E, compared to breast epithelial cells (MCF-10A), HR-positive breast cancer cells (MCF-7 and T47D), and HER2-positive breast cancer cells (SKBR3 and BT474), much higher level of lncBCMA is expressed in TNBC cells (MDA-MB-231 and MDA-MB-468), implying that lncBCMA may participate in TNBC metastasis since clinical observations have clearly demonstrated the stronger metastasis characteristic of TNBC than other subtypes of breast cancer[40–42].

To validate the speculation that lncBCMA may participate in TNBC metastasis, we employed *in situ* hybridization (ISH) to examine lncBCMA expression in the surgically resected tumors of TNBC patients ($n = 10$) with or without postoperative recurrence and metastasis. As shown in Fig. 1F, the patients ($n = 5$) with postoperative recurrence and metastasis within 3 years show higher lncBCMA expression in their tumors than that of the patients with no recurrence ($n = 5$). We further examined lncBCMA expression in the tumor samples of 129 TNBC patients (Supporting Information Table S3). It can be found that high lncBCMA expression is correlated with poor overall survival (OS) (Fig. 1G) and disease-free survival (DFS) (Fig. 1H) of TNBC patients. More importantly, lncBCMA shows much higher expression in the tumors of patients with postoperative recurrence and metastasis ($n = 42$) than that of the patients with no recurrence ($n = 87$) (Fig. 1I). The results described above indicate that high lncBCMA expression is positively correlated with metastasis and poor prognosis of TNBC patients.

3.2. *High lncBCMA expression inhibits eEF1A1 ubiquitination and promotes TNBC cell proliferation, migration, and invasion*

After validation of the correlation between lncBCMA expression and prognosis of TNBC patients, we next investigated the

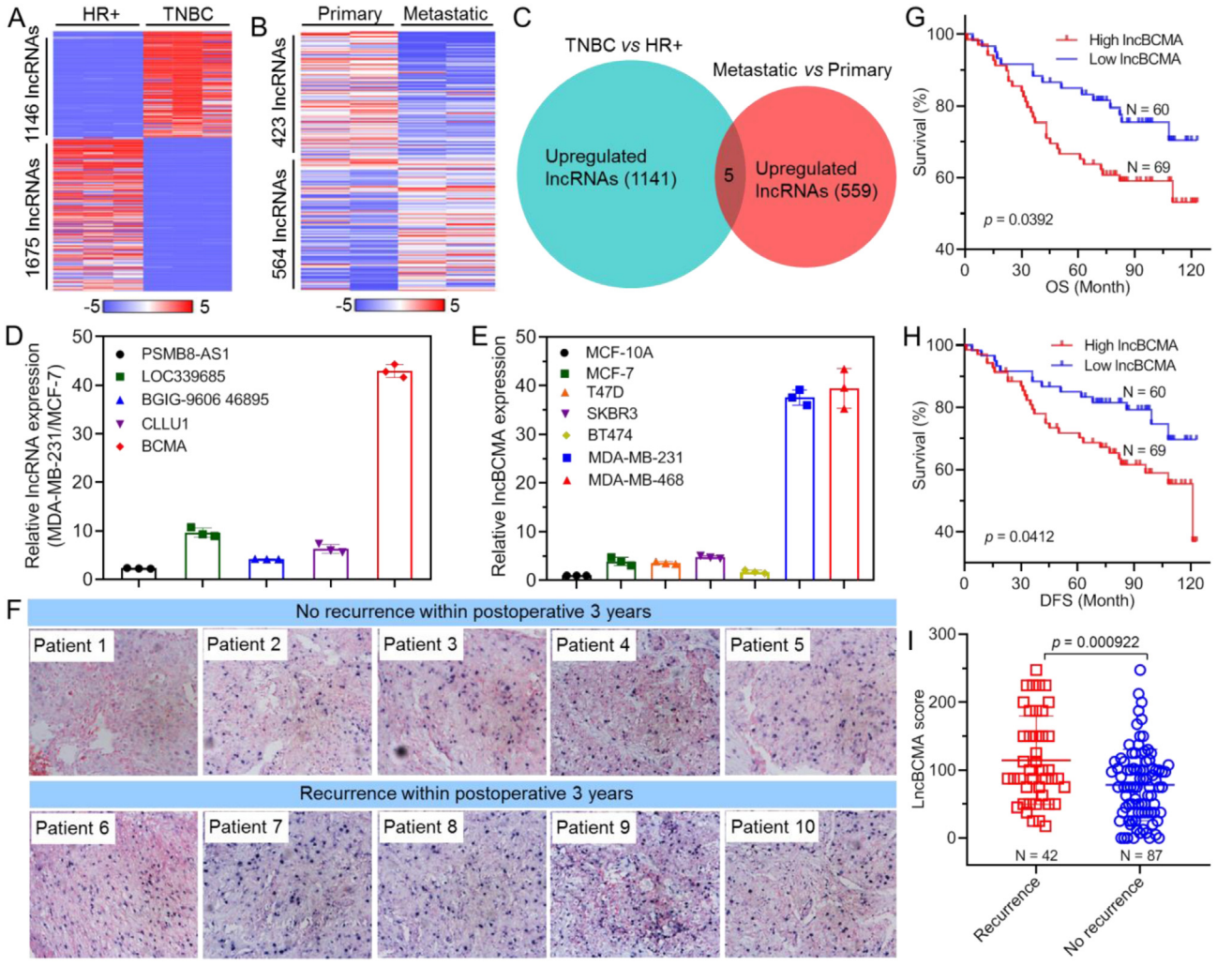

Figure 1 High lncBCMA expression is closely associated with metastasis and poor prognosis of TNBC patients. (A) The heatmap of lncRNAs that are up-regulated or down-regulated over 5-fold in the surgically resected tumors of HR-positive breast cancer patients ($n = 3$) and TNBC patients ($n = 3$). (B) The heatmap of lncRNAs that are up-regulated or down-regulated over 5-fold in the primary tumors and postoperative metastatic tumors in the lungs of two matched TNBC patients. (C) The number of overlapped lncRNAs that are up-regulated in the tumors of TNBC patients. (D) qRT-PCR analysis of the expression level of the overlapped lncRNAs shown in (C) in MDA-MB-231 and MCF-7 cells. Mean ± SD, $n = 3$. (E) qRT-PCR analysis of lncBCMA expression in breast epithelial cells (MCF-10A), HR-positive breast cancer cells (MCF-7 and T47D), HER2-positive breast cancer cells (SKBR3 and BT474), and TNBC cells (MDA-MB-231 and MDA-MB-468). Mean ± SD, $n = 3$. (F) ISH assay of lncBCMA expression in tumor tissues of TNBC patients with ($n = 5$) or without recurrence and metastasis ($n = 5$) within postoperative 3 years. (G, H) OS (G) and DFS (H) of TNBC patients ($n = 129$) with different level of lncBCMA expression in their tumors. (I) LncBCMA score determined by ISH assay in the tumor tissues of TNBC patients with (mean ± SD, $n = 42$) or without postoperative recurrence and metastasis (mean ± SD, $n = 87$).

biological function of this lncRNA in regulating TNBC metastasis. The cellular location of lncBCMA was first examined using nuclear/cytoplasm fractionation (Fig. 2A) and this lncRNA is predominately positioned in the cytoplasm, which is further verified by fluorescence *in situ* hybridization (FISH, Supporting Information Fig. S1). Having determined the intracellular location, we next silenced lncBCMA expression using the Lipo3k/siBCMA complexes (Fig. 2B and C). As shown in Fig. 2D and E, silencing lncBCMA expression in MDA-MB-231 cells could dramatically inhibit their proliferation (Fig. 2D) and clone formation (Fig. 2E). More importantly, the ability of migration and invasion is significantly suppressed after silencing lncBCMA expression in MDA-MB-231 cells (Fig. 2F). The similar tendency could be also found after silencing lncBCMA expression in MDA-MB-468 cells (Supporting Information Fig. S2). In contrast, up-regulating lncBCMA expression using the Lipo3k/lncBCMA-expressing plasmid complexes (Fig. 2G) could significantly enhance the proliferation (Fig. 2H), colony formation (Fig. 2I), migration, and invasion (Fig. 2J) of MCF-7 cells. All these results strongly demonstrate that lncBCMA plays a crucial role in TNBC growth and metastasis. To further validate this statement, Luc-expressing MDA-MB-231 cells with lentivirus-mediated stable lncBCMA silencing by shRNA were intravenously injected into healthy mice to evaluate their ability to form metastatic nodes *in vivo*. It can be found that silencing lncBCMA expression could significantly inhibit the formation of metastatic nodes and their

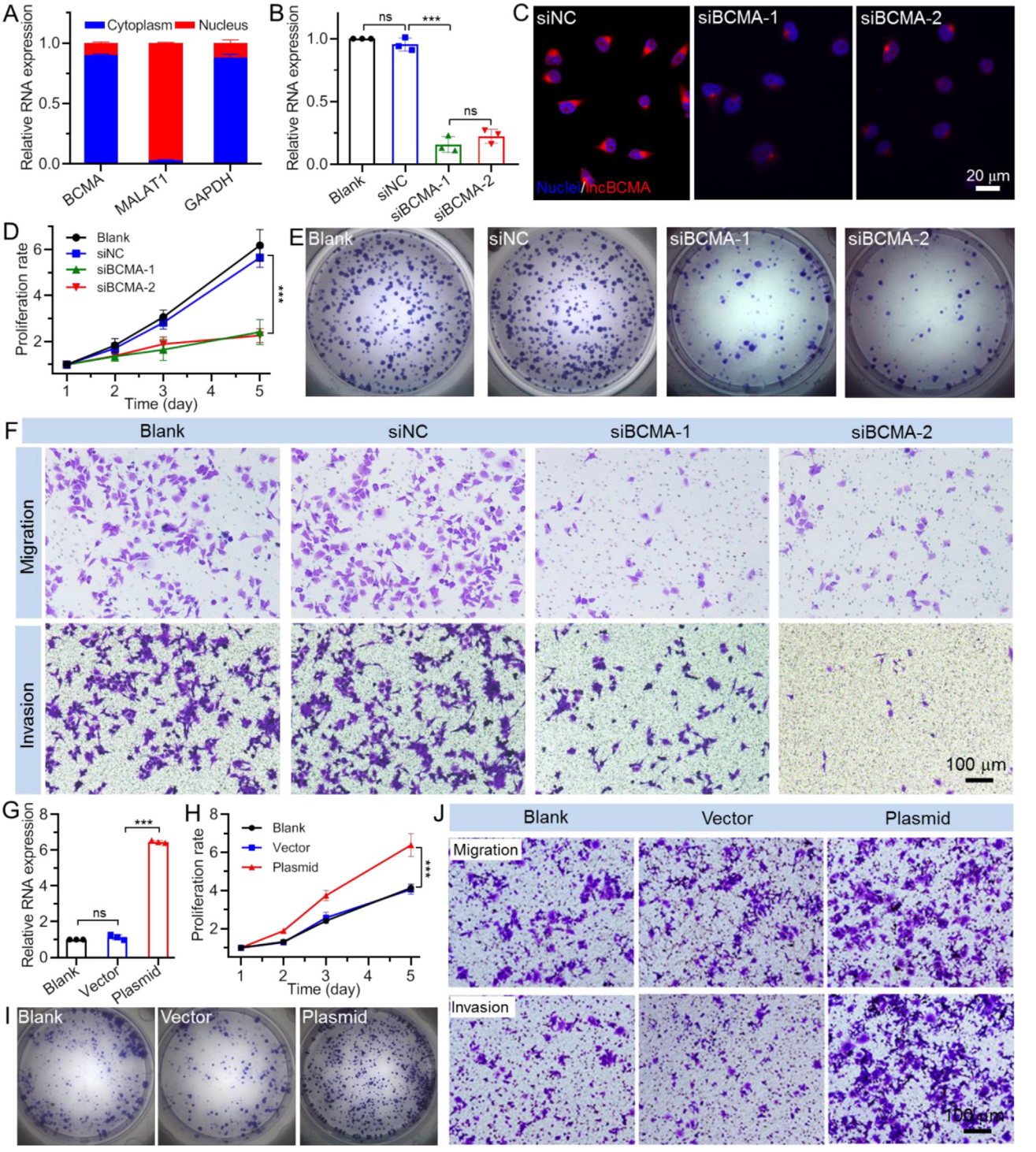

Figure 2 LncBCMA is predominately localized in the cytoplasm and high lncBCMA expression promotes TNBC cell proliferation, migration, and invasion. (A) The level of lncBCMA expression in the cytoplasm and nuclei of MDA-MB-231 cells determined by nuclear/cytoplasm fractionation. Mean ± SD, $n = 3$. (B, C) qRT-PCR (B, mean ± SD, $n = 3$, $^{***}P < 0.001$, ns, no significance.) and FISH analysis (C) of lncBCMA expression in MDA-MB-231 cells treated with the complexes of Lipo3k and siBCMA at a siRNA dose of 30 nmol/L. Scale bar, 20 μm. (D–F) Proliferation (D, mean ± SD, $n = 3$), colony formation (E), and migration and invasion (F, scale bar, 100 μm) of MDA-MB-231 cells treated with the complexes of Lipo3k and siBCMA at a siRNA dose of 30 nmol/L. (G) qRT-PCR analysis of lncBCMA expression in MCF-7 cells treated with the complexes of Lipo3k and lncBCMA-expressing plasmid at a plasmid dose of 1 μg/mL. Mean ± SD, $n = 3$. (H–J) Proliferation (H, mean ± SD, $n = 3$), clone formation (I), and migration and invasion (J, scale bar, 100 μm) of MCF-7 cells treated with the complexes of Lipo3k and lncBCMA-expressing plasmid at a plasmid dose of 1 μg/mL.

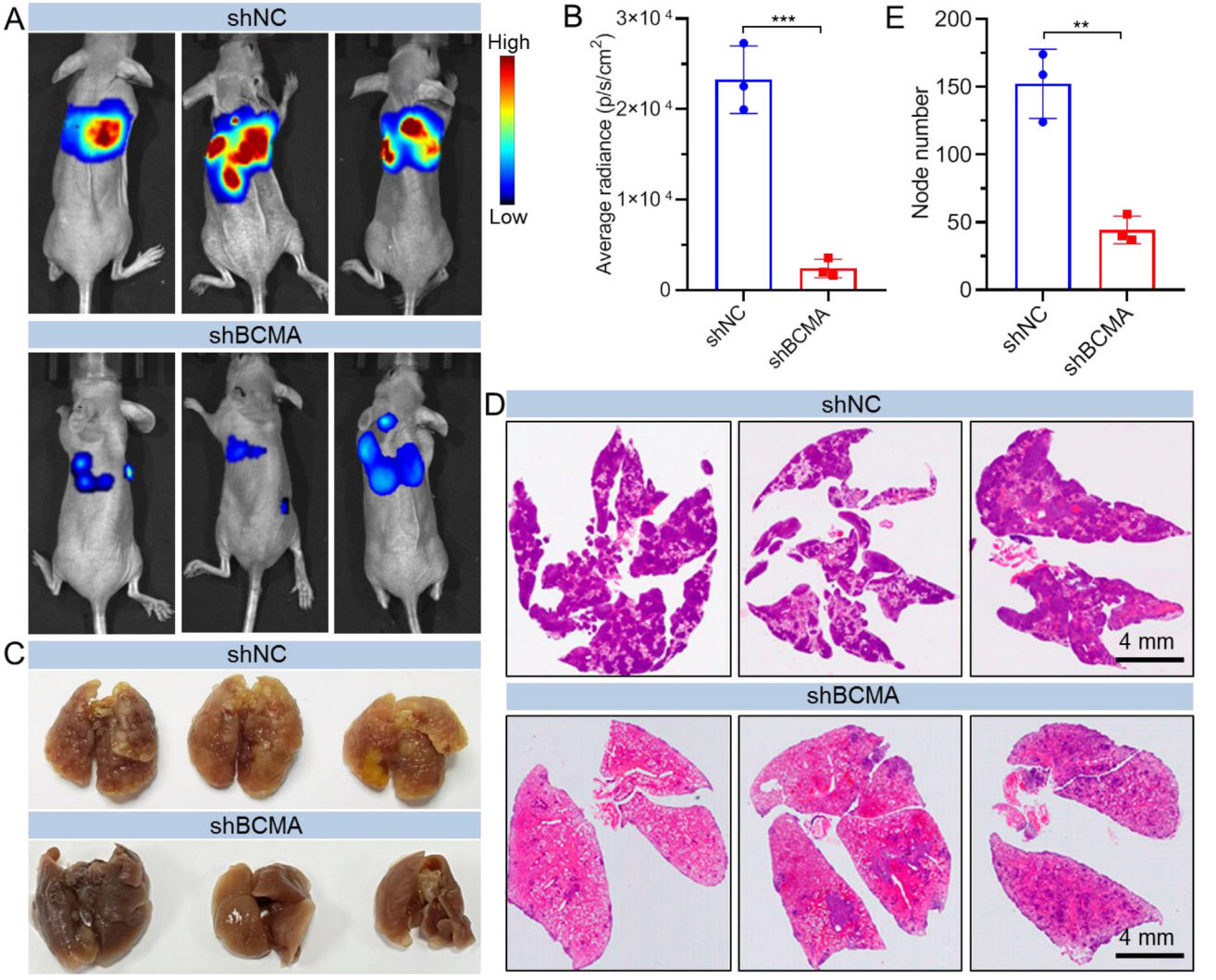

Figure 3 High lncBCMA expression promotes TNBC growth and metastasis. (A) Bioluminescence images of mice at Day 16 post intravenous injection of Luc-MDA-MB-231 cells with lentivirus-mediated stable lncBCMA silencing by shRNA. (B) Average radiance of tumor burden determined by bioluminescence images shown in (A). Mean ± SD, $n = 3$, $^{***}P < 0.001$. (C, D) Photo images (C) and hematoxylin-eosin (H&E) staining (D) of collected lungs of mice at Day 16 post intravenous injection of Luc-MDA-MB-231 cells with lentivirus-mediated stable lncBCMA silencing by shRNA. Scale bar, 4 mm. (E) Number of metastatic nodes in the lungs counted from the H&E staining shown in (D). Mean ± SD, $n = 3$, $^{**}P < 0.01$.

growth, as demonstrated by the weak bioluminescence (Fig. 3A and B), less metastatic node number (Fig. 3C–E and Supporting Information Fig. S3), and prolonged survival time (Supporting Information Fig. S4).

Having confirmed the biological function of lncBCMA to regulate TNBC growth and metastasis, we next explored its intrinsic regulatory mechanism. The proteins binding to lncBCMA were first examined by RNA pulldown as protein–lncRNA interaction is an important way for lncRNAs to regulate cellular behaviors[15–17]. From the image shown in Fig. 4A, one specific protein band (50–70 kDa) could be observed in the lncBCMA pulldown complexes. Subsequent mass spectrometry (MS, Supporting Information Fig. S5) detection indicates that eEF1A1 is the specific protein interacting with lncBCMA. The specific binding of lncBCMA to eEF1A1 is further validated by RNA pulldown followed by Western blot analysis (Fig. 4B) and RNA immunoprecipitation (RIP) assay (Fig. 4C). To identify the binding site of lncBCMA to eEF1A1, truncated lncBCMA with different length was used for RNA pulldown assay and the sequence nt 160–318 of lncBCMA is determined as the eEF1A1-binding site (Supporting Information Fig. S6). It is known that eEF1A1 is a classic and highly conserved GTP-binding protein in eukaryotic cells that widely involves various cellular behaviors (*e.g.*, proliferation, migration, and invasion) *via* regulating protein translation elongation and cytoskeleton formation[43–46]. Numerous researches have revealed that eEF1A1 is aberrantly up-regulated in many cancer types and high eEF1A1 expression could promote tumor cell proliferation, invasion, and migration[27–31]. We also examined the influence of eEF1A1 on the behaviors of MDA-MB-231 cells *via* using siRNA to silence eEF1A1 expression. The results show that silencing eEF1A1 expression in MDA-MB-231 cells could significantly inhibit their proliferation, migration, and invasion (Supporting Information Fig. S7). With this information, we next validated whether lncBCMA promotes TNBC

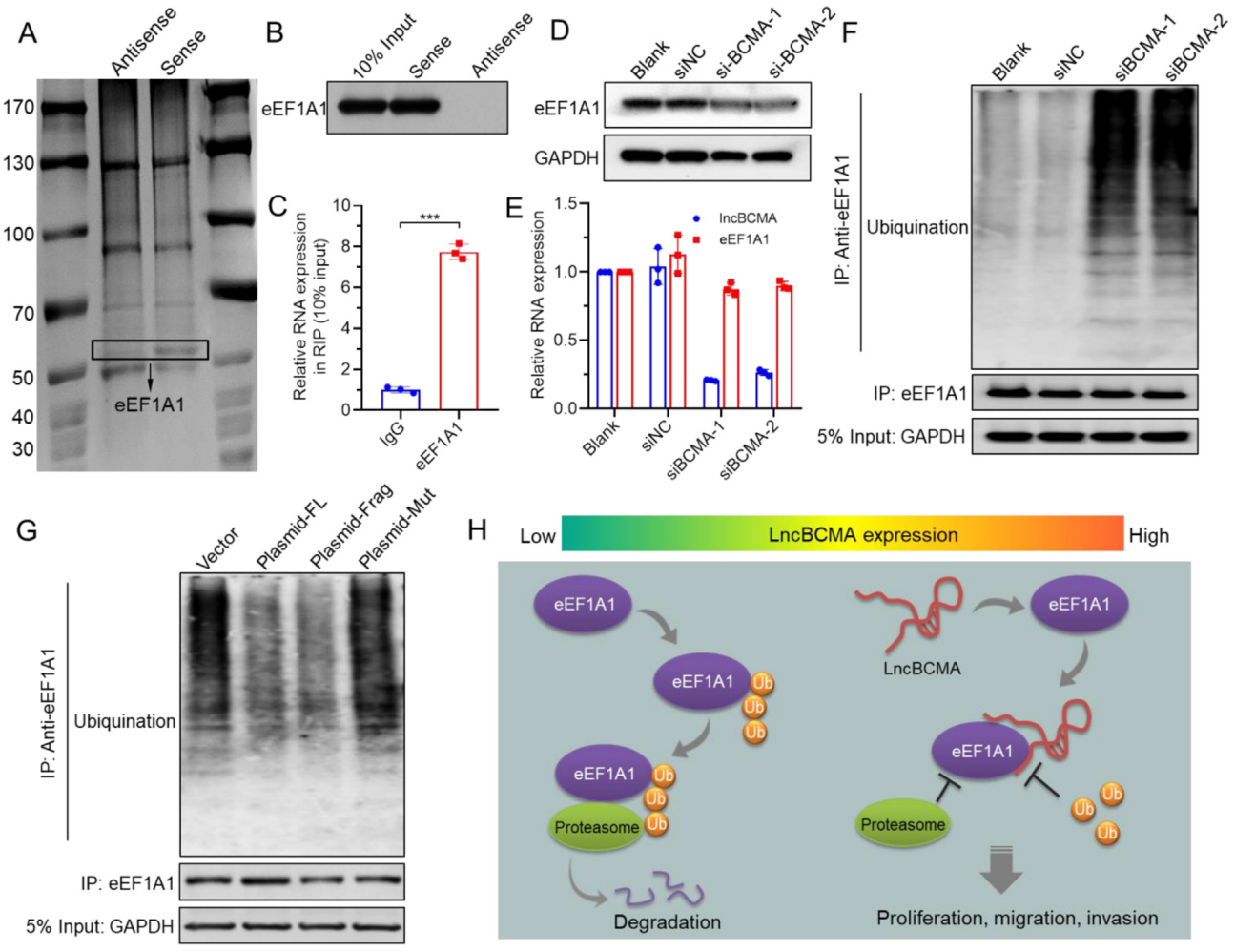

Figure 4 High lncBCMA expression promotes TNBC cell proliferation, migration, and invasion *via* antagonizing eEF1A1 ubiquitination. (A, B) The silver staining (A) and Western blot analysis (B) of RNA-protein binding complexes after RNA pulldown of lncBCMA in MDA-MB-231 cells. (C) RIP of eEF1A1 in MDA-MB-231 cells. LncBCMA was retrieved by eEF1A1 or IgG and then examined by qRT-PCR. Mean ± SD, $n = 3$, $^{***}P < 0.001$. (D, E) Western blot (D) and qRT-PCR analysis (E, mean ± SD, $n = 3$) of the protein and mRNA level of eEF1A1 in MDA-MBA-231 cells treated with the complexes of Lipo3k and siBCMA at a siRNA dose of 30 nmol/L. (F) Western blot analysis of the level of eEF1A1 ubiquitination in MDA-MB-231 cells treated with the complexes of Lipo3k and siBCMA at a siRNA dose of 30 nmol/L. (G) Western blot analysis of the level of eEF1A1 ubiquitination in MCF-7 cells treated with the complexes of Lipo3k and lncBCMA-expressing plasmid (Plasmid-FL), the plasmid of eEF1A1-binding sequence (Plasmid-Frag), or the lncBCMA-expressing plasmid with the mutated eEF1A1-binding sequence (Plasmid-Mut) at a plasmid dose of 1 μg/mL. (H) Schematic illustration of the molecular mechanism of lncBCMA to promote TNBC cell proliferation, migration, and invasion *via* antagonizing eEF1A1 ubiquitination.

growth and metastasis *via* regulating eEF1A1 expression, we examined eEF1A1 expression in MDA-MB-231 cells after silencing lncBCMA expression. As shown in Fig. 4D and E, silencing lncBCMA expression could significantly down-regulate the protein level of eEF1A1 (Fig. 4D), but does not affect the mRNA level of eEF1A1 (Fig. 4E). This result suggests that lncBCMA could regulate the eEF1A1 expression at a post transcriptional level such as ubiquitination, as previous studies have clearly demonstrated that eEF1A1 can be degraded *via* the classic ubiquitin−proteasome pathway[27]. To verify this speculation, we examined the level of eEF1A1 ubiquitination in MDA-MB-231 cells. As shown in Fig. 4F, much higher level of eEF1A1 ubiquitination could be observed after silencing lncBCMA expression. In contrast, up-regulating the full length or eEF1A1-binding site (sequence nt 160−318) of lncBCMA in MCF-7 cells could significantly suppress eEF1A1 ubiquitination (Fig. 4G). Nevertheless, if mutating the eEF1A1-binding site of lncBCMA and then up-regulating its expression, the level of eEF1A1 ubiquitination is still very high in MCF-7 cells (Fig. 4G). All these results indicate that lncBCMA-1 could stabilize eEF1A1 protein expression by antagonizing its ubiquitination to promote the proliferation, migration, and invasion of TNBC cells (Fig. 4H).

3.3. Targeted inhibition of lncBCMA expression suppresses TNBC cell proliferation, migration, and invasion

Based on the important biological function of lncBCMA, targeted inhibition of its expression could be expected to accomplish effective TNBC therapy. Nucleic acids such siRNA could be used to achieve this goal due to their unique characteristic of silencing

any target genes, especially the "undruggable" portions of human genome[47]. However, naked siRNA is susceptible to serum nucleases and could be rapidly cleared by renal filtration. In addition, endosomal entrapment is also a key barrier to restrict the therapeutic effect of nucleic acids[32,34,48,49]. To address these issues, we herein constructed an endosomal pH-responsive NP platform for *in vivo* siBCMA delivery. This nanoplatform is composed of the polymer Meo-PEG-*b*-PDPA (pK_a ~6.34) (Supporting Information Figs. S8−S10)[37,38] and cationic compound G0-C14 (Supporting Information Fig. S11). As shown in Fig. 5A, this amphiphilic polymer could self-assemble into stable NPs with hydrophilic PEG shells and hydrophobic PDPA cores that encapsulate the siRNA/G0-C14 complexes formed *via* electrostatic interaction[50]. Herein, the sequence 1 of siBCMA (*i.e.*, siBCMA-1) was encapsulated into the NPs as it has the similar silencing ability as sequence 2 of siBCMA (Fig. 2B). As shown in Fig. 5B, the spherical NPs (*i.e.*, NPs(siBCMA-1)) are well-dispersed and show an average size of ~70 nm. For these

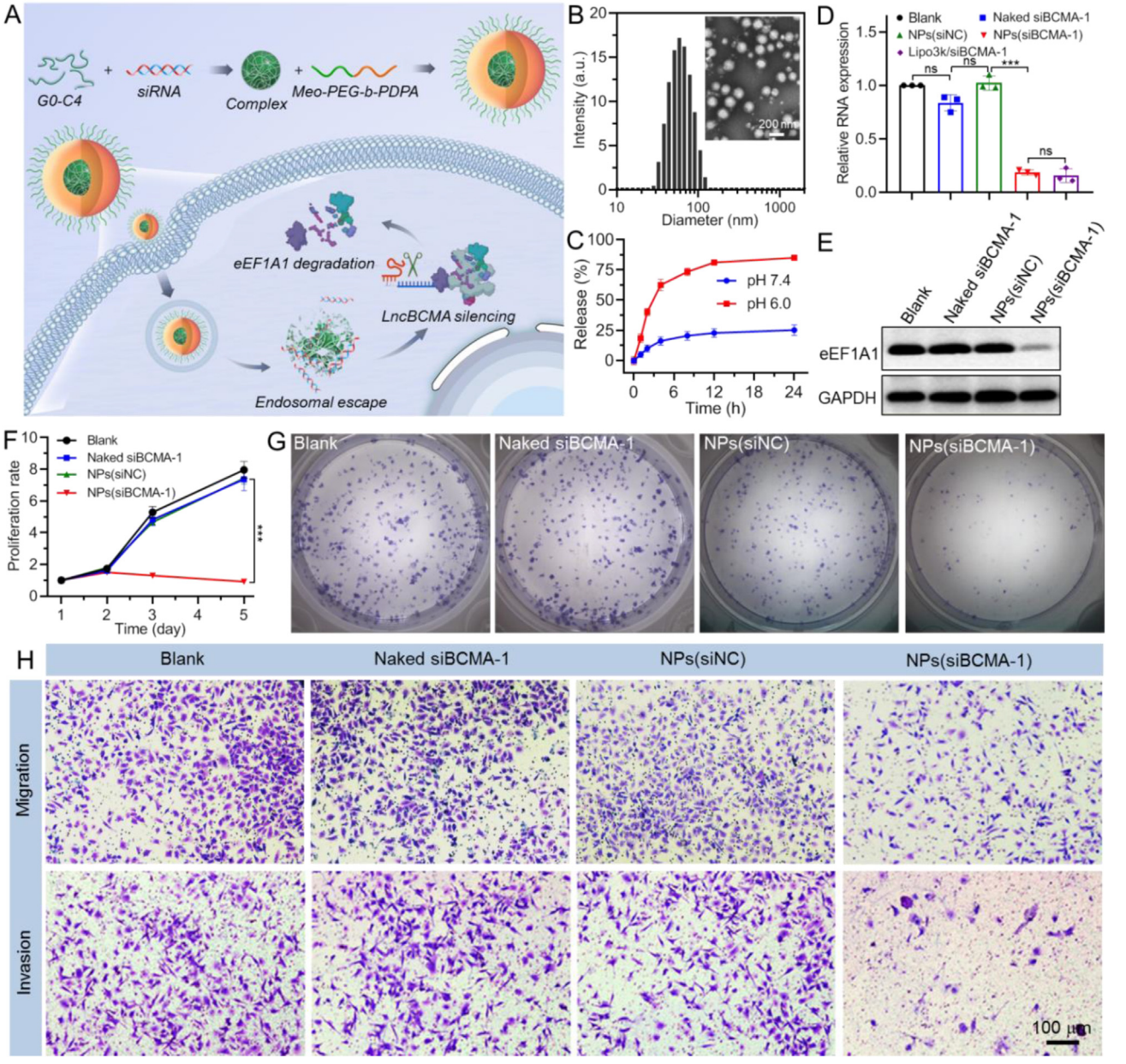

Figure 5 NPs-mediated lncBCMA silencing suppresses TNBC cell proliferation, migration, and invasion. (A) Schematic illustration of the NPs(siBCMA-1) made with the endosomal pH-responsive polymer Meo-PEG-*b*-PDPA and cationic lipid G0-C14, and this nanoplatform to use its endosomal pH response to improve the endosomal escape of encapsulated siRNA for efficiently silencing lncBCMA expression and promoting eEF1A1 ubiquitination. (B) Size distribution and morphology of the NPs(siBCMA-1) in aqueous solution. Scale bar, 200 nm. (C) The profile of siBCMA release from the NPs(siBCMA-1) incubated in PBS solution at different pHs. Mean ± SD, $n = 3$. (D, E) qRT-PCR analysis of lncBCMA expression (D, mean ± SD, $n = 3$) and Western blot analysis of eEF1A1 expression (E) in MDA-MBA-231 cells treated with the NPs(siBCMA-1) at a siRNA dose of 30 nmol/L ns, no significance; ***$P < 0.001$. (F−H) Proliferation (F, mean ± SD, $n = 3$), clone formation (G), and migration and invasion (H, scale bar, 100 μm) of MDA-MB-231 cells treated with the NPs(siBCMA-1) at a siRNA dose of 30 nmol/L.

siRNA-loaded NPs, they show a good stability (Supporting Information Fig. S12) and strong ability to protect siRNA from degradation by RNase (Supporting Information Fig. S13) at a physiological pH of 7.4. In comparison, because the PDPA polymer could be protonated at an acidic pH (*e.g.*, pH 6.0) below its pK_a, the NPs could disassemble into amorphous aggregates (Supporting Information Fig. S14)[51], thus leading to fast siRNA release (Fig. 5C). More importantly, because the protonated PDPA polymer could induce "proton sponge" effect to improve the endosomal escape of encapsulated siRNA into the cytoplasm (Supporting Information Fig. S15)[37,38], lncBCMA expression in MDA-MB-231 cells could be down-regulated by round 80% after treatment with the NPs(siBCMA-1) at a siRNA dose of 30 nmol/L (Fig. 5D and Supporting Information Fig. S16). Due to this lncBCMA silencing, eEF1A1 expression is dramatically inhibited (Fig. 5E), thereby leading to the weakened proliferation (Fig. 5F), clone formation (Fig. 5G), and migration and invasion (Fig. 5H) of MDA-MB-231 cells.

3.4. NPs-mediated systemic siBCMA-1 delivery inhibits TNBC growth and metastasis

We finally assessed the ability of NPs(siBCMA-1) to silence lncBCMA expression *in vivo* and inhibit TNBC growth and metastasis using the MDA-MB-231 orthotopic tumor model[52,53]. As shown in Fig. 6A, after intravenous injection of NPs(siBCMA-1) into the orthotopic tumor-bearing mice, due to their long blood circulation and high tumor accumulation (Supporting Information

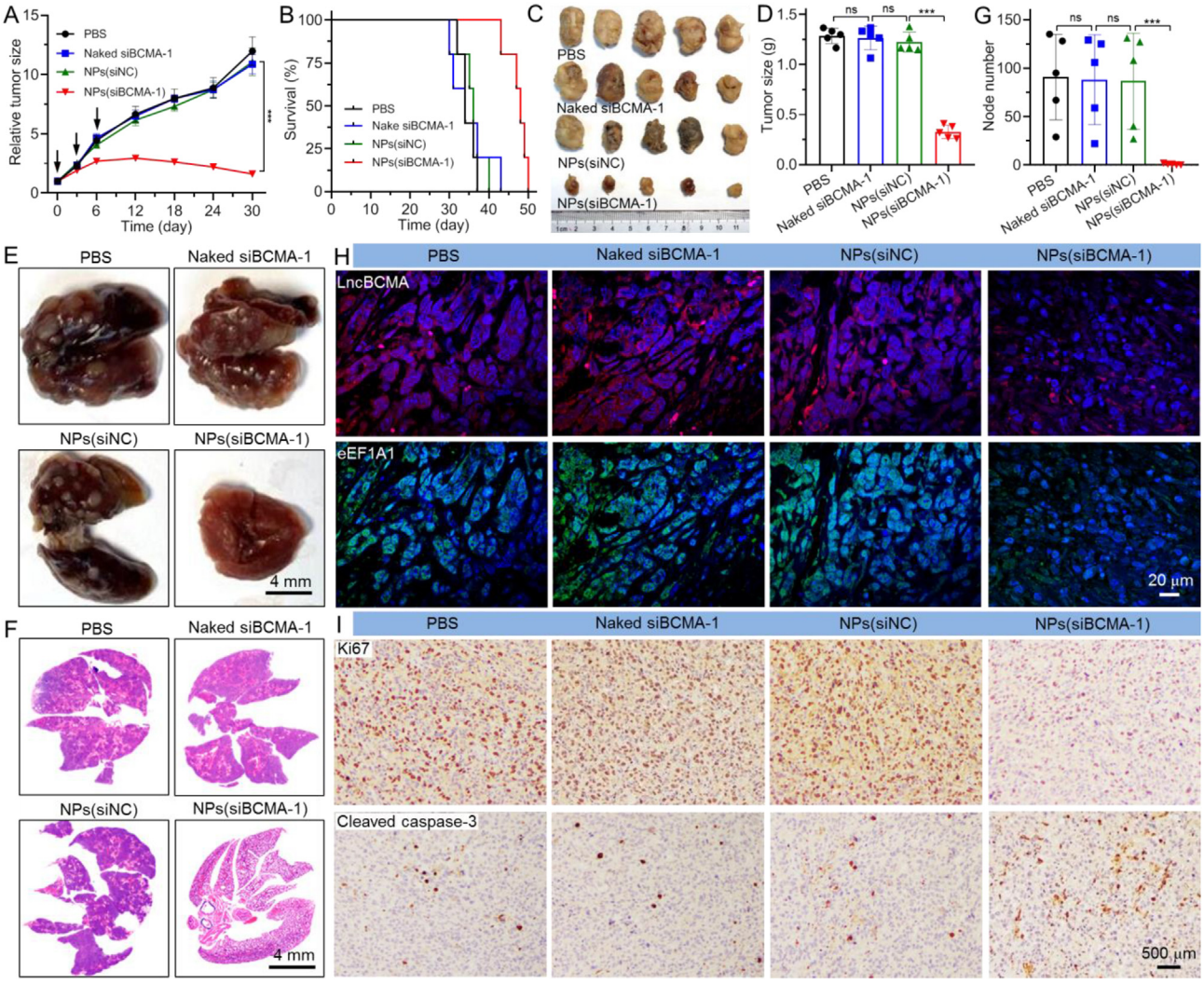

Figure 6 NPs-mediated lncBCMA silencing inhibits TNBC growth and metastasis in orthotopic tumor model. (A) Tumor growth of MDA-MB-231 orthotopic tumor-bearing mice treated with PBS, naked siBCMA-1, NPs(siNC), and NPs(siBCMA-1). The intravenous injections are indicated by black arrows. Mean ± SD, $n = 5$. (B−D) Survival rate (B), photo image (C) and weight (D, mean ± SD, $n = 5$) of collected tumors of MDA-MB-231 orthotopic tumor-bearing mice after systemic treatment with the formulas shown in (A). ns, no significance; $^{***}P < 0.001$. (E, F) Representative photo images (E) and H&E staining (F) of collected lungs of MDA-MB-231 orthotopic tumor-bearing mice after systemic treatment with the formulas shown in (A). Scale bar, 4 mm. (G) Number of metastatic nodes counted from H&E staining of the lungs of MDA-MB-231 orthotopic tumor-bearing mice after treatment with the formulas shown in (A). Mean ± SD, $n = 5$. (H) The expression of lncBCMA determined by FISH and eEF1A1 determined by IF analysis of the collected tumors shown in (C). Scale bar, 20 μm. (I) The expression of Ki67 and cleaved caspase-3 determined by IHC analysis of the collected tumors shown in (C). Scale bar, 500 μm.

Fig. S17)[54], the NPs(siBCMA-1) could dramatically suppress the tumor growth. Within an evaluation period of 30 days, the tumors increase by less than 2-fold in their size. However, for the mice treated with PBS, naked siBCMA-1 or the NPs loading scrambled siRNA denoted NPs(siNC), the average tumor size increases by more than 12-fold (Supporting Information Fig. S18). In addition, within a long-term evaluation period of 50 days, the NPs(siBCMA-1) could dramatically prolong the mouse survival (Fig. 6B) and effectively inhibit the tumor growth (Fig. 6C and D). More importantly, the administration of NPs(siBCMA-1) could significantly suppress the tumor metastasis in the lung (Fig. 6E−G and Supporting Information Fig. S19). In comparison with the mice received the treatment with PBS, naked siBCMA-1 or NPs(siNC), nearly no metastatic tumor nodes could be detected in the lungs of mice treated with the NPs(siBCMA-1). The histological analysis of tumor slides (Fig. 6H and I) also demonstrates that the NPs(siBCMA-1) are the most effective in silencing lncBCMA expression, suppressing eEF1A1 expression, and inhibiting tumor growth indicated by less proliferation (Ki67) and more apoptosis (cleaved caspase-3).

We also established the PDX tumor model to further evaluate the therapeutic effect of NPs(siBCMA-1). As shown in

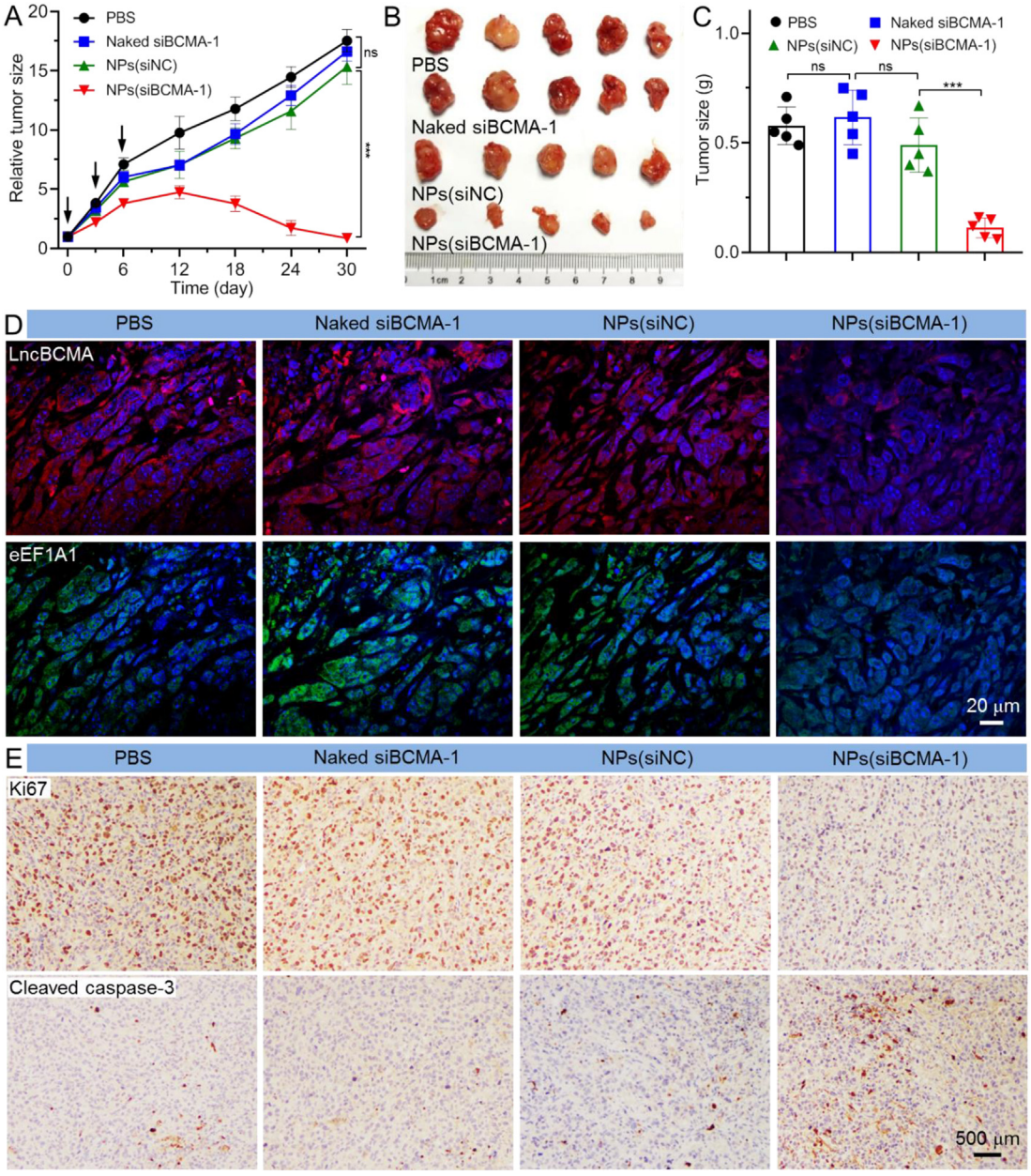

Figure 7 NPs-mediated lncBCMA silencing inhibits TNBC growth and metastasis in PDX tumor model. (A) Tumor growth of PDX tumor-bearing mice treated with PBS, naked siBCMA-1, NPs(siNC), and NPs(siBCMA-1). The intravenous injections are indicated by black arrows. Mean ± SD, $n = 5$. (B, C) Photo image (B) and weight (C, mean ± SD, $n = 5$) of collected tumors of PDX tumor-bearing mice after systemic treatment with the formulas shown in (A). ns, no significance; $^{***}P < 0.001$. (D) The expression of lncBCMA determined by FISH and eEF1A1 determined by IF analysis of the collected tumors shown in (B). Scale bar, 20 μm. (E) The expression of Ki67 and cleaved caspase-3 determined by IHC analysis of the collected tumors shown in (B). Scale bar, 500 μm.

Fig. 7A–C, the tumor growth is significantly inhibited after intravenous injection of NPs(siBCMA-1) into the PDX tumor-bearing mice. Within an evaluation period of 30 days, the average tumor size increases by around 1.5-fold. By contrast, the tumors of mice treated with PBS, naked siBCMA-1 or NPs(siNC) increase around 15-fold (Supporting Information Fig. S20). Similar as the results of orthotopic tumors, histological analysis also demonstrates that the NPs(siBCMA-1) are the most effective in suppressing the expression of lncBCMA and eEF1A1 in PDX tumor tissues (Fig. 7D) and inhibiting the growth of PDX tumors (Fig. 7E). Notably, the administration of NPs(siBCMA-1) does not influence the mouse weight (Supporting Information Fig. S21) and no apparent histological change could be observed in the tissues of major organs (Supporting Information Fig. S22), implying the low *in vivo* toxicity of NPs(siBCMA-1). This result is further verified by blood routine analysis (Supporting Information Fig. S23), in which the main parameters of liver and kidney function are in the normal range.

4. Discussion

Cancer metastasis is a complex process involving multiple steps (*e.g.*, detachment of cancer cells from the primary tumor, invasion and traveling to different sites, and proliferation) that are closely associated with multiple gene alternations[1–3,9,12]. As an important and highly conserved GTP-binding protein, eEF1A1 is a component of polypeptide translation machinery, which can bind GTP and deliver aminoacylated-tRNA to the mRNA-programmed ribosome during protein translation elongation process[43–45]. Emerging evidence has shown that eEF1A1 is aberrantly up-regulated in many types of cancers (*e.g.*, breast cancer, liver cancer, colorectal cancer, pancreatic cancer, etc.) and high eEF1A1 expression could promote tumor growth and metastasis *via* multiple regulatory mechanisms, including promoting cytoskeletal organization, suppressing p53 activity, and alleviating endoplasmic reticulum (ER) stress[27–31]. However, as one type of GTP-binding proteins that are hard to be targeted, there are no clinically available inhibitors for eEF1A1. Herein, we discovered a completely new lncRNA (*i.e.*, lncBCMA) and molecular mechanism study shows that high lncBCMA expression could enhance eEF1A1 stability *via* antagonizing its ubiquitination to promote the proliferation, migration, and invasion of TNBC cells. Therefore, targeted inhibition of lncBCMA expression could be considered as an effective strategy for TNBC therapy. In addition, directly targeting lncBCMA may also avoid side effects due to the high tissue-specificity of lncRNAs[26,55].

LncRNAs are widely involved in cancer development and progression[13–17]. However, their clinical translation is far from expected due to the difficulty in regulating lncRNA expression *in vivo*. RNA interference (RNAi) technology has been considered as an effective strategy as it can silence any target genes[47]. In particular, with the rapid development of nanotechnology, NPs-mediated RNAi has achieved great advances in recent years[56–61]. We had previously developed a series of NPs-mediated siRNA delivery systems for cancer therapy[37–39,62,63]. These NPs could respond to various biological stimuli (*e.g.*, pH, hypoxia, and redox) to enhance *in vivo* gene silencing. Particularly, the endosomal pH-responsive NPs could use their pH-responsive characteristic to dramatically enhance endosomal escape and therapeutic effect of various biomacromolecules (*e.g.*, siRNA, mRNA, and protein)[37,38]. Therefore, we herein employed this type of nanoplatform for targeted inhibition of lncBCMA expression and evaluated its ability to suppress TNBC growth and metastasis. Our experimental results show that this RNAi nanoplatform could efficiently silence lncBCMA expression *in vivo* and significantly inhibit TNBC tumor growth and metastasis.

5. Conclusions

In summary, we have identified a new functional lncRNA (*i.e.*, lncBCMA) that plays an important role in regulating TNBC tumor growth and metastasis. Molecular mechanism study reveals that this lncRNA could stabilize eEF1A1 expression *via* antagonizing its ubiquitination to promote the proliferation, migration, and invasion of TNBC cells. Using the endosomal pH-responsive NPs to *in vivo* deliver siBCMA could effectively silence lncBCMA expression and promote eEF1A1 ubiquitination, thereby leading to a dramatic suppression of tumor growth and metastasis. The lncBCMA could be used to predict the prognosis of TNBC patients and the NPs-mediated lncBCMA silencing could be a promising strategy for the treatment of metastatic TNBC.

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Author contributions

Ke Yang, Lei Xu, Yan Nie, Herui Yao, and Xiaoding Xu conceived and designed the experiments; Ke Yang, Lei Xu, Ying Xu, and Qian Shen performed the experiments; Ke Yang, Ying Xu, Qian Shen, and Yunfang Yu performed bioinformatics analyses; Ke Yang, Ying Xu, and Tao Qin collected tissue samples; Ke Yang, Lei Xu, Yan Nie, Herui Yao, and Xiaoding Xu analyzed the data and co-wrote the manuscript. Ke Yang, Lei Xu, and Ying Xu contributed equally to this work. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2022.12.004.

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