

ORIGINAL ARTICLE

Baculovirus vectors for antiangiogenesis-based cancer gene therapy

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Baculovirus is an insect virus that is non-pathogenic to humans and has emerged as a promising gene therapy vector. Since solid tumor growth/metastasis critically relies on angiogenesis and hEA, a fusion protein comprising human endostatin and angiostatin, exhibits potent antiangiogenic and antitumor efficacy in mouse models; this study aimed to evaluate the feasibility of baculovirus for hEA expression and antiangiogenesis-based cancer gene therapy. Toward this end, we constructed Bac-hEA that mediated transient hEA expression and Bac-ITR-hEA that exploited the adeno-associated virus inverted terminal repeats (ITRs) for prolonged hEA expression. Western blot and ELISA analyses showed that both Bac-hEA and Bac-ITR-hEA expressed hEA in transduced mammalian cells, yet Bac-ITR-hEA only marginally prolonged the hEA expression. In comparison with Bac-hEA, nonetheless, Bac-ITR-hEA significantly enhanced the hEA expression level that concurred with augmented antiangiogenic properties, as demonstrated by cell proliferation, migration and tubule network formation assays. Importantly, intratumoral injection of Bac-ITR-hEA into prostate cancer mouse models, when compared with Bac-hEA, exerted stronger antiangiogenic effects *in vivo*, more potently inhibited tumor growth and significantly prolonged mouse survival. This study collectively supported the notion that hEA is an effective antiangiogenic protein and proved the potential of baculovirus as a vector for antiangiogenesis-based cancer therapy, which may be combined with chemotherapy, radiotherapy or gene therapies using other vectors.

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Introduction

Cancer has been one of the major causes of deaths for decades. Although standard therapies (for example, surgery, chemotherapy and radiotherapy) have been in place, each therapy has its respective drawbacks and restrictions, thus entailing the development of novel cancer therapies. Since the progression of primary tumors beyond 1–2 mm³ and metastatic tumors critically depend on angiogenesis (the formation of new blood vessels from pre-existing vasculatures) to supply oxygen and nutrition,¹ inhibition of angiogenesis represents an appealing anticancer approach. To date, a multitude of antiangiogenic agents targeting tumor vasculature, including the monoclonal antibody bevacizumab, have shown antitumor potentials in clinical studies. Among the various

angiogenesis inhibitors, endostatin blocks endothelial cell proliferation, migration and tubule network formation, and inhibits tumor growth and angiogenesis in a variety of animal tumor models.² Conversely, angiostatin, an amino-terminal fragment of plasminogen, shows potent antiangiogenic and antitumor effects.³ Furthermore, a human endostatin and angiostatin fusion protein (hEA) exhibits prolonged half-life, imposes greater antiangiogenic effects and stronger antitumor efficacy in mouse models.⁴

However, for optimal efficacy the antiangiogenic inhibitor must be continuously present to offset constant secretion of proangiogenic stimuli, thereby necessitating high-dose, frequent and repeated administration of the antiangiogenic protein.⁵ In this regard, long-term supply of the inhibitors by gene transfer should improve clinical efficacy.⁵ Gene delivery can be achieved via various vectors including adenovirus (Ad), adeno-associated virus (AAV), lentivirus and others.⁶ It has been shown that AAV expressing both angiostatin and endostatin gives rise to suppression of primary and metastatic tumors,⁷ while Ad expressing the *hEA* fusion gene exerts potent

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antiangiogenic effects and suppresses the tumor growth in mouse models.⁴ Furthermore, injection of vaccinia virus that expresses hEA inhibits and delays the tumor progression.⁸ However, these vectors also possess various drawbacks. For example, *in vivo* injection of AAV may trigger hepatocellular carcinoma⁹ while Ad mounts potent immune responses, which could provoke severe side effects. Production of AAV and Ad vectors is cumbersome and expensive, possibly hindering their widespread applications in the clinical settings. Furthermore, the majority of human population has been exposed to Ad and AAV; thus, pre-existing immunity may limit the efficacies of these vectors.^{10,11}

In contrast to these human pathogens, baculovirus is an insect virus but it can efficiently transduce a plethora of mammalian cells for transgene expression. Since baculovirus is non-pathogenic to humans and causes minimal toxicity in mammalian cells, recombinant baculovirus construction and handling can be performed in Biosafety Level 1 facilities. Moreover, large-scale production of baculovirus vectors can be readily achieved by infecting insect cells.^{12,13} These attributes prompted the development of baculovirus as a vector for *in vitro* and *in vivo* gene delivery, development of cell-based assays, surface display of eukaryotic proteins, vaccine delivery, tissue regeneration and protein production.^{14–17} Moreover, recently recombinant baculovirus expressing *Diphtheria* Toxin A has been exploited to suppress the glioma xenograft growth in the rat brain.¹⁸ Another baculovirus expressing the tumor antigen also confers protective antitumor effects in mice with brain tumors,¹⁹ implicating the potential of baculovirus in cancer gene therapy.²⁰ Despite the wide spectrum of applications, the non-replication nature of baculovirus results in genome degradation within the mammalian cells over time, leading to transient transgene expression and mitigating its potential for cancer therapies that necessitate sustained expression.

Given that antiangiogenic hEA represses tumor progression, this study primarily aimed to explore the potential of baculovirus vector for hEA expression and prostate cancer therapy. We constructed a baculovirus Bac-hEA that expressed hEA under the control of the cytomegalovirus immediate early (CMV) promoter. To circumvent the shortcoming of transient expression, we also exploited the AAV inverted terminal repeats (ITRs). The AAV genome encompasses *rep* and *cap* genes and the flanking left and right ITRs. The Rep proteins are expressed from the endogenous promoters and can recognize the ITR-flanking genome to mediate site-specific integration into the host chromosome.²¹ It has been shown that transgene expression can be prolonged using a hybrid baculovirus vector containing a gene cassette flanked by the AAV ITRs.²² Therefore, we constructed a hybrid baculovirus that harbored the CMV-hEA cassette flanked by the AAV ITRs and Bac-Rep expressing the Rep proteins. The hEA expression, antiangiogenesis *in vitro* and *in vivo* and antitumor effects conferred by these baculovirus vectors were evaluated.

Materials and methods

Cells and media

Human embryonic kidney cells (HEK293) were cultured in DMEM (Sigma, St Louis, MO) containing 10% fetal bovine serum (Hyclone, Logan, UT). Human umbilical vein endothelial cells (HUVECs) were cultured in M200 medium with low serum growth supplement (Invitrogen, Carlsbad, CA) and cultured to passage three for ensuing experiments. Mouse prostate cancer cells (TRAMP-C1, ATCC CRL-2730) were cultured in DMEM containing 10% fetal bovine serum, 5 $\mu\text{g ml}^{-1}$ bovine insulin (Sigma) and 1 $\times 10^{-8}$ M dihydrotestosterone (Sigma).

Preparation of recombinant baculoviruses

The baculovirus donor plasmid pBac-AAV-lacZ, which harbored the CMV-lacZ gene cassette flanked by the AAV ITRs, was constructed previously.²³ The baculovirus donor plasmid pBac-CMV5 was generated by inserting the CMV promoter into pFastBac Δ polh Δ p10 whose polyhedrin and p10 promoters were removed from pFastBac DUAL (Invitrogen) previously. The fusion gene *hEA* comprising the human endostatin and angiostatin was PCR amplified from pBLAST42-hEndo::Angio (InvivoGen, San Diego, CA) and cloned into pBlueScript II KS+ (Stratagene, La Jolla, CA). The *hEA* gene was then subcloned into pBac-CMV5 in between the CMV promoter and the downstream polyadenylation signal to yield pBac-hEA. In parallel, the *hEA* gene was digested by *Bam*HI/*Sal*I treatment and subcloned into pAAV-MCS (Stratagene) in between the CMV promoter and the polyadenylation signal. The CMV-hEA cassette was then subcloned into pBac-AAV-lacZ to replace the CMV-lacZ cassette by *Not*I digestion, so that the resultant pBac-ITR-hEA contained the ITRs-flanking CMV-hEA cassette.

To construct pBac-Rep, the AAV-2 *rep* gene under the control of endogenous promoter was PCR amplified from pAAV-RC (Stratagene) and cloned into pFastBac Δ polh Δ p10. pBac-luc was constructed by transferring the *luc* (luciferase) gene from pGEM-luc (Promega, Madison, WI) to pBac-CMV5 downstream of the CMV promoter.

The plasmids pBac-hEA, pBac-ITR-hEA, pBac-Rep and pBac-luc were used to generate the recombinant baculoviruses (Bac-hEA, Bac-ITR-hEA, Bac-Rep and Bac-luc, respectively) following the instructions of Bac-To-Bac system (Invitrogen). The viruses were propagated and stored following the standard protocols.²⁴ For *in vivo* administration, the virus was concentrated by sucrose-cushioned (25%, w/v) ultracentrifugation and resuspended in phosphate-buffered saline (PBS, pH 7.4). The virus titer was determined by end point dilution assay²⁴ and expressed as pfu (plaque forming units) per milliliter.

Western blot and ELISA

HEK293 cells were transduced as described previously²⁵ using the multiplicity of infection (MOI) as indicated in Results section. The supernatant was harvested at different times and subjected to 12% sodium dodecyl

sulfate-polyacrylamide gel electrophoresis. Following the protein transfer, western blot was performed using anti-human angiostatin MAb (1:500 dilution; R&D Systems, Minneapolis, MN) as the primary antibody and HRP-conjugated rabbit anti-goat MAb (1:10 000 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD) as the secondary antibody. The membranes were developed using the NEL chemiluminescence substrate (Perkin-Elmer, Waltham, MA).

The hEA concentration in the conditioned medium was measured using an enzyme-linked immunosorbent assay kit (R&D Systems) following the manufacturer's instructions.

Proliferation inhibition, transwell migration and tubule formation assays

HEK293 cells were transduced with Bac-hEA or Bac-ITR-hEA (MOI 20), continued to be cultured and the supernatant (conditioned medium) was harvested at 2 days post-transduction (dpt) for subsequent assays. To examine whether hEA inhibited the HUVECs proliferation, the conditioned medium was either undiluted or diluted ($2\times$ or $5\times$) using the M200 medium, and then used for HUVECs culture in 96-well plates (5×10^3 cells per well). Three days later, the cell growth was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and the OD (optical density) at 570 nm was read. The relative cell number was calculated by the formula: $OD_{\text{sample}}/OD_{\text{control}}$, where the control was the HUVECs cultured using the conditioned medium collected from the mock-transduced cells.

For migration assays, HUVECs were seeded to the gelatin-coated Transwell Cell Culture Inserts (BD Biosciences, Franklin Lakes, NJ) in the 24-well plates (5×10^4 cells per well) containing 400 μl M200 medium and 100 μl conditioned medium ($5\times$ dilution). After 4 h culture, the HUVECs migrating across the membrane of the insert were labeled with the 4',6-diamidino-2-phenyl-indole (DAPI)-containing mounting medium and observed under the fluorescence microscope. The micrographs were captured digitally, and the average number of migrated cells per field was counted using Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD) from five random fields.

For tubule network formation assays, 48-well plates were coated with Matrigel (200 μl per well, BD Biosciences) at 4°C and incubated at 37°C for 30 min. HUVECs suspended in M200 medium were mixed with the conditioned medium at a volumetric ratio of 4:1, dispensed into each well (2×10^4 cells per well) and incubated for 8 h. The tubule network formation was observed microscopically, photographed, and quantified using Image Pro Plus 6.0, by averaging the number of connecting branch points in 10 randomly chosen fields.

Tumor model and virus administration

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, National Science Council). To establish the tumors,

mouse prostate cancer cells TRAMP-C1 were mixed with PBS and injected subcutaneously into the back of C57BL/6J mice (1×10^6 cells per mouse). The width (W) and length (L) of the tumor were measured twice a week and the volume (V) was calculated by the formula: V (mm^3) = $L\times W^2\times(\pi/6)$. When the tumor volume reached $\approx 50\text{ mm}^3$ (≈ 14 days after cell inoculation), the concentrated baculovirus (60 μl) was injected intratumorally (3×10^9 pfu per mouse, $n=6$) every 3 days for three times. When the tumor volume reached 500 mm^3 , the mice were killed and recorded as dead.

In vivo bioluminescence imaging

To verify the baculovirus transduction and transgene expression within the tumor, Bac-luc harboring the luciferase gene was injected into the tumors once as described above and 150 μl per mouse of D-luciferin (Caliper Life Sciences, Hopkinton, MA) was injected intraperitoneally 2 days later. After 10 min, animals were anesthetized with 2.5% isoflurane/air mixture and the luciferase expression was detected by the IVIS spectrum imaging system (Caliper Life Sciences).

Immunohistochemical staining

The tumor specimens were removed from the mice when they reach 200 mm^3 . The cryostat sections (10 μm thick) were fixed in methanol for 5 min, washed with PBS and blocked with the blocking buffer (0.1% Tween-20, 0.1 g ml^{-1} bovine serum albumin, 1% goat serum in PBS, pH 7.4) for 30 min. The sections were then incubated with the rat anti-mouse CD31 MAb (1:200 dilution; BD Biosciences) at 4°C overnight. After three washes, the sections were incubated with Alexa 594-conjugated goat anti-rat IgG (1:200 dilution; Invitrogen) for 1 h at room temperature. The sections were examined using the confocal microscope and the images were captured. The microvessel density was calculated using Image Pro Plus 6.0 by dividing the CD31-positive pixels by the total pixels in five random fields within tumor area.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using the *In Situ* Cell Death Detection kit (Roche, Mannheim, Germany). Briefly, the sections were fixed with 4% paraformaldehyde in PBS for 20 min, washed and incubated in the permeabilization buffer (0.1% Triton X-100 and 0.1% sodium citrate) on ice for 2 min. Following two PBS washes and air dry, the sections were incubated with the TUNEL reaction mixture for 1 h, counterstained with DAPI (Vector Laboratories, Burlingame, CA) and photographed under a fluorescence microscope.

Statistical analyses

The survival curves and median survival were analyzed by the Kaplan–Meier survival test. For the survival data, the log-rank test was used to assess differences among groups. For other comparisons, statistical significance was assessed by Student's *t*-tests.

Results

Confirmation of baculovirus-mediated hEA expression

To explore the potential of baculovirus vector for antiangiogenic cancer gene therapy, we constructed Bac-hEA which harbored the *hEA* fusion gene driven by CMV promoter (Figure 1a) because hEA exerted antitumor effects in mouse tumor models.⁴ Since sustained therapeutic protein expression is desired for cancer therapy and hybrid AAV-baculovirus incorporating the ITR-flanking gene cassette was demonstrated to prolong transgene expression,²⁶ we also constructed Bac-ITR-hEA encompassing the hEA expression cassette flanked by AAV ITRs, and Bac-Rep expressing the AAV Rep protein (Figure 1a), hoping that the Rep-ITR interactions could effectively extend the hEA expression.

To confirm the hEA expression, HEK293 cells were mock transduced, singly transduced with Bac-hEA (MOI 20) or co-transduced with Bac-ITR-hEA (MOI 20) and Bac-Rep (MOI 0, 5, 10 or 20). Western blot analyses of

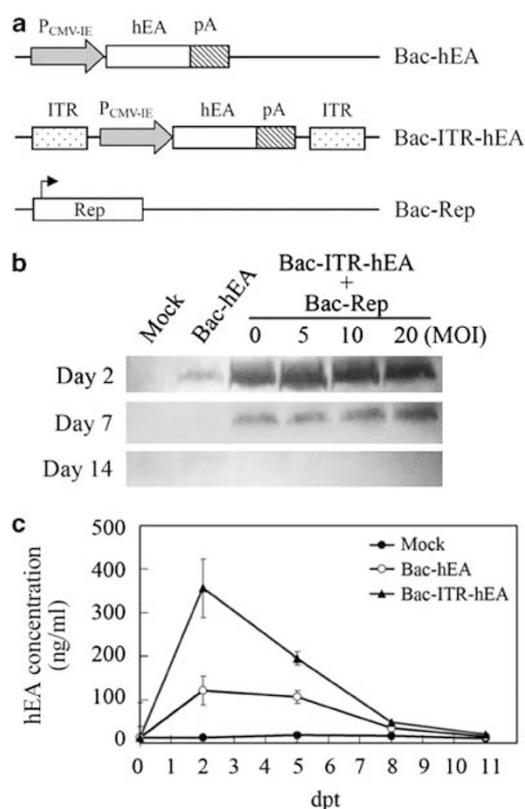


Figure 1 Baculovirus construction and confirmation of hEA protein expression. (a) Schematic illustration of the constructs. (b) Confirmation of hEA expression by western blot. (c) Quantification of hEA expression by ELISA. Bac-hEA harbored the *hEA* gene driven by the CMV promoter while Bac-ITR-hEA contained CMV-hEA cassette flanked by the ITRs. Bac-Rep contained the AAV *rep* gene driven by its endogenous promoter. HEK293 cells were mock transduced, singly transduced or co-transduced with Bac-ITR-hEA and Bac-Rep. The culture supernatants were collected at different times and analyzed by western blot using the anti-human angiostatin MAb or by ELISA using the anti-endostatin MAb. pA, the polyadenylation signal sequence.

the supernatants (Figure 1b) demonstrated that both Bac-hEA and Bac-ITR-hEA expressed hEA with an expected molecular mass of 58 kDa at 2 dpt, and intriguingly Bac-ITR-hEA resulted in considerably higher expression levels than Bac-hEA regardless of the Bac-Rep dosage. Conversely, Bac-hEA-mediated expression was transient, declining to undetectable levels at 7 and 14 dpt. The hEA expression mediated by Bac-ITR-hEA was prolonged to at least 7 days but ceased at 14 dpt, in a manner independent of Bac-Rep.

Since Bac-Rep failed to prolong the hEA expression, Bac-Rep was omitted in all subsequent experiments. The hEA expression levels were next quantified by mock transducing or transducing the cells with Bac-hEA or Bac-ITR-hEA, followed by ELISA analyses of the culture supernatants (Figure 1c). In agreement with the western blot data, mock transduction gave rise to barely detectable hEA whereas Bac-hEA and Bac-ITR-hEA transduction yielded transient hEA expression that culminated at day 2. The maximum hEA concentration conferred by Bac-ITR-hEA ($\approx 356 \text{ ng ml}^{-1}$) was ≈ 2.9 -fold that by Bac-hEA ($\approx 122 \text{ ng ml}^{-1}$), attesting that Bac-ITR-hEA augmented the expression of hEA. However, the expression duration was not significantly extended.

Confirmation of the antiangiogenic activities of hEA

Angiogenesis involves the proliferation and migration of endothelial cells and subsequent blood vessel formation. To attest the antiangiogenic activities of hEA, HEK293 cells were transduced as in Figure 1c and the supernatants were collected at 2 dpt for subsequent biological assays. For proliferation assays, HUVECs were cultured using the diluted ($5 \times$ and $2 \times$) or undiluted ($1 \times$) supernatants and the proliferation was measured by MTT assays 3 days later. The relative cell numbers (Figure 2a), as calculated by normalizing the data against that of the Mock-transduction group, revealed that the conditioned media from the Bac-hEA and Bac-ITR-hEA groups inhibited cell proliferation in a dilution-dependent manner and reduced the relative cell numbers to 72 and 56% compared with that of the Mock group, respectively.

The migration of HUVECs cultured in the conditioned medium ($5 \times$ dilution) was evaluated by the transwell assay. Figure 2b (upper panel) shows differences between the three groups with respect to the number of cells migrating through the membrane pore. Quantitative analyses of migrated cells (lower panel, Figure 2b) further depicted that the conditioned medium from the Bac-ITR-hEA group impeded HUVECs migration more potently than those from the Bac-hEA and Mock groups.

The tubule network formation assay (upper panel, Figure 2c) further illustrated that HUVECs cultured in the conditioned medium from the Mock group remained able to form a healthy tubule network, whereas the supernatants ($5 \times$ dilution) from the Bac-hEA and Bac-ITR-hEA groups suppressed the tubule network formation. Image analyses of the average number of branch points (lower panel, Figure 2c) further delineated more significant ($P < 0.05$) inhibition of tubule network formation in the Bac-ITR-hEA group than in the Bac-hEA and

Mock groups. Altogether, Figures 1 and 2 confirm that Bac-ITR-hEA, when compared with Bac-hEA, expressed higher levels of functional hEA that was capable of retarding the functions of endothelial cells.

Antitumor efficacies imparted by Bac-hEA and Bac-ITR-hEA
To evaluate the antitumor effects of the recombinant baculoviruses, mouse prostate cancer models were created

by subcutaneous injection of mouse TRAMP-C1 cells into the dorsal side of C57BL/6J mouse (1×10^6 cells per mouse). The *in vivo* baculovirus transduction was first assessed by intratumoral injection of Bac-luc (a recombinant baculovirus expressing luciferase) when the tumor volume reached 50 mm^3 , followed by bioluminescence imaging analyses. As illustrated in Figure 3a, intratumoral injection of baculovirus gave rise to effective transduction, as evidenced by the robust luciferase expression within the tumor at 2 days post-injection, which however disappeared at 8 days post-injection (data not shown).

Given the *in vivo* expression, we next injected PBS or the recombinant baculovirus (Bac-hEA or Bac-ITR-hEA) to the tumors (volume $\approx 50 \text{ mm}^3$, $n=6$ per group). Figure 3b depicts that the tumor in the PBS group quickly grew to $>500 \text{ mm}^3$ at 9 days post-injection. In contrast, Bac-hEA injection evidently suppressed the tumor growth, although the tumor growth recurred after day 9 and the tumor volume reached 500 mm^3 at day 18. Tumors treated with Bac-ITR-hEA remained $<100 \text{ mm}^3$ in volume for 9 days, which developed thereafter at a lower rate when compared with the Bac-hEA and PBS groups, and did not reach 500 mm^3 in size until day 27.

Concurrent with the tumor volume data, the life span (median survival) of the mice was significantly ($P<0.001$) prolonged from 9 days for the PBS group to 18 days for the Bac-hEA group (Figure 3c). Bac-ITR-hEA injection further extended the life span to 24 days ($P=0.002$ when compared with the Bac-hEA group), confirming that Bac-ITR-hEA was more effective in suppressing the prostate cancer growth than Bac-hEA.

Antiangiogenesis conferred by Bac-ITR-hEA

To examine whether the antitumor effects stemmed from the repression of angiogenesis, the *in vivo* experiments were repeated and mice were killed when the tumor volume reached 200 mm^3 . CD31-specific immunohistochemical staining of the tumor sections (Figure 4a) illustrated apparent formation of branching vessels within the tumors treated with PBS, indicating the occurrence of

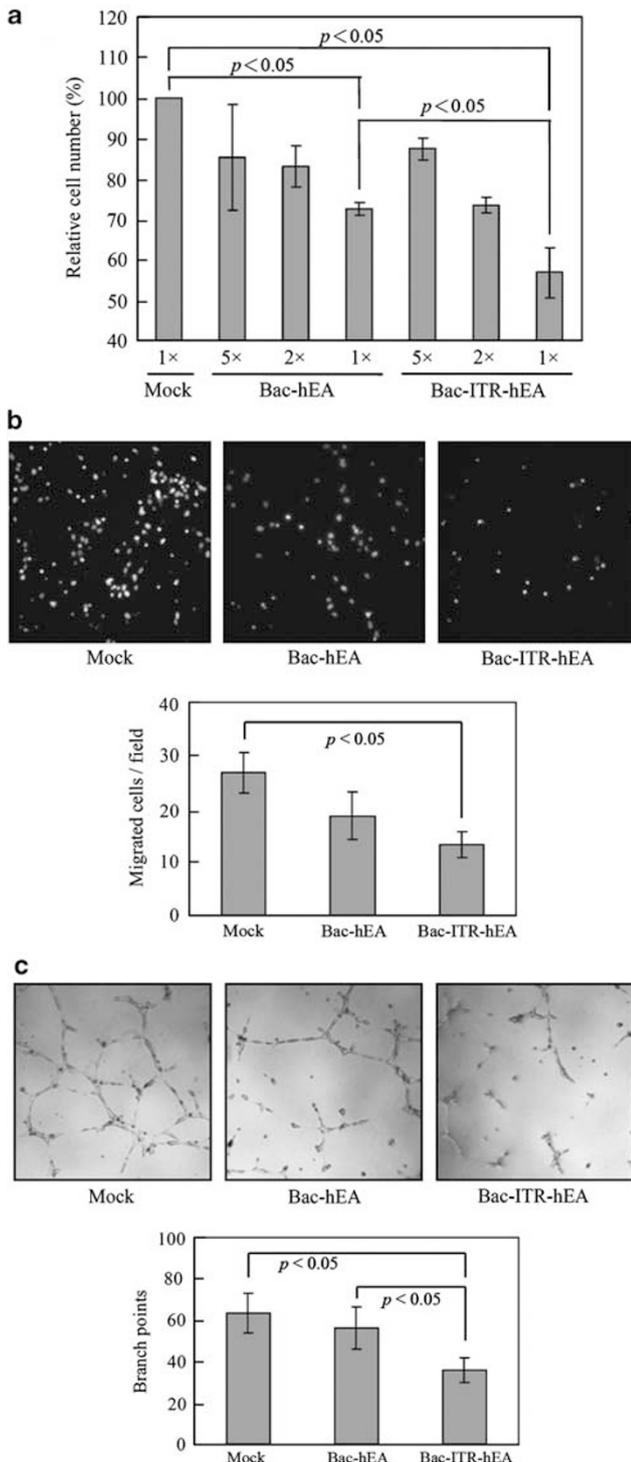


Figure 2 Antiangiogenic activity of baculovirus-expressed hEA. (a) Effects on cell proliferation. (b) Effects on cell migration. (c) Effects on tubule network formation. HEK293 cells were transduced as in Figure 1c and the conditioned medium was collected at 2 dpt for subsequent assays. For proliferation assays, HUVECs were cultured using the diluted ($5 \times$ and $2 \times$) or undiluted ($1 \times$) supernatants for 3 days and the proliferation was measured by MTT assays. The relative cell numbers were calculated by normalizing the OD_{570} data against that of the Mock-transduction group. For migration assays, HUVECs were cultured in cell culture inserts using the conditioned medium ($5 \times$) for 4 h. The cell migration was visualized by DAPI staining of cells and microscopy. The average numbers of migrated cells in five random fields were counted and analyzed by Image Pro Plus 6.0. For tubule network formation assays, HUVECs were cultured on Matrigel-coated 48-well plates with diluted conditioned medium ($5 \times$). The formation of tubule networks was examined after 8 h under the microscope and the images were captured digitally. The branch points in five random fields were counted and analyzed by Image Pro Plus 6.0.

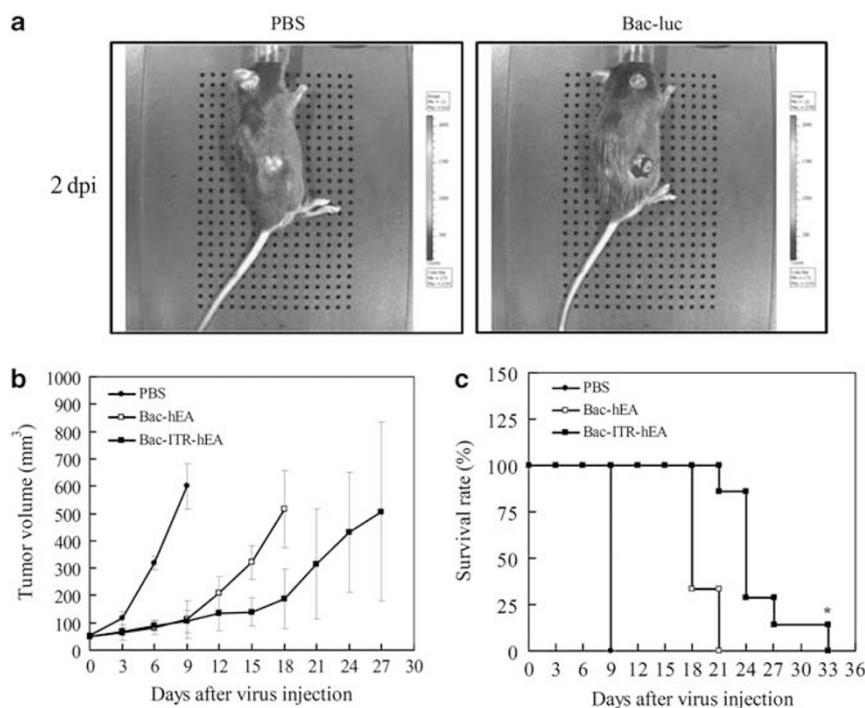


Figure 3 Antitumor efficacies conferred by Bac-hEA and Bac-ITR-hEA. **(a)** *In vivo* transgene expression. **(b)** Tumor growth curves. **(c)** Mouse survival. The tumors were established by subcutaneous implantation of TRAMP-C1 cells (1×10^6 cells per mouse) into 6-week-old C57BL/6J male mice ($n=6$ per group) and the tumor size was measured every 3 days. When the tumor volume reached $\approx 50 \text{ mm}^3$, the *in vivo* transgene expression was confirmed by injecting PBS or Bac-luc into the tumors, followed by bioluminescence imaging using the IVIS system on day 2 after injection. The antitumor effects were evaluated by intratumoral injections of PBS or baculovirus on days 0, 3 and 6 after the tumor size reached $\approx 50 \text{ mm}^3$. The mice were recorded as dead when the tumor volume reached 500 mm^3 . The mouse survival was calculated by Kaplan–Meier survival analysis. * $P < 0.005$ between Bac-ITR-hEA and Bac-hEA (and PBS) groups.

angiogenesis that accompanied the tumor growth. Bac-hEA attenuated the vessel formation but only to a limited extent. In marked contrast, the microvasculature structures appeared distinctly less organized and scarce in the tumors treated with Bac-ITR-hEA. Quantitative analysis of microvessel densities (Figure 4b) further confirmed that Bac-ITR-hEA gave rise to more significant ($P < 0.05$) inhibition of angiogenesis than the Mock and Bac-hEA groups.

Additionally, we examined whether tumors lack of adequate vascular networks underwent cell death. The TUNEL and DAPI stainings (Figure 5) revealed only mild cell death in the tumors treated with PBS or Bac-hEA, but the cells in the tumors treated with Bac-ITR-hEA underwent massive death. Figures 4 and 5 unravel that Bac-ITR-hEA mitigated the angiogenesis and induced cell death within the tumors more effectively than PBS and Bac-hEA.

Discussion

The overriding objective of this study was to evaluate the feasibility of baculovirus for antiangiogenesis-based cancer gene therapy. Toward this end, we constructed Bac-hEA that mediated transient hEA expression and the

hybrid baculoviruses, Bac-ITR-hEA and Bac-Rep, hoping that the cooperative interaction between the AAV Rep proteins and ITR would confer sustained hEA expression (Figure 1a). Indeed, both Bac-hEA and Bac-ITR-hEA were able to transduce mammalian cells for hEA expression. Regardless of Bac-Rep co-transduction, however, Bac-ITR-hEA only marginally prolonged the hEA expression and failed to extend the expression beyond 8 days (Figures 1b and c). The data markedly contrasted with the previous report that hybrid baculovirus vectors incorporating the AAV ITR-flanking cassette can prolong the transgene expression in rat brains to 90 days,^{22,26} presumably due to the disparity in the cell types being transduced. It should be noted that, despite prolonging the transgene expression *in vitro* and *in vivo*,²⁶ the AAV ITRs were unable to change the dynamics and transient nature of baculovirus-mediated expression, which would restrict its applications.

Nonetheless, Bac-ITR-hEA gave rise to a significantly higher hEA expression level than Bac-hEA (Figures 1b and c), which was concomitant with the augmented antiangiogenic properties as demonstrated in cell proliferation, migration and tubule network formation assays (Figures 2a–c). The data indicated that the flanking ITRs ameliorated the baculovirus-mediated expression, which agreed with the improved transgene expression mediated by the hybrid baculovirus²² and herpes simplex virus.²⁷

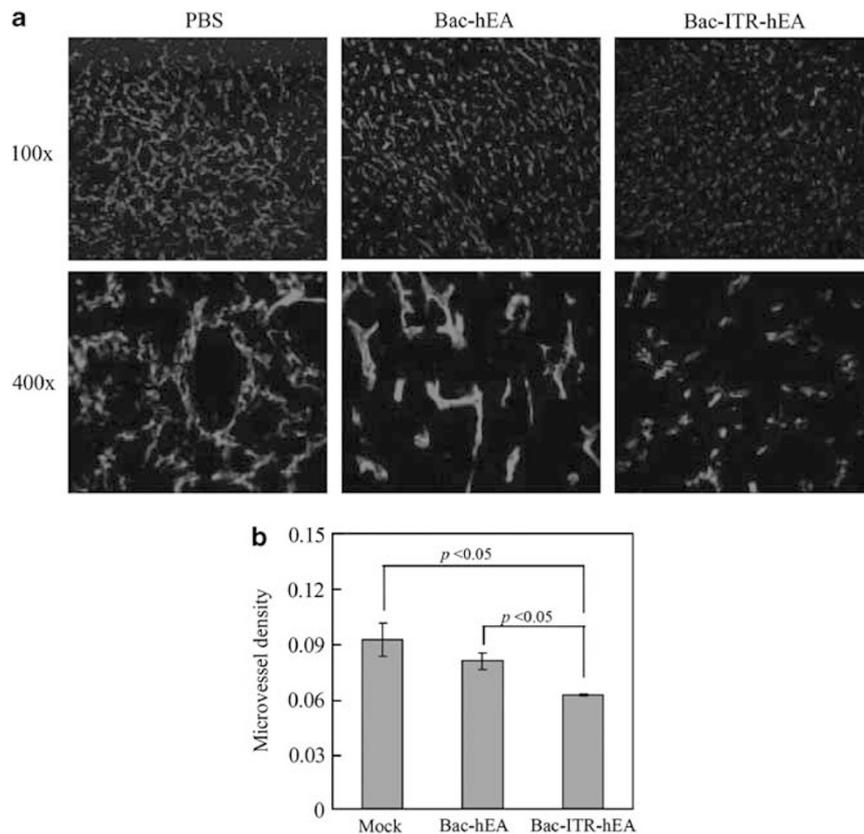


Figure 4 Bac-ITR-hEA conferred antiangiogenic effects. (a) CD31-specific immunohistochemical staining. (b) Microvessel density. Tumor specimens were removed when tumor volume reached $\approx 200 \text{ mm}^3$ and sectioned for CD31-specific immunohistochemical staining. The images were captured and the microvessel density was calculated by dividing the CD31-positive pixels by the total pixels in five random fields within the tumor. Magnification: $\times 100$ (upper panel); $\times 400$ (lower panel).

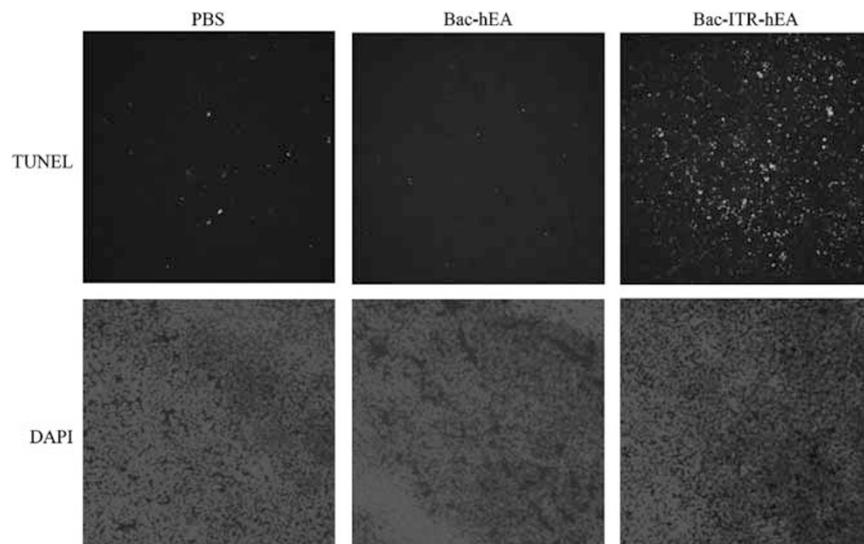


Figure 5 Bac-ITR-hEA induced cell death. The tumor sections obtained as in Figure 4 were subjected to TUNEL staining (upper panel) and DAPI counterstaining (lower panel). Magnification, $\times 200$.

The elevated expression may be ascribed to the intrinsic promoter activities of AAV ITRs, which function in concert with the CMV promoter to facilitate the recruitment of transcription factors.²²

Importantly, Bac-ITR-hEA, when compared with PBS and Bac-hEA, exerted stronger antiangiogenic effects *in vivo*, and more potently inhibited tumor growth and prolonged mouse survival from 9 days (PBS group) to 24

days (Bac-ITR-hEA group) (Figures 3–5), indicating that enhancement of the baculovirus-mediated hEA expression *in vivo* substantiated the antitumor effects. These data implicated strategies to potentiate the antitumor efficacy by either choosing stronger promoters such as CAG²⁸ or further improving the *in vivo* transduction efficiency. Although baculovirus transduces cancer cells *in vitro* at high efficiencies¹² and confers transgene expression within the tumors (Figure 3a), hereby baculovirus was injected directly into the established tumors ($\approx 50 \text{ mm}^3$) rich in vasculature; thus, intratumoral baculovirus transduction might have been mitigated as a result of baculovirus inactivation by serum complements as observed in multiple tissues.²⁹ Such problem may be alleviated by using complement inhibitors upon virus injection³⁰ and displaying decay accelerating factor (a protein shielding baculovirus from complement proteins) on the baculoviral envelope,^{31,32} which protect baculovirus from complement inhibition and improve *in vivo* baculovirus transduction.

Aside from the antiangiogenic effects elicited by hEA, *in vivo* administration of wild-type baculovirus triggers protective innate immunity against virus infections and tumors,³³ lending itself an ideal vector for antitumor therapy. Such baculovirus-induced antitumor effects can be at least partly explained by the baculovirus activation of dendritic cells and natural killer cells,³³ which inhibit the tumors in a transgene-independent manner.³⁴ Consequently, baculovirus has been exploited to treat cancers in liver,³³ skin,³⁴ lung³⁴ and brain.¹⁸ This study adds a new dimension to the applications of baculovirus vectors to prostate cancer treatment.

Altogether, this study demonstrates the proof-of-concept that baculovirus holds promise as a vector for antiangiogenesis-based cancer therapy and supports the assertion that hEA is a potent antiangiogenic factor to inhibit tumor growth.³⁵ Although the antitumor effects are temporary and subpar to completely lead to tumor regression, this is not uncommon as antiangiogenesis monotherapy fails to block tumor progression in the clinical studies.³⁶ Nevertheless, antiangiogenic agents can make the tumor more susceptible to eradication, enhancing the effectiveness of radiotherapy or chemotherapy, even at lower cumulative doses.³⁶ Therefore, in future studies baculovirus-mediated antitumor therapy should be attempted in conjunction with chemotherapy or radiotherapy. Furthermore, multiplex gene therapies targeting different mechanisms of angiogenesis by inserting a cocktail of several antiangiogenic, immunostimulatory and/or tumor-targeting suicide genes into the baculovirus vector can be explored. Meanwhile, the short-term inhibition of tumor growth at least partly stemmed from the transient expression of hEA. Disappointingly, the hybrid baculovirus harboring the ITRs was insufficient to confer persistent hEA expression, hence other approaches to extending transgene expression should be explored. For instance, appending the woodchuck hepatitis virus post-transcriptional regulatory element to the 3' end of the transgene increases the mRNA stability and prolongs protein expression;³⁷ therefore, future modifications of the baculovirus vector

by inserting the woodchuck hepatitis virus post-transcriptional regulatory element to the 3' end of *hEA* gene may be attempted. Conversely, stable transgene expression can be imparted by transposons such as *Sleeping Beauty*³⁸ and *PiggyBac*,³⁹ or by phage integrase such as ϕC31 .⁴⁰ These systems enable transgene integration into the chromosomes and may be incorporated into the baculovirus vector for long-term hEA expression. Alternatively, Bac-ITR-hEA may be administered intramuscularly because muscle provides a microenvironment more suitable for sustained expression and hEA can exert the antitumor effects via systemic administration.⁴ Finally, although *hEA* gene delivery can be achieved by either adenovirus³⁵ or vaccinia virus,⁸ the pre-existing immunity against these vectors in humans can attenuate the antitumor efficacies. In contrast, baculovirus is an insect virus in nature, and humans do not appear to possess pre-existing antibody and T cells specifically against baculovirus.⁴¹ As such, baculovirus can circumvent the pre-existing immunity problems and holds promise as an alternative vector in the prime-boost regime for prostate cancer gene therapy, by which adenovirus is used first, followed by the use of baculovirus or vice versa.

Conflict of interest

The authors declare no conflict of interest.

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