An Adenoviral Vaccine Encoding Full-Length Inactivated Human Her2 Exhibits Potent Immunogenity and Enhanced Therapeutic Efficacy without Oncogenicity

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Abstract

Purpose: Overexpression of the breast cancer oncogene HER2 correlates with poor survival. Current HER2-directed therapies confer limited clinical benefits and most patients experience progressive disease. Because refractory tumors remain strongly HER2+, vaccine approaches targeting HER2 have therapeutic potential, but wild type (wt) HER2 cannot safely be delivered in immunogenic viral vectors because it is a potent oncogene. We designed and tested several HER2 vaccines devoid of oncogenic activity to develop a safe vaccine for clinical use.

Experimental Design: We created recombinant adenoviral vectors expressing the extracellular domain of HER2 (Ad-HER2-ECD), ECD plus the transmembrane domain (Ad-HER2-ECD-TM), and full-length HER2 inactivated for kinase function (Ad-HER2-ki), and determined their immunogenicity and antitumor effect in wild type (WT) and HER2-tolerant mice. To assess their safety, we compared their effect on the cellular transcriptome, cell proliferation, anchorage-dependent growth, and transformation potential in vivo.

Results: Ad-HER2-ki was the most immunogenic vector in WT animals, retained immunogenicity in HER2-transgenic tolerant animals, and showed strong therapeutic efficacy in treatment models. Despite being highly expressed, HER2-ki protein was not phosphorylated and did not produce an oncogenic gene signature in primary human cells. Moreover, in contrast to HER2-wt, cells overexpressing HER2-ki were less proliferative, displayed less anchorage-independent growth, and were not transformed in vivo.

Conclusions: Vaccination with mutationally inactivated, nononcogenic Ad-HER2-ki results in robust polyclonal immune responses to HER2 in tolerant models, which translates into strong and effective antitumor responses in vivo. Ad-HER2-ki is thus a safe and promising vaccine for evaluation in clinical trials.

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as well as elicit strong polyclonal antibody and T-cell responses to multiple epitopes across the tumor-associated antigen of interest, yet possess a high safety profile. Of the many vaccine platforms utilized, recombinant adenoviruses show great promise of fulfilling these criteria in a cancer vaccine platform. Recombinant adenoviruses are widely used in clinical gene therapy and vaccine applications with an extensive safety profile and well-documented history of eliciting strong transgene-specific adaptive immune responses (6–9). Thus, to elicit maximal antitumor responses, we focused on using an adenoviral platform to target the well-validated breast cancer oncogene, HER2.

Although we wished to incorporate the HER2 gene into adenoviral vectors for therapeutic vaccination against HER2, the use of a wild-type HER2 (HER2-wt) oncogene raised serious safety concerns regarding its tumorigenicity (10, 11). HER2 is a well-documented member of the ErbB family of tyrosine-kinase receptors and functions in tandem with different binding partners to elicit multiple signaling pathways that enable tumorigenesis (12). HER2 is also known to bind and elicit signaling with other non-ErbB family members as well as to directly activate transcription by nuclear translocation and binding (13, 14). To potentially eliminate the oncogenic potential and maximize the immunologic potential encoded by full-length HER2, we constructed and tested the immunogenicity of several vec tors potentially ablated for HER2 kinase function. One vector contained a mutation in the HER2 ATP binding site (HER2-ki) whereas two others were truncated either before or after the transmembrane domain to completely eliminate the HER2 intracellular signaling domain. We hypothesized that these functionally inactivated oncogenes could be used in the adenoviral platform to elicit strong adaptive immune responses that would show efficacy against metastatic HER2+ mammary tumors in both naïve and tolerant preclinical models. We further hypothesized that strong overexpression of the most promising inactivated HER2 gene would be nononcogenic and thus safe for future clinical use.

Our results revealed that the recombinant adenoviral vectors expressing full-length HER2 inactivated for kinase function (Ad-HER2-ki) and expressing the extracellular domain of HER2 plus the transmembrane domain (Ad-HER2-ECD-TM) were highly effective in eliciting significant T-cell and antibody responses to HER2 in naïve mouse models compared with plasmid vaccination with HER2-ki constructs, thus validating the immunologic efficacy of the adenoviral platform. In contrast, we did not observe any induction of HER2-specific T-cell or antibody responses in HER2-ECD-vaccinated animals. Although the strong immune responses from both Ad-HER2-ki and Ad-HER2-ECD-TM vectors translated into significantly retarded tumor growth in naïve animals, our studies revealed that Ad-HER2-ki-vaccinated animals had the most significant HER2-specific T-cell responses as well as the most significant antitumor response. When animal models with tolerance to HER2+ were used, Ad-HER2-ki vaccination elicited only slightly diminished T-cell and antibody responses along with significant antitumor responses. Subsequent investigation into the oncogenicity associated with strong overexpression of HER2-ki revealed no evidence of its oncogenic functionality in terms of phosphorylation or transcriptional signature in primary human cells. We also found no evidence of its oncogenicity in enabling enhanced cellular proliferation, anchorage-independent growth, or transformation in vivo. Thus, our results strongly suggest that Ad-HER2-ki is an effective and safe vaccine that could show therapeutic efficacy in future clinical trials.

Materials and Methods

Cell lines. Tumor cell lines MCF-10a and 3T3 were obtained from the American Tissue Culture Collection (ATCC), and were maintained according to ATCC recommendations. The human-HER2 and control 4T1 cells were kindly provided by Dr. Michael Kershaw (Cancer Immunology Program, Peter MacCallum Cancer Centre, Victoria, Australia; ref. 15).

Adenoviral vector and cell line construction. The LTR-2/erbB2 plasmid was provided by Dr. L. E. Samelson (National Cancer Institute, Bethesda, MD) and HER2-ki (K753A) was created by quick-change mutagenesis. Adenoviral vectors were generated using standard cloning techniques as previously described (16, 17). Ad-Ras (H-Ras G12V) was kindly provided by Dr. Joseph Nevins, Duke University, Durham, NC. HER2+ cell lines were created through retroviral infection and/or plasmid transfection of 3T3 and MCF-10a cell lines and selection with hygromycin (500 ug/mL). Cell expression of HER2 was tested and confirmed in selected cells by Fluorescence-activated cell sorting (FACS) analysis using a
HER2-phycocerythrin-labeled antibody (BD Biosciences; Supplementary Table S3).

**Microarray and quantitative real-time-PCR assessments.**

Cellular RNA was extracted using TRI-Reagent (Molecular Reagents Center) and further purified by using a RNase kit (QIAGEN). RNA quality was assessed using an Agilent Lab-on-a-Chip 2100 Bioanalyzer (Agilent Technologies) and samples were processed for HG-U133+ v2.0 Affymetrix microarray hybridization according to standard protocols at the Duke Microarray Facility. Datasets were deposited at National Center for Biotechnology Information’s Gene Omnibus Express (GEO) in a MIAME-compliant form (along with complete details of all procedures) as accession number GSE13274. Datasets were analyzed using Genespring 7.2 and the Database for Annotation Visualization and Integrated Discovery (DAVID) using standard methods (18).

Real-time PCR was carried out with an ABI 7300 system using standard methods and intron spanning primers described in Supplementary Table S2. Expression differences were assessed using the comparative cycle threshold (Ct) method against GAPDH and β-actin control genes.

**Assessment of HER2-mediated phosphorylation, proliferation, and anchorage-independent growth in vitro.** Proliferation of stable cells was determined by MTT assay using 5,000 cells/well over the course of 3 d (against control counterparts) in 96-well plates. MTT growth assessments were done using a Bio-Rad plate reader after cell solidification in DMSO. Soft agar assays for stable and adenovirus-infected MCF-10A cells were done as described in O’Hayer and Counter, 2006, with adenovirus-infected MCF-10Aa cells incubated at a multiplicity of infection of 150 (19). Briefly, 50,000 cells/well were plated in 0.3% soft agar (on a base of 0.6% soft agar) and allowed to grow for a period of 2 wk in DMEM with 10% FCS (3T3 cells) or DMEM:F12 with 5% horse serum and mammary epithelial growth medium singlequots growth additives (Clonetics; MCF-10Aa cells). At the end of this time, colonies of >15 cells were counted and scored. Western blots of infected human mammary epithelial cells (HMEC) were done using standard procedures with HER2 phosphorylation assessed using a HER2-phospho specific antibody (20).

**Mouse experiments.** Experiments using BALB/c, NOD CB17-Prkdc SCID/J, and SCID-B6.129S7-Rag1 (tm1Mom) mice (obtained from Jackson Labs) were done in accordance with Duke Institutional Animal Care and Use Committee-approved protocols. Human HER2-transgenic mice (kindly provided by Dr. Wei-Zen Wei, Wayne State University, Detroit, MI; ref. 21) were crossed with BALB/c mice (Jackson Labs) to permit implantation of 4T1-HER2 tumors and genotyped by PCR as previously described (15). For HER2 measurement of 4T1-HER2 tumors, excised tumors were enzymatically digested as previously described (22, 23) and measured using a HER2-PE–labeled antibody (BD Biosciences). Stable 3T3 cells were injected s.c. into the flank of NOD-SCID mice (1 × 10⁶ or 1 × 10⁵ cells/animal) as previously described and measured after 28 d. Tumor measurements were made using calipers and volumes calculated using the formula \( V = \text{width} \times \text{width} \times (\text{length} / 2) \) whereas statistical differences were calculated using a mixed effects regression model using autoregressive covariance.

**Assessment of vector immunogenicity in vivo.** Immunogenicity experiments involved footpad injection of Ad-HER2-ki, Ad-HER2-ECD-TM, Ad-HER2-ECD, and Ad-LacZ vectors (2.6 × 10¹⁰ particles/mouse) in transgenic and naïve animals. Plasmid injection was done by injecting 100 μg of plasmid DNA in 50 μL of PBS i.m. as previously described. Fourteen days postinjection, mice were euthanized and splenocytes and sera were collected for analysis. IFN-γ ELISPOT assays (Mabtech Inc.) were done according to the manufacturer’s instructions using overlapping HER2 peptide mixes (2.6 μg/mL; BD Biosciences) as stimulating antigens and HIV-irrelevant overlapping peptide mixes as negative controls (BD Biosciences). Phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL) served as a positive control for splenocyte responsiveness. Antibodies were assessed by a flow cytometry adapted methodology reported by Piechocki et al. (24). Briefly, 3 × 10⁵ cells (mouse 4T1-HER2, HER2+; mouse 4T1, HER2−) were incubated with diluted (1:100, 1:400, 1:1,600) mouse serum for 1 h at 4°C, washed with 1% bovine serum albumin–PBS, stained with PE-conjugated antimouse IgG (Dako) for 30 min at 4°C, washed, and then samples were analyzed on a BD LSRII or BDFACSCalibur flow cytometer and results were represented as histograms or mean fluorescence intensity. Epitope mapping was done using spotted peptide arrays of 14 mer peptides overlapping by four amino acids representing the full length of the human HER2 protein or portions of the full-length human HER2 protein on cellulose membranes using a Spot Robot ASP 222 (Abimed) and done as described (25).

**Results**

Development of recombinant adenoviral vectors expressing inactivated forms of human HER2. To maximize adaptive immune responses against multiple epitopes of human HER2 while disabling its oncogenic potential, we developed three different adenoviral vectors, one encoding a full-length inactive form of human HER2 and two others encoding truncated forms of human HER2, either before or after the transmembrane region (and thus no intracellular signaling domain; Fig. 1A). The truncated forms of HER2 were produced by deleting the HER2 intracellular domain coding region after amino acid 634 for Ad-HER2-ECD-TM (nucleotide position 2084) and after amino acid 653 for Ad-HER2-ECD (nucleotide position 1959). To mutationally inactive human HER2, we mutated a key residue in the ATP binding region (K753A) to render the tyrosine kinase inactive (Fig. 1A; ref. 26). The K753A mutation had been previously shown to reduce HER2 phosphorylation, but its full impact on HER2 function and oncogenicity was undetermined at this time.

**Immunization with Ad-HER2-ki and Ad-HER2-ECD-TM but not Ad-HER2-ECD elicits strong functional T-cell and antibody responses in naive mice.** As previous studies had
reported widely differing efficiencies in the induction of HER2-specific immune responses, we first wanted to determine the strength of T-cell and antibody responses to the modified forms of HER2 using the adenoviral vaccine platform in comparison with the previously reported immunogenicity of HER2-ki plasmid-based vaccination (Fig. 1A; ref. 26). Naïve mice were vaccinated with a single dose of the vaccines and after two weeks, T-cell and antibody responses were assessed by ELISPOT assays and FACS assays (to show antibody binding to HER2+ human breast tumors), respectively. We found that Ad-HER2-ki and Ad-HER2-ECD-TM elicited highly significant responses compared with control vaccinations, but that Ad-HER2-ECD and plasmid-based HER2-ki vaccination elicited far weaker responses (Fig. 1B). ELISPOT assays showed that T-cell responses were strongest to epitopes in the extracellular domain of HER2 (ECD) and weakest to the intracellular and transmembrane domains (ICD and TM) in both Ad-HER2-ki and Ad-HER2-ECD-TM adenoviral vaccinations. As expected, T-cell responses to ICD domain
epitopes were only present in the Ad-HER2-ki–vaccinated animals and were not observed in animals vaccinated with Ad-HER2-ECD-TM, which was ICD truncated. Differences in T-cell responses between Ad-HER2-ki– and Ad-HER2-ECD-TM–vaccinated animals were mostly minor, but T-cell responses to the ECD and TM regions were slightly more robust in Ad-HER2-ki–vaccinated animals. In contrast, HER2-ki plasmid and Ad-HER2-ECD vaccination showed only weak responses to the ECD domain and ICD/TM were completely absent.

Our assessment of HER2-specific IgG responses mirrored our ELISPOT assessments, with both Ad-HER2-ki and Ad-HER2-ECD-TM vaccination eliciting highly significant levels of HER2-specific antibodies compared with control vector (Fig. 1C). Interestingly, plasmid-based vaccination with HER2-ki and adenoviral vaccination with HER2-ECD were both unable to elicit significant quantities of HER2-specific antibodies compared with control vaccinations.

Because antibody efficacy is a function of its targeted epitope, the antibody isotype, and the duration of the humoral response, we next investigated these aspects of our Ad-HER2-ki and Ad-HER2-ECD-TM vaccine-induced antibodies (VIA). Isotype-specific ELISA indicated the presence of multiple antibody isotypes from both vaccinations, with all IgG subtypes predominating, modest levels of IgM, and with low concentrations of IgA and IgE isotypes (data not shown). Epitope mapping revealed that Ad-HER2-ki elicited IgG antibodies that specifically bound multiple epitopes in the intracellular and extracellular regions of HER2 (Table 1), which Ad-LacZ did not elicit, thus showing a polyclonal HER2-specific adaptive response against full-length HER2. Lastly, we investigated the longevity of the polyclonal antibody response by immunizing wild-type C57BL/6 mice with Ad-HER2-ki and periodically assessing HER2-specific IgG antibody levels at different times postinjection by FACS (Fig. 1D). We found that peak antibody responses represent a titer of 1:25,000 based upon ELISA at day 78 (data not shown) and that although antibody responses gradually diminished, a single immunization resulted in considerable (roughly ~50%) HER2-specific antibody levels seven months after injection. Thus, vaccination using Ad-HER2-ki is sufficient to elicit robust levels of polyclonal antibodies that are part of a long-lived response that is significant at seven months postinjection.

In sum, the Ad-HER2-ki and Ad-HER2-ECD-TM vectors elicited significantly greater T-cell and antibody responses in comparison with Ad-HER2-ECD and HER2-ki plasmid-based vaccination.

**Ad-HER2-ki and Ad-HER2-ECD-TM vaccines are effective against HER2+ tumors in vivo.** Although both Ad-HER2-ki and Ad-HER2-ECD-TM were able to efficiently induce polyclonal T-cell and humoral immunity, it was unknown if these responses could effectively inhibit aggressive HER2+ tumor growth *in vivo*. To assess the antitumor effect of these vectors, an aggressive herceptin-resistant (ref. 15; Supplementary Fig. S1) HER2+ metastatic mouse mammary carcinoma line (4T1-HER2) was implanted in mice, which were subsequently vaccinated with either Ad-HER2-ki, Ad-HER2-ECD-TM, or with a control vector (Ad-LacZ; Fig. 2A). This approach effectively mimicked conditions seen in patients, as 4T1-HER2 cells were not solely dependent on HER2 for growth, but grew aggressively and could initiate metastases.

Furthermore, once the most promising vector was determined in the naïve model, that vector could be tested using the same approach in the more stringent HER2 transgenic mouse model that mimics the human HER2 tolerance seen in patients (Fig. 2A).

Using the naïve model, we found that mice receiving a single vaccination with either Ad-HER2-ki or Ad-HER2-ECD-TM showed significant retardation in tumor growth compared with the control Ad-LacZ (Fig. 2B). Comparison of the two vectors revealed that Ad-HER2-ki gave a significant protective advantage in blunting tumor growth compared with Ad-HER2-ECD-TM and a more significant advantage when compared with Ad-LacZ control animals. Assessment of T-cell responses and antibody responses in vaccinated animals revealed that although HER2-specific IgG responses were not significantly different T-cell responses showed significant differences (Fig. 2C and D).

In all tumor-bearing animals, HER2 peptide stimulation of splenocytes produced significant responses compared with unstimulated controls, indicating that aggressive HER2+ tumor growth had elicited a baseline level of T-cell activation in naïve animals against HER2 epitopes, which had been unable to curtail tumor growth. The domain-specificity of activated T-cell epitopes mirrored that of vaccinated non–tumor-bearing animals (Figs. 1B and 2C), in that ECD responses were dominant in all groups. Significantly, though, we observed that the dominant ECD-specific responses of Ad-HER2-ki and Ad-HER2-ECD-TM were significantly greater than control-vaccinated animals and directly correlated with the strength of the antitumor effect observed in vaccinated animals.

### Table 1. HER2-VIA antibody epitopes

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<tr>
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<td>ECD</td>
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<td>405-420</td>
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<td>1195-1211</td>
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<td>1229-1245</td>
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</table>
Although Ad-HER2-ki– and Ad-HER2-ECD-TM–vaccinated animals had significantly repressed tumor growth, tumors in these animals were not fully eliminated. We hypothesized that if HER2-specific responses were attributable for the diminished growth, then the 4T1 tumor outgrowths in these animals would have a reduced level of HER2 expression as a result of immunoselection induced by vaccination.

We further hypothesized that if the HER2-specific cytotoxic T-cells were the major selective pressure on tumors, then the level of HER2 expression in the remaining tumor cells would inversely correlate with the strength of anti-HER2 T-cell responses we observed in vaccinated animals. Tumors from vaccinated animals were excised and dissociated, and HER2 expression determined quantitatively by FACS.
Supporting the hypothesis, we found that HER2 expression was significantly diminished in both Ad-HER2-ECD-TM– and Ad-HER2-ki–vaccinated animals (compared with Ad-LacZ control), being lowest in Ad-HER2-ki–vaccinated animals (about 5% displaying expression comparable with control-vaccinated animals; Fig. 2D). Thus, HER2 expression was indeed found to be inversely associated with HER2-specific T-cell responses, strongly suggesting that HER2-specific T-cells were predominantly responsible for the efficacy of Ad-HER2-ki and Ad-HER2-ECD-TM vaccinations.

**Ad-HER2-ki vaccine effectiveness against HER2+ tumors in a tolerant in vivo model.** As tolerance to self-antigen is a major impediment to the successful clinical use of vaccines, we sought to determine if our most promising vaccine (Ad-HER2-ki) could be as effective in its immune induction and antitumor effects in a mouse with tolerance to human HER2+ (21). To first determine if immune responses elicited by Ad-HER2-ki vaccination were comparable in a tolerant setting, we vaccinated naïve and human HER2 transgenic animals with Ad-HER2-ki and assessed T-cell and antibody induction using ELISPOT and HER2-specific FACS. Our ELISPOT results revealed that HER2-transgenic animals produced nearly equivalent HER2-specific T-cell responses compared with naïve controls (Fig. 3A).

Similarly, FACS analysis of mouse serum for HER2-specific antibodies showed similar levels of specific binding to HER2+ cells (but not parental HER2- cells) between vaccinated HER2 transgenic mice, non-HER2 transgenic littermates, and wild-type C57BL/6 mice (Fig. 3B). As expected, serum from Ad-LacZ–vaccinated mice did not significantly bind to HER2+ cells, showing that antibody responses were HER2 specific in Ad-HER2-ki–vaccinated mice.

As immune responses in naïve and tolerant mice were nearly equivalent, we next wished to determine if the therapeutic effect elicited by the induction of HER2 immunity after Ad-HER2-ki vaccination was equivalent in tolerant animals. To determine therapeutic effect in tolerant animals, transgenic animals with tolerance to HER2+ were injected with the same dose of 4T1-HER2+ cells and vaccination with Ad-HER2-ki was given at 4 days postimplantation as previously done in naive counterparts (Fig. 3C). We found that as with immune responses, Ad-HER2-ki vaccination was again able to significantly blunt the growth of 4T1-HER2+ tumors with similar kinetics in tolerant animals. It should be noted, however, that it required repeated dosing to achieve these effects in tolerant mice, whereas a single vaccination was sufficient in wild-type (WT) mice (Fig. 2B). Indeed, tumors from Ad-HER2-ki–vaccinated HER2+ transgenic mice outgrew slightly quicker after the initial tumor regression compared with naïve Ad-HER2-ki vaccinated mice (Figs. 2B and 3C), despite three vaccinations. Thus, our results indicate that Ad-HER2-ki is a potent antitumor vaccine against aggressive non–HER2-dependent tumor growth in a tolerant preclinical model.

**Oncogenic signaling deficiency after overexpression of kinase inactivated HER2.** As our preclinical studies of immunogenicity and therapeutic effectiveness had revealed Ad-HER2-ki to be the most promising candidate for a human HER2 vaccine, we next wanted to determine if HER2-ki was truly nononcogenic. As strong overexpression of HER2 is known to be an early causative event in breast cancer, it was of the utmost importance to understand if
the single amino acid mutation could fully block phosphorylation and oncogenic signaling in the context of strong adenoviral overexpression.

To initially determine if there were differences between HER2-ki (kinase inactive) and HER2-wt (wild-type) expression and phosphorylation, we infected primary HMECs with Ad-HER2-ki or Ad-HER2-wt. Although infection with both vectors resulted in similar levels of high surface expression of HER2 as shown by FACS analysis and Western blot analysis (Fig. 4A, Supplementary Fig. S2), we could detect no evidence of HER2 phosphorylation in Ad-HER2-ki–infected HMECs, in contrast to Ad-HER2-wt–infected HMECs (Fig. 4B, Supplementary Fig. S2). We next examined the signaling consequences of this phosphorylation defect through a comparison of transcriptome profiles from Ad-HER2-wt–, Ad-HER2-ki–, and Ad-GFP control–infected HMECs. Using one-way ANOVA (P < 0.05 with Benjamini-Hochberg false discovery rate multiple testing correction) additionally filtered for genes with a >3-fold difference, we found that 423 genes were statistically different between the Ad-HER2-wt and Ad-GFP, whereas only 21 were different between Ad-HER2-ki and Ad-GFP (Fig. 4C). Subsequent investigation of nine representative gene targets by quantitative real-time PCR (Supplementary Table S1 and S2) revealed a high degree of array concordance and confirmed our transcriptome findings. A functional analysis of HER2-wt revealed significant overrepresentation and clustering into seven Gene Ontology (GO) groups: systems development, epidermal growth factor (EGF)-like, cytokine activity, chemotaxis, chemokine activity/GPCR activity, proliferation, and angiogenesis (Fig. 4D). These findings suggest that much of the oncogenic potential attributed to HER2 kinase activity could be mediated through inflammatory and chemotaxis pathways (an area currently under investigation in our lab), as well as through more established angiogenic and EGF typical pathways. In sum, immunoblot and transcriptome assessments indicate the vast majority of cellular pathway activation mediated by HER2-wt are critically dependent upon phosphorylation and kinase functionality, strongly suggesting a critical oncogenic signaling deficit in HER2-ki. Diminished proliferation and anchorage-independent growth of kinase-inactive HER2. Although immunoblots and transcriptome analysis revealed strong HER2-ki signaling deficiencies, it was unknown if these would diminish oncogenic function. To assess HER2 oncogenic function in vitro,
we constructed several stable HER2-wt and HER2-ki 3T3 fibroblasts lines that had different levels of HER2 expression (retrovirally selected lines expressed high levels of HER2 and are denoted as "high expression" whereas plasmid transfection selected lines expressed lower levels of HER2 and are denoted as "low expression"; Supplementary Table S3) and tested their proliferative and transformative capacity in vitro. We found that HER2-wt significantly augmented 3T3 proliferation in an expression-sensitive manner (Fig. 5A). In contrast, we found that strong HER2-ki expression significantly reduced cell proliferation, whereas weak HER2-ki expression had no effect on proliferation (Fig. 5A). Likewise, high-expression stable HER-wt 3T3 formed colonies in soft agar, whereas high-expression stable HER2-ki 3T3 cells formed colonies at a significantly reduced basal rate compared with control cells (Fig. 5A). Thus, HER2-wt enhanced cell proliferation and transformation in vitro, whereas HER2-ki inhibited proliferation and transformation in vitro. Although statistically significant, these colony formation differences were modest and only apparent in highly expressed lines. Thus, to elicit a more sensitive measure of in vitro transformation, EGF (an ErbB family growth factor) was used to enhance HER2 soft agar colony formation (27). Although EGF addition enhanced colony formation of all cell types (Fig. 5A and B), only HER2-wt cell displayed significantly enhanced anchorage-independent growth over control cells (Fig. 5A and B). Thus, even in the presence of EGF, HER2-ki displays
a significant transformation deficiency compared with HER2-wt.

Having determined a proliferative and transformative defect in HER2-ki expressing mouse fibroblasts, we extended our investigation to a more relevant human cell type using engineered immortalized (but nontransformed) human breast epithelial cells (MCF-10a) to express either HER2-wt or HER2-ki. As before, we found that HER2-wt cells grew at a significantly enhanced rate whereas HER2-ki cells displayed a significant repression of proliferation compared with control cells (Fig. 5C). Additionally, we determined that HER2-wt cells formed significantly more colonies in soft agar compared with HER2-ki cells and control cells (Fig. 5C).

Finally, we wished to explore if adenovirus-mediated delivery and oncogene overexpression impacted human cell transformation in vitro. Although unlikely, it remained possible that in the context of an inflammatory response and heavy overexpression of HER2-ki, the minor dysregulation observed in our microarray could impact transformative capacity. We infected MCF-10a cells with HER2-wt, HER2-ki, and control GFP adenoviral vectors, and assessed anchorage-independent growth in soft agar. Although adenovirus-mediated overexpression of HER2-wt elicited anchorage-independent growth in those cells, Ad-HER2-ki–infected cells were unable to form more colonies than Ad-GFP–infected cells or uninfected controls (Fig. 5D). Thus, adenovirus-mediated delivery did not significantly alter transformative capacity of HER2-ki.

Collectively these in vitro data show that the signaling defects conferred by the mutation of HER2-ki ablate the enhanced proliferation and anchorage-independent growth elicited by the HER2 oncogene. These defects were observed across different expression contexts and different cell types and thus strongly suggest that HER2-ki is a functionally ablated oncogene.

**Lack of tumorigenicity in immunodeficient xenografts.** Because HER2-ki had shown significant signaling and functional defects in vitro, we hypothesized that it would have a reduced oncogenic transformation potential in vivo. To assess this, stable HER2-ki, HER-wt, and control 3T3 cells were implanted in immunodeficient mice and tumor formation was assessed. Data from five independent experiments are summarized in Table 2, which shows that although all mice injected with 3T3 cells expressing the wild-type human HER2 gene (HER2-wt) developed tumors, mice injected with 3T3 cells expressing the kinase inactive gene (HER2-ki) did not develop tumors. Even at the highest concentrations of injected cells, the basal rate of spontaneous 3T3 transformation in control 3T3 cells proved higher than HER2-ki–expressing cells. Thus, the slightly greater transformation potential observed in control 3T3 cells suggests that HER2-ki expression has a negative effect on 3T3 transformation in vivo. These findings show that HER2 kinase function and downstream signaling are critical to its transformative capacity in vivo and further show that the transformative capacity of HER2-ki is ablated and its expression is safe in vivo.

**Discussion**

More than 20 years after its discovery, therapeutic strategies to target the human oncogene HER2 are achieving measures of efficacy in the clinic, through use of the HER2-specific monoclonal antibody trastuzumab and the HER2/EGF receptor selective kinase inhibitor lapatinib. However, many tumors have de novo resistance to these therapies and the majority of tumors that initially respond to them eventually become refractory to treatment. In spite of this, these tumors retain HER2 expression, thus permitting the use of alternative anti-HER2 approaches as potential adjunct therapies. Along these lines, several HER2 vaccines have been developed that utilize protein or peptide fragments of either the intracellular and/or extracellular domain. In our study, we compare the use of several possible inactivated HER2 vaccines and report the strong efficacy of an adenoviral vector that encodes a kinase-inactive full-length HER2 gene in an attempt to maximize immunotherapeutic anti-HER2 efficacy and concomitantly minimize HER2 oncogenic potential.

Although the oncogenic potential of HER2 is well documented, there have been few in-depth studies investigating the oncogenic effect of kinase-inactive HER2. HER2 is well known to signal in cooperation with the ErbB family of tyrosine kinase receptors, but has also been documented to translocate to the nucleus to activate genes such as Cox-2 (13). Other reports have also documented HER2 to be involved with other signaling receptor families (28), thus broadening the potential mechanisms whereby HER2 promotes oncogenesis. Our study shows that overexpression of a kinase inactive HER2 (HER2-ki) results in a complete block of HER2 phosphorylation and has a dramatic impact on cellular signaling as measured by transcriptome dysregulation, in spite of high levels of

<table>
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<th>3T3 clone</th>
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<th>Tumor formation at 28 d (1 x 10^5 cells)</th>
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<tr>
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<td>10/10 mice</td>
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</tr>
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<td>3T3-empty vector</td>
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<td>0/30 mice</td>
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</table>
HER2 expression. We also found that HER2-ki does not confer any proliferative or transformative potential to cells, in the context of different expression capacities, different cell lines, as well as in in vitro and in vivo settings. Although some genes were dysregulated by expression of HER2-ki and reversion mutations could potentially occur with the single HER2-ki mutation, our investigation strongly suggests that HER2-ki is a functionally inactivated oncogene and thus safe for use in humans.

Beyond safety, our study also shows that Ad-HER2-ki is able to induce strong adaptive immune responses against HER2 that translate into effective antitumor immunity in vivo. Although previous studies have used a variety of different vaccination strategies that target ErbB2/HER2, none have targeted the human full-length HER2 using an adenoviral vector and extended these findings to a HER2-tolerant model. Indeed, studies that have used similar vaccine strategies to target ErbB2/HER2 have all utilized the rat neu gene (HER2 homolog) in mice, which is recognized as a foreign transgene (neu) and thus more immunogenic than a self-antigen (29–32). A number of studies using the rat neu have also looked at prevention of spontaneous tumors in various rat neu-transgenic mouse models (32–36). Although the data show that these vaccine strategies can be effective in reducing or preventing the development of mammary tumors, the physiologic relevance of these tumor models has been questioned (37).

Additionally, the neuT model has been shown to be more responsive to DNA vaccination than the human HER2-transgenic model used in the present study, likely because the spontaneous neu tumors seem to be completely dependent upon neu for growth and are more sensitive to anti-neu antibodies (38). For these reasons, we chose to use the more rigorous tolerance model provided by the human HER2-transgenic mice using 4T1-HER2+ cells, an aggressive and metastatic mouse tumor that is not completely dependent on HER2 for growth. Vaccination with Ad-HER2-ki in this model elicited the generation of strong T-cell and antibody responses comparable with those elicited in naïve mice. Ad-HER2-ki vaccination was able to strongly limit HER2+ cancer growth and elicited strong selection against HER2 expression in residual tumor mass. Because 4T1 cells are not dependent on HER2, it is possible that HER2-dependent models would offer even stronger evidence of Ad-HER2-ki efficacy. Our results indicate that the induction of HER2-specific T-cells highly correlates with antitumor efficacy and anti-HER2 tumor selection in multiple vaccines, strongly suggesting that vaccine efficacy is dependent upon the strong induction of HER2-specific T-cells. Of relevance for future clinical studies, we were also able to show Ad-HER2-ki antitumor effects against an aggressive HER2+ breast cancer in a human HER2-tolerant mouse model that most closely resembles the state of immunologic tolerance to human HER2 that exists in cancer patients.

In sum, our study shows the importance of HER2 kinase function in oncogenesis as well as the safety and efficacy of Ad-HER2-ki and Ad-HER2-ECD-TM for therapeutic vaccination against HER2+ breast cancer. Based on these findings, we are actively pursuing the use of Ad-HER2-ki in clinical studies to determine the safety and efficacy of this approach in patients with HER2+ breast cancer. Although adenoviral vaccines in humans with pre-existing anti-Ad5 immunity have lacked efficacy, we recently described a modified Ad5 platform that overcomes this barrier (39). If successful, our strategy of targeting active oncogenes with inactive oncogene vaccines could become a significant therapeutic option in the treatment of different oncogene-dependent cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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