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Original Article

Overexpression of Adenovirus E1A Reverses Transforming Growth Factor-β-induced Epithelial-mesenchymal Transition in Human Esophageal Cancer Cells

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The epithelial-mesenchymal transition (EMT), a normal biological process by which epithelial cells acquire a mesenchymal phenotype, is associated with migration, metastasis, and chemoresistance in cancer cells, and with poor prognosis in patients with esophageal cancer. However, therapeutic strategies to inhibit EMT in tumor environments remain elusive. Here, we show the therapeutic potential of telomerase-specific replication-competent oncolytic adenovirus OBP-301 in human esophageal cancer TE4 and TE6 cells with an EMT phenotype. Transforming growth factor-β (TGF-β) administration induced the EMT phenotype with spindle-shaped morphology, upregulation of mesenchymal markers and EMT transcription factors, migration, and chemoresistance in TE4 and TE6 cells. OBP-301 significantly inhibited the EMT phenotype via E1 accumulation. EMT cancer cells were susceptible to OBP-301 via massive autophagy induction. OBP-301 suppressed tumor growth and lymph node metastasis of TE4 cells co-inoculated with TGF-β-secreting fibroblasts. Our results suggest that OBP-301 inhibits the TGF-β-induced EMT phenotype in human esophageal cancer cells. OBP-301-mediated E1A overexpression is a promising antitumor strategy to inhibit EMT-mediated esophageal cancer progression.

Key words: esophageal cancer, EMT, TGF-β, oncolytic adenovirus, E1A

The epithelial-mesenchymal transition (EMT) is a biological process by which epithelial cells lose their epithelial characteristics, acquire mesenchymal properties, and become motile during embryonic development and tissue repair [1, 2]. In epithelial cancer cells, the mesenchymal phenotype resulting from EMT introduces malignant properties such as migration, invasion, metastasis, and drug resistance [3]. EMT features in tumor tissues are highly associated with tumor metastasis and poor prognosis in patients with gastrointestinal cancers, including cancer of the esophagus [4], stomach [5], colon [6], and pancreas [7]. In esophageal cancer in particular, EMT has been shown to induce malignant potential, including invasion [8], angiogenesis [9], and chemoresistance [10].

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The EMT process is mainly induced through activation of EMT-promoting transcription factors such as the Snail and ZEB families [3, 11] and is an attractive therapeutic target for inhibiting tumor progression in cancer patients.

Recent evidence has suggested the involvement of the tumor microenvironment in tumor progression [12]. Normal stromal cells surrounding cancerous lesions have been shown to produce a variety of stimuli, including transforming growth factor-β (TGF-β), during tumor progression [13]. TGF-β is the main stimulating factor that induces the EMT process via activation of the EMT transcription factors of the Snail and ZEB families [14]. TGF-β-neutralizing antibody and small molecule inhibitors of TGF-β or TGF-β receptors have been developed as anti-EMT agents to disrupt the TGF-β signaling pathway [13]. However, none of these anti-EMT therapies has demonstrated much potency. Therefore, novel therapeutic strategies to inhibit EMT and EMT-related tumor progression are highly sought-after.

Oncolytic virotherapy is a promising antitumor strategy for the induction of tumor-specific cell death [15]. We previously developed a telomerase-specific replication-competent oncolytic adenovirus, OBP-301, which drives the expression of viral *E1A* and *E1B* genes under the control of the human telomerase reverse transcriptase (*hTERT*) promoter for tumor-specific virus replication [16]. OBP-301 has an antitumor effect against a variety of human cancer cells with telomerase activities [16, 17]. OBP-301 efficiently eradicates highly malignant gastric cancer cells with stem-like properties [18]. Moreover, OBP-301 exhibits an inhibitory effect in the development of lymph node metastasis [19, 20]. As EMT has been shown to play a crucial role in the development of malignant phenotypes with stemness and metastatic properties in various types of cancer [21-23], these findings suggest the therapeutic potential of OBP-301 against cancer cells undergoing EMT. However, whether OBP-301 affects the EMT program and EMT-related malignant phenotypes in human cancer cells remains unclear.

Adenoviral E1A is a multifunctional protein that plays a tumor-suppressive role in human cancer cells. Overexpression of E1A inhibits the proliferation of human tumor cells [24], and E1A protein has the therapeutic potential to sensitize human cancer cells to various chemotherapeutic agents [25]. Interestingly, overexpression of E1A induces epithelial characteristics in mesenchymal malignant tumor cells [24, 26], suggesting that it reverses the EMT-related malignant phenotype in epithelial cells. As OBP-301 induces the expression of E1A protein in virus-infected cancer cells in a telomerase-dependent manner, OBP-301-mediated E1A overexpression may have the potential to suppress the EMT-related malignant phenotype in human cancer cells.

In the present study, we investigated the therapeutic potential of telomerase-specific replication-competent oncolytic adenovirus OBP-301 against the TGF-β-induced EMT phenotype of human esophageal cancer TE4 and TE6 cells. First, the expressions of epithelial and mesenchymal markers and EMT-related transcription factors induced by TGF-β were assessed in TE4 and TE6 cells. Then, the effect of OBP-301 on the EMT-related malignant phenotype was evaluated via the expression of EMT-related markers and a cell migration assay. The underlying mechanism of OBP-301-mediated suppression of EMT cells was analyzed with respect to autophagy. Moreover, an orthotopic TE4 xenograft tumor model was used to evaluate the therapeutic potential of OBP-301 in the development of tumor growth and lymph node metastasis.

Materials and Methods

Cell lines. The human esophageal squamous cell carcinoma lines TE4 and TE6 were purchased from RIKEN BioResource Research Center (Tsukuba, Japan). Primary human esophageal fibroblasts, FEF3, were isolated from a human fetal esophagus as described previously [9]. TE4 cells stably transfected with the firefly luciferase (Luc) expression vector (TE4-Luc) were established as reported previously [27]. Cells were cultured for no longer than 5 months following resuscitation. TE4 and TE6 cells were maintained in RPMI1640 medium. FEF3 cells were maintained in Dulbecco's Modified Eagle Medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were routinely maintained at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant human TGF-β1, used to induce EMT in TE4 and TE6 cells, was obtained from Sigma-Aldrich (St. Louis, MO, USA). EMT was induced by 48-h treatment of TE4 and TE6 cells with TGF-β

(10 ng/ml). Chemotherapeutic agents 5-fluorouracil (5-FU), cisplatin, and docetaxel, used to evaluate the chemosensitivities of TGF-β-treated cells, were obtained from Sigma-Aldrich.

Recombinant adenoviruses. The recombinant tumor-specific replication-competent oncolytic adenovirus vector OBP-301 (Telomelysin) was constructed and characterized as previously reported [16, 17]. The E1A-intact wild-type adenovirus serotype 5 (Ad5) and E1A-defective adenovirus mutant dl312 were also used. The recombinant virus was purified by ultracentrifugation using cesium chloride step gradients; titers were determined by a plaque-forming assay using 293 cells. All viruses were stored at −80°C.

Morphological and histochemical phenotypes of TGF-β and adenovirus-treated cells. Cells were seeded in a 100-mm dish at a density of 3×10^5 cells/dish 24 h before treatment. To verify whether TGF-β induces EMT in TE4 and TE6 cells, cells were treated with TGF-β (10 ng/ml) for 0, 24, and 48 h. Cell morphological change was observed using an inverted microscope (IX71; Olympus, Tokyo). To evaluate the effect of OBP-301 in cells undergoing EMT, cells pretreated with TGF-β (10 ng/ml, 48 h) were infected with OBP-301 at multiplicities of infection (MOIs) of 0, 1, 5, 10, 20, and 50 plaque-forming units (PFU)/cell for 48 h. To compare the effect of several adenoviruses in cells undergoing EMT, cells pretreated with TGF-β (10 ng/ml, 48 h) were infected with Ad5 or dl312 at an MOI of 50 PFU/cell for 48 h, and their histochemical phenotypes were examined by Western blot analysis and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

Western blot analysis. Whole cell lysates were prepared in a lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Complete Mini; Roche Applied Science, Mannheim, Germany). Proteins (20 μg per lane) were electrophoresed on 10% SDS polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Health Care, Buckinghamshire, UK). The membranes were blocked with Blocking-One (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. The primary antibodies used were rabbit anti-E-cadherin monoclonal antibody (mAb) (3195; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-N-cadherin mAb (13116; Cell Signaling Technology), rabbit anti-vimentin mAb (5741; Cell Signaling Technology), mouse anti-Ad5 E1A mAb (554155; BD Bioscience, Franklin Lakes, NJ, USA), and mouse anti-β-actin mAb (A5441; Sigma-Aldrich). The secondary antibodies used were horseradish peroxidase-conjugated antibodies against rabbit IgG (NA934; GE Healthcare) or mouse IgG (NA931; GE Healthcare). Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Prime; GE Healthcare).

Quantitative real-time reverse transcription-PCR analysis. Total RNA was extracted from cells using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). After synthesis of cDNA using 200 ng of total RNA, the mRNA expressions of *N-Cadherin*, *Vimentin*, *Snail*, *Slug*, and *Zeb1* were determined by quantitative RT-PCR using the Applied Biosystems StepOnePlus™ real-time PCR system. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with reference to *glyceraldehydes-3-phosphate dehydrogenase* mRNA expression.

Migration assays. A 24-well Transwell chamber plate with an 8-μm pore size filter membrane (BD Bioscience, Bedford, MA, USA) was used. Cells were seeded in serum-free medium in the top chamber with a non-coated membrane at a density of 2×10^4 cells/well for migration assays. Medium supplemented with 10% FBS as a chemoattractant was added in the lower chamber. After incubation for 24 h, the cells on the lower surface of the membrane were fixed and stained with crystal violet. The number of cells migrating through the membrane was counted under a light microscope.

For the wound-healing assay, cells were seeded in Culture-Insert 2 Wells (ibidi, Martinsried, Germany) at a density of 1.5×10^4 cells/well for 24 h before treatment with TGF-β (10 ng/ml). After 48 h (TE4) or immediately (TE6) after ligand stimulation, OBP-301 (10 or 100 MOI, respectively) was added, and 24 or 36 h later, respectively, the migration capability was assessed by measuring the occupancy of the cell-free space using ImageJ software.

Cell viability assay. Cells were seeded in 96-well plates at a density of 10^3 cells/well 24 h before treatment with TGF-β (10 ng/ml) for 48 h. To evaluate the chemosensitivity in cells undergoing EMT, TGF-β-pretreated TE4 and TE6 cells were treated with chemotherapeutic agents (Sigma-Aldrich), including 5-FU (50 and 20 μM, respectively), cisplatin (10 μM), and docetaxel (0.5 and 4 nM, respectively) for 72 h.

Similarly, to evaluate the OBP-301 sensitivity in cells undergoing EMT, TGF-β-pretreated cells were infected with OBP-301 at MOIs of 0, 1, 5, 10, 20, 50, or 100 PFU/cell for 72 h. Cell viability was determined using a Cell Proliferation Kit II (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol.

Coculture of TE4 and FEF3 cells. TE4 and/or FEF3 cells were seeded in 100-mm dishes at a density of 5×10^5 cells/dish (n = 3). Seventy-two h after seeding, supernatants were collected and analyzed using a Human TGF-β1 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

In vivo orthotopic TE4-Luc xenograft tumor model. Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. To evaluate the therapeutic effect of OBP-301 on tumor growth and lymph node metastases of TE4 cells stimulated with TGF-β, we performed two treatment protocols, including pretreatment before inoculation and treatment after inoculation. In the treatment protocol before inoculation, TE4-Luc cells were treated with OBP-301 (10 MOI) for 24 h. OBP-301-pretreated or nontreated TE4-Luc cells (2×10^6 cells each) were suspended with FEF3 cells (5×10^5 cells) in an equal volume of Matrigel (20 μL) and were inoculated into the abdominal esophagus of 6-week-old female BALB/c-nu/nu mice (n = 3). In the treatment protocol after inoculation, TE4-Luc cells (2×10^6 cells) were suspended with FEF3 cells (5×10^5 cells) in an equal volume of Matrigel (20 μL) and were inoculated into the abdominal esophagus of 6-week-old female BALB/c-nu/nu mice (n = 3). Mice were intratumorally injected with OBP-301 (1×10^8 PFU) or PBS once a week for 2 cycles. To monitor the luminescence intensity, the substrate luciferin (VivoGlo Luciferin; Promega, Madison, WI, USA) was intraperitoneally injected. The luminescence intensities of tumors and lymph nodes were measured weekly for 4 to 5 weeks after inoculation by an IVIS Lumina imaging system (Xenogen IVIS Lumina II; Caliper LifeSciences, Hopkinton, MA, USA). The number of metastatic lymph nodes with luminescence in the abdominal cavity was counted.

Statistical analysis. Data are expressed as mean values ± standard deviation (SD). Significant differences were assessed using Student's *t* test. Statistical significance was defined as a *p* value of less than 0.05.

Results

TGF-β induces the EMT phenotype in human esophageal cancer TE4 and TE6 cells. TGF-β is one of the most important activators in the induction of EMT [13, 14]. To investigate the effect of OBP-301 in the induction of EMT, we first assessed whether indeed TGF-β induces EMT in two human esophageal cancer cell lines, TE4 and TE6. When TE4 and TE6 cells were treated with TGF-β (10 ng/ml) for 48 h, TGF-β-treated TE4 and TE6 cells showed spindle-shaped morphological changes with low cell-cell attachments compared to nontreated cells, which retained the high cell-cell attachments typical of epithelial cells (Fig. 1A). Western blot analysis demonstrated that TGF-β treatment increased the expression of mesenchymal marker proteins N-cadherin and vimentin in TE4 and TE6 cells while the expression of epithelial marker protein E-cadherin was not changed (Fig. 1B). Consistent with increased mesenchymal marker proteins, the expressions of N-cadherin and vimentin mRNAs were significantly increased after treatment with TGF-β (Fig. 1C). Moreover, RT-PCR revealed that TGF-β treatment significantly increased the mRNA expression of EMT transcription factors slug and zeb1 in TE4 and TE6 cells and snail in TE4 cells (Fig. 1D). These results suggested that TGF-β-treated TE4 and TE6 cells exhibited the EMT phenotype and could be used to evaluate the effects of OBP-301 on EMT in cancer cells.

OBP-301 inhibits the TGF-β-induced EMT phenotype in TE4 and TE6 cells. To investigate whether OBP-301 inhibits the TGF-β-induced EMT phenotype in human esophageal cancer cells, TE4 and TE6 cells pretreated with TGF-β (10 ng/ml, 48 h) were further infected with OBP-301 at different doses for 48 h. Western blot analysis demonstrated that OBP-301 infection dose-dependently decreased the expression of N-cadherin and vimentin proteins, consistent with the expression of adenoviral E1A protein in TGF-β-treated TE4 and TE6 cells (Fig. 2A). In contrast, the expression of E-cadherin protein was not changed or only slightly decreased in TGF-β-treated TE4 and TE6 cells, respectively, after OBP-301 infection (Fig. 2A). Quantitative RT-PCR analysis demonstrated that OBP-301 infection significantly decreased the mRNA expressions of EMT-related markers N-cadherin, vimentin, snail, slug, and zeb1 in TE4 and TE6 cells (Fig. 2B and C). These results suggest that OBP-301 inhibits the TGF-β-induced EMT

histochemical phenotype in human esophageal cancer cells.

E1A accumulation is involved in adenovirus-mediated EMT suppression. Adenoviral E1A protein has been shown to have tumor-suppressive potential in a variety of cancer cells [25]. To investigate the role of E1A in OBP-301-mediated EMT suppression, we compared the effects of E1A-intact Ad5 and E1A-deleted dl312 in the EMT induction of TGF-β-treated TE4 and TE6 cells. The expression of N-cadherin was suppressed by infection with Ad5, but not dl312 (Fig. 3A). Consistent with EMT suppression, the expression of adenoviral E1A protein was increased by infection with Ad5, but not dl312 (Fig. 3A). Quantitative RT-PCR analysis demonstrated that infection with Ad5, but not dl312, significantly decreased the mRNA expressions of N-cadherin and slug in TE4 and TE6 cells (Fig. 3B and C). These results suggest that adenovirus infection induces E1A accumulation, resulting in EMT suppression, in TGF-β-treated cancer cells.

OBP-301 suppresses EMT-mediated cell migration in TE4 and TE6 cells. EMT induces a malignant phenotype with migratory properties during tumor progression [28]. To investigate the effect of OBP-301 on the EMT-mediated malignant phenotype, we analyzed the migratory properties of TE4 and TE6 cells using transwell chamber and wound healing assays. Transwell chamber assays demonstrated that TGF-β treatment significantly enhanced the migratory properties of TE4 and TE6 cells (Fig. 4A). TGF-β-enhanced migration of

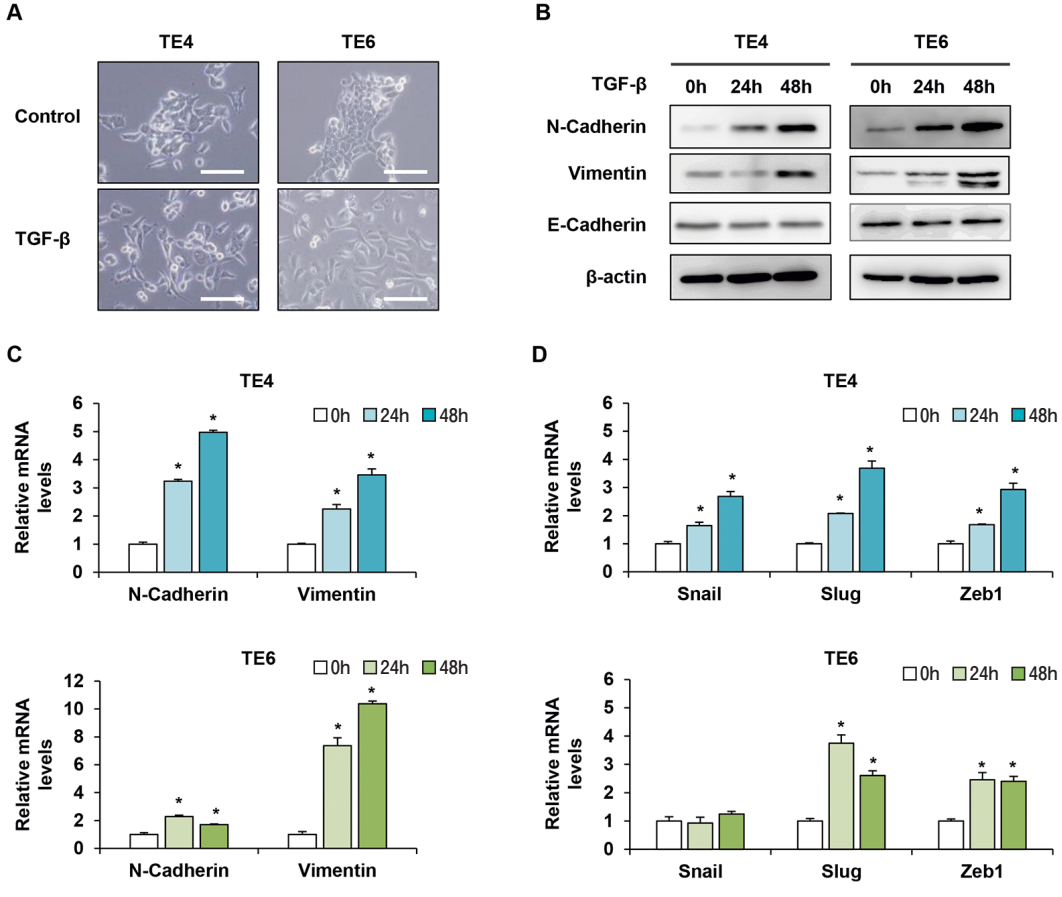

Fig. 1 TGF-β-induced EMT phenotype in esophageal cancer cells. **A**, Morphology of TE4 and TE6 cells cultured with or without TGF-β (10 ng/ml) for 48 h. Scale bars: 100 μm; **B**, Protein expression of mesenchymal markers (N-cadherin and vimentin) and epithelial marker (E-cadherin) in TE4 and TE6 cells treated with TGF-β (10 ng/ml) for 48 h. β-actin was used as a loading control; **C**, mRNA expression of N-cadherin and vimentin in TE4 and TE6 cells treated with TGF-β (10 ng/ml) for 48 h; **D**, mRNA expression of EMT transcription factors (snail, slug, zeb1) in TE4 and TE6 cells treated with TGF-β (10 ng/ml) for 48 h. The expression level at 0 h was set at 1.0. Data are expressed as mean values ± SD (n = 3). *: $P < 0.05$ (vs. 0 h).

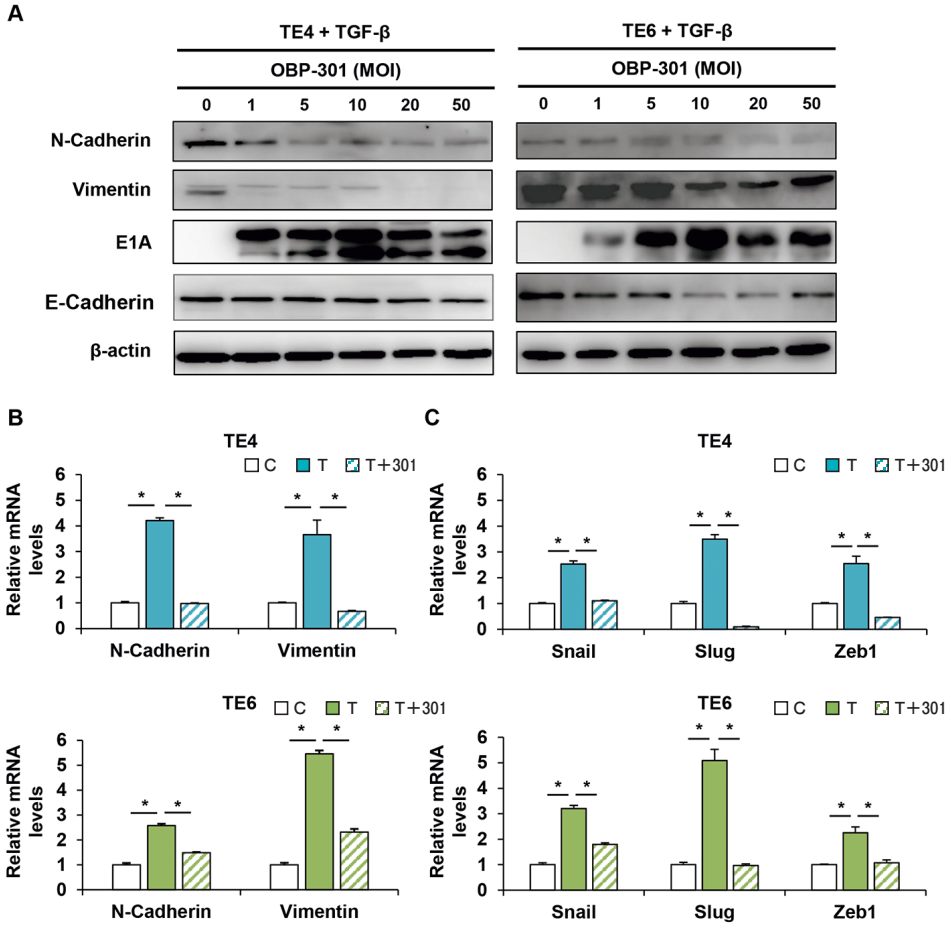

Fig. 2 Suppression of TGF-β-induced EMT markers by OBP-301 in esophageal cancer cells. A, Protein expression of mesenchymal markers (N-cadherin and vimentin), epithelial marker (E-cadherin), and adenoviral E1A in TE4 and TE6 cells after infection with OBP-301 at the indicated doses for 48 h following TGF-β treatment (10 ng/ml). β-actin was used as a loading control; B, mRNA expression of N-cadherin and vimentin in TE4 and TE6 cells after culture with or without OBP-301 (20 and 100 MOI, respectively) for 48 h following TGF-β treatment (10 ng/ml, 48 h); C, mRNA expression of EMT transcription factors (snail, slug, zeb1) in TE4 and TE6 cells after culture with or without OBP-301 (20 and 100 MOI, respectively, 48 h) following TGF-β treatment (10 ng/ml, 48 h). Data are expressed as mean values $\pm$ SD (n=3). *: $P<0.05$.

TE4 and TE6 cells was significantly inhibited after infection with OBP-301 for 24 h (Fig. 4A). Likewise, wound-healing assays demonstrated that TGF-β treatment induced cell migration, and OBP-301 infection attenuated the TGF-β-induced migratory properties of TE4 and TE6 cells (Fig. 4B). However, OBP-301 infection for 24 h did not decrease the viability of TE4 and TE6 cells treated with TGF-β (Fig. 4C). These results suggest that OBP-301 inhibits EMT-related migration in human esophageal cancer cells.

Chemoresistant EMT cells are sensitive to the cytopathic effects of OBP-301 via autophagy induction. EMT-mediated drug resistance is a major cause of tumor recurrence and poor prognosis [29]. We investigated whether TGF-β-induced EMT is associated with chemoresistance in TE4 and TE6 cells. When TE4 and TE6 cells were treated with TGF-β for 48 h, they exhibited significantly more resistance to the chemotherapeutic agents 5-FU, cisplatin, and docetaxel compared to nontreated cells (Fig. 5A). In contrast, OBP-301 infection significantly suppressed the viability of TGF-β-treated TE4 and TE6 cells compared to nontreated cells (Fig. 5B). These results suggest that OBP-301 eliminates cancer cells undergoing EMT that are refractory to chemotherapy.

We previously demonstrated that OBP-301 induces

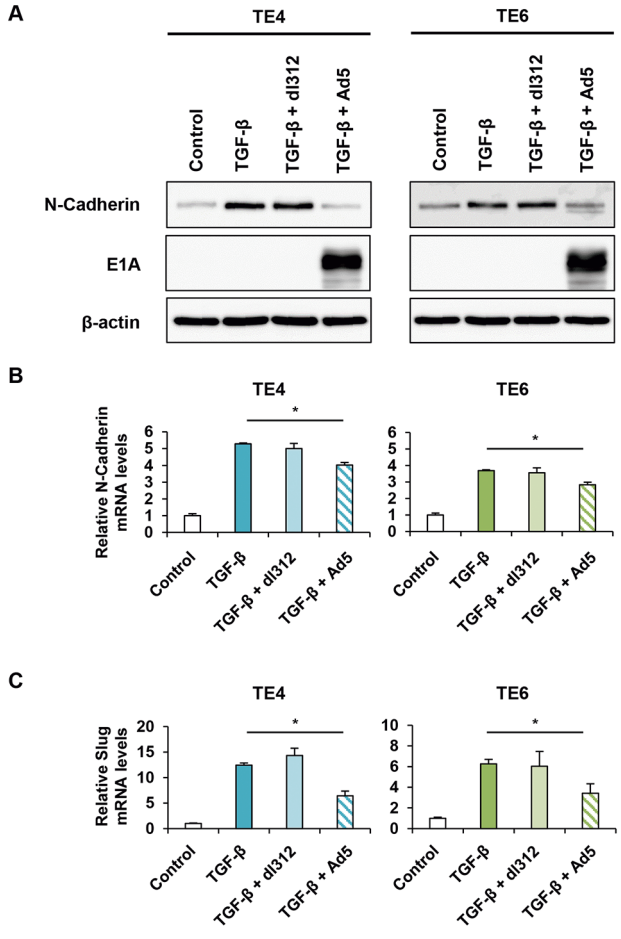

Fig. 3 Role of E1A in adenovirus-mediated EMT suppression. **A**, Protein expression of N-cadherin and E1A in TE4 and TE6 cells after infection with Ad5 and dl312 at MOI of 50 PFU/cell for 48 h following TGF-β treatment (10 ng/ml, 48 h). β-actin was used as a loading control; **B**, mRNA expression of N-cadherin in TE4 and TE6 cells after infection with Ad5 and dl312 at MOI of 50 PFU/cell for 48 h following TGF-β (10 ng/ml, 48 h); **C**, mRNA expression of EMT transcription factor slug in TE4 and TE6 cells after infection with Ad5 and dl312 at a MOI of 50 PFU/cell for 48 h following TGF-β treatment (10 ng/ml, 48 h). Data are expressed as mean values ± SD (n=3). *: $P<0.05$.

autophagy-related cell death in human cancer cells [30]. Here, we investigated the status of autophagy in TGF-β-treated TE4 and TE6 cells. When TE4 and TE6 cells were treated with TGF-β for 48 h, expression of the autophagy-related marker LC3-II was increased in a time-dependent manner (Fig. 5C). Moreover, when TE4 and TE6 cells were simultaneously treated with TGF-β and OBP-301 for 48 h, the expression of LC3-II was further increased in a dose-dependent manner (Fig. 5D). These results suggest that TGF-β induces autophagy, resulting in an OBP-301-sensitive phenotype, in esophageal cancer cells.

In vivo antitumor effect of OBP-301 on orthotopic TE4 tumors containing TGF-β-secreting fibroblasts. We recently established an orthotopic TE4 xenograft tumor model with lymph node metastases by co-inoculating TE4 cells and normal esophageal fibroblast FEF3 cells [27]. As stromal fibroblasts are normally TGF-β-secreting cells in the tumor microenvironment [13], we hypothesized that FEF3 cells induce EMT in TE4 cells, probably via TGF-β secretion. We first investigated the amount of extracellular TGF-β in conditioned medium after monoculture or coculture with TE4 and FEF3 cells for 72 h. As expected, coculture of TE4 and FEF3 cells induced a significantly higher concentration of extracellular TGF-β in conditioned medium compared to monocultures of TE4 or FEF3 cells (Fig. 6A). Moreover, when TE4 cells were cocultured with FEF3 cells for 96 h, the expression of vimentin mRNA was significantly increased in TE4 cells (Fig. 6B). These results suggest that co-incubation of TE4 cells with FEF3 cells induces the EMT program in the former by secretion of TGF-β from the latter.

Next, we investigated whether OBP-301 inhibits tumor growth and lymph node metastasis of TE4 cells using an orthotopic TE4 xenograft tumor model with FEF3 cells. To investigate the effect of OBP-301 in the tumorigenicity of TE4 cells co-inoculated with FEF3 cells, TE4-Luc cells pretreated with OBP-301 (10 MOI, 24 h) were co-inoculated with FEF3 cells into the abdominal esophagus of nude mice. OBP-301-pretreated TE4-Luc cells did not develop any tumors in mice (Fig. 6C and D). In contrast, to investigate the effect of OBP-301 on lymph node metastasis of TE4 cells co-inoculated with FEF3 cells, TE4-Luc tumors were treated with intratumoral injection of OBP-301 or PBS once a week for two cycles. OBP-301 treatment decreased tumor growth and the number of lymph node metastases compared to PBS treatment, although the difference was not significant (Fig. 6E, F and G). Histological analysis demonstrated the involvement of stromal tissues in esophageal tumors (Fig. 6G). These results suggest that OBP-301 inhibits the tumor growth and lymph node metastasis of TE4 cells within the tumor microenvironment containing TGF-β-secreting FEF3 cells, but may be less effective against established tumors.

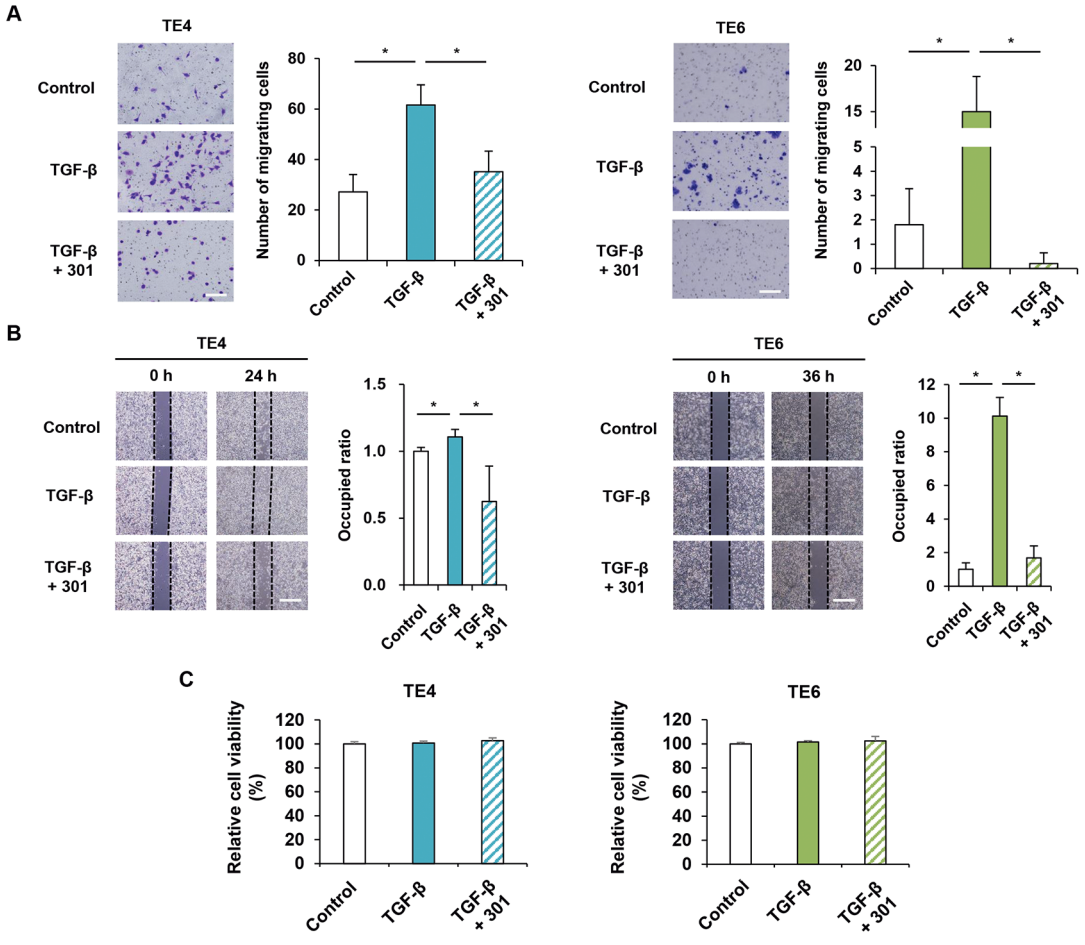

Fig. 4 Suppression of TGF-β-enhanced migration by OBP-301 in esophageal cancer cells. **A**, Migration ability of TE4 and TE6 cells infected with OBP-301 (10 and 100 MOI, respectively) and TGF-β (10 ng/ml) for 24 h. Scale bar: 200 μm; **B**, Scratch wound healing assay of TE4 and TE6 cells after OBP-301 infection (10 and 100 MOI, respectively) for 24 and 36 h, respectively, following TGF-β treatment (10 ng/ml, 48 h). Data are expressed as mean values ± SD (n=3). Scale bar: 500 μm. *: $P<0.05$; **C**, Relative cell viability was assessed for TE4 and TE6 cells treated with OBP-301 (10 and 100 MOI, respectively) and TGF-β (10 ng/ml) for 24 h. Data are expressed as mean values ± SD (n=5).

Discussion

Adenoviral E1A is a multifunctional protein that plays a tumor-suppressive role in human cancer cells [24]. OBP-301 induces an antitumor effect in mesenchymal types of tumor cells via accumulation of E1A [31]. OBP-301-mediated E1A overexpression further enhances the antitumor effects of p53 gene therapy and chemotherapy in mesenchymal types of tumor cells via suppression of anti-apoptotic p21 and MCL1 expression, respectively [32, 33], suggesting the involvement of E1A overexpression in the antitumor effect of OBP-301. In this study, OBP-301, a telomerase-specific oncolytic adenovirus, inhibited the TGF-β-induced EMT program and EMT-mediated mesenchymal malignant phenotype, including migration, chemoresistance, tumor growth, and lymph node metastasis, in human esophageal cancer cells. The accumulation of adenoviral E1A was involved in OBP-301-mediated EMT suppression, and TGF-β-induced autophagy was involved in cells' sensitivity to OBP-301. Thus, OBP-301 is a promising antitumor agent to inhibit EMT and induce autophagy-related cell death in tumor cells through the accumulation of E1A, especially under a TGF-β-enriched microenvironment (Fig. 7).

OBP-301 inhibited the TGF-β-induced EMT pro-

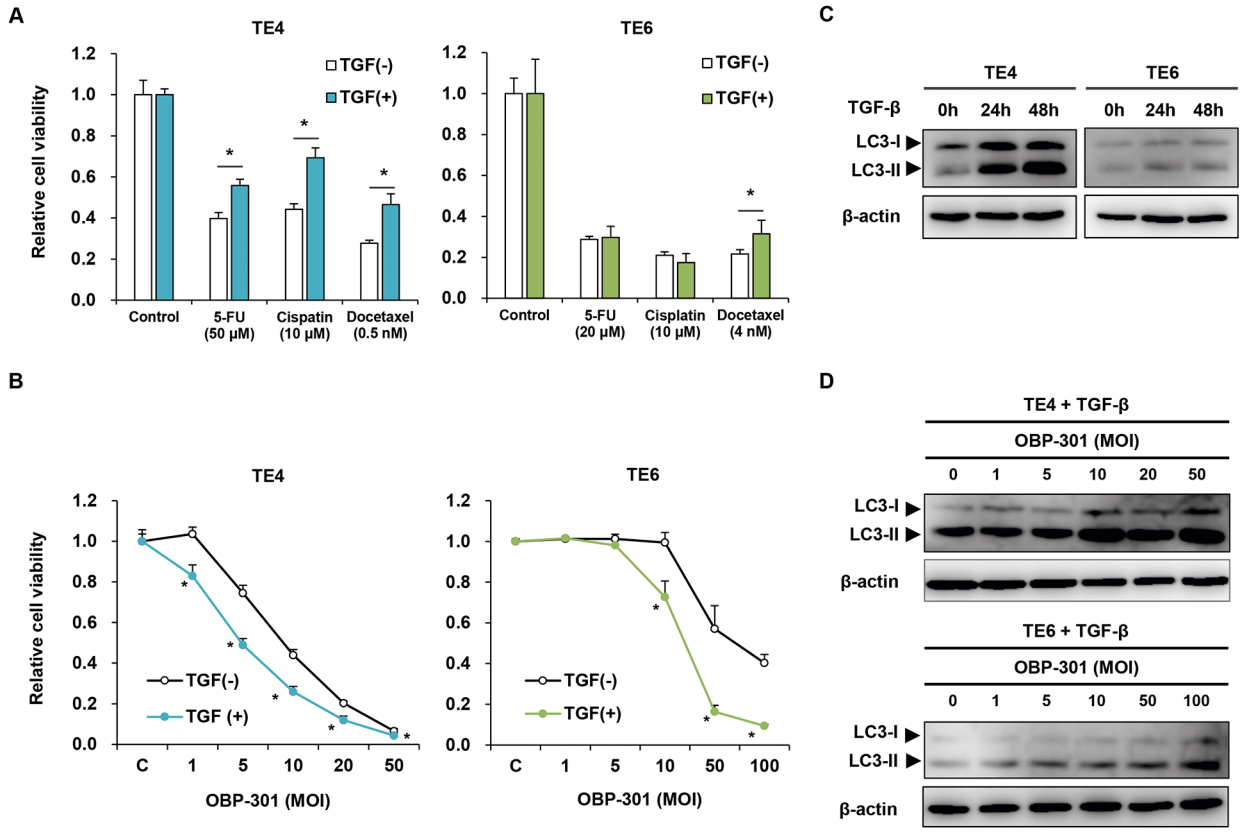

Fig. 5 TGF-β-treated esophageal cancer cells exhibit chemoresistant and OBP-301-sensitive characteristics. **A**, Relative cell viability was assessed in TE4 and TE6 cells treated with 5-FU (50 and 20 μM, respectively), cisplatin (10 μM), and docetaxel (0.5 and 4 nM, respectively) for 72 h following culture with or without TGF-β (10 ng/ml, 48 h); **B**, Relative cell viability was assessed in TE4 and TE6 cells treated with OBP-301 at the indicated doses for 72 h following culture with or without TGF-β (10 ng/ml, 48 h). Data are expressed as mean values ± SD (n=5). *: $P<0.05$; **C**, Protein expression of autophagy markers (LC3-I and LC3-II) in TE4 and TE6 cells treated with TGF-β (10 ng/ml) for 48 h; **D**, Protein expression of autophagy markers (LC3-I and LC3-II) in TE4 and TE6 cells after infection with OBP-301 at the indicated doses for 48 h following TGF-β treatment (10 ng/ml, 48 h). β-actin was used as a loading control.

gram in TE4 and TE6 cells via suppression of mesenchymal markers and EMT transcription factors (Fig. 2). The molecular mechanism of OBP-301-mediated EMT impairment involves the accumulation of adenoviral E1A (Fig. 3), which has been shown to possess a tumor-suppressive function [25]. Frisch *et al.* [26] demonstrated that E1A induces epithelial characteristics in mesenchymal types of malignant tumor cells. De Groot *et al.* [34] showed that E1A suppresses TGF-β signaling downstream in normal keratinocytes via downregulation of JunB, which is an EMT transcription factor, and Gervasi *et al.* [35] reported that JunB inhibition abrogates the TGF-β-induced EMT process in normal mammary epithelial cells. In contrast, E1A has been shown to induce the hypoacetylation of histone H3K27 in human normal fibroblasts [36]. Chang *et al.* [37] demonstrated that JunB is associated with super-enhancers and areas of highly acetylated H3K27 in the TGF-β-induced EMT program of human cancer cells. Although the underlying mechanism of E1A-mediated EMT suppression remains unclear, OBP-301 may at least in part suppress the TGF-β-induced EMT program via accumulation of viral E1A.

Tumor cells undergoing EMT possess the characteristics of migration, invasion, and chemoresistance [3]. TGF-β treatment induced migration and chemoresistance in TE4 and TE6 cells (Figs. 4 and 5). Interestingly, despite their chemoresistance, TGF-β-treated TE4 and TE6 cells were more susceptible to OBP-301 than non-treated cells (Fig. 5). In the mechanism of TGF-β-

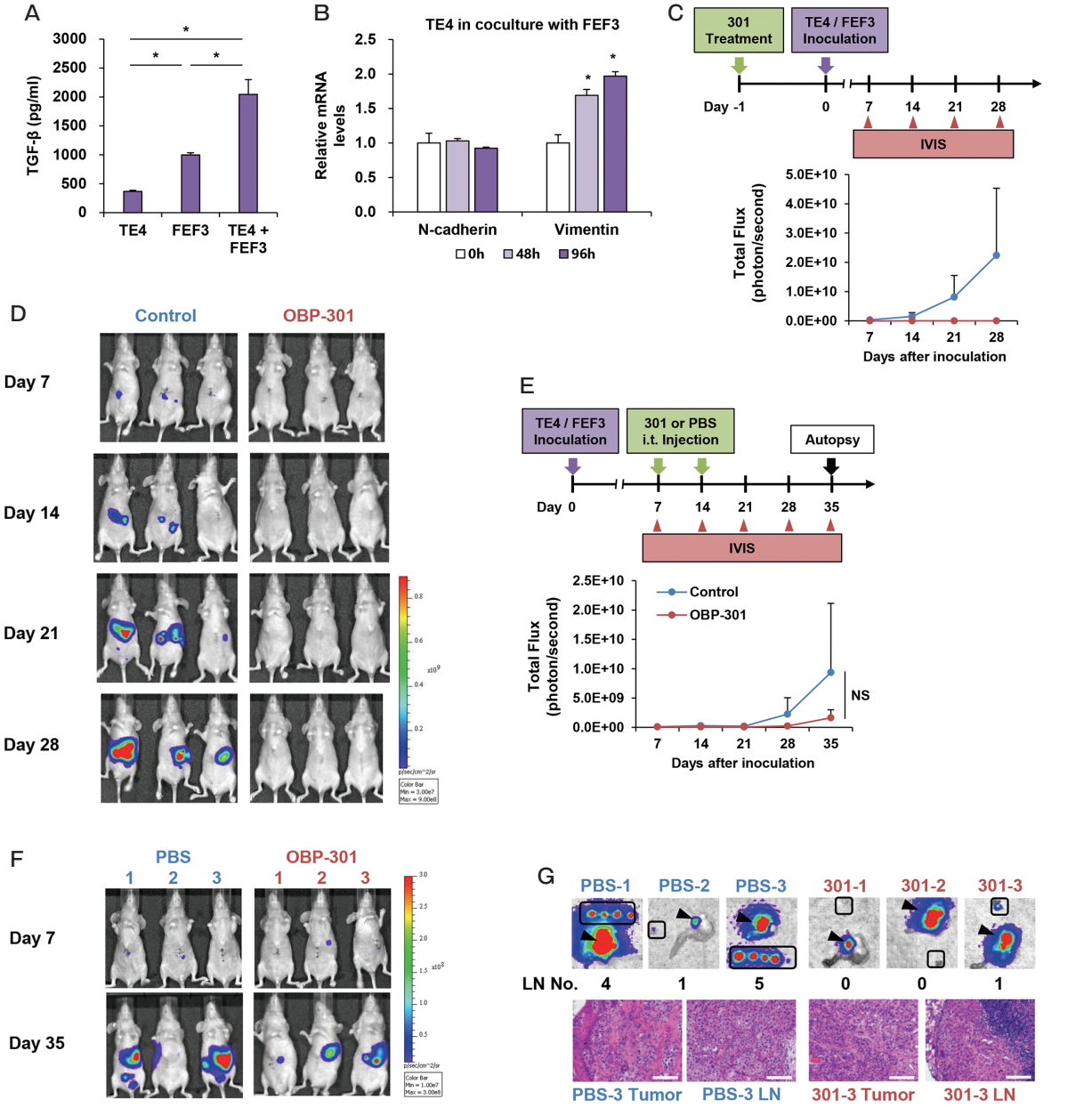

Fig. 6 OBP-301 inhibits tumor growth and lymph node metastasis in an orthotopic TE4 xenograft tumor model. **A**, TGF-β amounts in conditioned medium were analyzed with a Human TGF-β1 Quantikine ELISA kit (R&D Systems); **B**, mRNA expression of N-cadherin and vimentin in TE4 cells cocultured with FEF3 cells for 96 h. Data are expressed as mean values ± SD (n = 5). *: $P < 0.05$; **C**, After treatment with OBP-301 (10 MOI) (green arrow), OBP-301-pretreated or -nontreated TE4-Luc (2×10^6) cells were co-inoculated with FEF3 cells (5×10^5 cells) into the abdominal esophagus of nude mice (purple arrow). Luminescence in the tumors was analyzed using the IVIS system at days 7, 14, 21, and 28 after tumor inoculation (red arrowheads). Data are expressed as mean values ± SD (n = 3); **D**, Photographs of mice at days 7, 14, 21, and 28 after tumor inoculation; **E**, TE4-Luc (2×10^6 cells) were co-inoculated with FEF3 cells (5×10^5 cells) into the abdominal esophagus of nude mice (purple arrow). TE4 tumors were treated with intratumoral (i.t.) injection of OBP-301 (1×10^8 PFUs) or PBS at days 7 and 14 after tumor inoculation (green arrows). The luminescence in tumors and lymph nodes was analyzed using the IVIS system at days 7, 14, 21, 28, and 35 after tumor inoculation (red arrowheads). Data are expressed as mean values ± SD (n = 3); **F**, Photographs of mice at days 7 and 35 after tumor inoculation; **G**, Upper photographs represent TE4 tumors (black arrowheads) and lymph node tissues (black squares) with luminescence. Lower photographs represent the histological findings of TE4 tumors and lymph node tissues in PBS-3 and 301-3 cases. Scale bars, 100 μm.

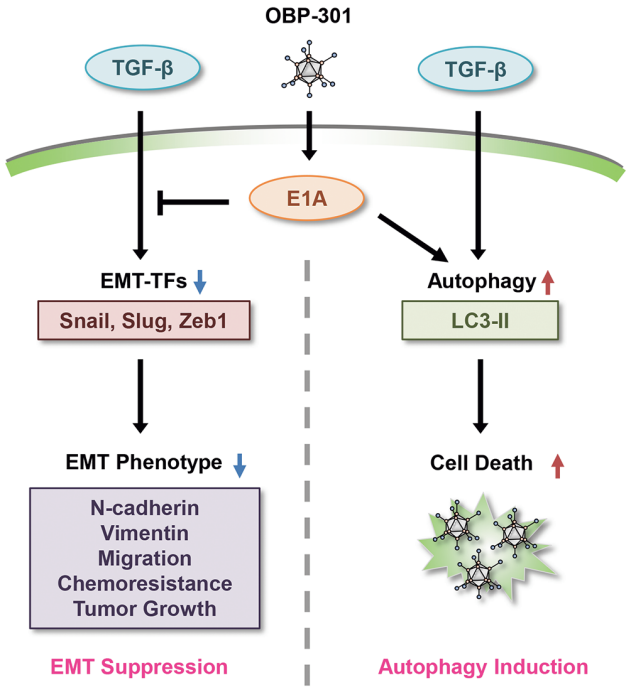

Fig. 7 Outline of OBP-301-mediated EMT suppression and autophagy induction in TGF-β-stimulated esophageal cancer cells.

induced sensitivity to OBP-301, TGF-β treatment induced autophagy, which is associated with OBP-301-mediated lytic cell death (Fig. 5). Autophagy is a fundamental process that promotes survival under various environmental challenges, such as nutrient deprivation [38]. Recently, it has been suggested that there is a relationship between TGF-β-induced EMT and autophagy. In the context of chemotherapy, TGF-β-induced autophagy and nutrient recycling has been associated with reduced chemotherapy-mediated nutrient deprivation and apoptosis, resulting in the chemoresistance of EMT cells [39]. In contrast, OBP-301 infection induces massive autophagy, contributing to cell death rather than cell survival, in cancer cells [30,40]. Thus, OBP-301 may have the therapeutic potential to overcome EMT-related chemoresistance in esophageal cancer cells via induction of autophagy-related cell death.

The typical tumor microenvironment supports malignant tumor growth, invasion, metastasis, and chemoresistance in various cancer types [12]. Cancer-associated fibroblasts promote tumor progression via the secretion of several cytokines, including TGF-β [41]. We recently reported that normal fibroblasts, or FEF3 cells, promote the tumor growth, migration, invasion, and lymph node metastasis of TE4 cells *in vitro* and *in vivo* [27]. In this study, we confirmed that FEF3 cells induce the EMT phenotype in TE4 cells via secretion of TGF-β (Fig. 6). In *in vivo* experiments with TE4- and FEF3-coinoculated tumors, OBP-301 suppressed the tumor growth and lymph node metastasis of TE4 cells (Fig. 6). These findings are consistent with our previous reports showing that OBP-301 suppresses tumor growth and lymph node metastasis in an orthotopic human colorectal cancer xenograft tumor model [19,20]. Although OBP-301 has the therapeutic potential to eliminate telomerase-positive cancer cells, the effect of OBP-301 is conversely attenuated in telomerase-negative normal fibroblasts [16]. As cancer-associated fibroblasts have recently emerged as attractive targets to improve the therapeutic potential of antitumor therapy [41], a combination of cancer stroma-targeting therapy with OBP-301 treatment may be an attractive strategy to inhibit the tumor growth and lymph node metastasis of esophageal cancer.

In conclusion, we demonstrated that a telomerase-specific oncolytic adenovirus, OBP-301, inhibits the TGF-β-mediated EMT program and EMT-related malignant phenotype in human esophageal cancer cells via E1 accumulation. As the development of anti-EMT therapies is urgently needed, oncolytic adenovirus-mediated E1A overexpression may be a way to inhibit EMT-related esophageal cancer progression. Further understanding of oncolytic adenovirus-mediated inhibition of EMT will improve this form of virotherapy.

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