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Examination of HER3 targeting in cancer using monoclonal antibodies


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Contributed by Michael Sela, December 11, 2014 (sent for review August 4, 2014)

The human EGF receptor (HER/EGFR) family of receptor tyrosine kinases serves as a key target for cancer therapy. Specifically, EGFR and HER2 have been repeatedly targeted because of their genetic aberrations in tumors. The therapeutic potential of targeting HER3 has long been underestimated, due to relatively low expression in tumors and impaired kinase activity. Nevertheless, in addition to serving as a dimerization partner of EGFR and HER2, HER3 acts as a key player in tumor cells’ ability to acquire resistance to cancer drugs. In this study, we generated several monoclonal antibodies to HER3. Comparisons of their ability to degrade HER3, decrease downstream signaling, and inhibit growth of cultured cells, as well as recruit immune effector cells, selected an antibody that later emerged as the most potent inhibitor of pancreatic cancer cells grown as tumors in animals. Our data predict that anti-HER3 antibodies able to intercept autocrine and stroma–tumor interactions might strongly inhibit tumor growth, in analogy to the mechanism of action of anti-EGFR antibodies routinely used now to treat colorectal cancer patients.

Significance

The human EGF receptor (EGFR/HER) family plays critical roles in tumor progression. Therefore, several therapies intercepting these receptors were developed and clinically approved. Importantly, patients treated with such therapeutics often develop resistance, and in some cases this resistance has been associated with activation of HER3. Potentially, HER3 blockade might overcome patient resistance. Hence, antibodies to HER3 have been developed by us and other researchers. However, it has remained unclear which antibody attributes are required for effective tumor inhibition. To address this issue, we generated several monoclonal antibodies, which were tested in vitro and in tumor-bearing animals. Our results suggest that anti-HER3 antibodies able to intercept stroma–tumor interactions, as well as accelerate HER3 degradation, might inhibit tumor growth better than other antibodies.
The new mAbs displayed a variety of biochemical and biological attributes, such as binding affinity to HER3, ability to displace NRG and block downstream signals, sort HER3 for degradation, and recruit natural killer cells. Ultimately, the Abs were tested in vitro and in tumor-bearing animals for their ability to inhibit growth of cancer cells. We focus on an especially potent tumor-inhibitory Ab that can displace NRG, robustly sort HER3 for intracellular degradation, and also recruit immune effector cells.

Results

Generation of mAbs Against the Extracellular Domain of HER3. To extend our previously described set of Abs to HER3 (24), a fusion protein combining the extracellular domain of HER3 and the Fc domain of a human IgG1 (denoted IgB3; Fig. S1A) was produced and used for the immunization of mice. We screened hybridoma supernatants by ELISA on IgB3-coated microplates and also performed negative selection on human IgG-coated microplate (Fig. S1B). The positive hybridoma supernatants were checked for their capacity to bind with the native form of HER3 by using an immunoprecipitation assay (Fig. S1C), and the corresponding hybridomas were subcloned. Twelve positive hybridomas were selected. Partial nucleotide sequencing of cDNAs encoding the heavy and light chains identified four distinct groups of Abs. These Abs were isotyped and identified as IgG1 molecules with kappa chains (Fig. S1D). Our subsequent studies used six mAbs: four from the new generation (NG33, NG83, NG140, and NG533) and two from the previous generation (XC90 and XC252) (24).

Interestigly, the data reported in Fig. S1E indicate that NG83 was able to recognize the denatured form of HER3 in Western blots.

The Generated Abs Recognize Specifically and with High Affinity the Native Form of HER3. A comparison of the capacity of purified mAbs to bind with a native IgB3 was performed by using ELISA (Fig. L4). The EC50 of our mAb for IgB3 binding ranged between 0.21 nM (XC252) and 16.8 nM (NG140). Next, we compared mAb ability to bind to the native form of the receptor using FACS and NIH/3T3-R2R3 cells, which co-overexpress ectopic HER2 and HER3 (25) (Fig. 1 B and C). To define the affinity of each Ab to HER3, we used the Tag-lite technology (Fig. S2). Each Ab was labeled with the d2-dye. By using fluorescence resonance energy transfer and measuring binding of the labeled mAbs to cells presenting Lumi4(Tb)-labeled HER3, we determined individual Kd values and reported them in Table 1. The values we obtained correlated with the patterns of HER3 binding determined by FACS (Fig. 1 B and C), although they differed from the patterns of IgB3 binding (Fig. L4). As expected, we were unable to define the affinity of mAb NG533, because its ability to bind with the native form of HER3 was barely detectable using either FACS (Fig. 1 B and C) or immunoprecipitation (Fig. 1D). As expected by the nanomolar range of HER3 recognition by the new mAbs, we confirmed using FACS their specificity to HER3 and not to the other members of the HER/ERBB family (Fig. S3).

Specific mAbs Trigger HER3 Degradation and Cell-Mediated Cytotoxicity. The mAbs’ capacity to down-regulate HER3 was determined by using Western blotting. Gastric cancer N87 cells were treated for 3 h with each mAb (10 µg/mL), and HER3 expression levels were analyzed by using Western blotting (Fig. 2 A and B). NG33 induced more extensive degradation of HER3 compared with other mAbs. Next, we evaluated whether the effect observed in N87 cells was cell-type-dependent. Hence, we tested different cancer cell lines and observed similar patterns of receptor degradation (Fig. 2C). In addition, we compared the pattern of HER3 degradation using NG33 (10 µg/mL) to the signal obtained using NRG (20 ng/mL; Fig. S4). Interestingly, NG33 induced faster and more extensive receptor degradation than did NRG. Furthermore, the mAb ability to lead to ADCC was determined by using BxPC3-luc cells, which were incubated with the studied mAbs and with human mononuclear cells. Cell killing is reported in Fig. 2D. Note that we used trastuzumab as a positive control and observed medium to high signals with three mAbs: NG33, NG83, and NG533. In conclusion, the series of mAbs displayed variation in terms of Ab activities, and NG33 stood out as a relatively strong inducer of both ADCC and HER3 degradation.

Ab NG33 Competes with NRG for Binding to HER3. A competition assay between mAbs to HER3 and d2-labeled NRG molecules was used to identify mAbs able to reduce NRG binding to HER3 (Fig. 3A). NIH/3T3-R2R3 cells overexpressing an ectopic HER3 were treated with increasing concentrations of each mAb, under conditions that avoid HER3 internalization. Thereafter, the cells were incubated with the labeled NRG. The results indicated that NG33 was the only mAb able to effectively displace NRG. Notably, NG140 weakly competed with NRG, and both XC90 and XC252 slightly enhanced NRG binding. To check whether the effect on NRG binding has an impact on the phosphorylation of HER3 and downstream signaling, we treated N87 cells with each mAb for 20 min, followed by a short stimulation with NRG (Fig. 3B). Evidently, NG33 completely prevented NRG-induced HER3 phosphorylation and subsequent AKT and ERK activation. Similarly, XC252 was able to partly decrease the phosphorylation level of HER3, but this result might not be attributed to direct competition with NRG.

NG33 Inhibits NRG-Induced Migration and Proliferation of Cancer Cells. Next, we studied the impact of NG33, our NRG competitor mAb, on ligand-induced cell proliferation and migration. First, we evaluated the effect of NG33 on NRG-induced migration of ovarian cancer cells (Fig. 3C). The results confirmed that NG33 treatment reduced NRG-induced migration. Second, NG33’s ability to influence survival of different cancer cells was...
NG33 is as Effective as Trastuzumab in Inhibiting Growth of HER2-Overexpressing Cancer Cells both in Vitro and in Animals. To examine NG33’s ability to decrease cancer cell growth, we focused on N87, a gastric cancer cell line overexpressing HER2 and co-expressing EGFR and HER3. The comparison of the geometric mean taken from the FACS experiment showed that N87 cells express 2.2-fold more HER2 than HER3 (Fig. 4B). Hence, we compared two different ways to decrease N87 cell growth: One approach used mAb NG33 to HER3, and the other used trastuzumab, a clinically approved therapeutic mAb directed against HER2. The effects of the Abs were compared both in vitro, by using a colorimetric assay on NRG-stimulated N87 cells, and in vivo, by using N87 cell xenographs (Fig. 4C and D). The in vitro comparison was extended to additional cell lines (Fig. 4A). The results obtained indicated that NG33 is as effective as trastuzumab in decreasing cancer cell growth in vitro and N87 tumorigenic growth in animals (P < 0.05).

Improvement of NG33’s in Vitro Effects by Combinations with Other mAbs Directed to HER3. To try and improve the effects of NG33, our most potent mAb, we combined it with another anti-HER3 Ab. First, we determined which Ab of our anti-HER3 series could target an epitope distinct from that targeted by NG33. For this determination, we used a Lumi4(Tb)-labeled NG33 and IgB3-coated microplates (Fig. 5A). The results indicated that XC90 was the only mAb able to compete with NG33; NG140 and XC252 did not alter NG33 binding, and, interestingly, mAb NG83 potentiated NG33 binding to IgB3. These observations were corroborated by using a labeled form of XC252 (Fig. 5B). In the next step, we tested mAb combinations for their ability to degrade HER3 (Fig. 5C) and also decrease phosphorylation of HER3, AKT, and ERK (Fig. 5D). The HER3 degradation assays identified the mixtures NG33+NG83 and NG33+NG140 as suitable combinations. However, the analysis of NRG-induced phosphorylation of HER3, ERK, and particularly AKT favored the combinations NG33+XC252 and NG33+NG140.

Table 1. $K_D$ determinations using the Tag-Lite technology

<table>
<thead>
<tr>
<th>mAbs</th>
<th>$K_D$, nM</th>
<th>SD, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG33</td>
<td>2.96</td>
<td>0.66</td>
</tr>
<tr>
<td>NG83</td>
<td>&gt;24</td>
<td>—</td>
</tr>
<tr>
<td>NG140</td>
<td>6.12</td>
<td>—</td>
</tr>
<tr>
<td>NG533</td>
<td>Un.</td>
<td>—</td>
</tr>
<tr>
<td>XC90</td>
<td>2.30</td>
<td>0.02</td>
</tr>
<tr>
<td>XC252</td>
<td>1.09</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Cells were transfected with HER3-SNAP-Tag and labeled with BG-Lumi4(Tb), a SNAP-tag substrate. After incubation with increasing concentrations of the indicated d2-labeled mAb directed to HER3, the $K_D$ was determined from binding curve fitting. The binding curve was obtained by measuring Tr-FRET between the donor Lumi4(Tb) and the acceptor d2-dye. Un., undetermined.

The Combination of NG33 with a Second, Noncompetitive mAb Enhances the Inhibitory Effect on Cancer Cell Proliferation in Vitro and, to Some Extent, also in Animals. As a prelude to in vivo studies, we examined single mAbs, or their combinations with NG33, for their capacity to inhibit proliferation of BXPC3 pancreatic cells, which highly express NRG (26) (Fig. 6A–C). Decreasing mAb concentrations were used, and a colorimetric assay was performed after 3 d of treatment. NG83 and NG140 used alone did not interfere with cell growth. However, at high concentrations, the combinations of NG33 and either NG83 or NG140 showed stronger inhibitory effects than NG33 alone (Fig. 6A and B); conversely NG33 alone induced 58% inhibition of cell survival, and in combination with NG83 or NG140, the mAb induced 73% or 60% inhibition, respectively (P < 0.0001). By contrast, the combination of NG33 and XC252 showed no additive impact on cell growth (Fig. 6C), probably due to the relatively strong inhibition imposed by XC252 when singly applied.

To select an in vivo tumor model, a pilot animal experiment was conducted by using a mixture of our stronger in vitro inhibitors, NG33 and XC252, and a series of tumor cell lines (gastric, N87; lung, A549; pancreatic, BXPC3; ovarian, OVCAR-5; and head and neck, CAL-27; Fig. S5). This experiment was performed with only three mice per group. Mice were injected twice a week with the mAb combination or with saline. The best responder of these in vivo models was the pancreatic BXPC3 xenograft. Hence, the efficacy of three different mAb combinations was evaluated on the respective xenograft. This second animal study was performed on groups of seven or eight mice, which were treated once every 3 d with saline, single mAbs, or a mAb combination (total: 0.2 mg per injection). Tumor growth curves are reported in Fig. 6E. The ability of NG33 to decrease tumor growth, compared with saline, was confirmed (P < 0.0001, after 3 wk of treatment). In this animal model, the other mAbs, NG83, NG140, and XC252, showed no statistically significant ability to decrease tumor growth when singly administered. However, the combination of NG83 (Fig. 6D) or NG140 (Fig. 6E) with NG33 showed a clear trend toward an improvement of NG33’s antitumor efficacy. These trends did not reach statistical significance, but similar results were also obtained in a second experiment. Notably, in line with the in vitro study, the...
The anti-HER3 mAb NG33 decreases NRG-induced phosphorylation of HER3, Akt, and Erk, as well as NRG-induced cell migration. (A) NIH3T3-R2R3 cells coexpressing human HER2 and HER3 were plated on black microplates and incubated for 45 min at 4 °C with increasing concentrations of mAbs to HER3. After washing, we added a fluorescently labeled NRG and incubated for 30 min at 4 °C. Fluorescence intensities (at 670 nm) were determined after three washes. (B) The ability of the indicated mAbs to inhibit NRG-induced phosphorylation of HER3, Akt, and Erk was studied by using N87 cells, which were treated at 37 °C for 20 min with the indicated mAbs (10 μg/mL). NRG (20 ng/mL) was added to the cells and incubated for 10 min. The cells were then lysed, and equal quantities of protein lysates were resolved by using electrophoresis and immunoblotting, as indicated. (C) The capacity of mAb NG33 to inhibit NRG-induced migration was tested by using OVCAR-5 cells that were seeded in the upper compartment of migration chambers. The lower compartment of each chamber was filled with medium supplemented with NRG (10 ng/mL). After 24 h, cells that reached the lower side of the filter were fixed, permeabilized, and stained by using Giemsa. Signals of triplicates were quantified. **** P < 0.0001 (ANOVA and post hoc tests).

In an effort to enhance the antitumor action of our most potent Ab, NG33, we combined it with a second Ab, for example, NG83 or NG140, which resulted in relatively small improvements of the inhibitory effect. This result is rather surprising, considering the clearly additive or synergistic effects induced by mAb combinations targeting EGFR (19) or HER2 (20, 21). Considering the relatively weak difference between mAbs used alone and mAb combinations at inducing HER3 degradation, one might speculate that the frequent overexpression of EGFR and HER2, but rare overexpression of HER3, underlays the weak additive effects observed when combining anti-HER3 mAbs. One important example, an Ab combination targeting EGFR, called Sym004, is currently being tested in clinical trials that recruited colorectal cancer (37) and non-small-cell lung cancer (38) patients. Interestingly, although one of our mAbs, XC252, accelerated HER3 degradation and decreased BXPC3 proliferation in vitro, this mAb did not enhance the ability of NG33 to inhibit BXPC3

combination of NG33 with XC252 (Fig. 6F) was clearly as efficient as NG33 alone.

In summary, by generating a set of mAbs to HER3 and testing them in vitro for the ability to inhibit NRG binding, enhance HER3 degradation, retard downstream signaling, recruit immune effector cells, and arrest growth of cancer cells in vitro, we selected NG33 as the most promising candidate for animal studies. As expected, NG33 emerged from our animal tests as the best inhibitor of pancreatic tumor cells that secrete NRGs and express HER3. Our attempts to enhance NG33’s anti-cancer effects by combining it with other, noncompetitive mAbs to HER3 yielded only limited added benefit. Hence, it is conceivable that NG33’s therapeutic potential is due to an ability to inhibit NRG-mediated growth and migration of tumor cells in response to stromal cues.

Discussion

Because several lines of evidence have implicated HER3 in tumorigenesis (27–29), and because this binder of multiple NRG isoforms participates in the development of resistance to some cancer therapies (14–17), a few anti-HER3 mAbs have been generated (23, 27, 28, 30). Several studies, including those performed in our laboratory, previously described a strategy to enhance the antitumor activity of mAbs by combining two Abs directed to nonoverlapping epitopes of the shared antigen, for example, EGFR (19, 31) or HER2 (20, 21). Their antitumor activities were quite limited, we tend to attribute the superiority of NG33 to the blockade of autocrine loops involving HER3 and the many NRG isoforms it can bind. It is worthwhile mentioning that the NRGs are highly expressed in carcinomas (34). Moreover, the mechanism of action of cetuximab, an anti-EGFR Ab used to treat colorectal cancer, has been attributed to blockade of EGFR-specific ligands like amphiregulin (35). Likewise, ovarian tumors might depend on an autocrine loop involving HER3 and NRG1 (36).

To study the effects of single mAbs on tumor growth, we selected BXPC3 human pancreatic tumor cells, because of their high expression levels of NRG (26). Accordingly, when singly applied, our NRG-competitive NG33 Ab better than the other mAbs, inhibited BXPC3 tumors (Fig. 6). Importantly, NG33 not only displaced NRG better than the other mAbs; it also induced stronger ADCC and more extensive degradation of HER3. Because other Abs induced some degradation and only weakly elevated ADCC, but their antitumor activities were quite limited, we tend to attribute the superiority of NG33 to the blockade of autocrine loops involving HER3 and the many NRG isoforms it can bind. It is worthwhile mentioning that the NRGs are highly expressed in carcinomas (34).

In an effort to enhance the antitumor action of our most potent Ab, NG33, we combined it with a second Ab, for example, NG83 or NG140, which resulted in relatively small improvements of the inhibitory effect. This result is rather surprising, considering the clearly additive or synergistic effects induced by mAb combinations targeting EGFR (19) or HER2 (20, 21). Considering the relatively weak difference between mAbs used alone and mAb combinations at inducing HER3 degradation, one might speculate that the frequent overexpression of EGFR and HER2, but rare overexpression of HER3, underlays the weak additive effects observed when combining anti-HER3 mAbs. One important example, an Ab combination targeting EGFR, called Sym004, is currently being tested in clinical trials that recruited colorectal cancer (37) and non-small-cell lung cancer (38) patients. Interestingly, although one of our mAbs, XC252, accelerated HER3 degradation and decreased BXPC3 proliferation in vitro, this mAb did not enhance the ability of NG33 to inhibit BXPC3
family for their tumorigenic growth and also for evasion of the cytotoxic effects of chemotherapy.

Materials and Methods

Generation of mAbs to HER3. Mice immunization, fusion between myeloma cells and splenocytes, and the subsequent hybridoma subcloning were performed as described (24). Hybridoma supernatant screening, using ELISA, was performed on 96 well-plates coated with IgG3 (1 μg/mL) or with a human IgG molecule (1 μg/mL). The plates were blocked with PBS containing BSA (1%; weight/vol) and incubated for 1 h with hybridoma supernatants, followed by a second incubation (60 min) with HRP-labeled anti-mouse IgG and subsequent detection using 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). The second step of the screening was performed by immunoprecipitation. Anti-mouse IgG agarose beads were incubated first with 100 μL of hybridoma supernatant and subsequently with whole-cell lysate from HER3-expressing T47D cells. The mAbs directed to HER3 were then isolated by using the BSA Clonotyping System/HRP kit (SouthernBiotech).

Tag-lite HER3 Binding Assay. A Tag-lite plasmid coding for HER3 fused to SNAP-tag (CISbio bioassays) was transiently expressed in HEK-293 cells. Cells were plated and labeled 24 h after transfection with 200 nM SNAP-Lumi4-Tb substrate (donated by CISbio bioassays) in Tag-lite labeling medium (1 h at 37 °C). Abs (100 μL at 1–2 mg/mL) were labeled with the d2 dye (acceptor). A set of 16 twofold serial dilutions spanning from 0.006 nM to 200 nM of labeled Ab (Ab-d2) were prepared in Tag-lite labeling medium. The specific signal was obtained by mixing cells (10,000 cells in 10 μL per well), 5 μL of Ab-d2 from the serial dilution and 5 μL of Tag-lite labeling medium. The nonspecific signal was obtained by mixing 10 μL of cells with 5 μL of the corresponding unlabeled Ab (300 nM) and 5 μL of Ab-d2 from the serial dilution. After overnight incubation at 20 °C, time-resolved (TR) fluorescence

tumors. Lack of added benefit might be attributed to the relatively weak activity of XC252 to trigger ADCC in vitro. Notably, a study that combined three mAbs to HER2 concluded that enhanced ADCC might explain synergistic mAb effects on tumor growth (39).

We previously proposed that a “surface lattice” formed by transmembrane receptors cross-linked by two or more Abs might underlay the enhanced ability of certain mAb combinations to accelerate receptor degradation in vitro and inhibit tumor growth in animals (18, 20, 21). According to this model, the aggregated receptors are recognized by the endocytosis machinery and are subsequently sorted for degradation in lysosomes in a dynamin-independent manner (19). Cross-linked HER3 molecules might deviate from the lattice model. For one, HER3 harbors a defective kinase domain (8), and it is uncoupled from GRB2, an adaptor needed for robust endocytosis of EGFR (40, 41). Endocytosis and degradation of EGFR family members are controlled by their kinase activity, which recruits the CBL ubiquitin ligase (42). By contrast, HER3 needs no ligand or autophosphorylation to undergo endocytosis (43), which is frequently followed by rapid recycling back to the plasma membrane (44). Moreover, HER3 down-regulation involves both a deubiquitinating enzyme, USP8, and a ubiquitin ligase, Nrdp1 (45). HER3 also interacts with NEDD4, a HECT family E3 ubiquitin ligase (46). These differences, along with the relatively low expression levels of HER3, might explain the lack of benefit of combining two anti-HER3 Abs.

In summary, our set of anti-HER3 Abs reduces the attractiveness of the option of combining two or more Abs to improve the anti-tumor effects of Abs to NRG receptors. Independently, our studies strengthen the possibility attributing to NRGs and their low affinity receptor, HER3, a driving role in tumor progression. This role is analogous to the mechanism underlying the therapeutic effect of anti-EGFRs such as panitumumab in colorectal cancer (35, 47).

Future studies should address the intriguing possibility that different types of carcinoma depend on distinct members of the EGF/NRG

Fig. 6. Both in vitro and in animals, combinations of noncompetitive mAbs to HER3 only weakly enhance the inhibitory effects of the respective single Abs. (A–C) Proliferation assays using MTT were performed on BXPC3 cells (5,000 cells per well). Cells were plated on the day before and treated for 72 h with the indicated mAbs. Increasing concentrations of the indicated mAbs (either alone or in combination) were used in medium supplemented with 1% serum and NRG (10 ng/mL). An irrelevant IgG fraction was used as control. (D–F) CD1-nude mice were grafted s.c. with 5 × 10⁶ BXPC3 cells. Once tumors became palpable (after 13 d), the mice were randomized into groups of eight animals and treated once every 3 d for 5 wk. The control group (CTRL) was treated intraperitoneally (IP) with 200 μL of PBS. The other groups were treated with the indicated mAbs, either alone or in combinations, at a final concentration of 0.2 mg/0.2 mL saline per mouse. Body weight was determined once a week, and the tumors were measured twice a week. Shown are average tumor sizes from seven or eight mice (±SEM).

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was measured on a Phosphor Imager reader. The E655/E620 ratio was computed, and values, measuring TR-FRET, were plotted against AB concentration.

ADCC Assays. ADCC was evaluated by using a luciferase assay. BXPC3-Luc cells (4,000 per well) were preincubated in microplates for 30 min with the Abs. Thereafter, Ficoll-purified human peripheral blood mononuclear cells from buffy coats were added at a 10:1 effector to target cell ratio (E:T). After 24 h at 37 °C, the supernatant was removed, and luciferase (Promega) was added. Bioluminescence was determined by using the Wallac Trilux 1450 Microbeta liquid scintillation and luminescence counter (Perkin-Elmer). The percentage of cellular cytotoxicity was calculated by using the following formula: percentage of specific lysis = [bioluminescence in experimental point – basal bioluminescence]/[bioluminescence in total lysis – basal bioluminescence] × 100. Basal bioluminescence was obtained when BXPC3-Luc cells were incubated with hPBMC alone. Likewise, bioluminescence in total lysates was obtained after a 30-min incubation of BXPC3-Luc in the presence of SDS (0.1%).

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**Tumorigenic Growth in Mice.** All animal studies were approved by the Weizmann Institute’s Review Board. Tumor bearing CD1 nude mice were reared into groups of eight mice and divided 1:1 into the treated (received a single s.c. injection with 2 × 105 cells) versus control (received 10% DMSO intraperitoneally as a control) group. Tumors were measured twice a week, with tumor volume calculated as width × length × depth/2. Animals were euthanized when tumor size reached 1,500 mm³. **Tumorigenic Growth in Mice.** All animal studies were approved by the Weizmann Institute’s Review Board. Tumor bearing CD1 nude mice were reared into groups of eight mice and divided 1:1 into the treated (received a single s.c. injection with 2 × 105 cells) versus control (received 10% DMSO intraperitoneally as a control) group. Tumors were measured twice a week, with tumor volume calculated as width × length × depth/2. Animals were euthanized when tumor size reached 1,500 mm³.