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ORIGINAL PAPER

A novel role mediated by adenoviral E1A in suppressing cancer through modulating decorin

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Abstract

Oncolytic adenovirus is an emerging alternative to current therapeutics. The adenoviral E1A, the first protein expressed upon oncolytic adenoviral infection, has been identified as an antitumor agent, but the mechanisms of its tumor inhibition ability are unclear enough. Decorin is ubiquitous in the extracellular matrix (ECM), which regulates multiple functions through interaction with ECM. Here, we intended to explore the effects of adenoviral E1A on the tumor extracellular matrix during gene therapy. We demonstrated that reduced decorin expression was found in patients with lung cancer. The adenoviral E1A or a mutant adenoviral E1A with Rb-binding ability absent (E1A 30–60aa, 120–127aa deletion) could increase the expression of decorin and down-regulate VEGF, two members of tumor ECM, involved in both vasculogenesis and angiogenesis. E1A/mE1A-mediated suppressing the migration and invasion ability of tumor cells was depended on decorin. E1A interacted with decorin directly and induced the proteasomal degradation of VEGF. In addition, E1A or mE1A can inhibit tumor growth in a subcutaneous lung cancer xenograft model. It suggested that decorin might be a crucial mediator among ECM components for adenoviral E1A-mediated antitumor activities. These studies on adenovirus E1A provide a new mechanism for the emerging therapies of tumor gene therapy.

Keywords Adenoviral E1A · Decorin · VEGF · Extracellular matrix · Lung carcinoma

Introduction

Lung cancer is the most common malignant tumor, and it is the major cause of cancer-related mortality [1]. Currently, great progress has been made in the treatment of non-small cell lung cancer (NSCLC). However, further investigations to identify potent therapeutic targets are critical for developing more effective treatment strategies for lung cancer patients [2].

The licensing of talimogene laherparepvec (T-Vec) is an important beginning for the treatment of oncolytic viruses, followed by the emergence of various vectors based on oncolytic adenovirus [3].When infected with oncolytic adenovirus, the early region 1A (E1A) gene of human adenovirus type 5 is the first to be expressed [4]. A large number of experiments have shown that E1A can inhibit the occurrence of tumors, and E1A suppresses tumor growth through many mechanisms. E1A inhibits cancer metastasis by decreasing cellular miR-520 h level and TWIST [5]. E1A can inhibit the expression of heat shock protein 5 (HSPA5), which is overexpressed in advanced cancer, to suppress the migration and invasion of TNBC cells [6]. When Mdm4 is present, E1A can form a ternary complex with p53, resulting in p53 being stable independent of p14ARF without degradation [7]. Our previous research showed that E1A can induce tumor cell apoptosis in the manner of p53-independent to act as an

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anticancer agent [8]. In addition, E1A improved the sensitization of cancer cells to chemotherapeutic drugs [9, 10].

Decorin is a proteoglycan that is present in the extracellular matrix and contains a core protein and a single-chain aminopolysaccharide [11, 12]. Studies have shown that decorin not only regulates the activity of various cell growth factors, but also participates in the assembly of extracellular matrix [13]. The deregulation of decorin was found in many types of tumors [14]. Connective tissue and ECM block drug administration or affect the efficacy of other gene therapy agents in solid tumors [15]. Choi et al. [16] reported that decorin can cause a decrease in ECM components in tumor tissues, and it also significantly reduces lung metastasis caused by B16BL6 melanoma cells. In addition, decorin can be used as an adjuvant to overcome TGF-β-mediated immunosuppression [17, 18]. These studies show that decorin provides a powerful treatment measure for overcoming tumor.

Based on the function of decorin or adenoviral E1A in cancer progression, we intended to investigate the relationship between decorin and the anticancer effects of E1A. In the present study, we proved that adenoviral E1A upregulated decorin expression and decreased VEGF expression in lung cancer cells. E1A interacted with decorin directly and induced the proteasomal degradation of VEGF. In vivo, E1A-induced decorin significantly reduced cell migration and invasion. Furthermore, compared to the control group, there was higher decorin expression, less collagen fibers and lower CD31 expression in tumor mass treated with E1A. These results indicated the adenoviral E1A played a crucial role in tumor suppression through modulating decorin or VEGF in ECM to change the tumor characteristics.

Results

The expression of decorin is reduced in lung cancer, and decorin had tumor-suppressing ability in vitro

First, we tested the expression of decorin in various cell lines. As shown in Fig. 1a, the expression level of decorin in cancer cells lines is much lower than that in normal cells. For further exploration, we tested the expression of decorin in tumors and adjacent non-tumor tissues by immunohistochemistry. The tissue chip with lung cancer specimens and the paired adjacent paracancerous ($n = 15$) was purchased from Outdo Biotech (Shanghai, China), and decorin expression was detected by immunohistochemistry analysis. Consistent with the data from Western blotting, the relative expression of decorin in the lung cancer specimens was significantly weakened compared with non-tumor tissues (Fig. 1b, c).

Because low decorin expression was related to advanced cancer [19–21], decorin may play important roles in the progression of lung cancer. We established an adenovirus-based system to functionally over-expressed decorin in two kinds of lung tumor cells (H1299 and A549) to explore the functional role of decorin. The tumor-suppressing ability of decorin was investigated by MTT assay. The cells used were infected with Ad-vector or Ad-decorin at a MOI = 0.5, 2, 10, 20, 50 for 48 h, respectively. As shown in Fig. 3d, it showed that Ad-decorin suppressed A549 and H1299 cancer cells growth efficiently compared to the control group (Ad-vector). These results indicated that decorin had a role in inhibiting human lung cancer cells growth.

E1A increased the expression of decorin and decreased VEGF

The adenoviral E1A had been reported to suppress cancer metastasis both in vitro and in vivo [22, 23]. However, the specific role of decorin in the treatment of cancer with E1A is unclear. Based on the role of decorin in blocking cancer progression, we intended to investigate whether decorin acted as a target of E1A in tumor cells.

Firstly, we added the appropriate dose of adenoviral E1A or mE1A, which is a mutant E1A with deletion of Rb-binding domain, to investigate whether E1A has a direct effect on decorin or VEGF in H1299 and A549 cells. Quantitative RT-PCR results showed that E1A/mE1A increased decorin mRNA expression level. The VEGF mRNA level was also detected but no significant variation (Fig. 2a). The Western blotting data confirmed that up-regulation of decorin (loading samples were from conditioned medium in cells infected with adenoviral vector Ad-DC315, Ad-DC315E1A or Ad-DC315-mE1A, since decorin was a small secreted protein) and down-regulation of VEGF protein by E1A/mE1A were dose dependent (Fig. 2b). ELISA was performed to detect the secreted decorin and VEGF in the conditioned medium. Decorin expression was higher in E1A/mE1A overexpressed cells compared with the vector control groups; moreover, the VEGF level was prominently lower than control (Fig. 2c). These data provided evidence to show E1A may directly regulate decorin and VEGF.

Decorin was critical for E1A-mediated inhibition of tumor cell growth

Additionally, aimed to determine the potent role of decorin on E1A's migration/invasion inhibition ability, we constructed E1A/mE1A-stably expressed cell lines (H1299-E1A, H1299-mE1A, A549-E1A and A549-mE1A) with E1A/mE1A lentivirus, in which also included EGFP expression (Fig. 3a). We performed quantitative RT-PCR and immunoblotting to determine that there were higher decorin and E1A level in E1A/mE1A-stably expressed cell lines (Fig. 3b, c). The migration and invasion of E1A/

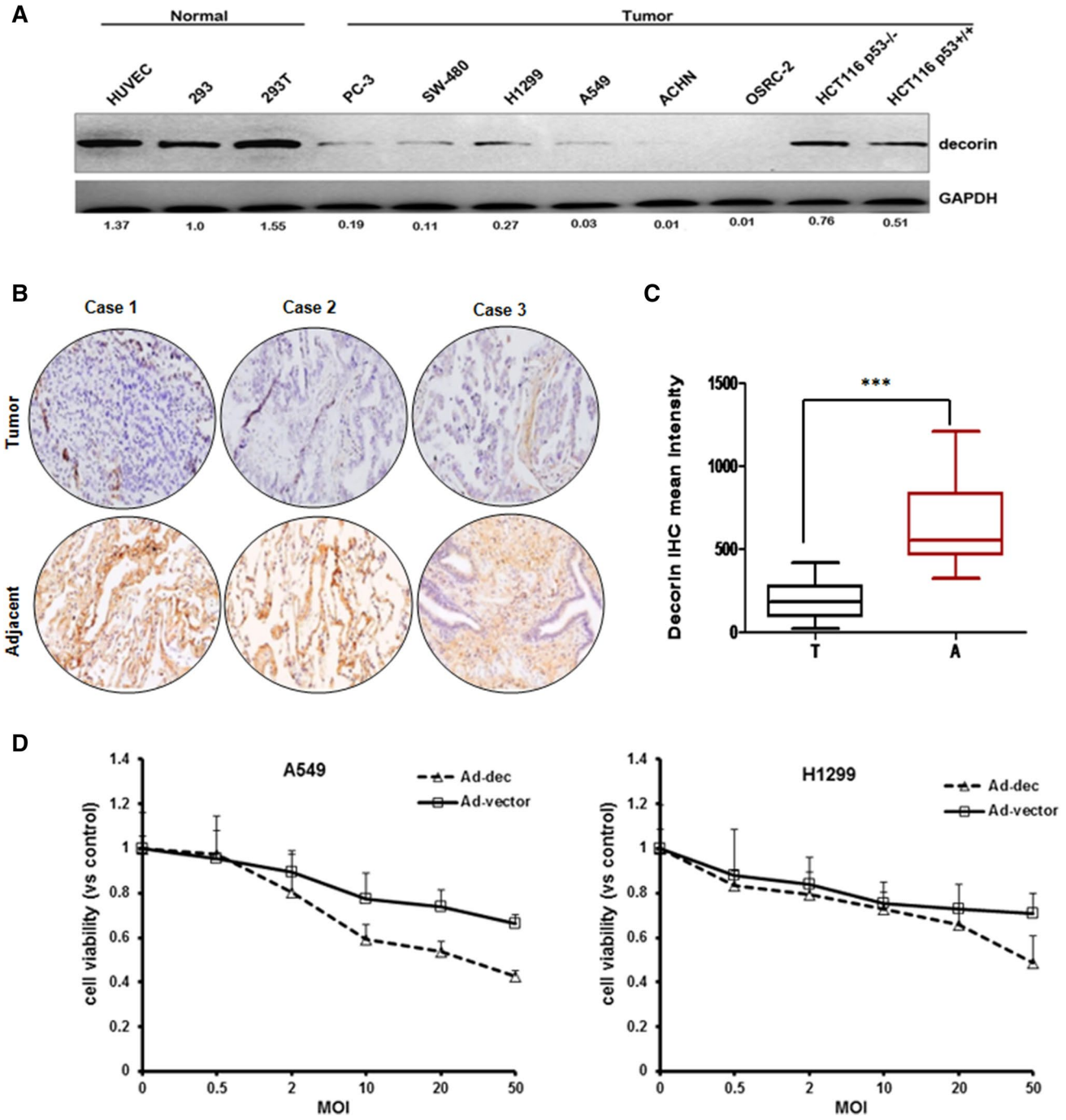


Fig. 1 Decorin expression was attenuated in cancer cell lines and the lung cancer specimens. **a** Decorin protein expression in different cell lines. HUVEC, human umbilical vein endothelial cells; PC-3, human prostate cancer cell lines; SW-480, human colorectal adenocarcinoma cell lines; ACHN and OSRC-2, human renal cancer carcinoma cell lines. Quantification was done using ImageJ software. **b** Representative decorin immunohistochemical staining in tumor tissue cores and adjacent noncancer tissues. **c** Statistical analysis of immunohistochemical (IHC) staining intensity. Box-and-whisker plots show the median (horizontal line), interquartile range (box) and 5th to 95th percentiles of the data. T, tumor tissues ($n=15$); A, adjacent noncancer tissues ($n=15$). ***$p<0.001$. **d** Decorin had tumor-suppressing ability in lung cancer cell lines. Ad-decorin has antitumor activity against human lung cancer cells (A549 and H1299). A549 and H1299 cells were infected with Ad-decorin or Ad-vector at the indicated dose (0.5, 2, 10, 20, 50) for 4 days. Cell viability was measured by MTT assay. The value of MOI = 0 was set at 1. Cell viability data were expressed as mean values ± SD ($n=6$)

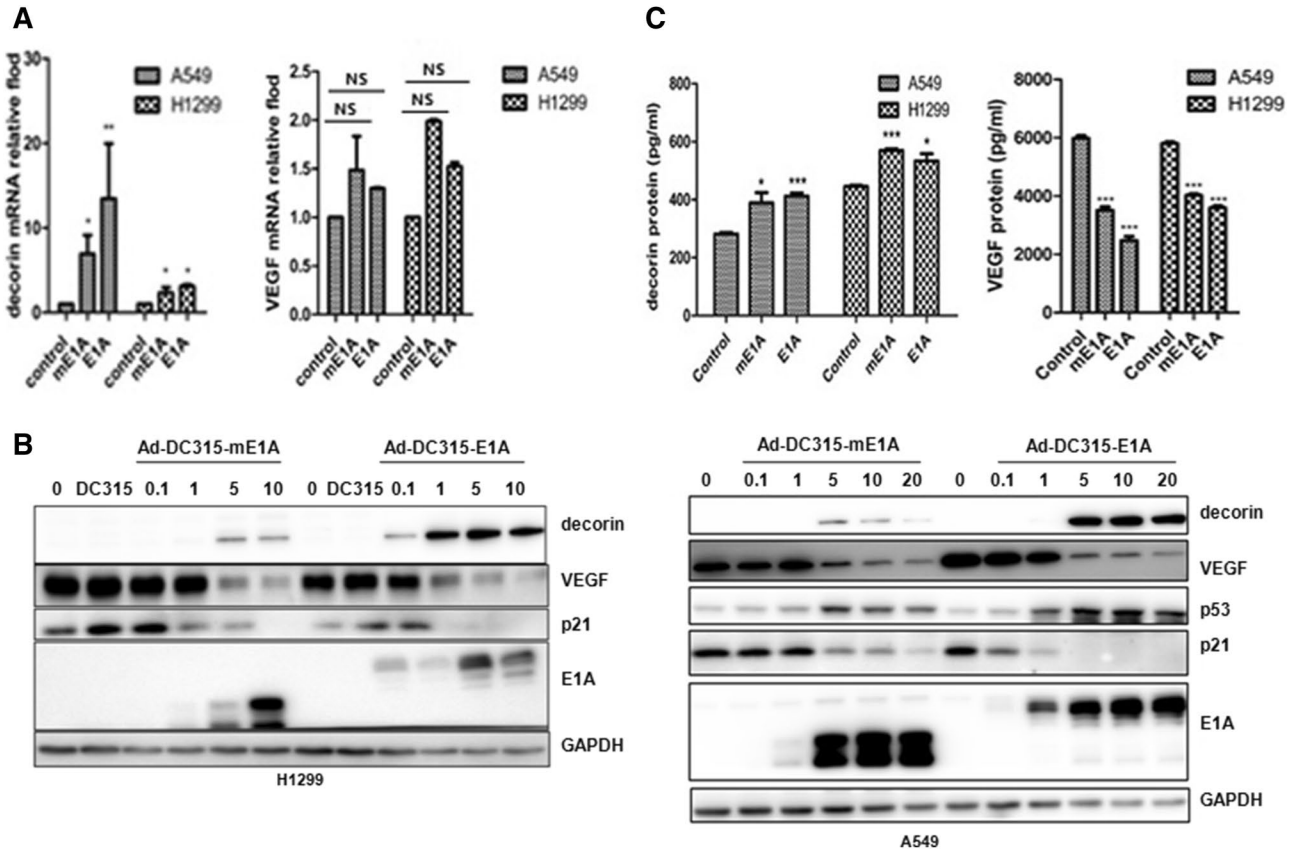


Fig. 2 E1A increased the expression of decorin and decreased VEGF. **a** A549 and H1299 were infected with Ad-DC315-mE1A or Ad-DC315E1A at an MOI of 10 for 24 h, and mRNA expression levels of decorin (left) and VEGF (right) were analyzed by qRT-PCR. Relative expression levels were calculated by normalization to the values for the housekeeping gene beta-actin. Triplicate wells were used for each experiment, and results are from at least two independent experiments. Values are shown as means standard errors of the means. $*p<0.05$; $**p<0.01$; NS, not significance. **b** H1299 and A549 cells were infected with Ad-DC315-E1A, Ad-DC315-mE1A or Ad-DC315 at the indicated MOI for 48 h. The level of p53, p21, decorin, VEGF and E1A proteins was analyzed by Western blotting, in which for decorin detect, the loading samples were from conditioned medium in cells infected with Ad-DC315-E1A, Ad-DC315-mE1A or Ad-DC315 at the indicated MOI. GAPDH was assayed as a loading control. **c** Cells were treated as in (**b**) and then supernatants were collected. ELISA was used to detect the protein expression of decorin and VEGF

mE1A-stable cell lines were also detected. Migration and invasion ability of E1A/mE1A-stable cell lines was attenuated comparing to blank cells (Fig. 3d). These data showed that E1A/mE1A had migration and invasion inhibition ability in vitro, which was consistent with other reports.

Next, we tested the tumorigenic of the E1A/mE1A-stabled expression cell lines in vivo. The same amounts of cells (H1299-vector, H1299-E1A, H1299-mE1A, A549-vector, A549-E1A and A549-mE1A) were injected subcutaneously in a xenograft tumor model. Tumor appearance and growth were monitored daily after inoculation. The tumor size was measured every week until the end of the experiment. As shown in Fig. 4a, tumors formed by E1A/mE1A-stabled expression cell lines exhibited an intense reduction than that control cells. It suggested that the tumorigenicity of E1A or mE1A overexpression cell lines was delayed overwhelmingly compared to the control group. Next, we wanted to determine whether E1A's migration/invasion inhibition ability was dependent on decorin, we performed a transfection assay with decorin siRNA. As shown in Fig. 4b, siRNA 1175 and 619 efficiently knock down decorin expression in E1A/mE1A-stabled expression cell lines, but not affect the expression of E1A or mE1A. Transwell was assayed using the combine siRNA1175 and 619 in the E1A stabled cell lines with silenced decorin; the cell migration and invasion ability was detected. As shown in Fig. 4c, d, there was much more migrated or invaded cells for E1A/mE1A-stabled expression cell lines when cells were decreased decorin by siRNA transfection. It indicated that the knock down of decorin attenuated E1A-mediated inhibition of cell migration. We also tested the role of decorin in E1A-mediated metastasis inhibition by injecting established stable cell lines (H1299-vector, H1299-E1A, H1299-mE1A, A549-vector, A549-E1A and A549-mE1A) into the tail vein of mice. The

data showed that the number of lung metastatic colonies in mice carrying E1A or mE1A was significantly reduced compared with the control group (Fig. 4e). These observations showed that decorin was a potent target of E1A-mediated antitumor activity.

E1A could bind to decorin and induced the proteasomal degradation of VEGF

E1A has no enzymatic or specific DNA-binding capabilities and carries out its functions by binding to multiple cellular regulatory proteins [24]. Then, we want to know whether E1A could interact with decorin or VEGF through a binding manner or not. A549 cells were infected with adenovirus Ad-E1A or vector Ad-dc315 for 48 h. Total cell lysates were immunoprecipitated using anti-E1A antibody and subsequently immunoblotted for anti-decorin or anti-VEGF antibody. As shown in Fig. 5a, an efficient interaction between E1A and decorin was observed, bur not VEGF. Then, we further examined whether E1A regulated VEGF stability. A549 and H1299 cells were infected with Ad-E1A, Ad-mE1A or Ad-dc315 (MOI = 10) for 24 h and then were treated with the protein synthesis inhibitor cycloheximide (CHX), and VEGF expression was measured by Western blot analysis. As shown in Fig. 5b, E1A expression markedly reduced the half-life of VEGF (< 4 h). It suggested that the expression of E1A decreased VEGF protein stability.

Evaluation on antitumor efficacy of E1A through decorin and/or VEGF expression in vivo

Finally, 4-week-old female nude mice from the Animal Center were subcutaneously inoculated with A549 cells (5×10^6 cells each). When the subcutaneous tumor diameter of mice was about 5 mm, the mice were randomly divided into four groups (six mice in each group), and the mice were injected with PBS, Ad-DC315 (vector), Ad-E1A and Ad-mE1A (virus injection dose was 1×10^9 PFU) for treatment and were injected three times a week every other day. Tumor size was measured every 7 days during treatment. The results showed that the inhibition effect of tumors by Ad-E1A and Ad-mE1A treatment groups was significantly higher than that of PBS group and Ad-DC315 treatment group (Fig. 6a). Tumor section slides were prepared for immunohistochemistry assay. As shown in Fig. 6b, the expression of decorin in Ad-E1A and Ad-mE1A groups was significantly up-regulated compared with the other two groups. Then, we used antibodies specific for adenovirus hexon protein to study viral distribution within the tumor mass. We found that Ad-E1A or Ad-mE1A group was significantly more distributed in the tumor mass than vector, based on the marked increased hexon expression.

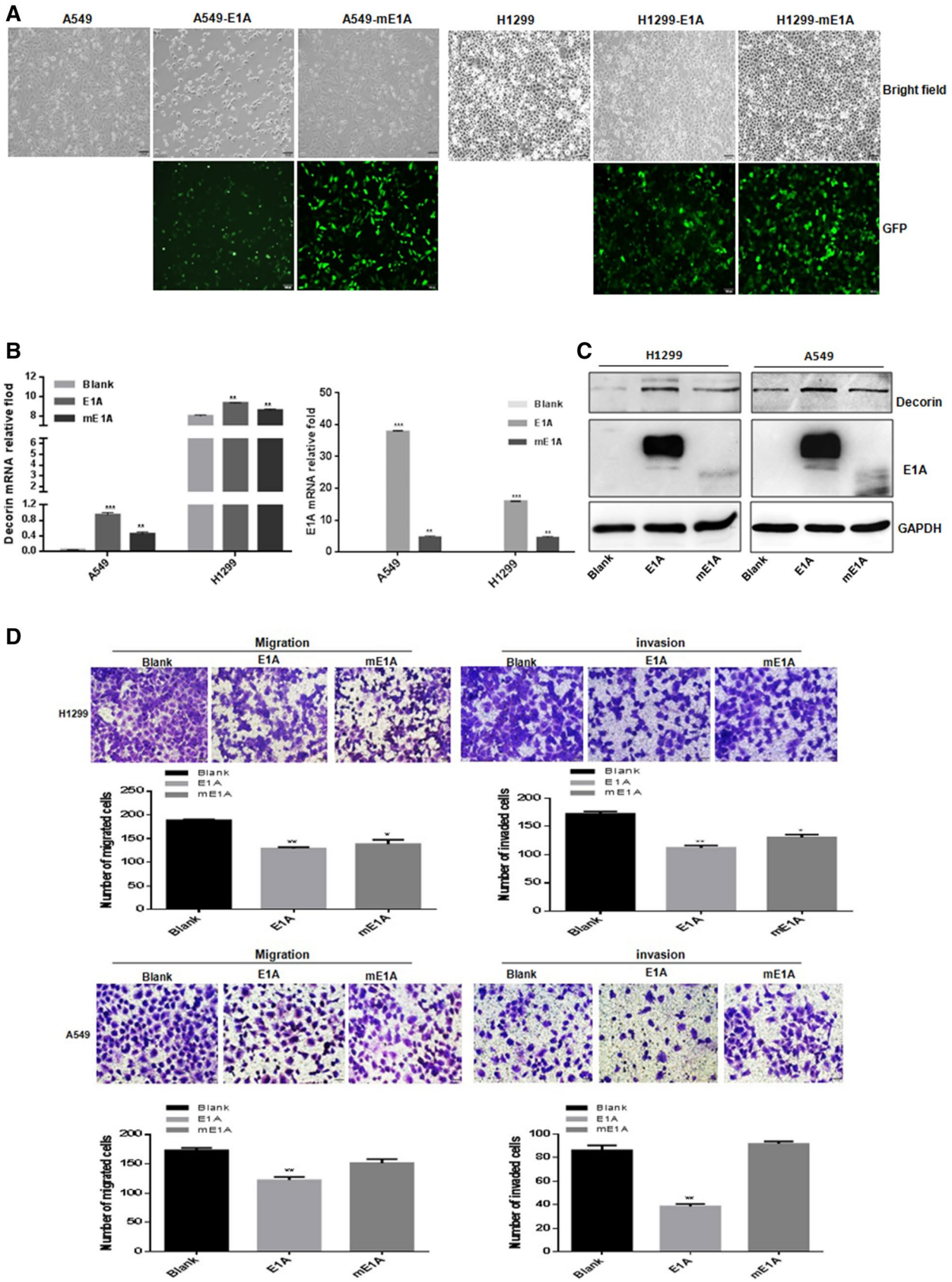
Tumor sections were subsequently stained by Masson's trichrome staining to analyze the distribution of collagen fibers in the cells (blue is collagen fibers). As shown in Fig. 6c, PBS-treated tumors contained higher levels of collagen fibers, whereas Ad-E1A or Ad-mE1A-treated tumors contained very little. For further study, we stained tumor tissue with a specific antibody to type I collagen, one of the ECM components. The results showed that type I collagen was more abundant in the PBS and vector-treated control tumor tissues, while immunopositive signals were less in the Ad-E1A or Ad-mE1A treated tumors (Fig. 6b), which was also consistent with the results of Massons's trichrome staining. Ad-E1A or Ad-mE1A changed ECM components in tumor mass. These results supported the antitumor effect of Ad-E1A or Ad-mE1A and the faster spread of the virus in tumor mass. We also detected the neoangiogenesis by CD31 staining within tumor. It showed that CD31 expression was decreased among Ad-E1A or Ad-mE1A treatment group compared to control groups (Fig. 6b). These data suggested the role of E1A/mE1A in suppressing cancer through changing the tumor characteristics.

Discussion

Many previous studies have indicated the antitumor effect of the human adenovirus E1A gene. E1A can induce tumor cell apoptosis and inhibit cancer cell metastasis [25]. Studies have shown that E1A can down-regulate several oncogenic signaling pathways, such as Her2/neu [26], EGFR [27, 28]. E1A also activated tumor suppressing pathways, such as p53 [7], [29], [30], PP2A/C or FOXO3a [31]. Our previous studies also showed that E1A and CR2 partially deleted mutant E1A (mE1A) can effectively induce tumor cell apoptosis and inhibit tumor growth in a p53-independent manner [7]. In addition, studies have shown that E1A gene therapy combined with paclitaxel treatment significantly improved the sensitivity of tumor cells to paclitaxel and prolonged the survival rate in orthotopic animal models of breast and ovarian cancer [32, 33]. However, the target of E1A-mediated tumor progression inhibition was still unclear and needed further investigations.

Since the adenoviral E1A has a key role in adenoviral replication, proteins encoded by E1A play an important role in adenovirus replication because E1A-encoded proteins are indispensable for maintaining the viral life cycle [34]. E1A, also known as transcription factor, achieves its gene regulatory function by interacting with cellular transcription factors such as CREB, ATF, c-Jun or cellular cofactors such as p300 and CBP and inhibitors such as pRB [35].

There is much more research about tumor inner signaling pathways mediated by adenoviral E1A. However, the role of E1A to affect molecules in the extracellular matrix (ECM)


A
A549
A549-E1A
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H1299
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E1A mRNA relative fold
C
H1299
A549
Decorin
E1A
GAPDH
D
Migration
invasion
H1299
A549
Number of migrated cells
Number of invaded cells

◂**Fig. 3** E1A-stably expressed cell lines have attenuated tumor cell mobility. **a** E1A/mE1A-stably expressed cell lines (H1299-E1A, H1299-mE1A, A549-E1A and A549-mE1A) were constructed by infected with E1A/mE1A lentivirus, in which also included GFP expression. The E1A/mE1A-stably expressed cell lines had high expression level of GFP under a fluoresce microscopy. **b** qRT-PCR was performed to determine decorin and E1A mRNA expression in the E1A/mE1A-stably expressed cell lines and compared to untreated cells to calculate induction. **c** Decorin and E1A protein level was determined by Western blotting in the E1A/mE1A-stably expressed cell lines. GAPDH was used as a loading control. **d** The migration and invasion ability of E1A/mE1A-stable cell lines was evaluated by transwell assay, comparing to blank cells

was hardly reported. In the present work, we found that E1A/mE1A increased decorin and decreased VEGF expression (Fig. 2), which both are components of extracellular matrix (ECM). E1A might provide an antitumor activity through affecting the ECM of tumor mass. Comparing to the wild-type adenoviral E1A, the mutant adenoviral E1A with Rb-binding ability absent (E1A 30–60aa, 120–127aa deletion) had partially different behaviors in inhibiting cancer cell migration and invasion ability (Fig. 3d), and it might be associated with the Rb signaling pathway in cancer cells.

E1A increases the expression of decorin and down-regulates VEGF, two members of tumor ECM, involved in both vasculogenesis and angiogenesis.

Decorin is an extracellular matrix protein, which belongs to a family of small proteoglycans rich in leucine [36]. More and more studies have shown that decorin, as a ligand for various cytokines and growth factors, directly or indirectly participates in various biological processes of cancer cells. Our data showed that decorin expression is reduced in lung cancer specimens and decorin had tumor-suppressing ability and inhibited invasion and metastasis of lung tumor cells (Fig. 1). Decorin could act as a potential therapeutic agent for cancers and shows good prospects for clinical and research applications. Due to the functions of decorin or adenoviral E1A in cancer progression (Fig. 2), we examined the potential connections between decorin and E1A-mediated anticancer activities. Our results showed that E1A's migration/invasion inhibition ability was dependent on decorin (Fig. 4). E1A interacted with decorin directly (Fig. 5). It suggested E1A played a novel role in mediating ECM components.

Decorin has many functions; it can regulate the activity of cell growth factors and participate in the assembly of extracellular matrix. In the subcutaneous lung cancer xenograft model, injection of E1A or mE1A can significantly inhibit tumor growth (Fig. 6). There appeared to have higher decorin expression and be devoid of collagen fibers in tumor bed treated with E1A or mE1A, suggesting that the expression of collagen fibers in tumor tissues decreased with the increase in decorin (Fig. 6). We also found there were less collagen fibers and lower CD31 expression in tumor treated with E1A, compared to the control group. It indicated that E1A inhibited the angiogenesis in tumor mass might through down regulating associated molecular markers, such as VEGF.

In conclusion, we had clearly demonstrated that adenoviral E1A suppressed cancer cell growth and possessed its antitumor activities through regulating decorin and VEGF in ECM to change the tumor characteristics.

Materials and methods

Cell lines, plasmids and lung tissue specimens

H1299, A549, HCT116 p53 +/+, HCT116 p53 −/− and HEK (human embryonic kidney)—293, 293T and HUVEC (human umbilical vein endothelial cells) cells are from the Chinese Academy of Sciences. It was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin at 37 °C in a 5% CO_2 incubator.

The plasmid pGV227-decorin was purchased from GenePharma (Shanghai, China). Decorin fragment was inserted into pCA13 adenoviral vector (Microbix Biosystems) to get pCA13-decorin shuttle plasmid. Adenovirus E1A gene (NC_001405.1) was ligated with adenovirus shuttle vector pDC315 (Microbix Biosystems Inc.) after enzyme cutting, and the ligation product was named as pDC315-E1A. pDC315-mE1A was constructed by cloning 30–60 and 120–127 amino acid deficient E1A synthesized by Invitrogen company into pDC315. The vector pDC315 was used as a control. The detailed methods were descripted previously [26].

E1A or mE1A cDNA from the constructed plasmids pDC315-E1A or pDC315-mE1A was inserted into pCDH1-CMV-MSC-EF1-GFP-Puro lentivirus vector (System Biosciences) at EcoR I/BamH I sites. E1A or mE1A overexpression lentiviruses were generated by co-transfecting 293T cells with the other two packing vectors pMD2G and pSPAX2 and concentrated as described previously [20]. Stable decorin overexpression cell lines (A549, H1299) were generated by infected with lentivirus and selected with 2 μg/ml puromycin for about 2 weeks.

The decorin siRNA and non-specific control siRNA were purchased from GenePharma (Shanghai, China). According to the manufacturer's instructions, we transfected Decorin siRNA and non-specific control siRNA into A549 and H1299 cells using siLentFect lipid reagent (Bio-Rad, Hercules, CA, USA).

Lung tissue specimens had been purchased from Outdo Biotech (Shanghai, China). The clinicopathologic information of patients was obtained from the archive of the pathology department and confirmed by the medical record of

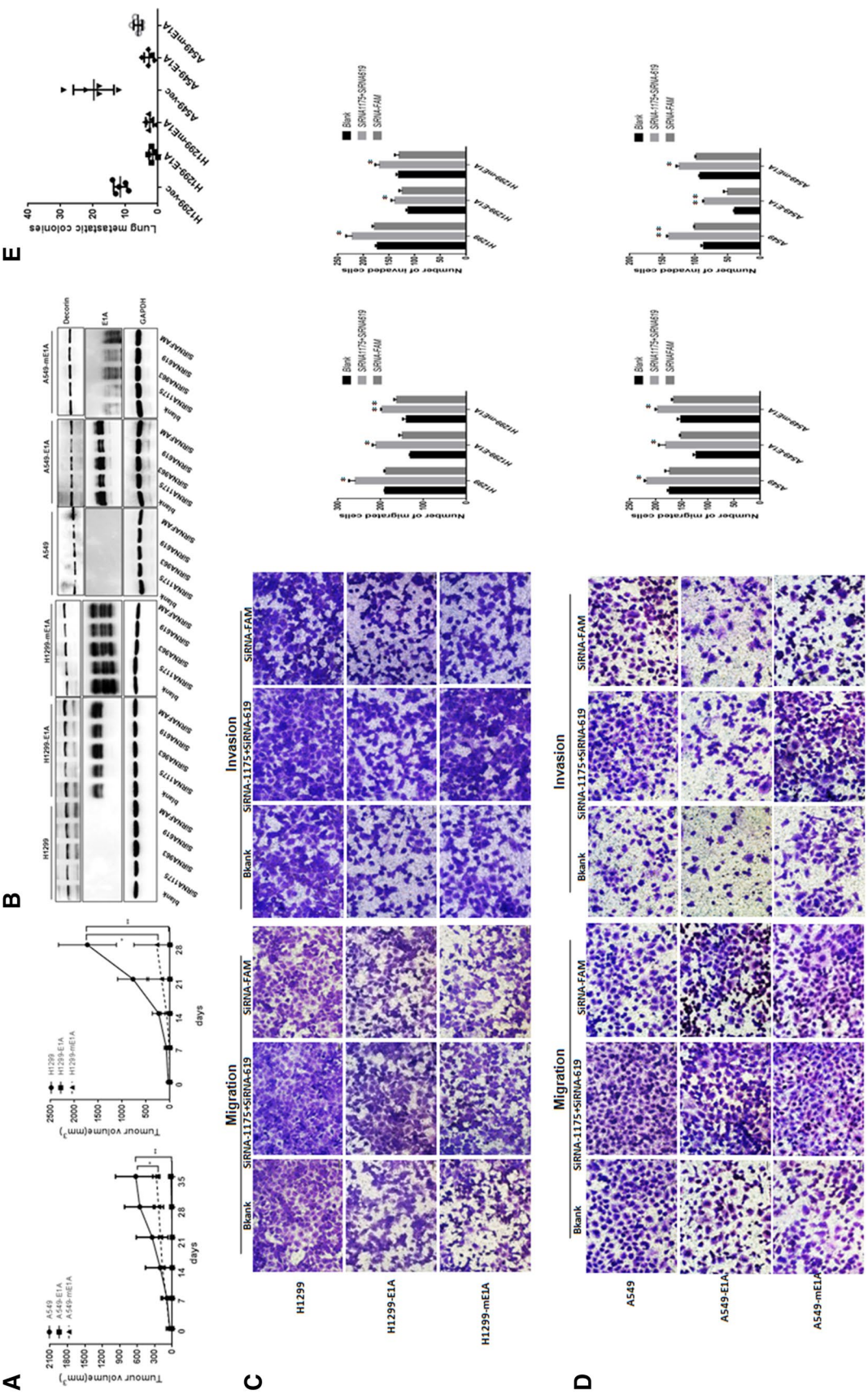

Fig. 4 Decorin was critical for E1A-mediated inhibition of tumor cell. **a** The tumorigenic of the E1A/mE1A-stabled expression cell lines in vivo. The same amounts of cells (H1299-vector, H1299-E1A, H1299-mE1A, A549-vector, A549-E1A and A549-mE1A) were injected subcutaneously in a xenograft tumor model. Tumor appearance and growth were monitored daily after inoculation. The tumor size was measured every week until the end of the experiment. **b** We performed a transfection assay with three synthetic decorin siRNAs (siRNA1175, siRNA963, siRNA619), respectively, in E1A, mE1A-stably expressed cell lines or the related blank cells. Western blotting was performed to detect the expression of decorin and E1A. **c**, **d** Transwell was assayed using the combine siRNA1175 and 619 (targeted to decorin) in the stabled cell lines with silenced-decorin; the cell migration and invasion ability was detected. **e** The effects of decorin on E1A-mediated metastasis inhibition in experimental metastasis assays. Mice were transplanted via tail vein injection with the indicated cells (H1299/vector, H1299/E1A, H1299/mE1A, A549-vector, A549-E1A and A549-mE1A). Lung metastatic colonies were counted with a stereoscopic microscope 8 weeks after injection

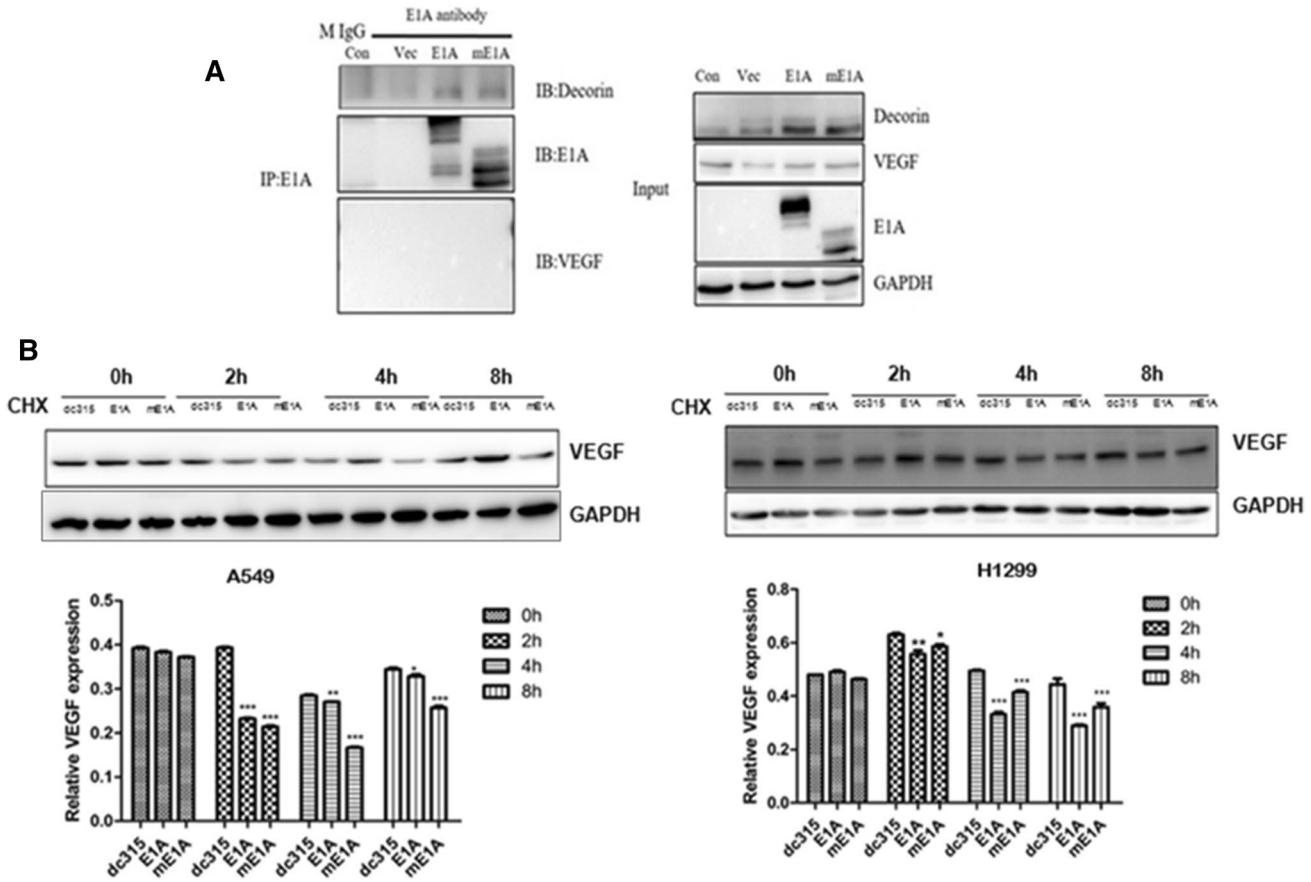

Fig. 5 E1A bound to decorin and induced the proteasomal degradation of VEGF. **a** A549 cells were infected with Ad-E1A, Ad-mE1A or vector Ad-dc315 for 48 h. Total cell lysates were immunoprecipitated using anti-E1A antibody and subsequently immunoblotted (IB) for anti-decorin or anti-VEGF antibody. **b** Determination of the protein stability of VEGF. A549 or H1299 cells were infected with Ad-E1A, Ad-mE1A or vector Ad-dc315 for 48 h. Then, cells were treated with 100 ug/mL CHX for the indicated times, and VEGF expression was analyzed by Western blot assay. The fold change in the protein expression was shown

Outdo Biotech, and informed consent was obtained from all patients. The patient studies were conducted in accordance with Declaration of Helsinki. We took lung cancer tissues and corresponding noncancerous tissues from 15 patients. Each array point had a diameter of 1.5 mm, and each point represented a tissue spot from one individual specimen.

Adenoviruses construction

The constructed shuttle plasmids pCA13-decorin, pDC315, pDC315-E1A and pDC315-mE1A were co-transfected into HEK-293 cells with adenoviral cytoskeleton plasmid pBHGE3 (Microbix Biosystems Inc.) through Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Specific primers for decorin or E1A (mE1A) were used by PCR to identify whether the plasmid construction was correct. HEK293 cells were infected with adenovirus Ad-decorin, Ad-DC315, Ad-DC315-E1A and Ad-DC315-mE1A with a density of about 80%, when half of the cells were floated; the virus was collected and the titer was measured by tissue culture infection dose 50 (TCID50) methods.

Cell proliferation assay

The cells were digested with trypsin and spread in 96-well plates with 10^4 cells per well. One day later, Ad-decorin with a multiplicity of infection (MOI) of 0, 0.5, 2, 10, 20 or 50 was added to the cells. Four days after infection, cell proliferation was analyzed by recording the absorbance of the sample at 570 nm using the MTT assay. The blank control groups were wells that added MTT and medium but no cells. The final data were the average of three independent experiments.

For cell growth assay, stably expressed or vector cell lines were counted on day 1 and plated into 12-well plate at the same amount. Then, the cell proliferation of cell lines was detected by cell counting for 4 continuous days and the cell growth curve was drawn according to the cell growth rate.

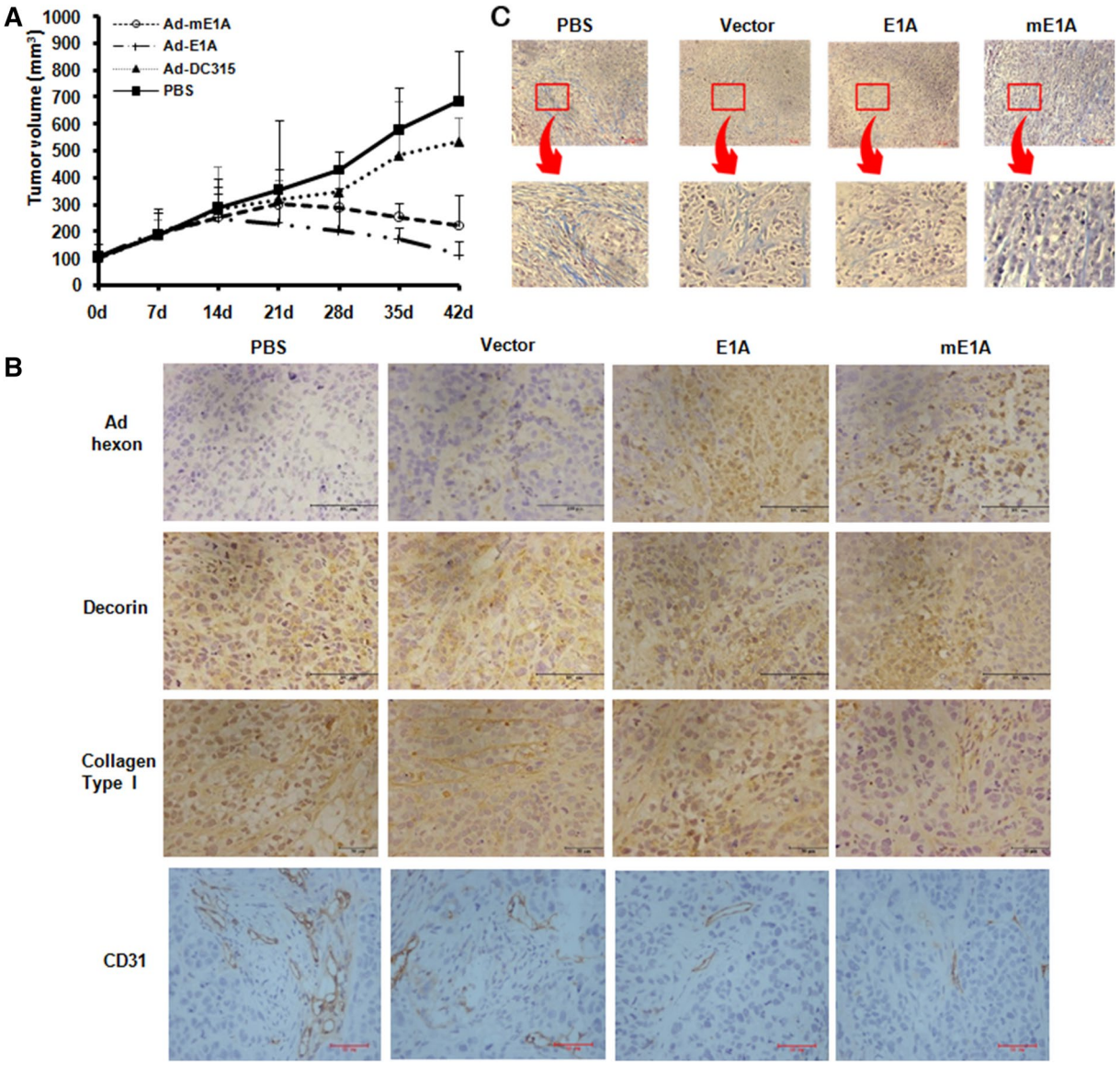

Fig. 6 The antitumor efficacy of Ad-E1A and Ad-mE1A in tumor xenograft nude mice. **a** A549 cells (5×10^6 cells per site) were inoculated into the flank of 4-week-old female BALB/c nu/nu mice. When the tumors reached 3–5 mm in diameter, Ad-DC315, Ad-E1A, Ad-mE1A or PBS was intratumorally injected on days 0, 2 and 4. Tumor growth is expressed as the mean tumor volume ± SD in each group ($n=6$). Statistical significance was determined using Student's t test. $^*p<0.05$, ** $p<0.01$. **b** Tumor section slides were prepared for immunohistochemistry assay. Immunohistochemical staining of adenoviral hexon protein (Ad hexon) to localize adenovirus in tumor tissue. Immunostaining for decorin, collagen type I and CD31 was performed. **c** Masson's trichrome staining of extracellular matrix (ECM) (blue staining) present in the tumor tissue sections

RNA isolation, reverse transcription and quantitative real-time PCR

Total cellular RNA was extracted using 1 ml of TRIzol reagent (Invitrogen) per well in six-well plates. 500 ng RNA was used for cDNA synthesis with HiScript® Reverse Transcriptase reagent Kit (Vazyme biotech, China). Samples were analyzed in triplicate by the Applied Biosystems 7500 PCR System. The targeted genes and endogenous housekeeping gene GAPDH were used as normalizing controls and amplified, using the PrimeScript™ RT reagent Kit (TaKaRa). The cycle number (Ct) was calculated, and the fold changes of gene expression were determined using the double ΔCt ($2^{-\Delta\Delta CT}$) method.

Transwell migration and invasion assays

Briefly, 1×10^5 cells were plated in the upper chamber of the chamber (Corning Costar; Lowell, MA), and 600 µl of medium containing 20% serum was added to the lower

chamber and fixed and stained after 24 h. Invasion assay was similar to migration, and 1×10^5 cells were inoculated in Matrigel—coated chamber and stained after 48 h.

Immunoblotting analysis

A549 and H1299 were seeded into six-well plates at 1×10^5 cells, and then the cells were infected with Ad-E1A or Ad-mE1A (or Ad-DC315) at MOI 10. The primary antibodies used were: anti-decorin (Abeam, USA), anti-VEGF (Beyotime, China), anti-p21 (Santa Cruz Biotechnology, USA), anti-p53 (Santa Cruz Biotechnology, USA), anti-E1A (clone M73, Merck Millipore) and anti-GAPDH (Santa Cruz Biotechnology, USA).

Quantification of decorin and VEGF expression

A549 and H1299 cells were seeded on a six-well plate at 1×10^5 cells per well, and then, cells were infected with Ad-E1A and Ad-mE1A with MOI of 20. After starvation treatment for 1.5 h, culture medium containing 20% serum was added for 24 h. After 24 h, the culture medium was removed, and the 600 µl serum-free culture medium was added for 24 h. Then, the supernatant of each well collected was centrifuged at 4000 rpm for 5 min to take the supernatant as samples. Decorin or VEGF expression was determined by ELISA according to the manufacturer's protocol for decorin (Abcam, USA) or VEGF ELISA kits (Dakewe, China).

Immunoprecipitation

Cells infected with different adenoviruses for 48 h were lysed with RIPA buffer and centrifuged at 4 °C to remove precipitation. 1 ug of the purified antibody was added to a test tube containing 1 mg of protein lysate and incubated overnight at 4 °C. The pre-washed Protein A agarose (20 µl of 50% bead slurry) was then added to the tube and incubated for 1–3 h at 4 °C. The mixture was washed 2–3 times with 500 µl of cold lysis buffer, centrifuged at 2000 rpm for 1 min, and subjected to immunoblot analysis with 20 µl of 5X SDS sample buffer.

Mouse xenografts

BALB/c nude mice (4–5 weeks old) were purchased from Beijing HFK Bio-technology. Animal welfare and laboratory procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals. Additionally, all animal experiments were approved by the Animal Care Committee of the Xuzhou Medical University, Xuzhou, China. First, A549 cells (5×10^6) were subcutaneously injected into the right side of the nude mice. When the tumor diameter reached about 5–6 mm, viruses with a dose of 1×10^9 PFU were injected into the tumor (PBS was injected as control), three times a week, and once every other day. Tumors were measured once a week, and their volume was calculated. Six weeks later, the subcutaneous tumor was removed, weighed and fixed in formalin and embedded in paraffin.

Next, for experimental metastasis assays, 1×10^6 A549 cells resuspended in PBS were injected through the tail vein to observe the tumor metastasis in nude mice. Lung metastatic colonies were counted by stereomicroscope after 8 weeks of injection.

Immunohistochemistry

Tumor tissue was fixed in 10% formalin, embedded in paraffin and cut into 3-mm sections. Deparaffinized tumor sections were treated with primary antibody for hexon, decorin, collagen type I or CD31. After incubation with a secondary antibody, tissue sections were then counterstained with hematoxylin. Masson's trichrome staining was used to analyze the distribution of collagen fibers (stained blue color). Representative sections were stained with Masson's trichrome and then observed by light microscopy.

Statistical analysis

Data were represented by the mean ± standard deviation (SD). Student's *t* test was used to compare differences between groups. Log-rank test was used to compare differences between groups in the survival rate of mice. Statistical significance was defined as a *P* value less than 0.05.

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Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Animal welfare and experimental procedures were carried out strictly in accordance with Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and all procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care Committee of the Xuzhou Medical University, Xuzhou, China.

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