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Immunocompetent Mouse Model of Breast Cancer for Preclinical Testing of EphA2-Targeted Therapy

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ABSTRACT

EphA2, a receptor tyrosine kinase, is elevated in many invasive human breast cancers, and the majority of EphA2 remains unphosphorylated. The successful attachment of ligand EphrinA1 present on the surface of adjacent cells to EphA2 initiates EphA2 phosphorylation leading to its turnover. In vivo efficacy of various approaches targeting EphA2 for breast cancer therapy is usually evaluated in nude mice bearing human breast cancer xenografts. In order to establish an immunocompetent mouse model of breast cancer for EphA2-targeted therapies, we evaluated a mouse breast cancer cell line (MT1A2) for EphA2 expression and phosphorylation. Overexpression of EphA2 was observed in MT1A2 cells and the majority of it remained unphosphorylated signifying that EphA2 in MT1A2 cells behaved similar to that of human breast cancer cells. Human adenovirus subtype 5 (HAd5) vectors expressing secretory forms of EphrinA1 were used for in vitro and in vivo targeting of MT1A2-derived EphA2. MT1A2 cells infected with HAd-EphrinA1-Fc (HAd expressing extracellular domain of human EphrinA1 attached to Fc portion of human IgG1 heavy chain) induced EphA2 activation and its turnover. This led to inhibition in MT1A2 cell colony formation in soft agar and cell viability in monolayer culture. In addition, MT1A2 cells-infected with HAd-EphrinA1-Fc failed to form tumors in syngeneic FVB/n mice at least 32 days post-inoculation. Moreover, intratumoral inoculation of FVB/n mice-bearing MT1A2-induced tumors with HAd-EphrinA1-Fc slowed the tumor growth. These results indicate that FVB/n mice-bearing MT1A2-induced tumors could serve as an immunocompetent model of breast cancer for EphA2-targeted therapeutic strategies.

INTRODUCTION

Disruption in normal cell cycle regulation leads to cancer or tumor formation. Investigators have spent considerable time and effort in search for cellular signals that are unique to cancer cells. EphA2 (130 kDa epithelial cell kinase; ECK), a receptor tyrosine kinase, is usually overexpressed and functionally altered in many metastatic human breast cancers.[1,2] In healthy breast epithelial cells EphA2 is associated with one of its Ephrin ligands (A1 to A5), predominantly 23 kDa EphrinA1. This binding leads to autophosphorylation of three tyrosine residues present in the intracytoplasmic domain of EphA2[3,4,5,6] and then activates or inhibits other kinases before getting internalized and degraded.[7,8,9]

Functionally altered EphA2 in invasive human breast cancer cells fails to bind to its ligand EphrinA1 leading to the accumulation of unphosphorylated EphA2.[1] The purified secretory-form of EphrinA1 (EphrinA1-Fc) protein[1] or EphrinA1-Fc expressed by an adenoviral vector[10] has been shown to activate EphA2 in breast cancer cells resulting in inhibition of their tumorigenic potential. Some of the EphA2-specific antibodies that bind to the extracellular domain of EphA2 could induce EphA2 phosphorylation leading to its degradation.[9] Therefore, EphA2 is an attractive target for potential breast cancer therapeutics.

Preclinical testing of various human breast cancer therapeutic approaches is usually carried out using human breast cancer xenografts in nude mice.[1,10,11,12] These models do not mimic the normal immunocompetent host. Moreover, immune-mediated therapeutic strategies cannot be tested effectively in nude mouse models. Transgenic mice having the polyoma virus middle T antigen (PyMidT) gene under the control of the long terminal repeat of mouse mammary tumor

virus develop adenocarcinoma of mammary epithelia[13] and offer a good immunocompetent model for breast cancer. PyMidT-induced mammary adenocarcinoma explants were grown in culture to isolate a continuous cell line (MT1A2) that could be used for developing tumors in syngeneic FVB/n mice.[13,14]

This manuscript describes that EphA2 is overexpressed in murine breast cancer cells (MT1A2), and the majority of it is not phosphorylated. Human adenovirus type 5 (HAd) vectors expressing secretory forms of EphrinA1 not only induced murine EphA2 activation but also led to inhibition of tumorigenic potential of MT1A2 cells in vitro. The main aim of this study is to demonstrate that MT1A2-induced tumors in syngeneic FVB/n mice could serve as an excellent immunocompetent breast cancer model for preclinical testing of breast cancer therapeutic approaches targeting EphA2.

MATERIALS AND METHODS

Cell culture and viruses

MT1A2, a murine breast cancer cell line was provided by Dr. William Muller, Department of Biology, McMaster University, Hamilton, Ontario, Canada. 293-EphrinA1-Fc, 293 cells stably expressing EphrinA1-Fc was kindly provided by Dr. B. C. Wang, Case Western Reserve University, Cleveland, OH. MT1A2, NIH-3T3 (a mouse fibroblast cell line), 293,[15] 293-EphrinA1-Fc and aggressive human breast cancer cell lines (MDA-MB-231 and BT549) 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, Pittsburgh, PA). MCF-10A (nontransformed human breast epithelial cell line) cells were grown as monolayer cultures as previously described.[10] The construction of HAd5 vectors: HAd-EphrinA1-Fc (HAd-ΔE1E3 expressing extracellular domain of human EphrinA1 attached to Fc portion of human IgG1 heavy chain), HAd-EphrinA1-Sc (HAd-ΔE1E3 expressing only extracellular domain of human EphrinA1), and HAd-ΔE1E3 (HAd having E1 and E3 deletions) is described elsewhere.[10] HAd5 vectors were grown in 293 cells, purified by cesium chloride-density gradient centrifugation,[16] and titrated in 293 cells by plaque assay.

Antibodies

EphA2-specific monoclonal (D7), anti-EphrinA1 polyclonal, anti- P-Tyr-specific, β-catenin-specific, E-cadherin-specific, goat-anti-mouse-FITC, goat-anti-rabbit-FITC, goat anti-mouse-HRP, goat anti-rabbit-HRP, and rabbit anti-mouse IgG antibodies were from the same sources as described.[10]

Purification of human EphrinA1-Fc

Cell supernatants containing EphrinA1-Fc were collected from 293-EphrinA1-Fc cells in monolayer cultures. EphrinA1-Fc was purified on a protein A sepharose column by affinity chromatography.

Western blot and immunoprecipitation
These assays were essentially performed as described elsewhere.[10] Human or murine cell lines were mock-infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 at a multiplicity of infection (m.o.i.) of 5 plaque forming unit (p.f.u.) per cell. At various times post-infection cells were harvested and cell extracts prepared for Western blot and immunoprecipitation analyses.

Immunofluorescence assay
Mock, HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3-infected NIH-3T3 or MT1A2 cell monolayers in sterile Lab-Tek eight-well chamber slides (Nalge Nunc International, Naperville, IL) were analyzed for EphA2- or EphrinA1-specific fluorescence by immunofluorescence assay (IFA) as described.[10]

Soft agar assay
NIH-3T3 or MT1A2 cell monolayers at approximately 80-90% confluency 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 as previously described.[10] A colony was defined as a cluster of 5 or more contiguous cells and colonies were counted 18 days post-infection.

Cell viability assay
NIH-3T3 or MT1A2 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 and cell viability assay was performed as described.[10]

In vivo studies
Ten-to-eleven-week old FVB/n mice were procured from the National Cancer Institute and were acclimated for 7 days before inoculation. Mice (7/group) were inoculated subcutaneously (s.c.) in the right axilla with $4x10^6$ MT1A2 cells infected with HAd-EphrinA1-Fc or HAd-ΔE1E3 at an m.o.i. of 5 p.f.u. per cell and harvested 12 h post-infection. Mice inoculated with mock-infected MT1A2 cells served as controls.

In another study, FVB/n mice (7 animals/ group) were first inoculated s.c. in the right axilla with 4×10^6 MT1A2 cells for development of MT1A2-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 with an overdose of an anesthetic when the tumors reached a volume of approximately 1000 mm^3 or on Day 48 post-inoculation.

Statistical analysis
The in vitro and in vivo data were analyzed statistically by Student's *t*-distribution and the significance was set at $P < 0.05$.

RESULTS

Status of EphA2 expression and phosphorylation in mouse breast cancer cells

In order to develop MT1A2-induced tumors in syngeneic FVB/n mice as an immunocompetent mouse model of breast cancer for EphA2 targeted strategies, the first thing was to determine whether EphA2 levels are elevated in MT1A2 cells, and an anti-human EphA2 antibody cross-reacts with mouse EphA2. The cell extract from MT1A2 was analyzed for EphA2 expression by Western blot and compared with EphA2 expression in NIH-3T3 cells. Human metastatic breast cancer cell lines (BT549 and MDA-MB-231) and the non-transformed human breast epithelial cell line (MCF-10A) served as controls. The anti-human EphA2 antibody cross-reacted with murine EphA2 and in MTIA2 cells EphA2 expression was significantly higher than that in NIH-3T3 cells (Fig. 1A) suggesting that EphA2 overexpression also occurs in murine breast cancer cells. As expected, EphA2 overexpression was observed in BT549 and MDA-MB-231 compared to MCF-10A (Fig. 1A).

In normal breast epithelial and other cell types, low levels of EphA2, predominantly in phosphorylated form (i.e., active form), are observed due to its association with ligand, mainly EphrinA1. In contrast, the majority of EphA2 in aggressive human breast cancer cells is unphosphorylated (i.e., inactive form). Treatment of human breast cancer cells in monolayer cultures with purified human EphrinA1-Fc induces EphA2 phosphorylation.[1] In order to determine phosphorylation status of EphA2 in MTIA2 cells and whether purified human EphrinA1-Fc will attach to murine EphA2 leading to its phosphorylation, MTIA2, NIH-3T3, MDA-MB-231, or MCF-10A cells in monolayer cultures were incubated with or without purified EphrinA1-Fc and levels of EphA2 expression and its phosphorylation were determined. The majority of EphA2 in MT1A2 cells was not phosphorylated but treatment of MT1A2 cells with EphrinA1-Fc resulted in efficient phosphorylation of EphA2 similar to that obtained with MDA-MB-231 cells (Fig. 1B) indicating a resemblance in the EphA2 status in murine and human breast cancer cells. As expected, EphA2 phosphorylation was observed both in MCF-10A and NIH-3T3 cells with or without EphrinA1-Fc treatment (Fig. 1B).

EphA2 expression and phosphorylation in HAd vector-infected cells

To determine whether EphrinA1-Fc or EphrinA1-Sc expressed by HAd vector in murine breast cancer cells would initiate EphA2 phosphorylation, MT1A2 or NIH3T3 cells were mock infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 and harvested at various times post-infection. Immunoprecipitated products obtained with an anti-EphA2 antibody were Western blotted using a P-Tyr-specific antibody. Both HAd-EphrinA1-Fc and HAd-EphrinA1-Sc induced EphA2 phoshorylation at 12 h post-infection, but in HAd-EphrinA1-Fc infected cells, low levels of EphA2 phosphorylation were evident even at 24 h post-infection (Fig. 2). This led to a significant reduction in EphA2 expression at 48 h post-infection in HAd-EphrinA1-Fc infected MT1A2 cells signifying the EphA2 turnover. High levels of EphA2 expression without detectable levels of EphA2 phosphorylation were observed in mock- or HAd-ΔE1E3-infected MT1A2 cells at various times post-infection (Fig. 2). On the other hand, low levels of EphA2 expression accompanied by steady EphA2 phosphorylation at various times post-infection were observed in NIH-3T3 cells irrespective of types of vector treatment (Fig. 2).

In healthy epithelial cells, E-cadherin is involved in cell-cell adhesions by homophilic interaction on neighboring cells.[17,18] It has been proposed that phosphorylation levels of tyrosine kinases maintain a balance between cell-extracellular matrix and cell-cell adhesions.[19] Apparently, MT1A2 cells seem to have better cell-to-cell adhesions compared to MDA-MB-231 cells (data not shown). To investigate whether infection of MT1A2 cells with HAd vectors will result in a change in levels of E-cadherin expression, various vector-infected cell extracts were monitored for E-cadherin expression by Western blot. There was no apparent change in levels of E-cadherin expression in MT1A2 cells irrespective of the type of vector treatment at various times post-infection (Fig. 2). Due to the fibroblastic nature of NIH-3T3 cells, E-cadherin expression was not detected in these cells (Fig. 2).

Subcellular localization of EphA2 or EphrinA1 expression in MT1A2 cells infected with HAd vectors

To explore whether EphrinA1 expression by HAd vectors will alter the subcellular localization of EphA2 or EphrinA1 in mouse breast cancer cells, MT1A2 or NIH-3T3 cells were mock infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3 and analyzed for EphA2 or EphrinA1 by IFA. In MT1A2 cells, the majority of EphA2 or EphrinA1 expression was noticed on the cell surface irrespective of the vector treatment (Figs. 3A & 3B). Furthermore, there were no visible differences in distribution and subcellular localization of EphA2 or EphrinA1 expression even in HAd-EphrinA1-Fc-infected MT1A2 cells compared to other treatment groups (Figs. 3A & 3B). As expected, in NIH3T3 cells, EphA2 or EphrinA1 expression was noticed on the cell surface irrespective of types of vector treatment and there were considerably lower levels of EphA2 expression compared to MT1A2 cells (Figs. 3A & 3B).

Inhibition in colony formation in soft agar by MT1A2 cells infected with HAd vectors

To investigate whether EphrinA1 expression by HAd vectors will affect the anchorage-independent growth of murine breast cancer cells, MT1A2 or NIH3T3 cells were mock-infected or infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3. Infected cells were harvested and seeded in the semi-solid agar matrix. Since NIH-3T3 cells are not tumorigenic, they were unsuccessful in forming colonies in soft agar irrespective of the treatment group. The number of colonies formed with MT1A2 cells following various treatments: mock-, HAd-EphrinA1-Sc-, HAd-EphrinA1-Fc-, or HAd-ΔE1E3-infected, were 139±17, 48.3±21, 33.6±10.7 and 85±5.9, respectively. MT1A2 cells infected with HAd-EphrinA1-Fc showed significant ($P<0.05$) reduction in number of colonies compared to mock- or HAd-ΔE1E3-infected group (Fig. 4) indicating that EphrinA1-Fc expression was responsible for inhibiting the colony forming ability of MT1A2 cells in soft agar.

Viability of MT1A2 cells infected with HAd vectors

To determine whether EphA2 activation by HAd vectors will affect survivability of mouse breast cancer cells, MT1A2 or NIH-3T3 cells were infected with HAd-EphrinA1-Sc, HAd-EphrinA1-Fc, or HAd-ΔE1E3, harvested at various times post-infection and live cells were counted. In MT1A2 cells infected with HAd-EphrinA1-Sc or HAd-EphrinA1-Fc, there was a steady decrease in cell viability with time reaching to 58.5±18 and 17.6± 0.4%, respectively at 96 h post-infection (Fig. 5B). The decrease in cell viability of MT1A2 cells-infected with HAd-EphrinA1-Sc or HAd-EphrinA1-Fc was significant ($P<0.05$), indicating that secretory forms of EphrinA1 (EphrinA1-Fc or EphrinA1-Sc) resulted in reduction of MT1A2 cell viability, with the effect

being more pronounced with HAd-EphrinA1-Fc. NIH-3T3 cells-infected with HAd-EphrinA1-Sc or HAd-EphrinA1-Fc served as controls (Fig. 5A).

Inhibition in tumorigenic potential of MT1A2 cells-infected with HAd-EphrinA1-Fc in vivo
The results of various in vitro experiments with HAd-EphrinA1-Fc were more promising than those obtained with HAd-EphrinA1-Sc, therefore, we used only HAd-EphrinA1-Fc for in vivo studies. To investigate whether activation of EphA2 in MT1A2 cells by HAd-EphrinA1-Fc will impede tumorigenic potential of these cells in vivo, syngeneic FVB/n mice were inoculated with HAd-EphrinA1-Fc-infected MT1A2 cells. Mice inoculated with mock- or HAd-ΔE1E3-infected MT1A2 cells served as controls. Palpable tumors were observed within 3 weeks post-inoculation in FVB/n mice inoculated with mock- or HAd-ΔE1E3-infected MT1A2 cells. HAd-EphrinA1-Fc-infected MT1A2 cells failed to initiate tumor formation during 4 weeks post-inoculation (Fig. 6A). However, palpable tumors were observed in 3 animals measuring 10.3, 33.2 and 247.1 mm^3 in volume on day 48 post-inoculation. The remaining three mice remained tumor-free until the end of the study, i.e. day 48 post-inoculation.

Inhibition in growth of MT1A2-induced tumors by HAd-EphrinA1-Fc
The failure of breast cancer cells infected with HAd-EphrinA1-Fc to form tumors in 3 out of 6 FVB/n mice suggested that EphA2 activation in mouse breast cancer cells inhibited the tumorigenic potential of these cells. In order to explore the potential of adenoviral vector-mediated activation of EphA2 in inhibiting growth of established tumors, FVB/n mice-bearing MT1A2-induced tumors were inoculated i.t. with PBS, HAd-ΔE1E3, or HAd-EphrinA1-Fc and the change in tumor volumes over time was monitored. There was decreased tumor growth at earlier time points in the group inoculated with HAd-EphrinA1-Fc compared to PBS or HAd-ΔE1E3 treated group (Fig 6B).

DISCUSSION

EphA2 offers a unique marker on breast cancer cells and is a potential target for breast cancer intervention. In general, in vivo testing of EphA2-targeted breast cancer therapeutic approaches are conducted in nude mouse models of breast cancer.[1,10] In order to develop an immunocompetent mouse model of breast cancer for preclinical testing of EphA2-specific therapies, the first and foremost requirement was to identify a mouse breast cancer cell line that mimics properties of human aggressive breast cancer cells, characterized by overexpression of EphA2 that is largely unphosphorylated. Due to non-availability of mouse EphA2-specific reagents, another important factor to consider was cross-reactivity of reagents specific to human EphA2 and its ligand EphrinA1 to their mouse counterparts.

Mouse breast cancer cells (MT1A2) were found to express elevated levels of unphosphorylated EphA2. In addition, purified human EphrinA1-Fc efficiently bound to EphA2 on the cell surface and efficiently activated murine EphA2. These results suggested that EphA2 activity in murine breast cancer cells was similar to that of metastatic human breast cancer cells. There was proficient phosphorylation of EphA2 leading to drastic decline in its expression levels in MT1A2 cells infected with HAd-EphrinA1-Fc. Furthermore, HAd-EphrinA1-Fc led to a decrease in the number of colonies formed in soft agar and cell viability of MT1A2 cells. Similar results were obtained with HAd-EphrinA1-Fc in MDA-MB-231, a human breast cancer cell line.[10]

Normal levels of E-cadherin expression were observed in MT1A2 cells and there was no change in its expression level in response to HAd-EphrinA1-Fc infection. In contrast, human breast cancer cells, such as MDA-MB-231, have been found to be E-cadherin-deficient[10,18] and expression of E-cadherin in MDA-MB-231 transfected with a plasmid containing the E-cadherin gene led to EphA2 phosphorylation similar to the normal breast epithelial cell line, MCF-10A.[18] In healthy epithelial cells, E-cadherin plays an important role in cell-cell adhesions by homophilic interaction on adjacent cells.[17,18] Our experiments have demonstrated that MT1A2 cells express E-cadherin, maintain cell-cell adhesions, and also express EphA2 and EphrinA1 on the cell surface, but the majority of EphA2 remains unphosphorylated suggesting that E-cadherin alone is not effective in mediating EphrinA1 interaction with EphA2.

The failure of HAd-EphrinA1-Fc-infected MT1A2 cells to form tumors in syngeneic FVB/n mice even until 32 days post-inoculation signifies the importance of EphA2 activation in inhibiting tumorigenic potential of these mouse breast cancer cells. The appearance of tumors in 3 out of 6 mice on day 48 post-inoculation strongly indicated that infection of MT1A2 cells with HAd-EphrinA1-Fc at an m.o.i. of 5 p.f.u./cell was not enough for complete inhibition of tumor-forming ability in FVB/n mice. Whereas, i.t. inoculation of FVB/n mice-bearing MT1A2-induced tumors with 1×10^9 p.f.u. of HAd-EphrinA1-Fc resulted in inhibition in the tumor growth, however the treatment was not enough for tumor regression. We have obtained similar results with inoculation of nude mice with MDA-MB-231 cells infected with HAd-EphrinA1-Fc, and i.t. inoculation of nude mice bearing human breast cancer xenografts.[10]

An immunocompetent mouse model of breast cancer based on MT1A2-induced tumors in syngeneic FVB/n mice has been utilized for preclinical testing of immunological approaches using adenoviral vectors.[13,20,21] Our in vitro and in vivo results with MT1A2 cells using HAd-EphrinA1-Fc and their comparison with the similar results obtained in MDA-MB-231 cells[10] indicate that FVB/n mice-bearing MT1A2-induced tumors could serve as an immunocompetent mouse model of breast cancer for preclinical testing of EphA2-targeted therapeutic strategies.

Circumvention of adenoviral vector immunity by the sequential administration of human and nonhuman adenoviral vectors has been proposed as an attractive possibility.[22,23,24,25,26,27] The immunocompetent breast cancer mouse model described in this paper could be a useful tool for testing circumvention of vector-specific immunity by alternative administration of human and non-human adenoviral vectors targeting EphA2. It will also offer an excellent model for preclinical testing of other gene therapy approaches where the vector entry into breast cancer cells is targeted through EphA2-specific interactions.

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FIGURE LEGENDS

Fig. 1. Status of EphA2 expression and phosphorylation in murine breast cancer cells. A) To determine overexpression of EphA2 in murine breast cancer cells, cell extracts from non-transformed human breast epithelial cells (MCF-10A), aggressive human breast cancer epithelial cells (MDA-MB-231 and BT549), murine breast cancer epithelial cells (MT1A2), and non-transformed murine fibroblast cells (NIH-3T3) were analyzed by Western blot using a human EphA2-specific antibody. B) To determine EphA2 expression and phosphorylation in murine breast cancer cells following incubation with soluble human EphrinA1-Fc, MCF-10A, MDA-MB-231, NIH3T3, or MTIA2 cells in monolayer cultures were treated with PBS (-) or 1 μg/mL of purified EphrinA1-Fc (+) for 5 minutes at 37° C. EphA2 levels were detected by Western blot analysis using a human EphA2-specific antibody. EphA2 phosphorylation levels were detected by Western blot analysis of immunoprecipitated EphA2 using a phosphotyrosine-specific antibody. Membranes were stripped and re-probed using a β-catenin-specific antibody to verify equal loading.

Fig. 2. EphA2 expression and phosphorylation and E-cadherin expression in murine breast cancer cells infected with HAd-EphrinA1-Fc or HAd-EphrinA1-Sc. MTIA2 or NIH-3T3 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 anti-E-cadherin 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 equal amounts of protein loading in each lane.

Fig. 3. Subcellular localization of EphrinA1 or EphA2 in murine breast cancer cells infected with HAd-EphrinA1-Fc or HAd-EphrinA1-Sc. NIH-3T3 or MTIA2 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 24 h post-infection cells were processed for IFA using A) an EphrinA1-specific antibody or B) an EphA2-specific antibody and a FITC-labeled secondary antibody. Cells were visualized under a fluorescence microscope and photographed at ×400.

Fig. 4. Inhibition in colony formation in soft agar by murine breast cancer cells infected with HAd-EphrinA1-Fc. MTIA2 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 of 5 or more cells were counted as a colony on Day 18 post-infection. **, $P<0.05$. Each treatment group represents the mean +/- SD from 3 wells.

Fig. 5. Viability of murine breast cancer cells infected with HAd-EphrinA1-Fc. NIH-3T3 or MTIA2 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 trysinization, stained with trypan blue, and the live cells were counted using a hemocytometer chamber and a

microscope. Each bar represents the percent viable cells compare to HAd-ΔE1E3 infected cells. Each time point represents the mean +/- SD from 3 wells.

Fig. 6. Inhibition in tumorigenic potential of MT1A2 cells by HAd-EphrinA1-Fc infection. A) MT1A2 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 inoculated s.c. in FVB/n mice in the right axilla. The development of tumors was monitored and their sizes were measured over time. B) FVB/n mice were inoculated s.c. in the right axilla with MT1A2 cells. Following the development of tumors, 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 percent increase or decrease in tumor volumes over time were compared to tumor sizes before i.t. inoculation. Each time point represents the mean +/- SD from 5-7 animals. *, $P<0.05$.

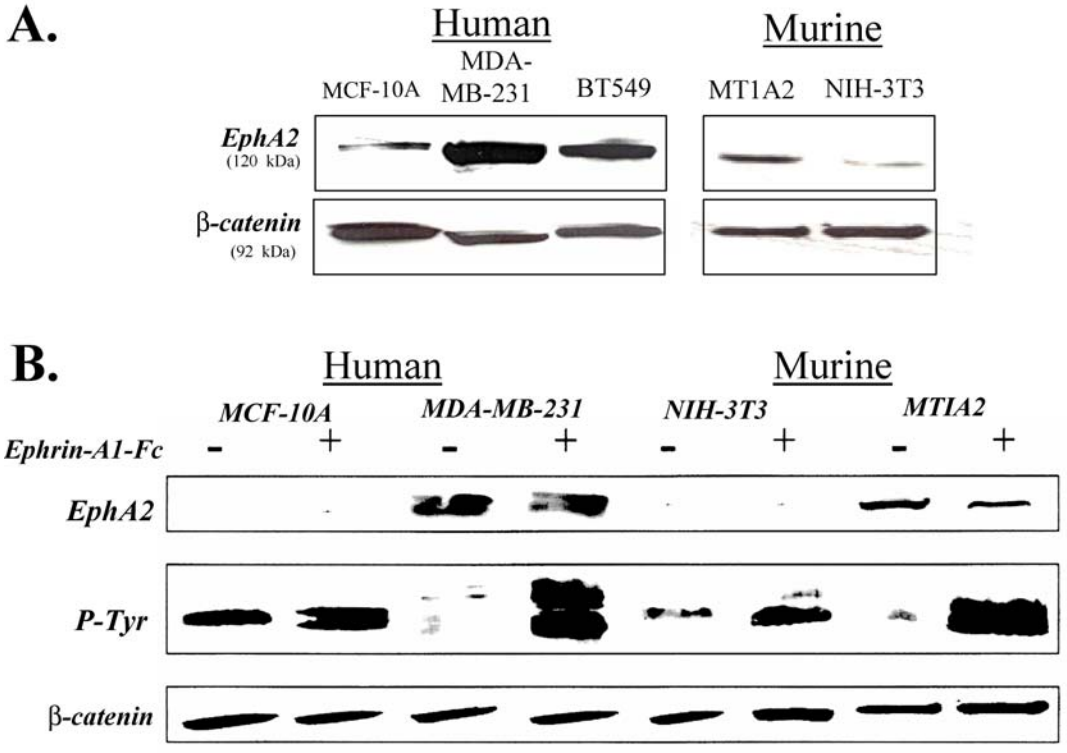
A.
Human
Murine
MCF-10A
MDA-MB-231
BT549
MT1A2
NIH-3T3
EphA2
(120 kDa)
β-catenin
(92 kDa)
B.
Human
Murine
MCF-10A
MDA-MB-231
NIH-3T3
MTIA2
Ephrin-A1-Fc
- + - + - + - +
EphA2
P-Tyr
β-catenin

FIG. 1

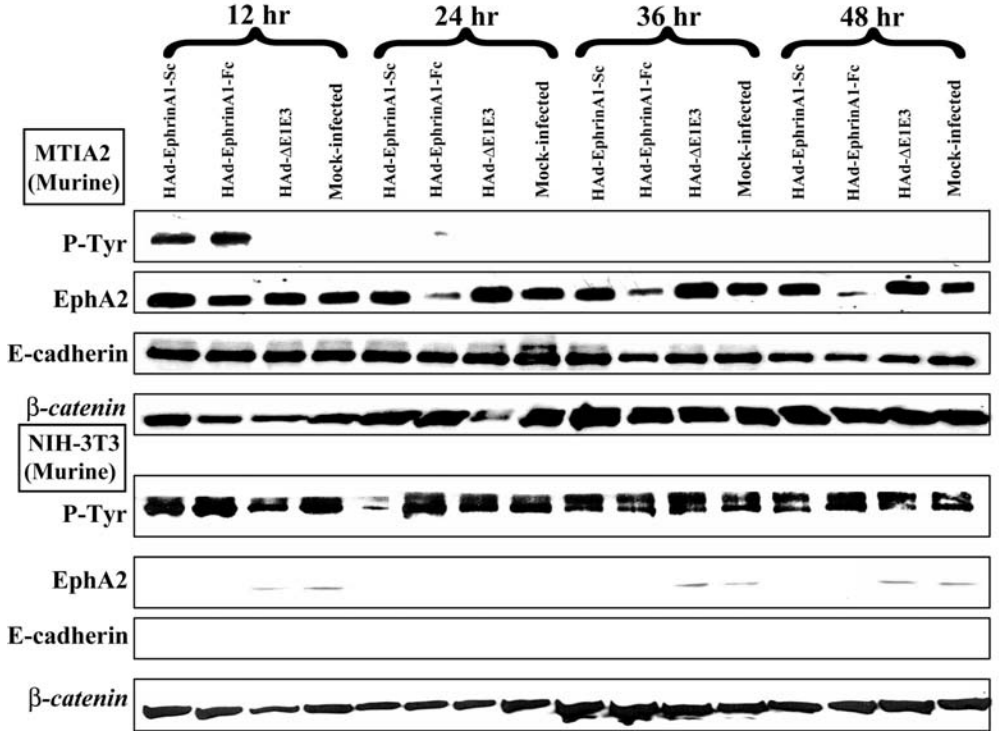
12 hr
24 hr
36 hr
48 hr
HAd-EphrinA1-Sc
HAd-EphrinA1-Fc
HAd-ΔE1E3
Mock-infected
MTIA2 (Murine)
P-Tyr
EphA2
E-cadherin
β-catenin
NIH-3T3 (Murine)
P-Tyr
EphA2
E-cadherin
β-catenin

FIG. 2

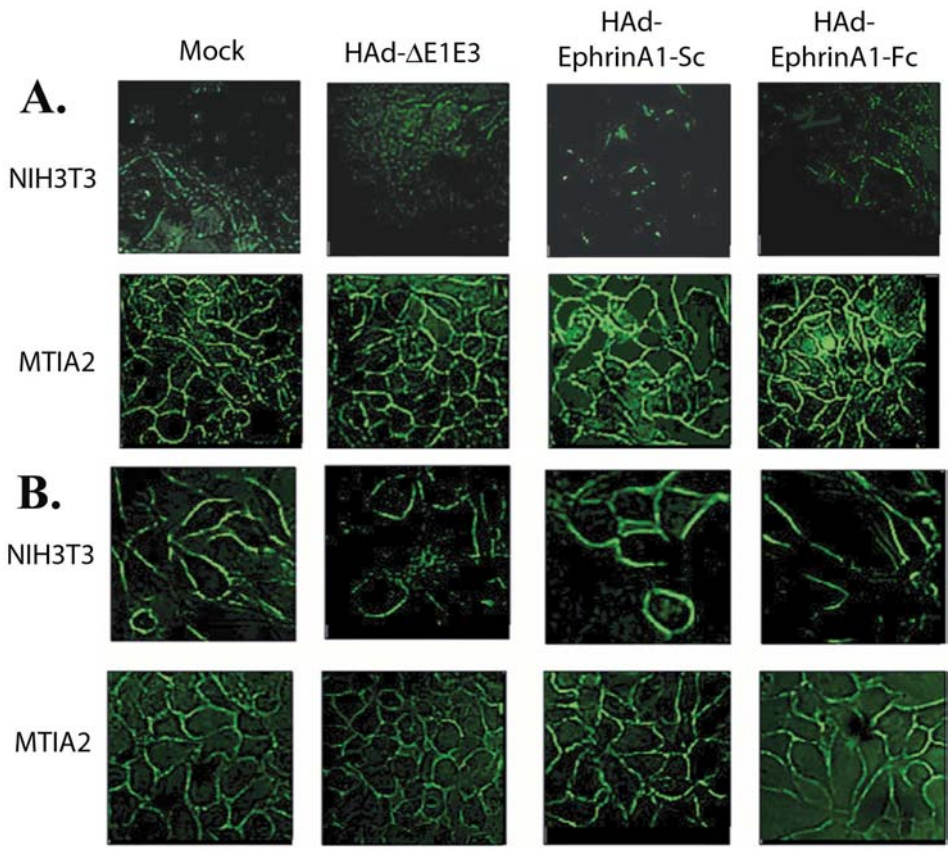
Mock
HAd-ΔE1E3
HAd-EphrinA1-Sc
HAd-EphrinA1-Fc
A.
NIH3T3
MTIA2
B.
NIH3T3
MTIA2

FIG. 3

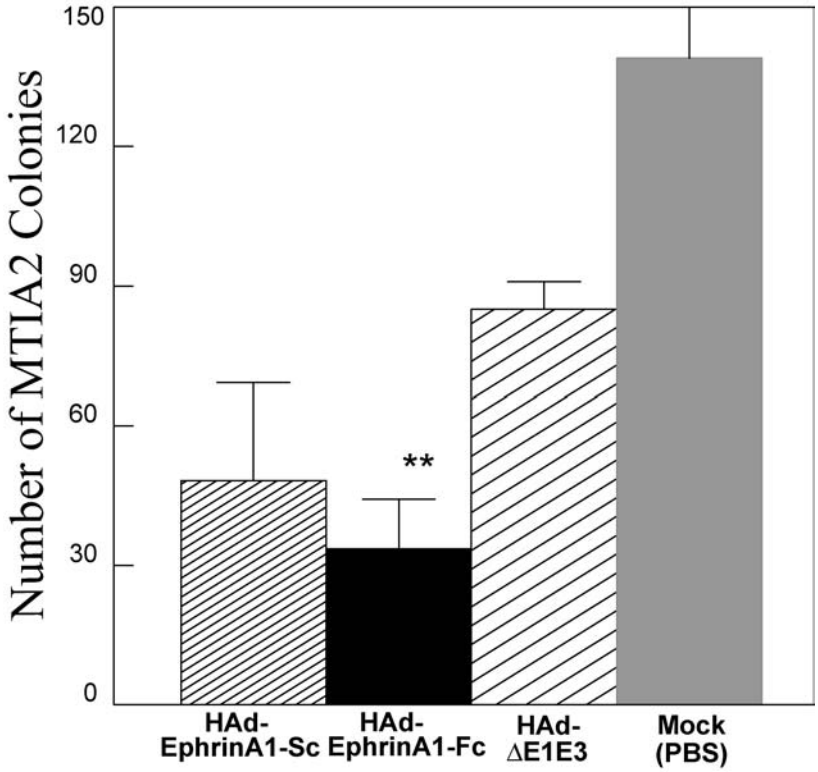
Number of MTIA2 Colonies
150
120
90
60
30
0
**
HAd-EphrinA1-Sc
HAd-EphrinA1-Fc
HAd-ΔE1E3
Mock (PBS)

FIG. 4

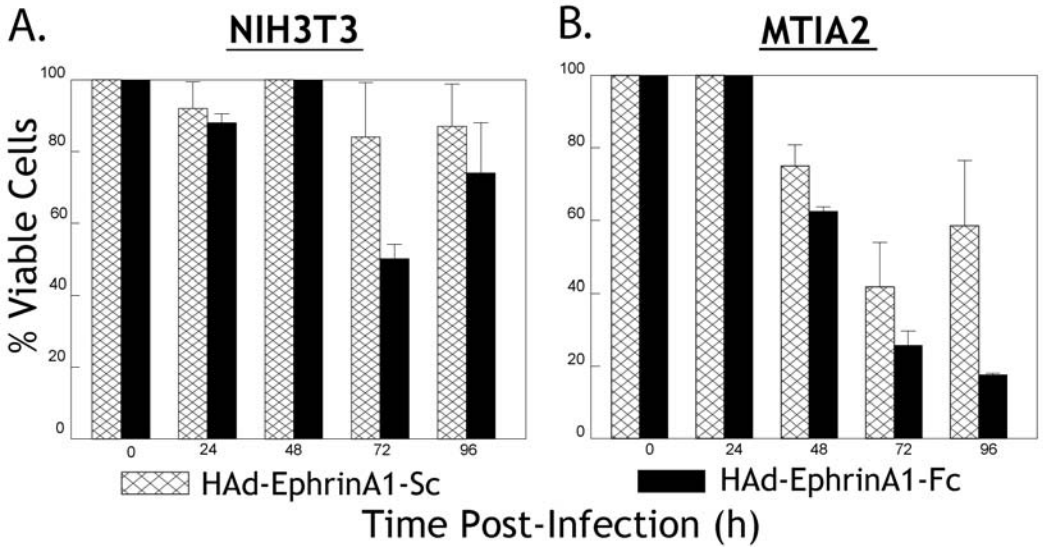
A.
NIH3T3
B.
MTIA2
% Viable Cells
100
80
60
40
20
0
0
24
48
72
96
HAd-EphrinA1-Sc
HAd-EphrinA1-Fc
Time Post-Infection (h)

FIG. 5

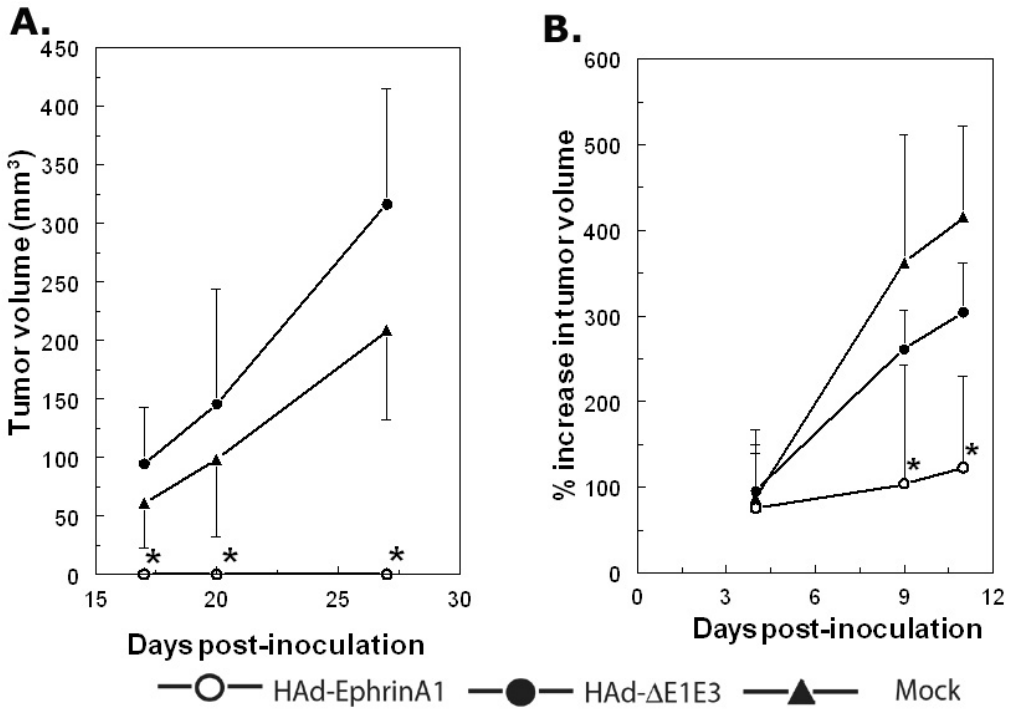
A.
B.
Tumor volume (mm3)
% increase in tumor volume
Days post-inoculation
Days post-inoculation
HAd-EphrinA1
HAd-ΔE1E3
Mock

FIG. 6

Table 1. Development of anti-vector humoral immune response in HAd5 vector-inoculated mice*

Mice No	i.t. inoculation with	HAd5-specific ELISA antibody titer
1	PBS	<50
2	PBS	<50
3	PBS	<50
4	PBS	<50
5	HAd-ΔE1E3	1600
6	HAd-ΔE1E3	800
7	HAd-ΔE1E3	400
8	HAd-ΔE1E3	800
9	HAd-EphrinA1-Fc	800
10	HAd-EphrinA1-Fc	800
11	HAd-EphrinA1-Fc	1600
12	HAd-EphrinA1-Fc	800

* FVB/n mice bearing MT1A2-induced tumors were inoculated i.t. either with PBS, HAd-ΔE1E3, or HAd-EphrinA1-Fc, blood samples were collected on Day 18 post-vector inoculation and were tested for the development of anti-vector humoral immune response by ELISA.