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Antibody Targeting of the EphA2 Tyrosine Kinase Inhibits Malignant Cell Behavior[1]

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ABSTRACT

EphA2 is a transmembrane receptor tyrosine kinase that is up-regulated on many aggressive carcinoma cells. Despite its overexpression, the EphA2 on malignant cells fails to bind its ligand, ephrinA1, which is anchored to the membrane of adjacent cells. Unlike other receptor kinases, EphA2 demonstrates kinase activity that is independent of ligand binding. However, ligand binding causes EphA2 to negatively regulate tumor cell growth and migration. Herein, we translate knowledge of EphA2 into strategies that selectively target malignant cells. Using a novel approach to preserve extracellular epitopes and optimize antibody diversity, we generated monoclonal antibodies that identify epitopes on the extracellular domain of EphA2. EphA2 antibodies were selected for their abilities to inhibit behaviors that are unique to metastatic cells while minimizing damage to nontransformed cells. A subset of EphA2 monoclonal antibodies were found to inhibit the soft agar colonization by MDA-MB-231 breast tumor cells but did not affect monolayer growth by nontransformed MCF-10A breast epithelial cells. These EphA2 antibodies also prevented tumor cells from forming tubular networks on reconstituted basement membranes, which is a sensitive indicator of metastatic character. Biochemical analyses showed that biologically active antibodies induced EphA2 phosphorylation and subsequent degradation. Antisense-based targeting of EphA2 similarly inhibited soft agar colonization, suggesting that the antibodies repress malignant behavior by down-regulating EphA2. These results suggest an opportunity for antibody-based targeting of the many cancers that overexpress EphA2. Our studies also emphasize how tumor-specific cellular behaviors can be exploited to identify and screen potential therapeutic targets.

INTRODUCTION

The most life-threatening forms of cancer often arise when a population of tumor cells gains the ability to colonize distant and foreign sites in the body (1). These metastatic cells survive by overriding restrictions that normally constrain cell colonization into dissimilar tissues (2). For example, typical mammary epithelial cells will generally not grow or survive if transplanted to the lung, yet lung metastases are a major cause of breast cancer morbidity and mortality. Recent evidence suggests that dissemination of metastatic cells through the body can occur long before clinical presentation of the primary tumor (2). These micrometastatic cells may remain dormant for many months or years after the detection and removal of the primary tumor (3). Thus, a better understanding of the mechanisms that allow for the growth and survival of metastatic cells in a foreign microenvironment is critical for the improvement of therapeutics designed to fight metastatic cancer.

One barrier to the development of antimetastasis agents has been the assay systems that are used to design and evaluate these drugs. For example, most conventional cancer therapies target rapidly growing cells. Despite this, cancer cells do not necessarily grow more rapidly but instead survive and grow under conditions that are nonpermissive to normal cells (2). Also, many standard cancer drug assays measure tumor cell growth or survival under typical cell culture conditions (*i.e.*, monolayer growth). However, cell behavior in two-dimensional assays often does not reliably predict tumor cell behavior *in vivo* (2, 4). Benign and malignant epithelial cells are often indistinguishable in monolayer culture and yet behave very differently from each other (and from their own behavior in two dimensions) when cultured on soft agar or Matrigel (4, 5). These differences are important, because soft agar assays and Matrigel behavior have been shown to be more predictive of *in vivo* behavior than cell behavior in monolayer (4–6). Thus, three-dimensional assays provide a new platform to investigate potential therapeutics.

The mechanisms by which metastatic cells subvert regulatory controls in three-dimensional culture remain largely unknown, but aberrant signal transduction is known to mediate metastatic progression (7). In particular, malignant cells often display elevated levels of tyrosine kinase activity (8–10). On the basis of evidence that tyrosine kinase activity is necessary for tumor cell growth, survival, and invasiveness, considerable effort has been devoted to therapeutic targeting of tyrosine kinases (11, 12). Monoclonal antibodies provide a particularly attractive way to target tyrosine kinases on malignant cells in that these enzymes have large extracellular domains and are often up-regulated in cancerous tissue (13). Promising new cancer treatments include antibodies directed against the extracellular domains of the receptor tyrosine kinases, HER2 and epidermal growth factor receptor (14–16). However, the relatively few tumors that overexpress these molecules limits the clinical application of these antibodies. Another setback can involve toxicity to normal tissues that express the target. Therefore, a need exists to identify molecules that are ubiquitously up-regulated in metastatic cancers but of which the targeting will not threaten normal tissue.

Previously, our laboratory identified tyrosine kinases that are selectively overexpressed or functionally altered in malignant cells (17). Much of our recent work has focused on EphA2, which is up-regulated in many aggressive cancer cells (18–22). We demonstrated recently that EphA2 is a powerful oncoprotein, sufficient to confer malignant transformation and tumorigenic potential on nontransformed mammary epithelial cells when overexpressed (19). Interestingly, EphA2 overexpression did not increase cellular growth rates of cultures in monolayer. Instead, the oncogenic potential of EphA2 was most apparent when using three-dimensional assays, such as behavior in soft agar and Matrigel (19).

Several features of EphA2 make it an excellent candidate for antibody-based targeting. First, the transmembrane receptor is found at high levels on tumor cells relative to surrounding epithelium (18, 19, 22). The highest levels of EphA2 are consistently found on the most aggressive tumor cells (18, 19). Also, multiple types of epithelial tumors (*i.e.*, breast, prostate, colon, lung, and skin) overexpress EphA2, and a very large percentage of cells within these tumors share this phenotype (18, 19, 22). Despite its overexpression, the EphA2 in malignant cells fails to bind its ligand (23), known as ephrinA1, which is anchored to the membrane of adjacent cells (24). Unlike other

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receptor tyrosine kinases, ligand binding is not necessary for EphA2 enzymatic activity (23). However, the defect in ligand binding in cancer cells is important, because binding to ephrinA1 causes EphA2 to negatively regulate tumor cell growth and migration (19, 23, 25). Thus, an antibody that has been designed to mimic the actions of ephrinA1 may have the potential to reverse metastatic behavior by activating the high levels of EphA2 on a tumor cell surface. Herein, we test a hypothesis that antibody targeting of EphA2 can restore normal cellular controls on growth in a foreign microenvironment.

MATERIALS AND METHODS

Cells and Antibodies. All of the mammary (MCF-10A, MCF^{EphA2}, BT474, and MDA-MB-231) and prostatic (PC-3) epithelial cells were cultured as described (18, 19, 23, 26). Antibodies for phosphotyrosine (4G10), β-catenin, and EphA2 (D7) were purchased from Upstate Biologicals, Inc. (Lake Placid, NY). Paxillin-specific antibodies were the gift of Dr. K. Burridge (University of North Carolina, Chapel Hill, NC). Additional EphA2 antibodies (EA2–5 and EN1) were screened from existing hybridomas (17) using the procedures detailed below. P3XBcl-2–13 cells were cultured as described (27).

DNA-based Immunizations. A plasmid (ecdEphA2-Fc; kindly provided by Dr. B. Wang, Case Western Reserve University, Cleveland, OH) encoding for a fusion of the extracellular domain of EphA2 linked to immunoglobulin heavy chain was used as the source of immunogen. DNA-based immunizations via the biolistics/RIMMS[4] procedure were performed as described previously (27). Briefly, purified plasmid DNA was precipitated onto gold particles using a calcium chloride method. Each cartridge was prepared to contain a total of 1.25 μg of DNA coated onto 0.5 mg of gold particles. Bullets were stored at 4°C in the dark. A 1-week-old female SJL mouse (The Jackson Laboratory, Bar Harbor, ME) was anesthetized with isoflurane for removal of fur and for immunization (27). DNA-coated gold particles were propelled into the epidermal tissue of the thoracic site using a helium-driven gene gun (PowderJect), regulated under 375 p.s.i. pressure. Two cartridges, containing a total of 2.5 μg of ecdEphA2-Fc plasmid, were delivered in overlaying shots to the thoracic site on days 0, 5, 7, and 9. On day 12, lymphocytes were harvested bilaterally from the axillary and brachial lymph nodes of the immunized mouse. Lymphocytes were fused with P3XBcl-2–13 cells at a ratio of 2.5:1 using a protocol reported previously (27).

FluorELISA. Cell surface binding by anti-EphA2 antibodies to the EphA2 receptor was monitored using modifications to a reported assay (27). Flat-bottomed tissue culture treated plates (96-well; Costar, Cambridge, MA) were treated with 100 μl of poly-L-lysine hydrobromide (Sigma, St. Louis, MO) diluted to 10 μg/ml in 0.1 M sodium phosphate (pH 8.0), for 1 h. Poly-L-lysine was removed from the wells before the addition of 100 μl of a cell suspension of MDA-MB-231 (positive for EphA2) or BT474 cells (negative controls) at a concentration of 3×10^4 cells/well. After incubation overnight at 37°C, 5% CO_2, the culture medium was gently removed, and 100 μl of supernatants from hybridomas were incubated on cells at room temperature for 1 h. The samples were washed three times with 1× Dulbecco's PBS (pH 7.1; Life Technologies, Inc., Grand Island, NY). Goat antimouse Alexa Fluor 488 antibody (100 μl; Molecular Probes, Eugene, OR), diluted to 2 μg/ml in PBS, was added for 1 h at room temperature. After washing cells with PBS, PBS containing 2% FCS (50 μl) was added to each well before observation using an inverted fluorescence microscope (Model DM-IRB; Leica, Deerfield, IL).

WB, IP, and Kinase Assays. Western blot analyses and IPs were performed as described previously (23). Briefly, detergent extracts of cell monolayers were extracted in Tris-buffered saline containing 1% Triton X-100 (Sigma). After measuring protein concentrations (Bio-Rad, Hercules, CA), 1.5 mg of cell lysate was immunoprecipitated, resolved by SDS-PAGE, and transferred to nitrocellulose (Protran; Schleicher and Schuell, Keene, NH). Antibody binding was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography (Kodak X-OMAT; Rochester, NY). To confirm equal sample loading, the membranes were stripped and reprobed with antibodies specific for paxillin or β-catenin. *In vitro* analyses of EphA2 enzymatic activity were performed as detailed previously (23).

Cell Growth Assays. Colony formation in soft agar was performed as described (19). For experiments with antibodies, an antibody (10 μg/ml) or a matched vehicle (PBS) was included in the top agar solutions. Colony formation was scored microscopically using an Olympus CK-3 inverted phase-contrast microscope outfitted with a ×40 objective. Clusters containing at least three cells were scored as a positive. The average number of colonies per high-powered field is shown. Ten separate high-power microscopic fields were averaged in each experiment, and the results shown are representative of at least three separate experiments.

To measure anchorage-dependent cell growth, 1×10^5 cells were seeded into each well of a six-well tissue culture-treated dish (Costar). After 3 or 7 days of incubation at 37°C, samples were harvested in a trypsin-EDTA solution (Life Technologies, Inc.) and counted using a hemacytometer (Olympus IX-70; Eugene OR). Each experiment was repeated at least three times, and representative results are reported. The statistical significance of all results was calculated using a Student's *t* test (Microsoft Excel, Seattle, WA), defining $P < 0.05$ as significant.

Cell Behavior in Matrigel. The behavior of cells on Matrigel was analyzed as described (19). Briefly, tissue culture dishes were coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) at 37°C before adding 1×10^5 MDA-MB-231 or MCF-10A cells that had been incubated on ice for 1 h with the indicated antibody (10 μg/ml) or vehicle (PBS). Cells were incubated on Matrigel for 24 h at 37°C, and cell behavior was assessed using an Olympus IX-70 inverted light microscope. All of the images were recorded onto 35-mm film (T-Max-400; Kodak).

Antisense Targeting of EphA2. To decrease EphA2 protein levels, MDA-MB-231 breast carcinoma cells were transiently transfected with phosphorothioate-modified antisense oligonucleotides that corresponded to a sequence that was found to be unique to EphA2 as determined using a sequence evaluation of GenBank ($^{5'}$-CCAGCAGTACCGCTTCCTTGCCCTGCG-GCCG-$^{3'}$). IAS oligonucleotides ($^{5'}$-GCCGCGTCCCGTTCCTTCACCAT-GACGACC-$^{3'}$) provided controls for the vehicle and transfection. The cells were transfected with oligonucleotides (2 μg/ml) using LipofectAMINE PLUS Reagent (Life Technologies, Inc.) according to the manufacturer's protocol. After transfection (24 h), the cells were divided. Half of the cells were seeded into soft agar, and the remaining cells were extracted and subjected to WB with the EphA2-specific antibody (D7). To control for sample loading, the membranes were stripped and reprobed with paxillin antibodies

RESULTS

Isolation and Characterization of EphA2 Antibodies. To generate monoclonal antibodies against the extracellular domain of EphA2, we combined gene gun-based immunizations with the RIMMS immunization protocol (Fig. 1*A*). Using a PowderJect gene gun, an SJL mouse was immunized with the pcDNA3-ecdEphA2-F_c expression plasmid. This plasmid encodes for a fusion of the extracellular domain of human EphA2 that has been linked to human immunoglobulin to facilitate secretion of the fusion protein. The PowderJect gene gun allowed transfection of epidermal cells *in vivo* (27), and consequently, the ecdEphA2-F_c protein was properly folded, modified, secreted, and then recognized by the host immune system. The mice were immunized with the plasmid every other day for a total of 12 days. This abbreviated schedule was intended to minimize immunodominance and, thus, to optimize the breadth of different B lymphocytes that could respond to the antigen (28).

After 12 days, the responding B lymphocytes were isolated from primary lymph nodes. The lymph node B lymphocytes were selected to increase the likelihood that class switching and affinity maturation had already taken place, which was particularly important given the abbreviated schedule (28). The use of lymph node B lymphocytes, rather than spleen cells, also increased the diversity of antigen recognition, because only a small population of lymph nodes cells are able to colonize the spleen (27). The lymph node B cells were fused with

[4] The abbreviations used are: RIMMS, repetitive immunization, multiple sites; EA antibodies, EphA2 activating antibodies; ecdEphA2-F_c, fusion protein consisting of the extracellular domain of EphA2 linked to IgG-F_c; ECM, extracellular matrix; EN antibodies, antibodies that do not stimulate EphA2; FluorELISA, fluorescence-based ELISA procedure; IAS, inverted antisense; IP, immunoprecipitation; WB, Western blot analysis.

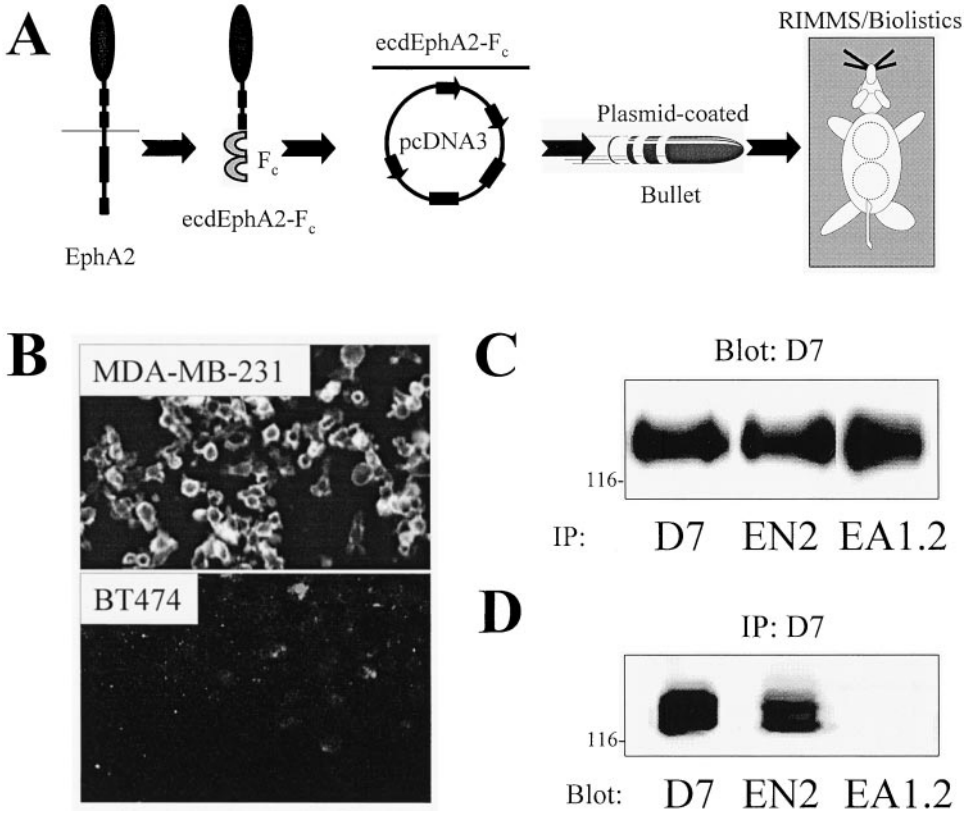

Fig. 1. Generation and characterization of EphA2 antibodies. *A*, shown is an overview of the experimental strategy that was used to generate monoclonal antibodies against the EphA2 receptor tyrosine kinase. Briefly, mice were immunized using the biolistics and RIMMS procedures with a plasmid that encodes for a fusion of the extracellular domain of EphA2 linked to immunoglobulin (See text for details). By transfecting epidermal cells with EphA2 *in vivo*, this strategy optimized the recognition of conformation-defined epitopes. *B*, representative results of a FluorELISA experiment with EA1.2 antibodies were viewed using epi-fluorescence microscopy (×40). Whereas EphA2-overexpressing MDA-MB-231 cells stained prominently, the EphA2-deficient line BT474 revealed low-level immunoreactivity. *C*, specificity for EphA2 was confirmed by immunoprecipitating cell lysates from MDA-MB-231 cells with each candidate antibody, followed by WB with the known EphA2 antibody, D7. *D*, the failure of some antibodies (shown here as *EA1.2*) to recognize EphA2 as measured by Western blot analyses revealed that some of antibodies generated by our approach recognized native or conformation-defined epitopes on EphA2. The relative mobility of molecular mass standards is shown on the *left*.

a myeloma (P3XBcl-2–13) that had been engineered to overexpress Bcl-2. The overexpression of Bcl-2 in the myeloma cells minimized apoptosis of the subsequent hybridomas during the fusion and subcloning procedures (28). The resulting hybridoma cells were then divided into 48 different bulk cultures immediately after polyethylene glycol-mediated somatic fusion.

As a preliminary screen for EphA2-immunoreactivity, supernatants from bulk culture hybridomas were screened for immunoreactivity against EphA2. Our immunization strategy was designed to identify extracellular EphA2 epitopes on viable tumor cells. Thus, we used a fluorescence-based ELISA protocol (FluorELISA), which selects for antibody reactivity against live cells. This screening approach was preferable to Western blot analyses, which might have biased against antibodies that recognize conformation-restricted epitopes. FluorELISA identified 44 bulk hybridoma populations that stained EphA2-overexpressing tumor cells (MDA-MB-231) but not EphA2-deficient cells (BT474; Fig. 1*B*). Immunoreactivity was confirmed using fluorescence microscopy, which revealed a pattern of diffuse membrane staining that was consistent with our previous studies of EphA2 subcellular localization (data not shown; Refs. 19, 23). On the basis of strong immunostaining of target-positive but not target-deficient cells, three hybridoma bulk cultures were initially selected for subcloning by flow cytometry. Using supernatants from these subcloned hybridomas, FluorELISA analysis was repeated (the results of a representative subclone is shown in Fig. 1*B*).

The EphA2 specificity of hybridoma subclones was confirmed using multiple analyses (Table 1; Fig. 1, *C* and *D*). For example, monolayers of EphA2-overexpressing (MDA-MB-231) cells were extracted, and the lysates were immunoprecipitated with each candidate antibody (Fig. 1*C*). The resulting material was resolved by SDS-PAGE and subjected to Western blot analyses with a known EphA2 antibody (D7). The inverse experiment (immunoprecipitate using D7 and blot with candidate antibodies) indicated that a subset of the antibodies could not recognize EphA2 epitopes that had been ablated during SDS-PAGE electrophoresis (Fig. 1*D*). However, the EphA2 immunoreactivity of many of these antibodies (including EA1.2) was restored when the lysates were collected and resolved using nonreducing conditions (not shown). Finally, each candidate antibody was isotyped and found to be of the IgG 1 subclass.

EphA2 Antibodies Inhibit Tubular Network Formation. Recent studies have shown that tumor cell behavior within a three-dimensional microenvironment, such as Matrigel, can reliably predict the differentiation state and aggressiveness of mammary epithelial cells (4, 5, 29). Thus, we asked whether EphA2 antibodies would alter the behavior of mammary epithelial cells on Matrigel. Monolayer cultures of benign (MCF-10A; low EphA2 levels) or malignant (MDA-MB-231; high EphA2 levels) breast epithelial cells were incubated on Matrigel in the presence of EphA2 antibodies (10 μg/ml) or vehicle (PBS; Fig. 2). Within 24 h, nontransformed MCF-10A epithelial cells had organized into acinus-like spheres on Matrigel, which was con-

Table 1 *Overview of EphA2 monoclonal antibodies*

Two groups of EphA2 antibodies were identified in this study: EA and EN antibodies. The antibodies were evaluated for their technical applications as measured using WB, IP, and immunofluorescence staining (IF), as well as for their ability to inhibit tumor cell (MDA-MB-231, PC3) growth in soft agar or tubular network formation on Matrigel (MDA-MB-231, MCF^{EphA2}). The asterisk (*) signifies a differential ability of EA antibodies to recognize EphA2 during WB. EA antibodies generally did not recognize EphA2 under reducing conditions (see text for details).

Antibody	WB	IP	IF	EphA2 Activation	EphA2 Degradation	Soft Agar Inhibition	TN[a] Inhibition
EA Group (EA1.1, EA1.2, EA2, EA3, EA4, EA5)	*	+	+	+	+	+	+
EN Group (EN1, EN2)	+	+	+	−	−	−	−

[a] TN, tubular network formation.

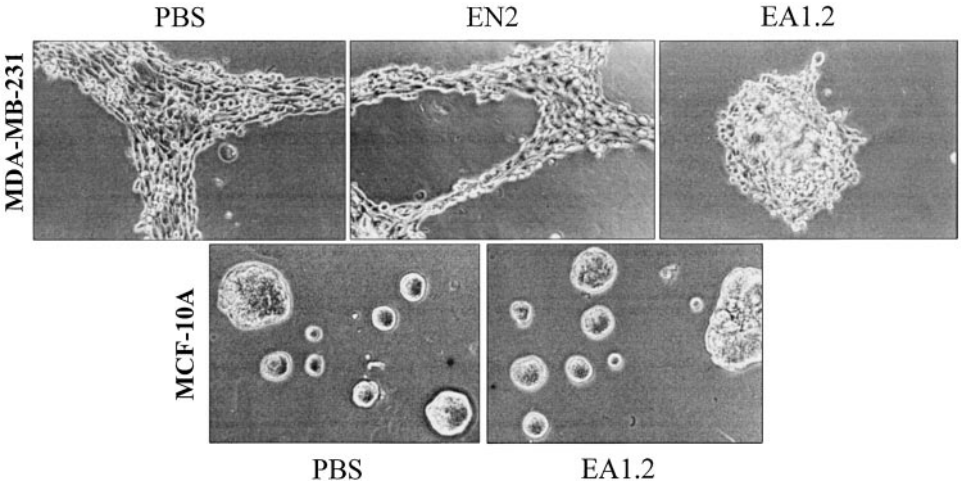

Fig. 2. EA1.2 antibodies prevent tubular network formation by aggressive breast cancer cells. MDA-MB-231 (*top*) or MCF-10A (*bottom*) cells were incubated with the indicated antibodies (10 μg/ml) or vehicle (PBS) before being plated onto three-dimensional basement membranes composed of Matrigel. Cell morphology was recorded 24 h after plating. MDA-MB-231 cells treated with vehicle (*top left*) or EN2 antibody (*top center*) organized into tubular networks that invaded atop and throughout the Matrigel. In contrast, treatment with EA1.2 caused MDA-MB-231 cells to form spherical clusters that resembled the differentiated behavior of nontransformed MCF-10A cells (*bottom left*). Note that EA1.2 did not alter the differentiated phenotype MCF-10A cells (*bottom right*).

sistent with their differentiated phenotype (4, 19). Although MCF-10A cells express EphA2 (19, 23), none of the EphA2 antibodies were toxic to nontransformed cells or altered their abilities to organize into differentiated structures (Fig. 2, *bottom panels*; data not shown).

Unlike the differentiated behavior of MCF-10A cells, MDA-MB-231 cells quickly assembled into tubular networks (Fig. 2, *top left*). These networks progressively invaded all throughout the Matrigel, which was consistent with previous evidence of their aggressiveness (5, 30). The formation of tubular networks was highly sensitive to EphA2 antibodies. For example, EA1.2 antibodies prevented MDA-MB-231 cells from organizing into tubular networks (Fig. 2, *top right*). Instead, EA1.2-treated MDA-MB-231 cells assembled into spherical structures that resembled the behavior of differentiated cells (albeit larger than structures formed by MCF-10A cells). This dramatic change in behavior was reproduced using EphA2-overexpressing MCF-10 cells (MCF^{EphA2}). Notably, MCF^{EphA2} cells demonstrated evidence of cell death in the presence of EA1.2 antibodies (not shown). In contrast, other EphA2 antibodies, such as EN2, did not prevent the formation of tubular networks. Our results suggest that certain EphA2 antibodies alter tumor cell interaction with the surrounding three-dimensional microenvironment in a manner that is consistent with a less invasive and more highly differentiated phenotype.

Selective Inhibition of Malignant Cell Growth. On the basis of evidence that EphA2 overexpression induces anchorage-independent tumor cell growth (19), we asked if antibody targeting of EphA2 could prevent tumor cell colonization of soft agar. MDA-MB-231 cells were suspended in soft agar for 7 days at 37°C in the presence of purified antibody (10 μg/ml) or vehicle control (PBS), administered at the time of suspension. Microscopic evaluation revealed that some EphA2 antibodies, such as EA1.2, inhibited at least 60% of soft agar colony formation as compared with vehicle-treated controls (Fig. 3*A;* Table 1; $P < 0.0003$). Other EphA2 antibodies, such as EN2, did not alter soft agar colonization ($P > 0.3$). EA1.2 similarly inhibited at least 60% of soft agar colonization by PC-3 prostate cancer cells ($P < 0.0003$; not shown). Control experiments confirmed that neither isotype-matched (IgG 1) controls (*e.g.,* antipaxillin) nor antibodies against intracellular epitopes on EphA2 (*e.g.,* D7) decreased soft agar colonization. On the basis of their differential effects on cell behavior in soft agar and Matrigel, we were able to distinguish two subsets of EphA2 antibodies. Those antibodies that actively inhibited tumor cell growth were termed EA antibodies, whereas those antibodies that were neutral to tumor cell growth were termed EN antibodies. The differences between EA and EN antibodies extended to multiple biological and biochemical outcomes (see Table 1). For the sake of brevity, only the results of experimentation with a single EN or EA antibody are reported, and these findings are representative of the two classes of EphA2 antibodies reported here.

In light of the decreased anchorage-independent growth, we asked whether EphA2 antibodies would alter the anchorage-dependent growth of benign epithelial cells. To test this, 1×10^5 nontransformed MCF-10A mammary epithelial cells were seeded into plastic tissue culture-treated dishes in the presence of EN2 or EA1.2 antibodies. Seven days after plating, the number of cells was assessed microscopically using a hemacytometer (Fig. 3*B*). None of the EphA2 antibodies significantly altered the growth rates of nontransformed epithelial cells. No inhibition of growth was observed at any dose of antibody

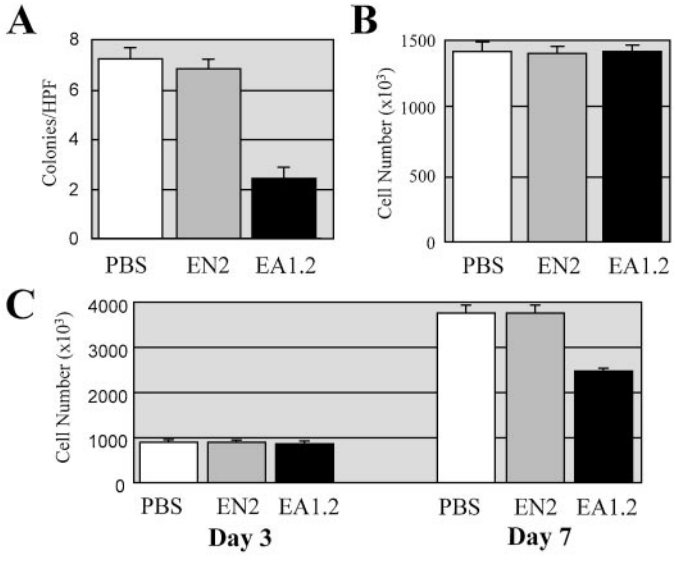

Fig. 3. EphA2 antibodies selectively inhibit malignant growth. *A,* MDA-MB-231 cells were incubated in the presence of either purified EphA2 antibodies (10 μg/ml) or vehicle (PBS) for 7 days at 37°C in soft agar. EA1.2 ($P < 0.0003$) but not EN2 antibodies ($P > 0.3$) significantly inhibited colony formation relative to vehicle treated controls. Results are reported as colonies per high-powered field (*HPF*). *B,* 2×10^5 MCF-10A cells were grown in monolayer culture for 7 days at 37°C in the presence of 100 μg/ml of the indicated EphA2 antibodies. Neither EA1.2 ($P > 0.34$) nor EN2 ($P > 0.22$) antibodies altered monolayer cell growth relative to the vehicle control. *C,* the monolayer growth of 2×10^5 MDA-MB-231 cells was evaluated microscopically after 3 or 7 days of incubation at 37°C in 100 μg/ml of the indicated antibodies. Note that EA1.2 antibodies did not significantly decrease tumor cell growth after 3 days in culture. However, after the samples had reached confluence, the growth-inhibitory effects of EA1.2 were detected; *bars,* ±SD.

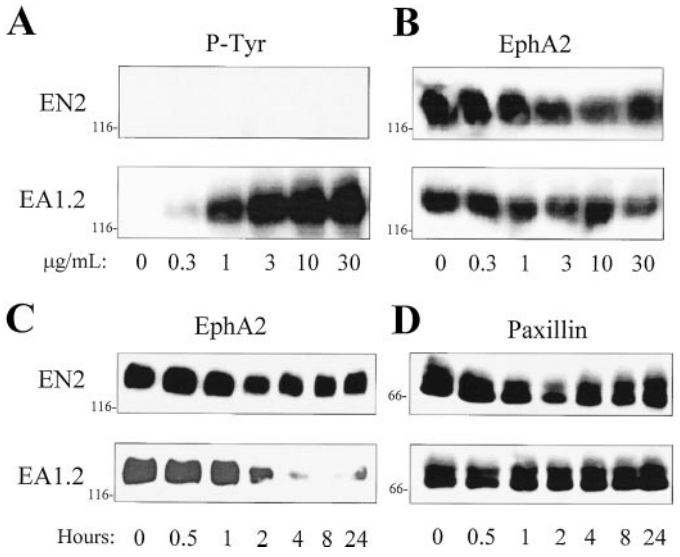

Fig. 4. Growth inhibitory antibodies promote tyrosine phosphorylation and degradation of EphA2. *A* and *B*, monolayers of MDA-MB-231 cells were incubated in the presence of the indicated amount of purified EphA2 antibodies for 8 min at 37°C. *A*, cell lysates were then immunoprecipitated with an EphA2-specific antibody (D7), resolved by SDS-PAGE, and subjected to WB with a phosphotyrosine-specific antibody (4G10). *B*, the membranes were stripped and reprobed with D7 as a loading control. The relative mobility of molecular mass standards is shown on the *left*. *C*, monolayers of MDA-MB-231 cells were incubated in the presence of 30 μg/ml of the indicated antibodies or vehicle (PBS) for 0–24 h at 37°C. Cell lysates were then resolved by SDS-PAGE and subjected to WB with the EphA2-specific antibody, D7. *D*, the membranes were stripped and reprobed with a paxillin antibody as a loading control. The relative mobility of molecular mass standards is shown on the *left*.

or time point (not shown). The failure to inhibit MCF-10A cells did not result from a lack of EphA2 on MCF-10A cells or from an inability to bind EphA2 on these cells, as determined by immunofluorescence microscopy (data not shown).

Identical studies with MDA-MB-231 cells revealed fundamental differences between how the EphA2-specific antibodies affect anchorage independent *versus* dependent cell growth. After 3 days in culture, none of the EphA2 antibodies had altered the anchorage-dependent (monolayer) growth of MDA-MB-231 cells (Fig. 3*C, left side*), which was similar to our findings with nontransformed epithelial cells. However after reaching confluence, the growth of EA1.2-treated cultures (as measured on day 7) was reduced by at least 30% in EA1.2-treated samples compared with vehicle or EN2-matched controls (Fig. 3*C*; $P < 0.08$). Identical monolayer growth assays using E1.2 antibody-treated PC-3 cells resulted in a similar pattern of growth, with inhibition of growth detected only after cells had reached confluence (data not shown). We reasoned that the decreased cell numbers at later times might have resulted from an inhibition of anchorage-independent growth, such as occurs after cells reach confluence. Altogether, our analyses indicate that EA antibodies selectively impair the malignant growth of carcinoma cells (*i.e.*, anchorage-independent growth) without inhibiting the anchorage-dependent growth or survival of nontransformed epithelial cells.

Antibody-dependent EphA2 Degradation. We then sought to determine the mechanism by which EphA2 antibodies, such as EA1.2, inhibit malignant behavior. We were able to eliminate that the biological activities of EphA2 antibodies reflected differential EphA2 binding, because, for example, EN2 and EA1.2 bound comparably well to EphA2-overexpresing tumor cells as measured using FluorELISA, immunofluorescence microscopy, and IP (Fig. 1*B;* data not shown). Therefore, we hypothesized that biologically active (EA) antibodies might have triggered EphA2 autophosphorylation, whereas biologically neutral (EN) antibodies did not. To study EphA2 stimulation, MDA-MB-231 cells were incubated with EN2 or EA1.2 antibodies for 8 min at 37°C (Fig. 4, *A* and *B*). EphA2 was then immunoprecipitated with D7 antibodies, resolved by SDS-PAGE, and subjected to WB with the phosphotyrosine-specific antibody 4G10. Treatment with EA1.2 consistently increased the phosphotyrosine content of EphA2 in a dose-dependent manner, whereas treatment with EN2 did not induce receptor autophosphorylation at any dose. Thus, the growth-inhibitory actions of EphA2 antibodies correlated with their abilities to stimulate EphA2 autophosphorylation.

Autophosphorylation often fates receptor tyrosine kinases to be internalized and down-regulated via proteolysis. To ask if the EA1.2 antibody down-regulates EphA2, MDA-MB-231 cells were treated with PBS, EN2, or EA1.2 antibodies for 0–24 h, and EphA2 protein levels were evaluated by WB. Biologically active antibodies, such as EA1.2, decreased EphA2 levels within 2 h, and low levels of EphA2 persisted for at least 24 h (Fig. 4, *C* and *D*). In contrast, the EN2 antibody did not alter the levels of EphA2, which was consistent with its inability to trigger EphA2 autophosphorylation (Fig. 4*C*). EphA2 was similarly degraded in response to other EA antibodies (data not shown). Thus, the growth-inhibitory actions of EphA2 antibodies related to receptor degradation.

With many receptor kinases, ligand-mediated autophosphorylation is necessary for intrinsic enzymatic activity (31), whereas the kinase activity of EphA2 does not require receptor autophosphorylation (23). To ask if antibody-mediated autophosphorylation would alter EphA2 enzymatic activity, MDA-MB-231 cells were treated in the presence of absence of EA1.1 antibodies as detailed above. Equal amounts of protein lysates (Fig. 5*A*) were then immunoprecipitated using D7 antibodies. One half of the immunoprecipitate was analyzed using *in vitro* autophosphorylation assays. Despite dramatic differences in phosphotyrosine content, EphA2 demonstrated comparable levels of enzymatic activity in control and EA1.1-treated samples (Fig. 5*B*). Over time, the amount of EphA2 enzymatic activity decreased in samples that had been treated with EA antibodies. Western blot analyses of the remaining sample indicated that the reduced EphA2

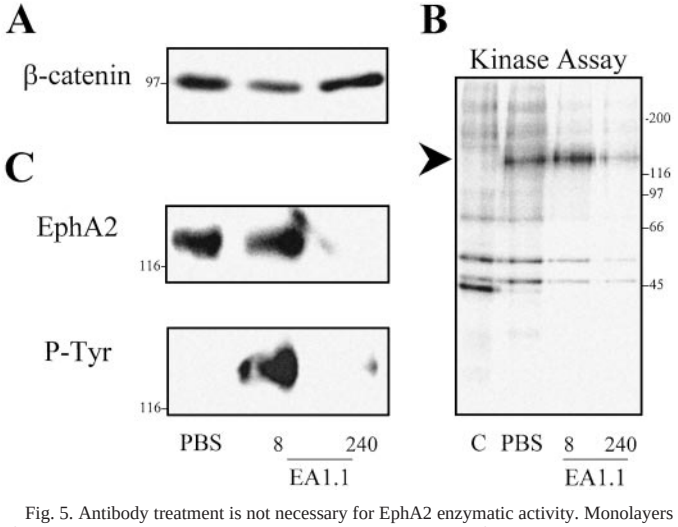

Fig. 5. Antibody treatment is not necessary for EphA2 enzymatic activity. Monolayers of MDA-MB-231 cells were incubated with EA1.1 antibodies for 8 or 240 min at 37°C or with PBS for 8 min (as a vehicle control). Extracts of cell lysates were subjected to Western blot analyses to determine EphA2 levels and to confirm equal sample loading (*A*) before IP of the remaining sample with D7 antibodies. Half of the immunoprecipitated EphA2 was subjected to an *in vitro* autophosphorylation assay (*B*), which was resolved by SDS-PAGE and subjected to autoradiography. A mock IP of cell lysates with an irrelevant antibody (denoted as "*C*") provided a control for nonspecific background. The *arrowhead* denotes the relative mobility of labeled EphA2. The remaining half of the sample was subjected to Western blot analyses with antiphosphotyrosine (4G10) and EphA2-specific antibodies to ensure EphA2 stimulation and equal sample loading, respectively (*C*). Note that the level of EphA2 enzymatic activity relates directly to the amount of EphA2 protein.

enzymatic activity related directly to lower levels of EphA2 protein (Fig. 5*C*). These studies verify that the enzymatic activity of EphA2 does not require receptor phosphorylation and indicate that EA antibodies ultimately serve to abrogate EphA2 enzymatic activity by degrading EphA2 (23).

We then asked if EN antibodies could prevent EA1.1-mediated EphA2 autophosphorylation and subsequent degradation (data not shown). However, preincubation in a 10-fold excess of EN2 did not prevent EphA2 autophosphorylation by EA1.1. Similarly, EN1 did not prevent EA1.1-mediated degradation of EphA2. ELISA-based epitope mapping studies revealed that EN1 and EN2 recognized different epitopes on EphA2 than those that were recognized by the different EA family members.

Decreased EphA2 Levels Block Tumor Cell Growth. Although the growth-inhibitory actions of EphA2 antibodies correlated with EphA2 down-regulation, it was unclear whether decreased levels of EphA2 were directly responsible for decreased tumor cell growth. An alternative possibility was that antibodies such as EA1.2 could transmit signals that decrease soft agar colonization. To distinguish between these possibilities, we developed an antisense oligonucleotide-based approach that decreased EphA2 expression in tumor cells independent of EphA2 activation (Fig. 6*A*). Monolayers of MDA-MB-231 cells were treated with 2 μg/ml of an antisense oligonucleotide that was specific for human EphA2. Western blot analyses confirmed that antisense oligonucleotides selectively decreased EphA2 expression in MDA-MB-231 cells, whereas an IAS did not (Fig. 6*A*). We then suspended MDA-MB-231 cells in soft agar and found that EphA2 antisense oligonucleotides decreased soft agar colonization by at least 60% as compared with matched controls (Fig. 6*B*). Consistent results with EphA2 antibodies and antisense oligonucleotides thus indicate that decreased EphA2 expression is sufficient to decrease tumor cell growth.

DISCUSSION

The major finding of this study is that monoclonal antibody targeting of EphA2 selectively inhibits malignant cancer cells. We also show that inhibitory antibodies induce EphA2 autophosphorylation and degradation, and that decreased EphA2 protein expression is sufficient to inhibit tumor cell growth. This study is the first to show that the metastatic behavior of tumor-derived, EphA2-overexpressing cancer cells can be reversed using monoclonal antibodies. Another novel aspect of our present study is that stimulation of EphA2 decreases malignant cell growth by decreasing EphA2. Thus, we propose that EphA2 overexpression is necessary for the metastatic behavior of these cells. Finally, we outline new procedures to generate and test monoclonal antibodies that have been directed against tumor cell antigens. In particular, recent understanding of tumor cell behavior in three-dimensional assays can be used to design approaches that selectively inhibit malignant cells while minimizing toxicities to normal cells.

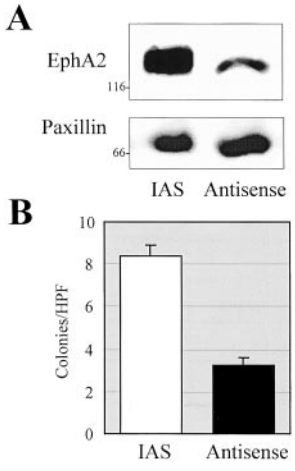

Fig. 6. Decreased EphA2 protein levels are sufficient to reduce tumor cell colonization of soft agar. Monolayers of MDA-MB-231 cells were transfected with 2 μg/ml of EphA2 antisense or IAS oligonucleotides at 37°C for 24 h. *A*, WB of whole cell lysates with EphA2-specific D7 antibody confirms that transfection with antisense oligonucleotides decreases EphA2 protein levels. The membranes were stripped and reprobed with paxillin antibodies as a loading control. The relative mobility of molecular mass standards is shown on the *left*. *B*, MDA-MB-231 cell monolayers, treated with antisense oligonucleotides as detailed above, were suspended in soft agar for 7 days before microscopic analysis of colony formation. Note that colony formation by MDA-MB-231 cells was significantly impaired by EphA2 antisense oligonucleotides as compared with the IAS control ($P < 0.002$). Results are reported as colonies per high-powered field (*HPF*); *bars*, $\pm$SD.

The mechanism of EphA2 antibody action involves EphA2 stimulation. At first glance, this seems counter-intuitive, because activated receptor tyrosine kinases usually transmit signals that promote cell growth and invasiveness (31, 32). In contrast, the enzymatic activity of EphA2 does not require receptor autophosphorylation (23). Consistent with this, *in vitro* and *in vivo* phosphopeptide mapping studies confirm that a predicted "activation loop" tyrosine is not phosphorylated in EphA2.[5] Phosphorylation of such activation loop motifs liberates the enzymatic activity of other receptor tyrosine kinases, but this motif does not appear to regulate EphA2 (31, 32). Ongoing studies are seeking to determine the structural basis that allows for constitutive EphA2 enzymatic activity.

Rather than triggering enzymatic activity, biologically active antibodies induce autophosphorylation-mediated EphA2 degradation. Thus, antibody stimulation of EphA2 disposes of a powerful oncoprotein. Consistent with this, the interaction of EphA2 with its cognate ligand, ephrinA1, similarly induces EphA2 degradation.[6] A related mechanism has been proposed to explain the biological actions of Herceptin, which stimulates HER2 autophosphorylation and degradation (33). Whereas tyrosine phosphorylated EphA2 negatively regulates tumor cell growth, unphosphorylated EphA2 functions as a powerful oncoprotein (19). One possible explanation for these dramatic differences in behavior is that tyrosine phosphorylation of EphA2 could cause it to interact with different substrates. Consistent with this hypothesis, tyrosine phosphorylated EphA2 localizes to different cellular sites than unphosphorylated EphA2 (23).[5] Indeed, ongoing studies are seeking to contrast substrates that are phosphorylated by EphA2 before and after treatment of tumor cells with EA antibodies.

We cannot exclude that antibody stimulation of EphA2 also transmits signals that actively inhibit tumor cell growth. Indeed, ligand-mediated activation links EphA2 to downstream signals that decrease tumor cell attachment to underlying ECM (23, 25). Because ECM attachments transmit signals that promote cell growth and survival (34), decreased ECM attachments could also contribute to decreased tumor cell growth. Although consistent results with EphA2 antibodies and antisense strategies suggest that decreased EphA2 decreases tumor cell growth, future investigation could distinguish the relative contributions of EphA2 signaling *versus* EphA2 degradation to the biological consequences of EphA2 antibodies.

EphA2 provides an attractive antigen for monoclonal antibody targeting. High levels of EphA2 apply to many human cancers, including metastatic melanomas, breast, prostate, and colon carcinomas (18, 19, 21–23, 35–37). Furthermore, EphA2 stably binds its

[5] M. S. Kinch. Phosphopeptide mapping of the EphA2 kinase, manuscript in preparation.

[6] J. Walker-Daniels and M. S. Kinch. Activation-dependent degradation of the EphA2 kinase, manuscript in preparation.

ligands in nontransformed epithelial cells; unstable cell-cell adhesions, in particular those caused by the loss of E-cadherin function, prevent ligand binding in metastatic cells (23). Differential ligand binding could expose EphA2 epitopes on tumor cells that are sterically occluded by ligand in nontransformed cells. Even if the antibodies were to bind and stimulate EphA2 on nontransformed epithelial cells, they would likely transmit signals that are normally conveyed by endogenous ligands. We suggest the gross EphA2 overexpression on malignant cells exaggerates the biochemical and biological outcomes of antibody stimulation.

Our studies identify differences in the growth regulation of nontransformed and malignant epithelial cells that can be exploited using EphA2 antibodies. EphA2 antibodies selectively inhibit the anchorage-independent growth of metastatic cells, whereas the anchorage-dependent growth of nontransformed epithelial cells remains unchanged. The selective inhibition of transformed cell growth is even more dramatic, because the nontransformed MCF-10A cells that were used in our studies had levels of EphA2 that are higher than would likely be encountered *in vivo*.[7]

Another outcome of our study is that three-dimensional assays of tumor cell behavior (soft agar colonization and Matrigel behavior) provided the most sensitive ways to screen antitumor agents. Three-dimensional assay systems can model certain behaviors that are unique to tumor cells, which cannot be accurately conveyed by monolayer assay systems (4, 6). Had our studies relied on monolayer assays of tumor cell growth, we may have incorrectly concluded that antibody targeting of EphA2 has no effect on metastatic behavior. Similarly, the oncogenic potential of overexpressed EphA2 becomes most apparent when studied using soft agar and Matrigel (19). Thus, our results support the use of three-dimensional assays for the preclinical evaluation of oncogenes and antitumor agents.

Our demonstration that EphA2 antibodies inhibit tubular network formation is interesting in light of recent evidence that network formation can predict vasculogenic mimicry, a mechanism by which metastatic cells can undergo vascularization independent of endothelial cells (38, 39). Consistent with this, EphA2 is highly overexpressed in metastatic melanoma cells that are capable of vasculogenic mimicry, and antisense targeting of EphA2 prevents tubular network formation (20). An implication of our present study is that, in addition to their impact on tumor cell growth, EphA2 antibodies might have value for inhibiting tumor angiogenesis via inhibition of vasculogenic mimicry. Future investigation could determine whether EphA2 antibodies inhibit tumor neovascularization, either alone or in combination with approaches that target endothelial cells.

An important technical feature of our present study is the application of biolistics and RIMMS to generate antibodies against cell surface proteins. Gene gun-based immunizations increased the sensitivity of antigen recognition and helped generate antibodies with conformation-defined epitopes, because the antigen was synthesized, folded, and secreted *in vivo* (27). By minimizing immunodominance, the RIMMS strategy increased the diversity of responding B lymphocytes (40, 41). We have generated previously antibodies against EphA2 using protein antigens (17); however, the use of plasmid-based transfections can increase the efficiency of identifying epitopes that are exposed *in vivo*. In addition to their successful application to EphA2 here, it is likely that the biolistics/RIMMS combination can be applied to other antigens where conformation-defined or weakly immunogenic epitopes are desired.

These studies suggest that the EphA2 on tumor cells can be targeted using monoclonal antibodies to selectively inhibit tumor cells. On the basis of its overexpression, EphA2 might also serve as a target for small molecule inhibitors of enzymatic activity. However, strategies that ablate EphA2 enzymatic activity could impart undesired toxicities, because ligand-bound EphA2 negatively regulates malignant growth, and because EphA2 regulates other physiological processes, such as vision (42). Ultimately, improved strategies to target EphA2 should have significant therapeutic value by optimizing damage to the many tumor cells that overexpress EphA2 while minimizing injury to normal tissues. The information conveyed here might also help to identify and target other antigens that are overexpressed or functionally altered on tumor cells.

[7] M. S. Kinch, unpublished observations.

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