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Decreased Tumorigenic Potential of EphA2-Overexpressing Breast Cancer Cells Following Treatment with Adenoviral Vectors That Express EphrinA1

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ABSTRACT

The EphA2 receptor tyrosine kinase is frequently overexpressed in invasive breast cancer cells. Moreover, these malignant cells have unstable cell-cell contacts, which preclude EphA2 from interacting with its ligand, EphrinA1, which is anchored to the membrane of adjacent cells. This defect is important because ligand binding causes EphA2 to transmit signals that negatively regulate tumor cell growth and survival, whereas the absence of ligand binding favors these same behaviors. In our present study, human adenoviral type 5 (HAd) vectors were engineered to express secreted-forms of EphrinA1. These vectors were used to infect MDA-MB-231 human breast cancer cells, or MCF-10A human breast epithelial cells providing matched controls. Infection with HAd-EphrinA1-Fc (HAd vector expressing extracellular domain of human EphrinA1 attached to Fc portion of human IgG_1 heavy chain) caused increased EphA2 activation and turnover and consequently decreased tumor cell viability in soft agar assays. Consistent with this observation, infection of MDA-MB-231 cells with HAd-EphrinA1-Fc prevented tumor formation in xenograft models. Furthermore, therapeutic modeling via intratumoral inoculation revealed that HAd-EphrinA1-Fc significantly inhibited subsequent tumor growth as compared to matched controls. These results suggest that targeting of EphA2 with adenoviral vectors may have therapeutic value.

Key Words: EphA2, EphrinA1, breast cancer, adenoviral vectors, gene therapy, delivery vehicle

INTRODUCTION

Changes in the expression or function of receptor tyrosine kinases have been linked to many types of cancers, most notably breast cancer.[1] Tyrosine kinases transmit powerful signal that are essential for the growth, survival and invasiveness of aggressive cancer cells. For instance, the EphA2 receptor tyrosine kinase is overexpressed in many highly aggressive breast cancers.[2] High levels of EphA2 have been documented in many different cancers[2,3,4,5,6] and this overexpression has been attributed to many different transcriptional and post-translational modifications that frequently arise in cancer. For example, EphA2 expression is governed in part by p53 and Ras, both of which are frequently altered in cancer.[7,8] These changes are important because high levels of EphA2 are sufficient to promote a tumorigenic phenotype,[2] which clearly demonstrate a role for EphA2 overexpression in cancer.

Much recent evidence has shown that EphA2 functions very differently in normal versus malignant cells.[9,10] For example, EphA2 may function in normal cells as a negative regulator of cell growth and migration to mediate contact inhibition at high cell density. These outcomes appear to arise from the fact that EphA2 binds a ligand, known as EphrinA1[11,12,13] which is anchored to the membrane of adjacent cells. The biochemical consequences of ligand binding include EphA2 autophosphorylation[11,12,13] which mediates active signaling that decreases extracellular matrix (ECM) attachment and importantly, increases EphA2 turnover. Consequently, normal epithelial cells generally have low levels of EphA2 protein, which is itself tyrosine phosphorylated. However, tumor cells generally have unstable cell-cell contacts and this decreases EphA2-EphrinA1 binding.[14] Consequently, the EphA2 in malignant cells is not phosphorylated, which alters the subcellular localization and function of EphA2 in a manner that favors tumor cell growth and survival.[2] Compounding this, decreased ligand-mediated turnover causes EphA2 to accumulate at the cell surface. However, restoration of EphA2 stimulation can be achieved using artificial ligands[15] and monoclonal antibodies[16] and these treatments negatively regulate tumor cell growth and survival.

Human adenovirus (HAd)-based vectors have been the focus of considerable interest in the last few years, based on their potential application as delivery vehicles for human gene therapy.[17,18,19,20] The results of animal studies and clinical trials in humans for cancer therapy have further increased interest in adenoviral-based therapy.[21,22,23,24,25,26] Based on the differential ligand binding that EphA2 demonstrates in normal versus malignant cells, we describe herein the engineering of HAd type 5 (HAd5) vectors that express secreted forms of EphrinA1 and show that these vectors can negatively regulate tumor cell growth in vitro and in vivo.

RESULTS

Generation of HAd5 vector containing EphrinA1-Fc or EphrinA1-Sc

Two variants of EphrinA1 constructs: i) a secretory-form consisting only of extracellular domain (lacking native GPI-anchorage) genetically conjugated to the Fc portion of IgG_1 (EphrinA1-Fc), and ii) the extracellular domain of EphrinA1 lacking GPI-anchorage and without Fc portion of IgG_1 (EphrinA1-Sc) were used to generate HAd-EphrinA1-Fc and HAd-EphrinA1-Sc, respectively (Fig.1). These vectors were generated using *Cre* recombinase-mediated site-specific recombination.[27] Similarly, an empty vector (HAd-ΔE1E3) was also generated to serve as a negative control. These vectors were purified by cesium chloride density-gradient centrifugation and DNA was isolated from purified virions. To determine whether HAd vectors contained the correct insert, DNA samples were digested with *HindIII, XhoI, or KpnI* and the resultant fragments were separated by agarose gel electrophoresis. HAd-EphrinA1-Fc, HAd-EphrinA1-Sc, and HAd-ΔE1E3 produced the expected restriction patterns suggesting the authenticity of these vectors (data not shown). For further characterization of these vectors, total cellular RNA was isolated from vector-infected cells and analyzed by Northern blot using an EphrinA1- specific

radiolabeled probe. The major bands showing hybridization with the probe were approximately 1.8 and 1.4 kb in RNA samples isolated from HAd-EphrinA1-Fc- or HAd-EphrinA1-Sc-infected cells, respectively suggesting that EphrinA1-Fc and EphrinA1-Sc were expressed in infected cells (Fig. 2). The appearance of EphrinA1-Sc-like signal in both the HAd-ΔE1E3 and HAd-EphrinA1-Fc lanes may represent endogenous EphrinA1.

EphrinA1 expression in HAd vector-infected cells

To detect the Fc portion of IgG_1 attached with EphrinA1, 293 or MDBK cells were mock-infected or infected either with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 for 24 or 48 h. Cell extracts and supernatants were analyzed by Western blot analyses to detect the Fc portion of EphrinA1-Fc. In HAd-EphrinA1-Fc-infected 293 cell lysates and supernatants, EphrinA1-Fc was detected at both 24 and 48 h time points (Fig. 3A). On the other hand, in MDBK cells (without E1 complementation), most EphrinA1-Fc was restricted to supernatants. EphrinA1-Fc expressed in HAd-EphrinA1-Fc infected cells migrated at the level similar to EphrinA1-Fc purified from 293 cells stably expressing EphrinA1-Fc. No specific bands were detected in cell lysates or supernatants of 293 or MDBK mock-infected or infected with HAd-EphrinA1-Sc or HAd-ΔE1E3. These results suggest that HAd-EphrinA1-Fc can induce efficient expression and secretion of EphrinA1-Fc.

To confirm the expression of EphrinA1 by vectors containing EphrinA1, 293 cells were mock-infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 and harvested at 24 and 48 h post-infection. Supernatants and cell extracts were analyzed for EphrinA1 expression by Western blot analyses. In HAd-EphrinA1-Fc infected cells, EphrinA1-Fc (approximately 65 kDa) expression was observed both in cell extracts and supernatants at 24 and 48 h post-infection (Fig. 3B), whereas in HAd-EphrinA1-Sc infected cells, EphrinA1-Sc (approximately 25 kDa) expression was detected in the cell extract and the supernatant at 48 h post-infection. These results confirmed expression of EphrinA1-Fc or EphrinA1-Sc by HAd-EphrinA1-Fc or HAd-EphrinA1-Sc, respectively.

EphA2 expression and phosphorylation in vector-infected cells

Transduction efficiencies of 293, MDA-MB-231 and MCF-10A cell lines with a HAd5 vector were 91, 82 and 63% respectively as determined using a HAd5 vector (HAd-GFP) expressing green fluorescent protein (GFP) as a reporter[28] suggesting that HAd5 vectors will efficiently transduce both MDA-MB-231 and MCF-10A cells. To ask if EphrinA1 expression by HAd vectors might alter EphA2 expression or function, MDA-MB-231 or MCF-10A cells were mock-infected or infected either with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3. Cell lysates were collected at various times post-infection and subjected to Western blot analyses with antibodies specific for EphA2, E-cadherin, or β-catenin. Immunoprecipitated products obtained with an anti-EphA2 antibody were also subjected to Western blot analysis using an anti-phosphotyrosine (P-Tyr) antibody (Fig. 4). EphA2 became phosphorylated efficiently within 12 h in HAd-EphrinA1-Fc cells and this persisted for at least 24 h. Notably, the levels of EphA2 phosphotyrosine content decreased by 36 h and further analyses of whole cell lysates indicated that this outcome was the result of increased EphA2 turnover. EphA2 activation and degradation was also observed in HAd-EphrinA1-Sc-infected cells though the timing and efficiency of these responses were considerably lower than results with cells infected with HAd-EphrinA1-Fc.

The majority of EphrinA1 and EphA2 in mock- or HAd-ΔE1E3-infected MDA-MB-231 cells was present as intracellular aggregates as detected by immunofluorescence assay (IFA). Whereas in HAd-EphrinA1-Sc, or HAd-EphrinA1-Fc-infected breast cancer cells, EphrinA1 and its derivatives were diffusely distributed in the cytoplasm and also on the cell surface (data not shown). However, in HAd-EphrinA1-Fc-infected cells, the intensity of EphA2 immunoreactivity in IFA was drastically reduced (data not shown), which was consistent with the increased EphA2 turnover observed by Western blot

analyses (Fig. 4). As expected, MCF-10A cells expressed moderate levels of EphrinA1 and distributed on the cell surface and none of the experimental conditions altered EphrinA1 or EphA2 distribution significantly in IFA (data not shown).

It has been demonstrated that expression of E-cadherin in MDA-MB-231 cells improved EphA2 activation.[29] We wanted to know whether activation of EphA2 enhances expression of E-cadherin in MDA-MB-231 cells. The levels of E-cadherin expression in breast cancer cells were below the detection level at all times, irrespective of the type of vector treatment (Fig. 4) suggesting that EphA2 activation does not restore E-cadherin expression to normal levels. No visible change in E-cadherin expression was observed in MCF-10A with any of the HAd vector infection (Fig. 4).

Inhibition of soft agar colonization and viability of breast cancer cells by HAd vectors

Cancer cells can survive and grow in semi-solid matrices (e.g., soft agar), and this behavior distinguishes malignant from non-transformed cells. For many cancer cell lines, soft agar colonization relates to metastatic potential.[30] To ask if HAd vectors that express EphrinA1 might alter this phenotype, MDA-MB-231 or MCF-10A cells were mock-infected or infected either with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 prior to suspension in semi-solid agar. Consistent with previous findings, MCF-10A cells could not colonize whereas MDA-MB-231 cells efficiently colonized soft agar (256±5, 257±66 colonies by mock and HAd-ΔE1E3 controls, respectively) (Fig. 5A). Notably, HAd-EphrinA1-Fc infection dramatically reduced colony formation. HAd-EphrinA1-Sc infection did not inhibit colony formation as efficiently as HAd-EphrinA1-Fc, which was consistent with its decreased biochemical activity (Fig. 4)

We then related the inhibitory effects of HAd-EphrinA1-Fc with cell viability and observed a steady decline in MDA-MB-231 cells viability over time, with 11± 0.2% viability at the 96 h time point (Fig. 5B). The decline observed using HAd-EphrinA1-Sc was less dramatic but this construct was also able to significantly ($P<0.05$) inhibit MDA-MB-231 cell viability. These results suggest that the decreased soft agar colonization of HAd-EphrinA1-Fc represents active tumor cell killing.

Inhibition in tumorigenic potential of breast cancer cells-infected with HAd-EphrinA1-Fc in nude mice

To evaluate the potential effect of targeted EphrinA1-Fc expression in vivo, vector-infected MDA-MB-231 cells were evaluated for their tumorigenic potential in nude mice. In animals inoculated with mock- or HAd-ΔE1E3-infected cells, palpable tumors were apparent within 2 weeks post-implantation and steadily increased thereafter (Fig. 6A). Infection with control HAd-ΔE1E3-infected cells did not significantly alter tumor volume relative to mock-infected cells. In dramatic contrast, MDA-MB-231 cells that had been infected with HAd-EphrinA1-Fc failed to establish tumors in nude mice (Fig. 6A). These animals remained tumor-free throughout the study (48 days) and post-mortem analysis did not reveal the presence of tumors at the site of inoculation (data not shown).

Inhibition in growth of MDA-MB-231-induced tumors by i.t. inoculation with HAd-EphrinA1-Fc

Although the results above were encouraging, we considered that preventative studies using cells that had been treated with HAd-EphrinA1-Fc prior to implantation did not impart a high degree of physiological relevance. To evaluate these constructs within a more therapeutically relevant setting, our next line of investigation utilized animals bearing established tumors. Once the tumors reached a volume of 50-100 mm^3, the mice were treated i.t. with either PBS, HAd-ΔE1E3, or HAd-EphrinA1-Fc. Tumor volume was then tracked over time. Whereas the vector control (HAd-ΔE1E3) did not differ significantly from mock controls (Fig 6B), HAd-EphrinA1-Fc treatment decreased tumor growth. The decrease in tumor growth was durable, lasting at least 11 days following treatment.

DISCUSSION

The major finding of our present study is that targeting of malignant cells with adenoviral vector EphrinA1-Fc is sufficient to decrease tumorigenic potential in vitro and in vivo. We also relate the efficacy of this targeting strategy to EphA2 activation and degradation.

These findings have implications for many different cancers that overexpress EphA2 including metastatic melanoma, breast, prostate, colon, lung, ovarian, and esophageal cancers.[2,4,5,6] Moreover, EphA2 in these cells is not tyrosine phosphorylated and thus accumulates at the cell surface. Importantly, overexpression of EphA2 observed in these cells occurs without a change in the binding motif for its ligand, EphrinA1. Rather, the defect appears to reside at the level of decreased cell-cell stability. In particular, stable EphA2-EphrinA1 binding requires the proper expression and functioning of E-cadherin. Consistent with this, the defect in EphA2 phosphorylation and stability can be corrected using purified forms of EphrinA1-Fc.[3,8]

If our present findings can be extended to analogous situations in human, then adenoviral based targeting of EphA2 could provide therapeutic benefit for these conditions. Consistent with this idea, recent studies have shown that EphA2 on these tumors can be targeted using monoclonal antibodies[9,31] and peptides.[15] The adenoviral approach offers the advantages over purified protein-based approaches mainly due to the ability of adenoviral vectors to express high levels of EphrinA1 continuously and over longer periods of time. EphrinA1-Fc has the potential to engage and stimulate multiple different EphA (and some EphB) family members, which could be an issue for therapeutic specificity or toxicity. Yet, in this light, it is notable that no gross toxicities were observed in the xenograft modeling. Future studies will be necessary to evaluate the specificity and toxicity issues associated with adenoviral-based EphrinA1-Fc targeting. Since HAd-EphrinA1-Fc-infected cells efficiently secrete EphrinA1-Fc, it was anticipated that EphrinA1-Fc would have "bystander effect." A significant inhibition in cell viability at an m.o.i. of 5 p.f.u. per cell is an indirect evidence of bystander effect of EphrinA1-Fc.

We did detect differences in the efficiency of adenoviral delivery of EphrinA1-Fc and EphrinA1-Sc. The levels of EphrinA1-Fc expression and secretion in HAd-EphrinA1-Fc-infected cells were considerably higher than comparable studies with EphrinA1-Sc. These differences directly related to the relative efficacy of either construct in vitro, which is consistent with the specificity of this system. We did not attempt to determine the reason for differences in their levels of expression and secretion.

The fact that HAd-EphrinA1-Fc treatment was sufficient to inhibit established tumors, which is consistent with a recent report where targeted stimulation of EphA2 was sufficient in inhibiting tumorigenic potential.[9] It is particularly notable that we were able to achieve a powerful anti-tumor response with a single intratumoral inoculation with 1×10^9 p.f.u. of HAd-EphrinA1-Fc, albeit this was not sufficient to achieve tumor shrinkage. HAd vectors expressing cytokine, chemokine or co-stimulatory molecules,[16,32,33] interferon-inducing protein, p202[34] or inoculation with vector expressing thymidine kinase followed by systemic administration of ganciclovir[35] have been tested in breast cancer therapeutics with encouraging results. These approaches could be combined with EphA2-targeting to augument the therapeutic effect.

We did not monitor EphrinA1-Fc expression in mice following intratumoral inoculation with HAd-EphrinA1-Fc, therefore, the duration of EphrinA1-Fc expression was not known. In addition, whether the cells that were killed were infected with HAd-EphrinA1-Fc and the exact mechanism of cell killing require further investigation. However, our preliminary results suggest that there was increased expression of caspase 3 in breast cancer cells infected with HAd-EphrinA1-Fc, whereas bax expression remained unchanged (Noblitt and Mittal, unpublished data). It will be worthwhile to determine the status of other

cellular proteins, such as low molecular weight phosphatase (LMW-PPT),[36] fibronectin, integrins, Src homology 2-containing phosphotyrosine phosphatase (SHP2), and FAK in response to HAd-EphrinA1-Fc infection in breast cancer cells.

MATERIALS AND METHODS

Cell culture and viruses

293 cells, HAd5 E1-transformed human embryonic kidney cells that support E1-deleted HAd replication,[37] were used primarily to grow recombinant E1-deleted HAd vectors. 293*Cre* cells (a gift from Merck, Inc., Whitehouse Station, NJ) not only support replication of E1-deleted HAd but also produce Cre-recombinase,[38] which catalyzes site-specific homologous recombination in the presence of *loxP* sites.[27] The MT1A2 cell line was derived from polyoma virus middle T antigen (PyMidT)-induced mammary adenocarcinoma in transgenic mice.[32] This cell line was kindly provided by Dr. William Muller, Department of Biology, McMaster University, Hamilton, Ontario, Canada. 293, 293*Cre*, MT1A2, MDBK (Madin Darby Bovine Kidney cell line), and MDA-MB-231 (aggressive human breast cancer cell line) were grown as monolayer cultures in Eagle's minimum essential medium (MEM) (Gibco BRL, Gaithersburg, MD) supplemented with 5-10% reconstituted fetal bovine serum (FetalClone III; Hyclone, Logan, UT), 50 μg/ml gentamicin sulfate (Fisher Scientific, Pittsburgh, PA) and 50 μg/ml amphotericin B (Fisher Scientific). MCF-10A (nontransformed human breast epithelial cell line) cells were grown as monolayer cultures in MEM supplemented with 5 % FetalClone III, 50 μg/ml gentamicin sulfate, 50 μg/ml amphotericin B, 10 μg/ml insulin (Sigma-Aldrich, Inc., St. Louis, MO), 50 μg/ml epidermal growth factor (Upstate Biotechnology, Lake Placid, NY) and 0.50 μg/ml hydrocortisone (Sigma-Aldrich). HAd5 vectors were grown in 293 cells and virus-infected cell pellets were used for preparation of virus stocks. For in vivo studies, purified virus preparations generated by cesium chloride-density gradient centrifugation.[39] Virus stocks and purified virus preparations were titrated in 293 cells by plaque assay.

Antibodies

The EphA2-specific monoclonal antibody (D7) was generated by Dr. M. S. Kinch[40] and anti-EphrinA1 polyclonal antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. An antibody specific for P-Tyr was acquired from Upstate Biological, Inc., Waltham, MA, and β-catenin-specific, and E-cadherin-specific antibodies were obtained from Transduction Laboratories, San Diego, CA. FITC-labeled antibodies: goat-anti-mouse-FITC and goat-anti-rabbit-FITC were procured from Southern Biotechnology, Birmingham, AL, and Sigma-Aldrich, respectively. HRP-conjugated antibodies: goat anti-mouse-HRP and goat anti-rabbit-HRP were from Bio Rad, Hercules, CA, whereas, a rabbit anti-mouse antibody was acquired from Chemicon, Temecula, CA.

Construction of E1 shuttle plasmids containing EphrinA1-Fc or EphrinA1-Sc and generation of HAd5 vectors

Restriction endonucleases, T4 DNA ligase, T4 polymerase, and their appropriate buffers were purchased from New England Biolabs, Beverly, MA. Calf intestine alkaline phosphatase and its appropriate buffer were obtained from Life Technologies, Inc., Gaithersburg, MD.
Plasmid DNA were purified using Qiagen Plasmid Maxi Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol.

A 2.8 kb *NruI* and *XbaI* fragment containing the EphrinA1-Fc gene along with the cytomegalovirus (CMV) immediate early promoter was excised from pCDNA3-EphrinA1-Fc, pCDNA3 containing the EphrinA1 attached with Fc portion of IgG_1 (kindly provided by Dr. B. C. Wang, Case Western Reserve University, Cleveland, OH)**.** In order to obtain the EphrinA1-Sc (EphrinA1 without the transmembrane

anchor and Fc portion of IgG_1) construct along with the CMV promoter, pCDNA3-EphrinA1-Fc was digested with *NruI* and *BamH1*and a 1.2 kb fragment was isolated.

For generation of HAd5 vectors, the *Cre* recombinase-mediated site-specific recombination system was used.[27] To generate HAd5 E1 shuttle plasmids containing EphrinA1-Fc (2.8 kb *NruI-BamH1*fragment), or EphrinA1-Sc (1.2 kb *NruI-BamH1*fragment), these DNA fragments were inserted at an appropriate site in the shuttle vector pDC316 [plasmid containing the left end of HAd5 (4Kb) with 3.1 kb E1 deletion, a *lox*p site for site specific recombination in the presence of *Cre* recombinase, and an intact packaging signal (Ψ)][27] to produce pDC316-EphrinA1-Fc and pDC316-EphrinA1-Sc, respectively. 293*Cre* cells were cotransfected with pDC316-EphrinA1-Fc or pDC316-EphrinA1-Sc and pBHGloxΔE1,3Cre (plasmid containing almost entire HAd5 genome except the Ψ, E1 and E3 deletions, and the addition of a *lox*p site for site specific recombination) using the calcium-phosphate technique[41] to generate HAd-EphrinA1-Fc and HAd-EphrinA1-Sc, respectively by *Cre* recombinase-mediated site-specific recombination. The pDC316 and pBHGloxΔE1,3Cre were obtained from Microbix, Inc., Toronto, Ontario, Canada. Similarly, a vector having E1 and E3 deletions (HAd-ΔE1E3) was also constructed. HAd5 vectors were plaque purified in 293 cells.

Northern Blot Analysis

Total cellular RNA was isolated 24 h post infection using TRI reagent (Sigma-Aldrich) according to the manufacturer's protocol. Approximately 10 μg RNA was electrophoresed through 1.5 % agarose-formaldehyde gel and transferred to a Zeta-probe membrane (BioRad Laboratories) by capillary transfer.[42] The membrane was incubated at 80°C for 1 h and prehybridized at 42 °C for 2 h. Subsequently the membrane was hybridized overnight at 42 °C with an EphrinA1-specific probe labeled with [α^{32}P]-dCTP (Amersham Biosciences Corp, Piscataway, NJ) using random labeling kit (Invitrogen, Carlsbad, CA). The membrane was washed, exposed with a Phosphor Imager screen, scanned by Cyclone storage phosphor system and analyzed with OptiQuant image analysis software (Packard Instrument Company, Meriden, CT).

Western Blot Analysis

Cell lysates were fractionated on 10-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad) by electrophoresis as described elsewhere.[42] To avoid non-specific binding, membranes were blocked with phosphate-buffered saline (PBS), pH 7.2 containing 0.05% Tween 20 and 5% dry skimmed milk. For detecting phosphorylation of immunoprecipitated EphA2, membranes were blocked using 2% cold fish gelatin and 0.1% bovine serum albumin instead of dry skim milk. Membranes were probed with one of the following primary antibodies, specific for: D7 (EphA2), 4G10 (P-Tyr), E-cadherin or EphrinA1. After extensive washing, the membranes were then probed with either goat anti-mouse IgG antibody-conjugated with HRP or goat anti-rabbit IgG-HRP as appropriate. For detection of EphrinA1-Fc, a goat anti-human IgG antibody-HRP was used as a primary antibody. The membranes were soaked in a 1:1 ratio of Super Signal West Pico Stable Peroxide Solution: Super Signal West Pico Luminol/Enhancer (Pierce Co., Rockland, IL) for chemiluminescence and emitted light was captured by autoradiography (Kodak X-OMAT; Kodak, Rochester, NY) or enhanced chemiluminescence (Kodak Image Station; Kodak). Membranes were stripped and re-probed with a β-catenin-specific antibody to verify equal loading, and when feasible, membranes were cut into two pieces to avoid stripping.

Immunoprecipitation

Protein A sepharose (PAS) beads were treated with a rabbit anti-murine (RAM) IgG antibody to prepare RAM-attached to PAS (RAMPAS) as described.[16] Cell lysates and D7 antibody were added into microfuge tubes containing RAMPAS to allow specific binding of D7-EphA2 complexes to RAMPAS. Beads were washed extensively to remove the unbound proteins of the cell lysate. Immunoprecipitated

protein samples were loaded and fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and Western blot analysis was performed as described above.

Soft Agar Assay
MDA-MB-231 or MCF-10A cell monolayers at approximately 80-90% confluency were infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u./cell. Following 6 h of infection, cells were harvested by trypsinization and resuspended in 2X MEM supplemented with 10% FetalClone III. Warmed 0.6% agarose in water was then added in a 1:1 ratio to 2X MEM containing cells and the mixture was poured onto 0.3% agarose cushions in 6-well dishes. The agarose was allowed to solidify at room temperature followed by incubation at 37°C in a CO_2 incubator. Every 3rd day, each dish alternatively received 250 µl /well of 1X MEM supplemented with 10% FetalClone III or 1 ml/well of 2X MEM and 0.6% agarose (1:1 ratio). Twelve days post-infection, colonies were assessed microscopically and defined as clusters of contiguous cells. The mean and standard deviation were calculated from data obtained from 3 independent samples.

Cell Viability Assay
MDA-MB-231 or MCF-10A cells in 12-well culture plates were mock-infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u./cell. At 24, 48, 72 and 96 h post-infection, cells were harvested by trypsinization, stained with Trypan blue and the live cells were counted microscopically using a calibrated grid. Each bar represents the per cent viable cells compare to HAd-ΔE1E3 infected cells. Each time point represents the mean +/- SD from 3 wells.

In Vivo Studies
Ten-to-eleven-week old athymic BALB/c mice were obtained from the National Cancer Institute. All animals were acclimated for 7 days before inoculation. Mice (7 animals per group) were injected s.c. in the right axilla with $4x10^6$ MDA-MB-231 cells infected with HAd-EphrinA1-Fc or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u. per cell, harvested 12 h post-infection and mixed with Matrigel (1:1). Mice inoculated with mock-infected MDA-MB-231 cells provided matched controls.

In another experiment, nude mice (7 animals/ group) were inoculated s.c. in the right axilla with 4×10^6 MDA-MB-231 cells mixed with Matrigel (1:1) for development of MDA-MB-231-induced tumors. Following the development of 50-100 mg^3 tumors, intratumoral (i.t.) injections of PBS, or 1×10^9 p.f.u. of purified preparation of HAd-EphrinA1-Fc or HAd-ΔE1E3 were performed. Tumor volumes were calculated using the formula, $Tumor\ volume = \{Length(Width)^2\}/2$, for both control and treated mice to evaluate the extent of tumor size reduction or increase over time. Mice were sacrificed when the tumors reached a volume of approximate 700 mm^3 or on Day 48 post-inoculation.

Statistical analysis
Statistical analyses of in vitro and in vivo data were performed using Student's *t*-distribution, defining significance as $P < 0.05$.

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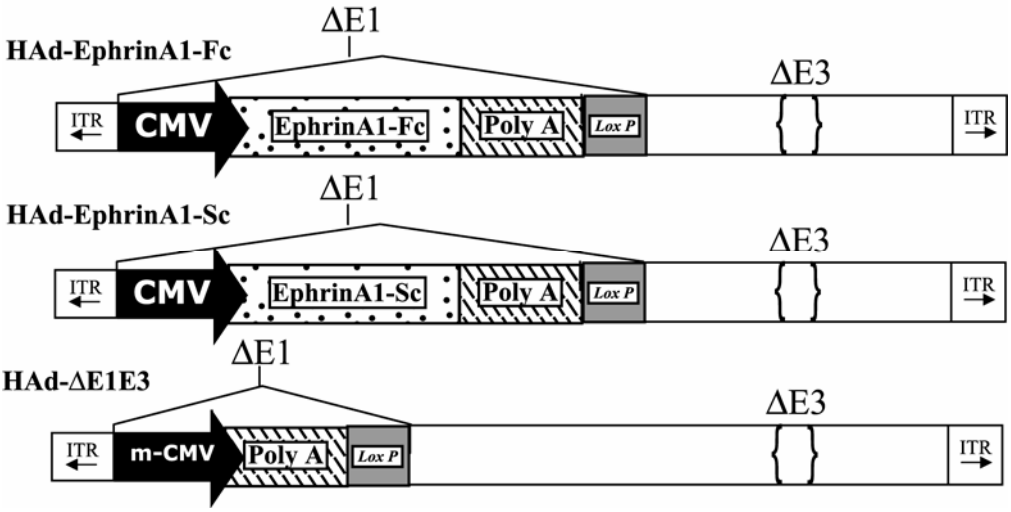

Fig. 1. Diagrammatic representation of the structure of HAd5 vectors. Three HAd vectors: 1) HAd-EphrinA1-Fc (HAd vector containing EphrinA1-Fc), 2) HAd-EphrinA1-Sc (HAd vector containing EphrinA1-Sc), and 3) HAd-ΔE1E3 (HAd vector having the m-CMV promoter, Poly A, and *loxP* site) were generated by *Cre* recombinase-mediated site-specific recombination. ITR, inverted terminal repeat; CMV, human cytomegalovirus immediate early promoter; m-CMV, mouse cytomegalovirus immediate early promoter; Poly A, simian virus 40 polyadenylation site, *Lox P*, *loxP* site for site-specific recombination by *Cre* recombinase; ΔE1, deletion in early region 1 (E1); ΔE3, deletion in early region 3 (E3).

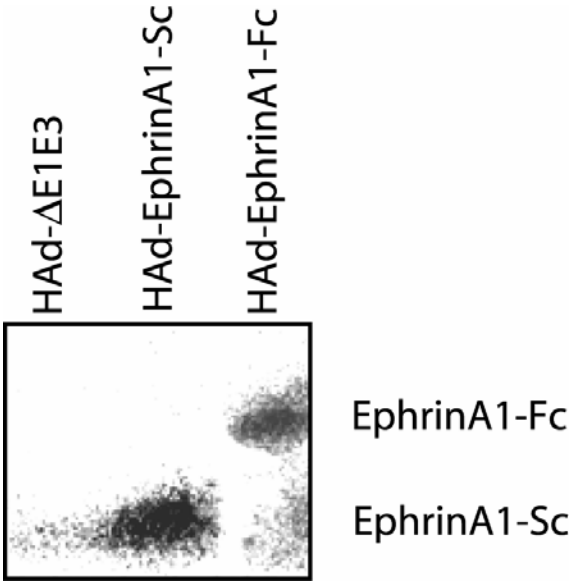

Fig 2. Detection of EphrinA1-specific messages in HAd vector-infected cells by Northern blot. Confluent monolayers of MTIA2 cells were infected with either HAd-ΔE1E3, HAd-EphrinA1-Sc, or HAd-EphrinA1-Fc at an m.o.i. of 5 p.f.u. per cell and at 24 h post-infection, the total RNA was isolated electrophoresed in formaldehyde-agarose gel and transferred to nylon membrane. The membrane was hybridized with random-primed ^{32}P-labeled EphrinA1-specific probe and signal detected by phosphor imager system. The arrows point to the positive band specific to either the EphrinA1-Fc or EphrinA1-Sc message.

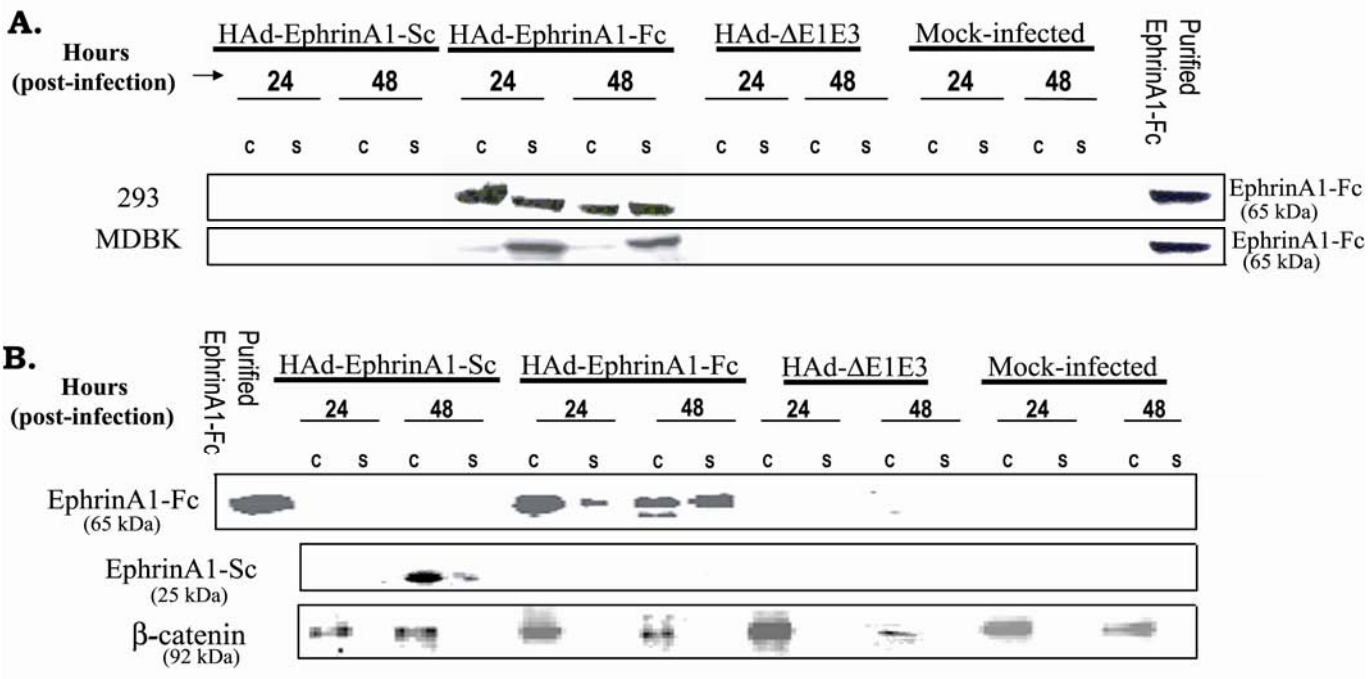

Fig 3. EphrinA1-Fc or EphrinA1-Sc expression in cells infected with HAd vectors. A) 293 or MDBK cells were mock-infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u. per cell. Cell supernatants and cell lysates were collected at 24 and 48 h post-infection. EphrinA1-Fc protein was detected by Western blot analysis using a goat-anti-human-HRP conjugated antibody. B) 293 cells were mock-infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u. per cell. Cell supernatants and cell lysates were collected at 24 and 48 h post-infection. EphrinA1-Fc (~65 kDa band), or EphrinA1-Sc (~25 kDa band) was detected by Western blot analysis using a rabbit anti-EphrinA1 antibody. The blot was overexposed to observe EphrinA1-Sc expression. Purified EphrinA1-Fc was loaded as a positive control. β-catenin expression was monitored to verify the amounts of protein loading in each lane. *C* = cell lysate; *S* = cell supernatant.

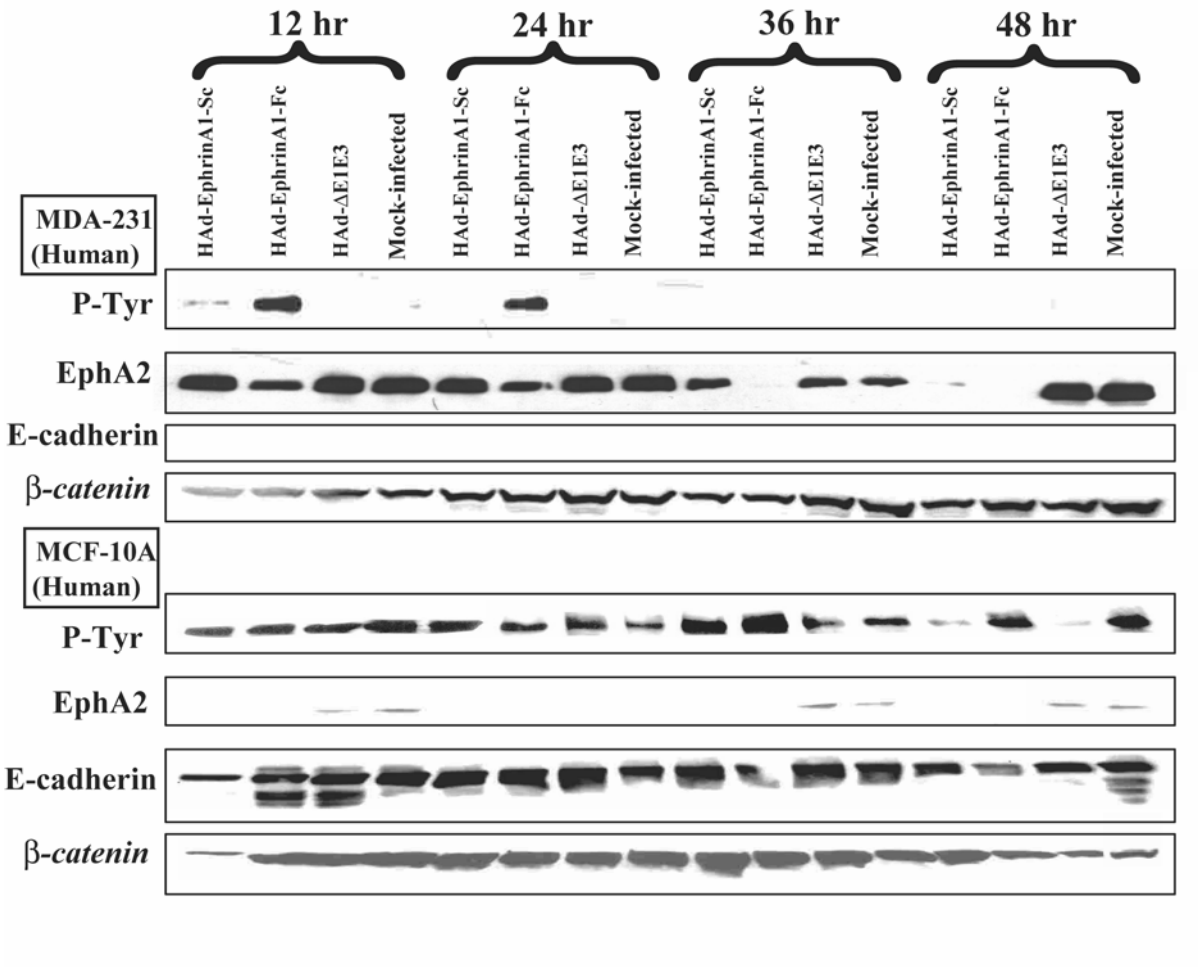

Fig. 4. EphA2 expression & phosphorylation and E-cadherin expression in human breast cancer cells infected with HAd-EphrinA1-Fc or HAd-EphrinA1-Sc. MDA-MB-231 or MCF-10A cells were mock-infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u./cell. Cells were harvested at 12, 24, 36 and 48 h post-infection and analyzed for expression of EphA2 or E-cadherin by Western blot using an anti-EphA2 or E-cadherin-specific antibody, respectively. For detection of EphA2 phosphorylation, EphA2 was first immunoprecipitated with an anti-EphA2 antibody, and then analyzed for tyrosine phosphorylation by Western blot using an anti P-Tyr antibody. β-catenin expression was monitored to verify the similar amounts of protein loading in each lane.

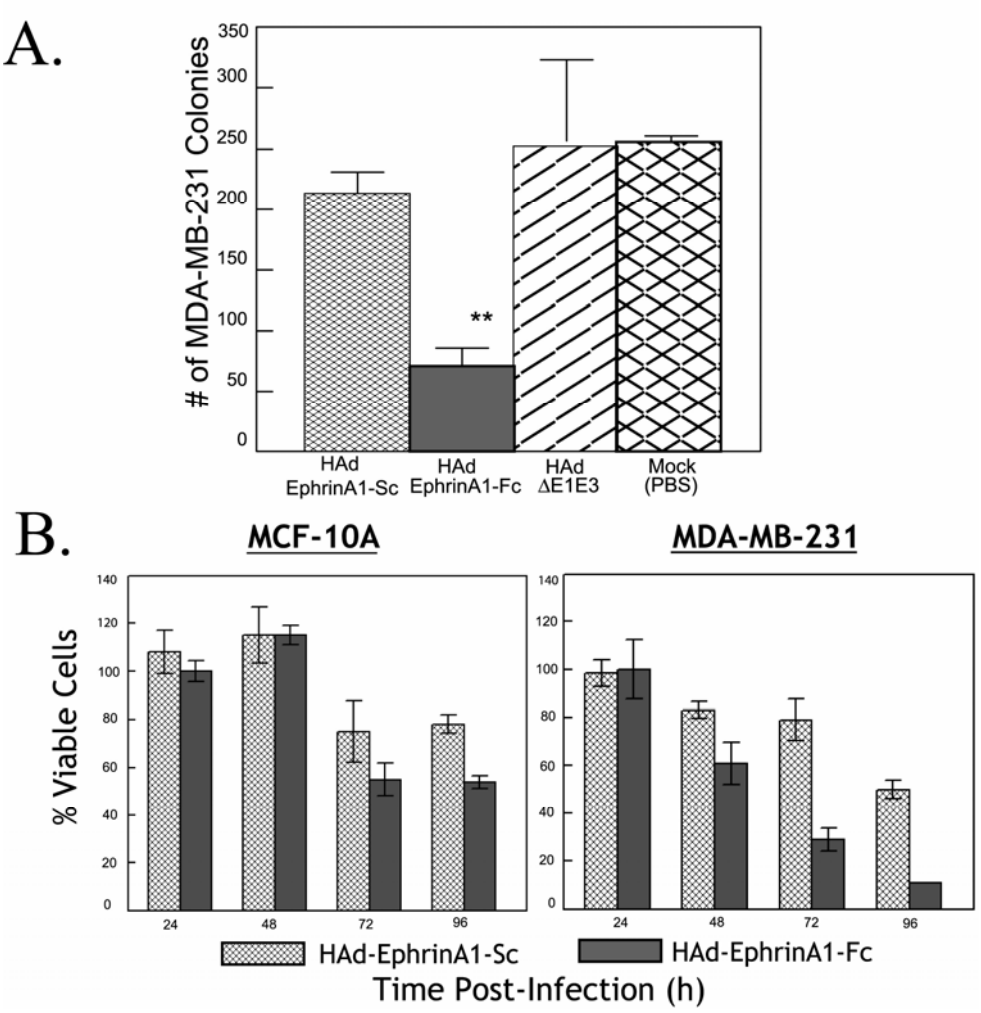

Fig 5. Inhibition in soft agar colonization and viability of human breast cancer cells infected with HAd vectors. A) MDA-MB-231 cells were mock infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u./cell. At 6 h post-infection, cells were trypsinized and seeded onto semi-solid agarose cushions in 6-well culture dishes. Cell clusters were counted as a colony on Day 12 post-infection. Each treatment group represents the mean +/- SD from 3 wells. B) MCF-10A and MDA-MB-231 cells were infected with HAd-EphrinA1-Fc or HAd-EphrinA1-Sc at an m.o.i. of 5 p.f.u./cell. At 24, 48, 72 and 96 h post-infection, cells were harvested by trypsinization, stained with Trypan blue and the live cells were counted. Each bar represents the per cent viable cells compare to HAd-ΔE1E3 infected cells. Each time point represents the mean +/- SD from 3 wells. **, $P<0.05$.

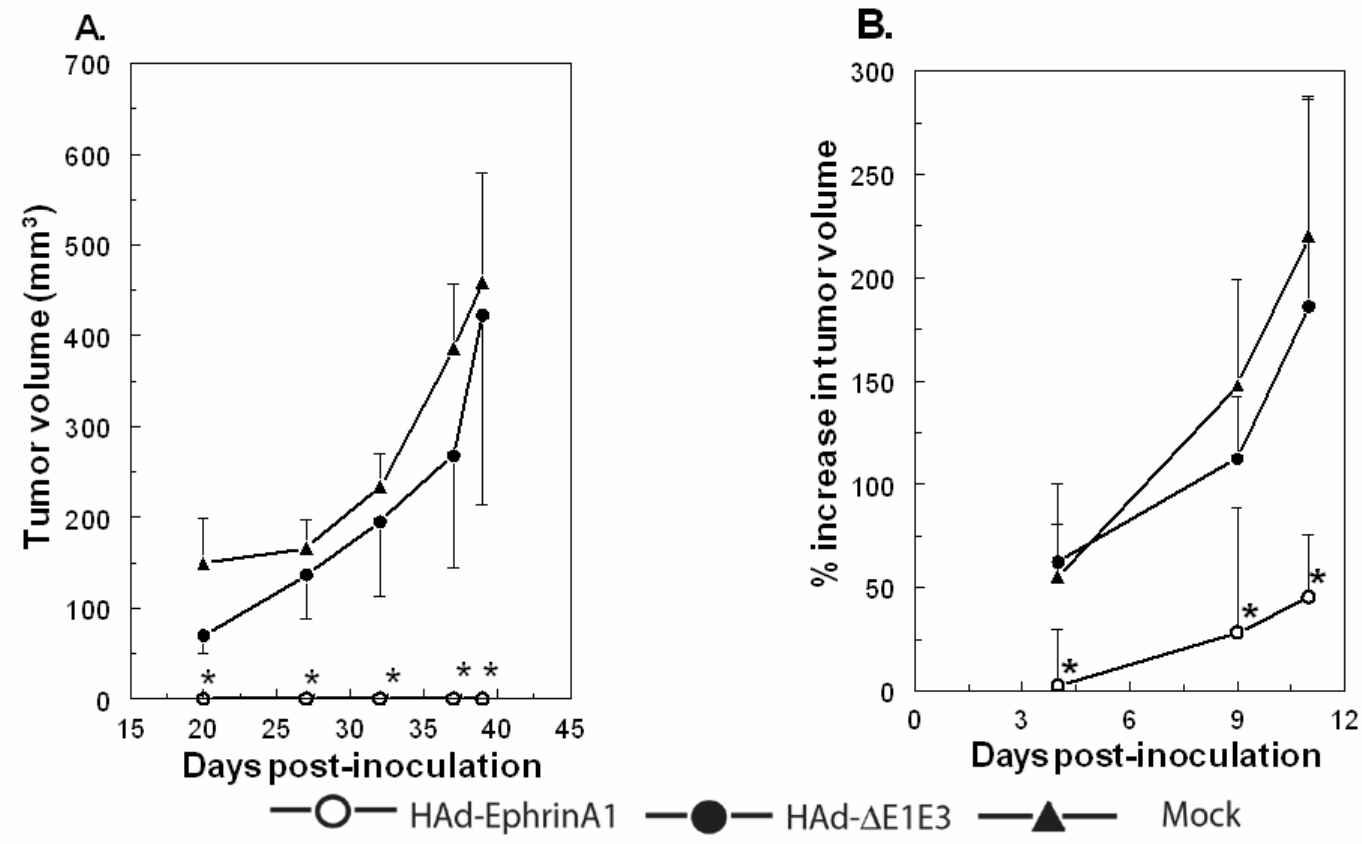

Fig. 6. Role of HAd-EphrinA1-Fc in inhibiting tumorigenic potential of human breast cancer cells in vivo. A) MDA-MB-231 cells were mock-infected or infected with HAd-EphrinA1-Fc or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u./cell. At 12 h post-infection, cells were harvested and mixed with Matrigel (1:1) and inoculated s.c. in nude mice in the right axilla. The development of tumors was monitored and their sizes were measured over time. B) Nude mice were inoculated s.c. in the right axilla with MDA-MB-231 cells mixed with Matrigel (1:1). Following the development of tumor, animals were inoculated i.t. either with PBS or 1×10^9 p.f.u. of purified preparation of HAd-EphrinA1-Fc or HAd-ΔE1E3. To evaluate the effect of the treatment, the per cent increase or decrease in tumor volumes over time compared to tumor sizes before i.t. inoculation. Each time point represents the mean +/- SD from 5-7 animals. *, $P<0.05$.