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A DNA vaccine against ERBB2 impairs chemical carcinogenesis in random-bred hamsters.

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ABSTRACT

Vaccines against oncoantigens halt early neoplastic lesions in several cancer-prone, genetically engineered mouse models, whereas their ability to prevent chemical carcinogenesis has not been explored. This is a significant issue since exposure to chemical mutagens is responsible for a substantial percentage of cancers worldwide. Here we show that the archetypal oncoantigen ERBB2 is transiently overexpressed in Syrian hamsters during the early stages of 7,12-dimethylbenz[α]anthracene (DMBA)-induced oral carcinogenesis. Repeated DNA vaccinations against ERBB2 significantly reduce the number, size and severity of oral lesions in a manner directly proportional to the anti-ERBB2 antibody response. These results support the prospects of vaccines as a fresh strategy in the management of individuals at risk for exposure to defined carcinogenic agents.
INTRODUCTION

Immunoprevention of tumors associated with microbial infections is expected to decrease the human tumor burden in the near future (1, 2). Vaccines against tumor antigens with a causal role in the promotion of carcinogenesis unrelated to infections (oncoantigens) (3) halt early neoplastic progression in several cancer-prone, genetically engineered mice due to their ability to target molecules with an essential role in tumor progression. They have a limited therapeutic potential, but are very effective in inhibiting the early stages of cancer. Whereas the potential of vaccines against oncoantigens to prevent tumors is being extensively studied in transgenic mouse models (4-7), their ability to interfere with the progression of chemical carcinogenesis is still mostly unexplored. Chemical pollutants and especially polycyclic aromatic hydrocarbons are widespread environmental contaminants responsible for many animal and human cancers (8).

The membrane tyrosine kinase receptor ERBB2 is an archetypal oncoantigen overexpressed by several carcinomas generally characterized by a more aggressive course in both humans and animals, whereas its expression is low or absent in normal adult tissues (9-12). Anti-ERBB2 vaccination protects against and cures mice with transplantable ERBB2+ tumors (13), and inhibits one of the most aggressive, metastasizing and lethal ERBB2-driven mammary carcinogenesis in transgenic mice (14) for the whole of their natural lifespan (15). In cancer patients, anti-ERBB2 vaccines elicit both antibody and cell-mediated response to ERBB2, while monoclonal (mAb) anti-ERBB2 antibodies display a significant therapeutic activity (16).

Spurred by Sun et al.’s observation of the expression of ERBB2 during oral carcinogenesis induced by a non-heterocyclic polycyclic aromatic hydrocarbon (7,12-dimethylbenz[a]anthracene, DMBA) in hamsters (17), we first evaluated the intensity and persistence of ERBB2 expression in a large group of random-bred hamsters during the
DMBA-induction of oral squamous cell carcinomas. This is one of the best characterized animal models of chemical carcinogenesis and closely recapitulates several features of the development of the corresponding human cancer (17-20). While ERBB2 is expressed only transiently during the early stages of DMBA carcinogenesis, we found that repeated boosts with a DNA vaccine anti-ERBB2 resulted in significant reduction of the number, size and severity of oral lesions in function of the anti-ERBB2 antibody titer. This finding may open a fresh scenario in the management of individuals exposed to a defined carcinogenic agent.

MATERIALS AND METHODS

Hamsters
One hundred and six, six-week-old male, random-bred Syrian golden hamsters (*Mesocricetus auratus*, Charles River Laboratories, Calco, Italy), of about 100 g were maintained in specific pathogen-free conditions with a 12 h light–dark cycle, and with rodent chow and tap water *ad libitum*. Their right cheek pouch was painted three times a week with a 0.5% solution of DMBA dissolved in mineral oil (Sigma-Aldrich, Milan, Italy) applied with No. 4 paintbrush. The amount of carcinogen and treatment delivered to each animal was rendered quite uniform by using the “wiped-brush” method (19, 20). The unilateral formation of tumors allowed the animals to eat and swallow normally.

Experimental plan
In the first experiment, thirty-six hamsters were treated with DMBA for twelve weeks (Fig. 1A). At the end of every week, three hamsters were randomly sacrificed. Pouches and major organs were inspected and processed for histopathological analysis.
In the next experiment, seventy hamsters were first electroporated in both tibial muscles with 50 µg of EC-TM plasmids coding the extracellular and transmembrane domains of rat ERBB2 receptor, or with the empty pcDNA3 plasmids (21) twenty-one and seven days before starting DMBA carcinogenesis induction (day 0), and then every three weeks (Fig. 2A). Every week hamsters were anesthetized and the pouches everted. The first onset of visible lesions, and their number/single cheek pouch (multiplicity) was evaluated. Each lesion was measured in the two perpendicular diameters, classified and photographed. The type, multiplicity and size of the lesions at the end of DMBA-induction period were used to elaborate an overall pathological score (PS) for each hamster (22). PS 0 for no lesions; 1 for each preneoplastic lesion; 2 for an exophytic lesion less than 1 mm in diameter; 3 to 8 for each exophytic lesion larger than 1 mm proportionally to the increase of their mean diameter. At the end of twelve weeks’ carcinogenesis induction blood was collected, hamsters were sacrificed and their pouches and major organs were processed for histopathological and molecular analyses. All experimental procedures were approved by the Institutional and National Animal Care Committee.

**Histology and immunohistochemistry**

Specimens were fixed in methyl-Carnoy’s fluid or 10% neutralized formaldehyde solution and embedded in paraffin, or fixed in pyridoxal phosphate and embedded in OCT. Four µm thick sections were stained with hematoxylin-eosin, or processed for immunohistochemistry. Several commercial anti-ERBB2 mAb were assayed for their ability to recognize Syrian hamster ERBB2 receptor on cheek pouches with and without preneoplastic and neoplastic lesions (c-erbB-2/Her2/neu Ab-17 and c-erbB-2/Her2/neu, LabVision, Fremont, CA; c-erbB-2 Oncoprotein and HercepTest™, Dako Cytomation, Milan, Italy; Her 2/ErbB2, Cell Signaling,
Positive controls were microarray slides of human ERBB2+ mammary carcinomas and the appropriate isotype antibody for each primary antibody used. Since the best results on formalin fixed-paraffin embedded samples were obtained with the anti-ERBB2 mAb c-erbB-2 Oncoprotein, this mAb was used for all the studies reported here. For p63 staining, the anti-human p63 protein clone 4A4 (Dako) was used at 1:50 dilution.

**Western Blot analysis**

Samples from normal epithelium, preneoplastic lesions and exophytic lesions were homogenized in RIPA buffer (Cell Signaling Technology, Pero, Italy). Forty μg of total proteins were separated on an 8% SDS–polyacrylamide gel, and electrotransferred to a Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Western blot analysis was performed with the mouse Ab-3 mAb (Oncogene Research Products, Cambridge, MA, USA). The membrane was then exposed to the appropriate horseradish peroxidase-conjugate secondary antibody (1:5000) (Sigma) for 1 h at room temperature, and labeled bands were detected with a commercial chemiluminescence kit (Immun-Start, Bio-Rad Hercules, CA, USA). Images of the immunoreactive bands were acquired with a Kodak Image Station 440CF (Kodak, Rochester, NY, USA).

**Plasmid electroporation**

pcDNA3 vectors coding the extracellular and transmembrane domains of rat HER-2 receptor (EC-TM) and the empty control plasmid (pcDNA3) were produced and used as we have previously described in detail (21). Briefly, anesthetized hamsters were electroporated by injecting 50 μg of plasmids in 40 μl of 0.9% NaCl supplemented with 6 mg/ml polyglutamate
into both tibial muscles. Electric pulses were applied by two electrodes placed on the shaved skin around the injection area covered with a conducting gel. Two square-wave 25 ms, 375 V/cm pulses were generated by a T820 electroporator (BTX, San Diego, CA, USA). Hamsters were electroporated twenty-one and seven days before starting DMBA carcinogenesis induction (day 0), and then every three weeks.

**Antibody response**

The titer of anti-ERBB2 antibodies was assessed by flow cytometry with BALB/c NIH-3T3 fibroblasts stably co-transfected with the wild-type rat ERBB2, mouse class I H-2K\(^d\) and B7.1 genes (BALB/c NIH 3T3-NKB cells) (23). FITC-conjugated goat antibodies specific for hamster IgG heavy and light chains (ImmunoKontact, Abingdon, Oxon, UK) were used to detect bound primary antibody. Normal hamster serum was used as a negative control, and the Ab-4 mAb (Oncogene Research Products, Cambridge, MA, USA) was the positive control. Cells were resuspended in PBS-azide-BSA containing 1 mg/ml of propidium iodide to gate out dead cells, and evaluated with a CyAn ADP (Dako Cytomation, Heverlee, Belgium) and the Summit 4.3 (Dako Cytomation) software. The specific 3T3-NKB binding potential of the sera was calculated as follows: \([\% \text{ positive cells with test serum}] \times \text{fluorescence mean}] \times \text{serum dilution (23). A total of } 10^4 \text{ viable cells were analyzed in each evaluation.}"

**Statistical analysis**

Differences in antibody titer, multiplicity and diameters of lesions were analyzed with one-way analysis of variance. Those in the % of lesion-free hamsters were evaluated with the Log-Rank (Mantel-Cox) test and Fisher’s exact test. Differences in the number of fast and intermediate progressors were evaluated with Fisher’s exact test, whereas distribution within
groups was assessed with Pearson’s chi-square test. Differences in the number of exophytic lesions classed in different stage groups were compared with the non-parametric Mann–Whitney Rank Sum test. All analyses were performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) with $P < 0.05$ as the significance cut-off.

RESULTS

ERBB2 expression throughout the DMBA-induced carcinogenesis

The right cheek pouch of random-bred Syrian hamsters was painted three times weekly for twelve consecutive weeks with a 0.5% solution of DMBA in mineral oil (Fig. 1A). Three randomly chosen hamsters were sacrificed at the end of every week to follow the lesion development. Direct inspection of the cheek pouch surface (Fig. 1B) disclosed early enlargement of blood vessels in the painted area, followed at week 4 by inflammation associated with a rough surface. Around week 7, preneoplastic lesions became evident, sometimes as corrugated or verrucous leukoplakias. Hereafter exophytic lesions appeared and grew. Histological analysis (Fig. 1C) revealed that, within four weeks of DMBA induction, a moderate inflammatory reaction was developed as well as the hyperkeratotic feature of the epithelium. At week 7, foci of intraepithelial dysplasia became evident, while the epithelium displayed hyperkeratotic and acanthotic features. Subsequently dysplastic lesions grew into intraepithelial carcinoma. The resulting exophytic lesions consisted of finger-like papillae covered by a multilayered epithelium with features of moderately differentiated, in situ keratinized squamous cell carcinoma, sometime progressing to invasive squamous cell carcinoma. ERBB2 expression was evaluated throughout the induction (Fig. 1D and E): it was absent or scarcely present in the basal layer of normal and inflamed epithelium, but became markedly enhanced in the hyperkeratotic and acanthotic epithelia of the leukoplakia patches,
then almost disappeared in intraepithelial and invasive carcinomas. Preliminary data in few specimens from different patients (3 normal mucosae, 6 dysplastic lesions, and 5 carcinomas) suggest that a similar pattern of transient ERBB2 expression is also a feature of human oral preneoplastic/dysplastic lesions and squamous cell carcinoma (Supplementary Fig. S1).

**DMBA-induced carcinogenesis is hampered by anti-ERBB2 DNA vaccine**

To determine whether induction of the immune response against ERBB2 impairs a chemically-driven carcinogenesis, forty hamsters were electroporated every three weeks with EC-TM plasmids (21, 24). Thirty control hamsters were electroporated with the empty pcDNA3 plasmids. In both groups, DMBA induction of cheek pouch carcinogenesis began one week after the second electroporation (Fig. 2A). The kinetics of the occurrence of preneoplastic lesions was delayed in hamsters electroporated with EC-TM (Fig. 2B) and their multiplicity was reduced (Fig. 2C). Ten weeks after the beginning of DMBA induction, preneoplastic lesions were present in 60% (18/30) of control hamsters, but in only 32.5% (13/40) ($p = 0.029$) of those electroporated with EC-TM plasmids. In these hamsters, lesion multiplicity was less than half (0.62 ± 0.15) of that of the controls (1.21 ± 0.23) ($P = 0.029$). The assessment of preneoplastic lesions was ended at week 10 since most of them then progressed to exophytic lesions. At week 11, in fact, all 30 control hamsters displayed one or more exophytic lesions (Fig. 2D). By contrast, 6 of the 40 hamsters electroporated with EC-TM (15%) ($P = 0.034$) remained free from exophytic lesions until week 12, when the experimental observation was ended. At this time, both the multiplicity (Fig. 2E) and the size (Fig. 2F) of the exophytic lesions were lower in EC-TM electroporated hamsters than in the controls.
Pathological stage of the exophytic lesions.

Since in human head and neck squamous cell carcinomas, overexpression of p63, a member of the p53 family, is widely associated with poor prognosis (25, 26), we assessed p63 expression in DMBA-induced lesions. Post-mortem pathological analysis showed that exophytic lesions of control hamsters displayed a diffuse p63 expression, while in the smaller lesions of EC-TM electroporated hamsters it was confined to basal and parabasal cell layers (Fig. 2G and H).

The presence of smaller exophytic lesions is of special interest since size is directly associated with a more advanced stage. Most of the smaller lesions were lined by dysplastic epithelium with areas of severe dysplasia, whereas the larger lesions were invasive, advanced squamous cell carcinomas. A pathological survey performed at the end of the experiment on 35 randomly chosen EC-TM and control hamsters displaying exophytic lesions showed that both dysplastic lesions and carcinomas were less advanced in those electroporated with EC-TM (Supplementary Table S1).

EC-TM immunized hamsters with the highest anti-ERBB2 antibody titer display hampered DMBA-carcinogenesis.

At the end of the induction the PS was assessed for each hamster by evaluating the stage of its cheek lesions. It was significantly lower in EC-TM immunized hamsters than in the controls ($P < 0.001$). The analysis was deepened by setting two arbitrary thresholds at PS 2.5 and 5.0 to classify hamsters as fast ($PS > 5$), intermediate ($2.5 < PS < 5$), and slow progressors ($PS < 2.5$) (Fig. 3A). The controls were mainly fast progressors (73%) and the other 27% were intermediate. No animal had a PS less than 2.5 at the sacrifice. Of the EC-TM electroporated hamsters, 27% were fast progressors, 25% intermediate progressors and 48% slow
progressors with a very indolent progression (Fig. 3B). Anti-ERBB2 antibody titer was nearly undetectable in the controls, whereas in the EC-TM group it was inversely correlated \((P < 0.05)\) to the PS value (Fig. 3C). Moreover, the time of appearance of exophytic lesions, the number of preneoplastic and exophytic lesions, and their diameters were markedly different between fast and slow progressors in the EC-TM hamsters (Supplementary Fig. S2).

**DISCUSSION**

These findings show that vaccination against an oncoantigen significantly impairs the progression of chemically induced oral carcinogenesis. The incidence, number and size of preneoplastic and exophytic lesions are reduced and their pathological stage is less advanced in hamsters immunized against ERBB2. Moreover, at the end of the observation period, all control hamsters displayed exophytic lesions and invasive cancer whereas six (15\%) of the ERBB2 immunized hamsters were still free from lesions. This protection is proportional to the intensity of anti-ERBB2 antibody response induced by the vaccine even if the titers in the slow progressors ranged from very low titers to very high. The presence of slow progressors with low anti-ERBB2 antibody titers can probably be ascribed to the induction of a stronger cytotoxic response against ERBB2\(^+\) target cells. Since our population was random-bred, vaccination was expected to elicit markedly different levels of cytotoxicity. The collaboration of antibody and cell-mediated cytotoxic responses in protection against ERBB2\(^+\) tumors has been clearly documented (27). We have previously shown that electroporation of the EC-TM plasmid in hamsters elicits a protective anti-ERBB2 antibody and cell-mediated cytotoxic response against ERBB2\(^+\) transplantable tumors (21). In the present study, a similar cytotoxic response was also elicited in the few ERBB2 vaccinated hamsters tested (data not shown). However, when random-bred hamsters are used,
differences in major histocompatibility antigens markedly affect the efficacy of T-cell killing of ERBB2+ target cell lines, and so the cytotoxic response of all the immunized hamsters was not compared.

Admittedly, random-bred hamsters are not a suitable model for fine dissection of the immune mechanisms activated by the vaccine because of the lack of specific reagents and standardized procedures. However, the immune mechanisms elicited by anti-ERBB2 DNA vaccine have been extensively studied in various mouse models of ERBB2 cancer. In wild-type BALB/c mice, the electroporation of EC-TM plasmids elicits an antibody and CD8+ T cell mediated immune response (28). In BALB/c mice transgenic for the ERBB2 oncogene (BALB-neuT mice), it inhibits the progression of ERBB2-driven carcinogenesis. However, in these mice vaccine elicits mostly, if not only, an antibody response (14, 29). The lack of cell-mediated cytotoxicity is due to the absence of CD8+ T cells reacting with rat ERBB2 with high avidity. In BALB-neuT mice, a form of split-tolerance allows anti-ERBB2 vaccine to induce only a CD4+ T cell activation and a significant antibody response to ERBB2 (30, 31). The protection elicited in these mice against autochthonous carcinomas rests on the multiple direct and indirect antitumor activities of vaccine-induced anti-ERBB2 antibodies.

The antibody response to ERBB2 is especially effective because ERBB2 is both the target antigen of the EC-TM vaccine and a membrane-exposed receptor regulating cell growth (3). EC-TM-induced anti-ERBB2 antibodies impede the progression of ERBB2 carcinogenesis by blocking the proliferation of ERBB2+ tumor cells (29). Inhibition of ERBB2 receptor dimerization and induction of internalization and recycling prevent the transduction of activating signals (14, 24). EC-TM induced antibodies reduce the basal level of Akt phosphorylation in ERBB2+ cells without impairing PI3K enzymatic activity, and induce an increase of PTEN phosphatase activity correlated with reduced PTEN tyrosine
phosphorylation (32). They also indirectly affect tumor growth by mediating antibody-dependent cell-mediated cytotoxicity (23).

ERBB2 is a tumor associated molecule with a causal role in cancer progression. However, it is a self tolerated molecule and triggering a response to it has to circumvent central and peripheral tolerance (4, 15). Data in mice (24) and hamsters (21) have shown that EC-TM plasmid electroporation is an effective way to break tolerance. However, the antibody response thus induced is short-lived and frequent electroporations are required for a sustained control of carcinogenesis (24). This requirement is also evident in human patients vaccinated against tumor-associated antigens (33). In the present study, EC-TM plasmids electroporations repeated every four weeks kept anti-ERBB2 antibody levels stable during the twelve weeks of the DMBA carcinogenesis induction (not shown).

The kinetics of the occurrence of preneoplastic lesions is markedly delayed in ERBB2 vaccinated hamsters. Their progression to exophytic lesions, and their growth is also delayed. Necroscopic pathological observations show that both the progression of preneoplastic lesions to severe dysplasia and that of squamous cell carcinomas to frankly invasive cancer are hampered. Our work on transgenic mice electroporated with EC-TM plasmids had shown that the immunity elicited hampered the progression of neoplastic lesions while it was unable to reduce the growth of existing lesions (15, 24, 31). This issue has not been directly addressed in the present experiments. However, it is probable that the effects we observed on more advanced lesions in ERBB2 vaccinated hamsters are mostly the result of immune inhibition of the progression of earlier stages of the lesions.

Present data extend to specific immunity previous findings showing that IL-12-boosted innate immunity effectively counteracts 3-methylcholanthrene carcinogenesis in mice through the release of cytokines and boosting of innate immunity mechanisms (34). They also endorse
the pioneering work of Thorbecke's group on vaccination with tumor cells (35) and on the central role of antibody (36) in preventing DMBA induced sarcomas in chickens. Since vaccines against oncoantigens are especially effective in inhibiting the progression of early stages of cancer (4), characterization of oncoantigens overexpressed during such stages along with refinement of vaccines to make them able to trigger innate and adaptive immune mechanisms, may form a fresh strategy for the management of individuals at high risk of cancer following exposure to a specific carcinogenic agent. However, caution is needed in directly assessing the significance of these results to humans. While the hamster cheek pouch model mimics many aspects of human cancer (e.g. multi-stage development, pathological and histological features, molecular alterations) it has some limitations (e.g. the human mouth does not have a similar structure and chemical induction is very quickly elicited in hamsters by a high carcinogen dose) (18). Moreover, studies addressing the expression of ERBB2 in human preneoplastic and neoplastic oral cavity lesions have provided inconclusive and conflicting results (37-43). Whether ERBB-2 is driving the early stage of carcinogenesis, and at which stage this driving role acquires critical significance are not yet clearly defined. The pathological evidence in supplementary Figure 1 suggests that the controversy on ERBB2 overexpression in human head and neck cancer may be partly due to its transient overexpression in critical stages of carcinogenesis. However, the samples analyzed are too few as a source of definitive conclusions. Nevertheless, the question of whether the ERBB2 expression in our model really mimics the human scenario, while important, is not a central issue of this study. Although ERBB2 expression in the human head and neck cancer is debated, our data suggest that an immune response against an oncoantigen, even if it is apparently transiently expressed only in a few stages of carcinogenesis, may hamper the progression of mutagen-induced carcinogenesis.
The differences we observed between control and ERBB2 immunized hamsters are relatively small and thus may seem of limited clinical significance. However, vaccine-induced protection was observed against a continuous and prolonged chemical induction of oral cancer that in eleven weeks causes the onset of oral exophytic lesions in all control hamsters. As evinced by necroscopic analysis at the twelfth week, most of them were lesions characterized by a severe grade of dysplasia or were invasive carcinomas (Supplementary Table S1). In this fast and aggressive model, our multiple protection data acquire a special significance since they provide a proof of concept on the potential of vaccination in hampering chemical carcinogenesis. In a slower and less aggressive chemical induction, more refined vaccines and more effective vaccination schedules may lead to a stronger immunity, and the protection afforded may acquire a true clinical significance. The experimental evidence that pharmacological (17) and vaccine-induced interference with an oncogene product impairs mutagen induced carcinogenesis provides a further demonstration on the efficacy of preventive maneuvers for cancer prevention and may spur the search for fresh immune, pharmacological and combined strategies to treat healthy persons at risk, for whom no active therapeutic option exists at present.
REFERENCES


Figure 1: Stages of DMBA-induced carcinogenesis. A, weeks when the right cheek pouch mucosa was painted with DMBA (blue arrows). B, appearance of DMBA painted area: enlargement of blood vessels (B1); inflammation and whitish granular appearance (B2); foci of leukoplakia (B3 and B4), exophytic lesions (B5 and B6). C, histological examination: moderate (C1) and marked inflammatory reaction (C2); focal dysplasia (C3 and C4); moderately differentiated, \textit{in situ} (C5) and invasive squamous cell carcinoma (C6). D, immunohistochemistry of ERBB2 expression: absent or scarcely present (D1 and D2); markedly evident in hyperplastic and dysplastic epithelia (D3 and D4); poorly evident and only persisting in limited areas of the squamous cell carcinomas (D5 and D6). E, Western blot analysis of ERBB2 expression evaluated on three hamster cheek pouches. Data were normalized with actin expression. (Original magnification: C1-2, D1-2 x400 and C3-6, D3-6 x200).

Figure 2: Anti-ERBB2 DNA vaccine hampers DMBA-induced carcinogenesis. A, starting two weeks before the cheek pouch painting with DMBA, hamsters were electroporated every three weeks with pcDNA3 or EC-TM plasmids (red arrows). Percent of hamsters free of preneoplasric lesions (B) and their multiplicity (C). Percent of hamsters free of exophytic lesions (D), their multiplicity (E) and their size (E). Expression of p63 in large exophytic and hyperkeratotic lesions from hamsters electroporated with empty pcDNA3 (G) or EC-TM (H) plasmids. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (Original magnification: G and H x100).

Figure 3: EC-TM immunized hamsters with the highest anti-ERBB2 antibody titer display
hampered DMBA-carcinogenesis. A, PS is lower in EC-TM immunized hamsters than the controls electroporated with empty pcDNA3 plasmids ($P < 0.001$). The 2.5 and 5 PS thresholds divide hamsters into fast (gray), intermediate (empty), and slow (green) progressors. B, The number of fast progressors is significantly lower among the hamsters immunized with EC-TM plasmids ($P < 0.001$). Slow progressors were only present among EC-TM electroporated hamsters. C, Among EC-TM electroporated hamsters the slow progressors (green square) displayed the highest titer of anti-ERBB2 antibodies, while those classed as fast progressors (gray square) the lowest ($P < 0.01$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. 