Ectodomain-Specific E-Cadherin Antibody Suppresses Skin SCC Growth and Reduces Tumor Grade: A Multitargeted Therapy Modulating RTKs and the PTEN–p53–MDM2 Axis

Sabine M. Brouxhon1,4, Stephanos Kyrkanides4, Veena Raja4, Andrew Silberfeld4, Xiaofei Teng4, Denise Trochesset2,3,4, Jason Cohen2,3, and Li Ma4

Abstract
Tumor cell survival consists of an intricate balance between cell growth and cell death pathways involving receptor tyrosine kinases [RTK; i.e., HER1-4, insulin-like growth factor-1 receptor (IGF-1R), etc.], MDM2, and the tumor suppressor proteins phosphatase and tensin homolog deleted on chromosome ten (PTEN) and p53. We recently demonstrated that shedded E-cadherin extracellular domain fragment (sEcad) is a valid oncogenic target that is significantly increased in human clinical skin squamous cell cancers (SCC) samples, UV-induced mouse tumors, and cells and promotes tumor cell proliferation, migration, and invasion by interacting and activating with the HER-phosphatidylinositol 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) axis. In resected human SCC tumors, we reported enhanced sEcad-HER1, sEcad-HER2, and sEcad-IGF-1R, but not FL–Ecad–RTK interactions. Here, we demonstrate that a sEcad antibody against the ectodomain of E-cadherin suppressed SCC growth and increased tumor differentiation in orthotopic cutaneous SCC xenografts by inhibiting proliferation and inducing apoptosis. A similar anti-sEcad antibody-induced inhibition of proliferation and induction of cell death was evident in PAM212 cells in vitro. Mechanistically, anti-sEcad administration upregulated an array of cell death pathways (i.e., Bad, active caspase-3, and cleaved PARP) and inhibited inhibitors of apoptosis (IAP; survivin, livin, etc.), RTKs (HER1, HER2, p95HER2, and IGF-1R), MAPK and PI3K/mTOR prosurvival signaling. Interestingly, in anti-sEcad mAb-treated tumors and PAM212 cells, this effect was associated with a profound increase in membrane, cytosolic, and nuclear levels of PTEN; enhanced cytosolic p53; and a decrease in MDM2 levels. Overall, our studies suggest that an antibody-based therapy against sEcad may be a novel therapeutic platform for cutaneous SCCs by hampering key proto-oncogenes (RTKs, IAPs, and MDM2) and activating potent tumor suppressor proteins (PTEN and p53) intricately linked to tumor growth and survival. Mol Cancer Ther; 13(7); 1791–802. ©2014 AACR.

Introduction
Despite increased public awareness and the implementation of various chemopreventative measures, cutaneous squamous cell cancers (SCC) are on the rise with an estimated 700,000 new cases diagnosed annually (1). More importantly, patients on immunosuppressive drugs exhibit a 65 to 100 times greater risk of developing SCCs compared with the general population, and these skin cancers carry an enhanced risk for nodal metastasis (2). Therefore, there is a clear and urgent need for the identification of novel targets and therapeutic strategies for these and standard cutaneous SCCs.

Authors’ Affiliations: Departments of1Emergency Medicine, 2Dermatology, and 3Pathology; and 4Health Sciences Center, Stony Brook University, Stony Brook, New York

Corresponding Author: Sabine M. Brouxhon, School of Medicine, Stony Brook University Health Sciences Center, Stony Brook, NY 11794. Phone: 631-632-8584; Fax: 631-632-8924; E-mail: Sabine.Brouxhon@stonybrook.edu

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E-cadherin belongs to a family of integral transmembrane glycoproteins that promote the establishment of calcium-dependent homotypic cell–cell adhesions. Using a chronic photocarcinogenesis model, we have previously demonstrated a sequential ultraviolet (UV)-induced loss of membrane-bound E-cadherin with progression from normal epithelium to dysplastic lesions, papillomas, and overt SCCs (3). Moreover, we have found a concomitant increase in E-cadherin mRNA and a progressive increase in the shedding of the E-cadherin extracellular domain fragment (termed sEcad), suggesting that posttranslational processing of E-cadherin via ectodomain shedding plays an important role in UV-induced skin carcinogenesis (4).

In many epithelial-derived cancers, heightened circulating levels of sEcad generally correlated with increased histopathologic grade, metastasis recurrence and signified a worse prognosis (5, 6). Along these lines, we and others have confirmed that sEcad is a valid oncogenic target in that it is selectively increased in human skin, breast, and prostate cancer tissues and cells, as well as HER2-positive breast tumors and bodily fluids from...
MMTV-PyMT genetically engineered mice (4, 7, 8). Importantly, irrespective of E-cadherin status, we have validated that sEcad is functionally involved in skin and breast cancers as it enhances cell proliferation, migration, and invasion, the latter via increasing the production of proinvasive metalloproteinases (MMP; ref. 4). Although the molecular mechanisms underlying sEcad-induced pro-oncogenicity are still under investigation, we have previously shown that sEcad interacts with multiple receptor tyrosine kinases (RTK), including the human epidermal growth factor receptors (HER1-3) and insulin-like growth factor-1 receptor (IGF-1R), leading to the phosphorylation of these RTKs and activation of downstream mitogen-activated protein kinase (MAPK)-phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and inhibitor of apoptosis (IAP) signaling (4, 7). Of note, the RTK-MAPK-PI3K/Akt pathway is one of the most potent driving forces promoting tumor progression, and this axis has emerged as one of the most concentrated areas in anticancer drug development.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN), the most frequently mutated or downregulated tumor suppressor gene in skin and other human and mouse cancers, negatively regulates the RTK and PI3K–Akt–mTOR axis through its lipid phosphatase activity as well as via protein–protein interactions (9, 10). Upon PTEN downregulation, heightened plasma membrane phosphatidylinositol 3-kinase (PI3K) recruited by PI3K activates Akt, which phosphorylates downstream mTOR, glycogen synthase kinase 3 and BAD signaling, resulting in an increase in cell proliferation and a decrease in apoptosis (11). In addition, PI3K/Akt activation promotes the phosphorylation and nuclear entry of an E3-ubiquitin ligase murine double minute 2 (MDM2) oncoprotein, which then interacts with the p53 tumor suppressor protein to facilitate its degradation (12, 13). Conversely, overexpression of PTEN inhibits PI3K/Akt and restricts MDM2 to the cytoplasm, thereby promoting p53 function and enhancing the sensitivity of tumor cells to drug-induced apoptosis (12).

Given that sEcad interacts and activates the RTK–MAPK–PI3K–Akt–mTOR axis suggests that binding of sEcad to RTKs may also inhibit PTEN and p53 tumor suppressor functions, thereby driving tumor growth and apoptosis resistance. Thus, the purpose of this study was to build an understanding of the mechanisms of action of a validated that sEcad is functionally involved in skin and breast cancers as it enhances cell proliferation, migration, and invasion, the latter via increasing the production of proinvasive metalloproteinases (MMP; ref. 4). Although the molecular mechanisms underlying sEcad-induced pro-oncogenicity are still under investigation, we have previously shown that sEcad interacts with multiple receptor tyrosine kinases (RTK), including the human epidermal growth factor receptors (HER1-3) and insulin-like growth factor-1 receptor (IGF-1R), leading to the phosphorylation of these RTKs and activation of downstream mitogen-activated protein kinase (MAPK)-phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and inhibitor of apoptosis (IAP) signaling (4, 7). Of note, the RTK-MAPK-PI3K/Akt pathway is one of the most potent driving forces promoting tumor progression, and this axis has emerged as one of the most concentrated areas in anticancer drug development.

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width (mm) \times 0.52. Mice were randomized to receive a single intraperitoneal injection of 10 mg/kg anti-sEcad mAb (DECMA-1, U885, custom order; Sigma), rat IgG1 (0116-14, custom order; Southern Biotech), or equivalent volumes of saline (200 μL). Palpable tumors were then monitored weekly until sacrifice at 20 days after treatment. Tumor data were statistically analyzed with one-way ANOVA, and Bonferroni post hoc analysis. P values <0.05 were considered significant. This study was conducted in accordance with NIH guidelines and protocols approved by the Institutional Animal Care and Use Committee at Stony Brook University.

Results

**Targeted mAb against sEcad suppresses tumor burden and decreases tumor grade in PAM212 orthotopic skin xenografts**

To test the *in vivo* therapeutic efficacy of anti-sEcad mAb treatment, 2 \times 10^6 PAM212 cells suspended in 100 μL of PBS, were inoculated subcutaneously in the flanks of female SCID mice. Mice bearing tumors, with an estimated volume of 100 mm^3 were subsequently randomized to receive a one-time injection of saline (n = 6), IgG (10 mg/kg, n = 6), or anti-sEcad mAb (10 mg/kg, n = 7) and tumor growth was monitored for an additional 20 days. Starting from 12 days, anti-sEcad treatment demonstrated a significant decrease in tumor volume and burden compared with controls (Fig. 1A and B). Moreover, blinded histopathologic analysis revealed increased squamous differentiation in anti-sEcad mAb-treated tumors, whereas control and IgG treatment showed a lack of squamous differentiation and marked infiltration into skeletal muscle and surrounding tissues (Fig. 1C). Immunohistochemical staining of tumors resected from each treatment group was then analyzed for proliferation and apoptosis. Consistent with the observed reduction in tumor growth, BrdUrd incorporation was significantly decreased (Fig. 1D), whereas apoptosis was dramatically increased (Fig. 1E) in mAb-treated mice. Collectively, these results demonstrate that anti-sEcad mAb therapy causes a potent antitumor effect in orthotopic PAM212 tumor xenografts by inhibiting tumor growth and inducing programmed cell death.

**Mice exhibit no evidence of toxicity to anti-sEcad mAb administration**

Clinical studies using the HER2 receptor inhibitor Herceptin has revealed unanticipated off-target effects ranging from asymptomatic left ventricular dysfunction to symptomatic congestive heart failure. Here, histologic assessment of tissues was conducted to determine whether or not the targeted mAb against the ectodomain of E-cadherin caused any degree of tissue damage, and/or inflammation or lesions. Although a more in-depth understanding of pharmacokinetics, pharmacodynamics, as well as toxicity profiles are required for this therapeutic, our preliminary findings show no histopathologic cytotoxicity in the heart, intestine, kidney, or liver of anti-sEcad mAb-treated animals at the dose used and time frame observed in this study (Fig. 2).

**Targeted mAb against sEcad inhibits cell proliferation and induces apoptosis in vitro**

Based on the antitumoral effects of anti-sEcad mAb therapy in PAM212 xenografts *in vivo*, we next explored whether the effects of mAb could be confirmed *in vitro*. Consistent with the *in vivo* findings, blockade of sEcad significantly decreased cell numbers (by ~72%, P < 0.001) and inhibited cell proliferation (by ~80%, P < 0.001) as assessed by using a hemocytometer and BrdUrd colorimetric ELISA (Fig. 3A and B). Moreover, using an apoptotic and necrosis cell death–based ELISA, we observed a dramatic increase in both cell death pathways upon anti-sEcad mAb treatment (Fig. 3C and D). Comparably, phase contrast images displayed many of the morphologic changes characteristic of apoptosis and necrosis, including apoptotic bodies, nuclear condensation, fragmentation, and loss of membrane integrity (Fig. 3E). Next, we assessed the effect of anti-sEcad mAb on the expression of specific proapoptotic proteins. Western blot analysis revealed amplified expression of Bad and caspase-3 (active) in anti-sEcad mAb–treated cells (Fig. 3F). Furthermore, mAb treatment increased cleaved PARP level in total cell lysates, but reduced the nuclear full length PARP (Fig. 3F). As increasing evidence indicates that IAPs are upregulated in many cancer types including cutaneous SCCs, we next examined the expression levels of survivin, XIAP, cIAP-1, and livin in these cells by Western blot analysis. Strikingly, a marked reduction in the expression levels of survivin, XIAP, cIAP-1, and livin were evident in anti-sEcad mAb–treated PAM212 cells (Fig. 3G). Taken together, these results demonstrate that anti-sEcad mAb administration inhibits cell proliferation, induces apoptosis, increases proapoptotic, and suppresses prosurvival signaling in PAM212 skin SCC cells.

**Targeted mAb against sEcad downregulates multiple receptor tyrosine kinases and downstream MAPK–PI3K prosurvival signaling**

Recent studies in our laboratory have demonstrated direct sEcad-HER and sEcad-IGF-1R interactions in human and mouse skin and breast cancer tissues and cells, the binding of which results in the phosphorylation of HER1-4 and activation of downstream MAPK–PI3K/Akt/mTOR signaling (4). Moreover, utilizing a variety of HER as well as MEK and PI3K inhibitors, we have confirmed that sEcad signals via this HER–MAPK–PI3K axis to promote skin SCC and breast cancer proliferation, migration, and invasion (4, 7). Because of the interdependent relationship between these pro-oncogenic pathways, we sought to determine whether monoclonal antibody targeting of sEcad may tip the balance toward cell death and away from pro-growth by downregulating this axis. Interestingly, immunoblot analysis confirmed that mAb induced the downregulation of phospho-HER1, HER2,
and IGF-1R, as well as the truncated p95HER2 receptor (Fig. 4A). Similarly, mAb-treated cells also demonstrated a similar reduction in total HER1, HER2, and IGF-1R levels in membrane fractions (Fig. 4B), which is consistent with our prior findings in breast (15). Furthermore, in PAM212 cells exposed to anti-sEcad mAb, downregulation of HERs and IGF-1R was associated with a marked reduction in Ras and phospho-MEK1/2 as well as PI3K, mTOR, and the mTOR substrate 4EBP1. However, phospho-Akt and ERK1/2 levels remained unchanged (Fig. 4C and D). Taken together, these data demonstrate that blockade of sEcad simultaneously interferes with multiple RTKs and inhibits MAPK-PI3K/mTOR/4EBP1 signaling, thereby tipping the balance away from cell survival in favor of tumor cell death.

Targeted mAb against sEcad enhances PTEN and p53 cellular levels while suppressing MDM2 in vivo and in vitro

The tumor suppressor gene PTEN is frequently mutated, deleted, or silenced in skin and other human cancers and directly antagonizes the oncogenic PI3K/Akt/mTOR pathway (16, 17), leading us to speculate that PTEN may be upregulated by anti-sEcad mAb therapy. Indeed, immunohistochemical staining of primary tumors from mice treated with anti-sEcad mAb demonstrated a dramatic increase in membrane, cytosolic, and nuclear PTEN staining in resected primary tumors (Fig. 5A). Because PTEN is well known to regulate p53 protein levels and activity, we hypothesized that anti-sEcad mAb-induced PTEN induction may result in enhanced p53 expression. This is particularly
important, because downregulation/mutation of the tumor suppressor protein p53 affects more than 50% of human cancers, and is of significant focus for chemotherapeutic drug design (18). Consistent with our previous studies in HER2-positive breast cancers (7), immunohistochemical analysis of p53 in mAb-treated PAM212 xenograft tumors revealed a robust increase in p53 immunostaining (Fig. 5A). Similarly, total levels of PTEN and p53 were markedly increased in anti-sEcad mAb-treated PAM212 cells by immunoblotting in vitro (Fig. 5B). PTEN-enforced p53 function has been shown to be a result of the restriction of MDM2 to the cytoplasm and its subsequent degradation (12, 19). When we evaluated MDM2 in tumors by immunohistochemistry, an extensive amount of MDM2 was observed in the nucleus of controls, whereas a marked reduction in staining was noted with mAb treatment (Fig. 5A). Moreover, Western blot analysis of total lysates from anti-sEcad mAb-treated PAM212 cells showed a marked reduction in MDM2 levels, cyclin D1, as well as c-Jun and NF-κB (Fig. 5B).

To investigate whether the membrane or cytosolic localization of PTEN, p53, or MDM2 was altered by anti-sEcad mAb treatment, we next performed subcellular fractionation and Western blotting. In untreated PAM212 cells, the majority of PTEN was in the cytosolic pool, whereas very low levels of PTEN were in the membrane or nuclear fractions (Fig. 5C). In contrast, MDM2 expression was only evident in membrane and cytosolic fractions, and very low levels of p53 were observed in the cytosolic pools (Fig. 5C). Upon mAb treatment, PTEN was markedly increased in all compartments, whereas MDM2 levels were reduced in both membrane and cytosolic fractions and accordingly p53 expression was dramatically increased in the cytosol (Fig. 5C). Collectively, these data demonstrate that the expression of PTEN and p53 are increased in anti-sEcad mAb-treated SCC xenografts and cells, suggesting that mAb-induced tumor suppression may be mediated by induction of PTEN, which subsequently stabilizes and amplifies p53 proapoptotic functions.

**Anti-sEcad mAb enhances PTEN-p53 colocalization and restricts MDM2 to the cytoplasm**

PTEN inhibits activation of the PI3K–Akt–mTOR signaling axis, restricts MDM2 to the cytoplasm, thereby segregating it away from p53 (19, 20). This PTEN-induced cytoplasmic localization of MDM2 promotes MDM2 degradation and sensitizes cancer cells to chemotherapy (19). In contrast, the PI3K–Akt–mTOR signaling axis promotes the phosphorylation and nuclear entry of MDM2, where it interacts with p53, inhibits its transcriptional activity, and targets it for degradation by the proteasome (20). Therefore, we next used a dual immunofluorescence colocalization assay to assess the potential interactions between PTEN and p53, as well as MDM2 and p53 based on spatial localization. In control and IgG-treated PAM212 cells, PTEN was predominately in a perinuclear/cytosolic location, with little immunostaining in the nucleus (Fig. 6A). Unlike PTEN, p53 showed diffuse nuclear staining and a punctate perinuclear staining pattern, the latter exhibited some overlap with PTEN (stained red), as confirmed by the yellow color in the merged image (Fig. 6A). In contrast, 16-hour mAb treatment resulted in a marked increase in cytosolic and nuclear PTEN immunostaining that colocalized with p53 (Fig. 6A). When we examined MDM2 immunostaining, our data demonstrated a greater degree of nuclear MDM2 in control and IgG-treated PAM212 cells, which colocalized with nuclear p53. However, in anti-sEcad mAb-treated cells, MDM2 was predominately localized to the cytoplasm and exhibited minimal overlap with p53 (Fig. 6B). To understand whether the colocalized PTEN and p53 interact, coimmunoprecipitation studies were performed using cytosolic fractions from mAb-treated PAM212 cells. As shown in Fig. 6C, 48 hours after...
treatment no significant association between PTEN and p53 was observed. Collectively these data demonstrate anti-sEcad mAb enhances PTEN-p53 colocalization and dissociates MDM2 away from p53 by restricting MDM2 to the cytoplasm.

**Discussion**

Although it is well accepted that membrane-bound E-cadherin is downregulated with UV-induced SCC progression, little is known about the role of the shed E-cadherin ectodomain fragment in this cancer type (4). Using a chronic photocarcinogenesis model, we have recently shown a sequential loss of membrane-bound E-cadherin and increased sEcad levels with progression from normal skin to dysplastic lesions and overt skin SCCs. Moreover, we have demonstrated that exogenous sEcad enhances SCC proliferation, migration, and invasion by interacting and activating with multiple RTKs (i.e., HER1, HER2, and IGF-1R) and promoting downstream MAPK-P38/Akt/mTOR signaling (4). Notably, these sEcad-induced functional effects were evident in SCC...
cells, irrespective of their FL-E-cadherin expression status, suggesting that intact E-cadherin was not necessary for these biological effects. Indeed, immunoprecipitation of HER1, HER2, and IGF-1R confirmed the presence of sEcad–RTK association, but minimal to absent FL–E-cadherin–RTK interactions, in human SCC and breast cancer specimens (4, 7). Altogether, these results suggest that sEcad is a valid oncogenic target and justify testing sEcad inhibitors as potential therapeutic agents in skin SCCs.

In this study, we demonstrate for the first time that targeted monoclonal antibody therapy against the EC4 domain of E-cadherin, which may bind cell surface bound intact E-cadherin, sEcad, or potentially sEcad-HER complexes, suppressed orthotopic PAM212 SCC growth in vivo, which is in agreement with our previous findings in HER2+ MMTV-PyMT mice (7). Similar findings were also reported in a MARY-X 643 human inflammatory breast cancer xenograft model, wherein an anti-EC2 domain-specific sEcad mAb decreased both the tumor size and number of lymphovascular emboli (21). In addition, intraperitoneal injection of an EC1-domain–specific anti-sEcad mAb prolonged survival in a HT29 ascites tumor xenograft model by enhancing host antitumor responses (22). Besides effects on tumor growth, this study provides further evidence that anti-sEcad mAb treatment altered tumor grade. Specifically, anti-sEcad mAb-treated tumors exhibited a black necrotic eschar (4 of 7 treated tumors) overlying the residual tumor and blinded histopathologic analysis demonstrated a more
differentiated squamous cell phenotype with keratin pearl formation. Immunohistochemical analysis further confirmed these anti-sEcad mAb-induced anti-oncogenic effects, with resected tumors demonstrating reduced proliferation along with induction of apoptosis. Consistent with the in vivo findings, sEcad blockade was an effective antitumor strategy in PAM212 SCC cells by inhibiting proliferation and inducing multiple cell death pathways, including apoptosis and necrosis, which is in line with previous studies in skin SCC neoplastic tumors in vivo. Scale bars, 10 μm. B, immunoblot and densitometry analysis of PTEN, p53, MDM2 cyclin D1, c-Jun, and NFkB levels in total lysates of PAM212 cells treated with control, IgG, or anti-sEcad mAb for 48 hours. C, PAM212 cells were treated with 400 μg/mL of IgG or anti-sEcad mAb for 48 hours and membrane, cytosol, and nuclear fractions were subjected to immunoblotting with the indicated proteins. Na+/K+ ATPase, G3PDH, and fibrillin served as corresponding fractionation loading controls. Data are presented as mean ± SEM.

\[ ^* P < 0.05; ^{**} P < 0.01 vs. C or IgG. \]

\[ n = 2 \text{ to } 3. \]

Figure 5. Anti-sEcad mAb enhances PTEN and p53 while inhibiting MDM2 in vivo and in vitro. A, treatment of SCID mice with 10 mg/kg of anti-sEcad mAb results in a significant increase in PTEN and p53 immunostaining and downregulation of MDM2 immunolevels in skin SCC neoplastic tumors in vivo. Scale bars, 10 μm. B, immunoblot and densitometry analysis of PTEN, p53, MDM2 cyclin D1, c-Jun, and NFkB levels in total lysates of PAM212 cells treated with control, IgG, or anti-sEcad mAb for 48 hours. C, PAM212 cells were treated with 400 μg/mL of IgG or anti-sEcad mAb for 48 hours and membrane, cytosol, and nuclear fractions were subjected to immunoblotting with the indicated proteins. Na+/K+ ATPase, G3PDH, and fibrillin served as corresponding fractionation loading controls. Data are presented as mean ± SEM.

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\[ n = 2 \text{ to } 3. \]
RTKs, including the HER family of receptors and IGF-1R, are integrally involved in tumor cell proliferation and survival by providing mitogenic and antiapoptotic responses to cells via activation of the MAPK-PI3K/Akt pathways. Here, our studies are the first to demonstrate that anti-sEcad mAb exerts cytostatic and cytotoxic effects by downregulating multiple RTKs, including HER1, HER2, and IGF-1R in PAM212 cells. These findings are consistent with our recent findings in MMTV-PyMT mice and MCF-7, BT474 parental, and BT474 Herceptin-resistant breast cancer cell lines, wherein anti-sEcad mAb downregulated all HER family members, including HER1-4. This is particularly important, because drugs that target multiple HERs or one HER family member and/or different RTK, such as IGF-1R, are actively being pursued in many cancer types so as to overcome tumor resistance after a single-targeting agent. Although, this multitargeted approach has yet to be fully exploited in skin SCCs, targeting of individual HER family members alone or in combination with other RTKs are currently...
under preclinical or clinical development. Specifically, cetuximab a monoclonal antibody targeting HER1, has been shown to play a beneficial role for patients with nonmelanoma skin cancers refractory to standard therapy (25, 26). Moreover, topical inhibition of HER2 in v-raf-transgenic mice before UV exposure resulted in a reduction in tumor size and tumor numbers (27). Similarly, dual inhibition of HER1 and HER2 effectively inhibited the promotion of skin tumors using a 2-stage carcinogenesis model (28). Finally, treatment of Colo16 skin SCC xenografts with the IGF-1R inhibitor A12 alone or in combination with cetuximab, resulted in a substantial inhibition of tumor growth (29). Altogether, these findings suggest that anti-sEcad mAb in combination with other RTK inhibitors or standard chemotherapeutic agents may be a plausible therapeutic modality as it downregulates multiple RTKs, thereby minimizing the compensatory upregulation of alternate RTK(s) that typically occurs with de novo or acquired resistance.

RTK signal through a common vertical mitogenic and prosurvival network that involves multiple crosstalk and feedback loops within the MAPK and PI3K/Akt/mTOR signaling pathways—thereby limiting therapeutic vulnerability and facilitating tumor cell resistance. To our knowledge, this is the first report to document an anti-sEcad mAb-induced downregulation of these MAPK and PI3K signaling pathways in skin SCC cells, including the downstream effectors Ras, MEK1/2, mTOR, and 4E-BP1. Why phosphorylation of Akt and ERK1/2 levels remained unchanged with antibody treatment needs further exploration, but may be related to timing or the existence of alternative activation pathways independent of PI3K or MEK (30, 31). Importantly, Ras is an upstream activator of the Raf/MEK1/2/ERK1/2 pathway and 21% of skin SCCs harbor activating Ras mutations (32, 33). Moreover, aberrant PI3K and Ras activation promotes key tumorigenic phenotypes that display enhanced mitogenesis, escape from apoptosis, and drug resistance (33, 34).

Although there is very little information on the role of MAPK or PI3K inhibitors in cutaneous SCC, combined EGFR and PI3K inhibition was reported to dramatically induce apoptosis in a panel of human skin SCC cells (33). PI3K inhibition was also shown to significantly block IGF-1-mediated epidermal proliferation and SCC promotion in DMBA-initiated BKSIGF-1 mice (35). More importantly, single-agent therapies targeting components of this axis, such as the mTOR inhibitor Rapamycin, were shown to effectively suppress the progression of cutaneous SCCs in preclinical mouse models (36, 37). Taken together, these findings have important clinical implications and raise the possibility that anti-sEcad mAb treatment may be a novel multitargeted approach for skin SCCs in which it downregulates two major downstream pro-oncogenic pathways known to promote skin SCC growth and survival.

The subcellular partitioning of many tumor suppressor proteins, including p53 and PTEN, is critical for their diverse biologic functions (38, 39). p53 is induced by a variety of proapoptotic stimuli, including growth factor withdrawal, irradiation, and chemotherapy. It is also a well-known transcription factor that transactivates a number of genes associated with various functions, including cell-cycle arrest and apoptosis (20). Mutations, involving gain-or loss-of-function of p53 are frequently seen in many cancers, including SCCs (40, 41). Autoregulation of p53 is conducted by MDM2, an E3 ubiquitin ligase, which is induced by nuclear p53 transcriptional activity. MDM2 translocates into the nucleus where it ubiquititates p53, resulting in its nuclear export and proapoptotic degradation (42, 43). Activation of PI3K/Akt prevents p53-dependent apoptosis by promoting the nuclear entry of MDM2 and inhibiting p53 transcriptional activity (44). In contrast, PTEN, also induced by nuclear p53, positively regulates p53 function by antagonizing PI3K, protects p53 from MDM2-mediated degradation, and promotes p53 transcriptional activity (42, 43, 45). Besides this nuclear function of p53, cytosolic p53 exerts transcription-independent biologic activities. For instance, cytoplasmic p53 induces apoptosis by promoting mitochondrial outer membrane permeabilization, thereby triggering the release of proapoptotic factors and activation of caspases and subsequent cleavage of PARP, one of the DNA-repairing enzymes (40, 46). In this study, anti-sEcad mAb induced an increase in total p53 levels, which was accompanied by an increase in active caspase-3 and cleaved PARP, and a decrease in nuclear PI3-Parp. Furthermore, 48-hour mAb treatment dramatically increased membrane, cytosolic, and nuclear PTEN levels, as well as cytoplasmic p53. In contrast, membrane and cytosolic pools of MDM2 were decreased following anti-sEcad administration. Moreover, we are the first to show enhanced PTEN and p53, concomitant with decreased MDM2 in anti-sEcad mAb-treated tumors in vivo and PAM212 cells in vitro. These findings suggest that one of the mechanisms mediating mAb-induced inhibition of cell proliferation and induction of apoptosis may be because of the positive regulation of p53 by PTEN, which then overrides the negative modulation of p53 by MDM2.

It is not surprising that mAb treatment upregulated PTEN while concomitantly decreased c-jun and NF-kB in our study as both c-jun and NF-kB have been demonstrated to inhibit PTEN transcription (43, 47, 48). Although the best known function of PTEN involves the dephosphorylation of PtdIns(3,4,5)P3 at the plasma membrane surface, cytoplasmic PTEN has been shown to inhibit cell proliferation (38). Moreover, nuclear PTEN has been proposed to regulate the cell cycle through the suppression of cyclin D1 activity (49). Indeed, cyclin D1 expression was found decreased with anti-sEcad mAb treatment in this study. Nuclear PTEN has also been demonstrated to act as a proapoptotic factor, suggesting an important nuclear function for PTEN in tumor suppression (50). Consistent with these findings, our immunofluorescence analysis revealed enhanced PTEN-p53 cytosolic and nuclear colocalization in sEcad antibody-treated cells. In contrast, MDM2 was restricted to the cytosol, whereas p53 was...
mainly localized in the nucleus, suggesting an escape of p53 from MDM2 mediated degradation. However, even though 16-hour sEcad mAb treatment enhanced the colocalization of MDM2 with p53, coimmunoprecipitation studies at the 48-hour time point did not demonstrate any significant association. This may be just related to timing or the sensitivity of the antibodies used in the specific assays because we also found a discrepancy between the presence of nuclear p53 expression by immunofluorescence and absence of nuclear p53 in Western blot analysis in mAb-treated PAM212 cells (the former was 16 hours and the latter was 48 hours after mAb administration). Nevertheless, these studies demonstrate that anti-sEcad mAb administration enhances membrane, cytosolic, and nuclear levels of PTEN, while simultaneously decreasing MDM2 levels and nuclear entry.

In conclusion, our results show that an antibody-based therapy against the extracellular domain of E-cadherin, which may bind cell surface bound E-cadherin, sEcad, or potentially sEcad–HER complexes, inhibits SCC tumor growth, promotes cell death, and inhibits prosurvival pathways, the latter by downregulating the RTK-MAF-Pi3K-IAP and modulating PTEN-p53 and MDM2 levels and subcellular distribution. Moreover, we show that anti-sEcad treatment exerts no untoward cytotoxicity to mice or end-organs at the dose and time period used in this study. These results suggest that anti-sEcad mAb therapy, either alone or in combination with standard chemotherapeutic agents may be an effective strategy for the treatment of cutaneous SCCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S.M. Brouxhon, S. Kyranides
Development of methodology: S.M. Brouxhon, L. Ma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Brouxhon, D. Trochesset, L. Ma
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): S.M. Brouxhon, S. Kyranides, D. Trochesset, J. Cohen, L. Ma
Writing, review, and/or revision of the manuscript: S.M. Brouxhon, D. Trochesset, J. Cohen, L. Ma
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Study supervision: S.M. Brouxhon
Other (design of experiments): L. Ma

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Ectodomain-Specific E-Cadherin Antibody Suppresses Skin SCC Growth and Reduces Tumor Grade: A Multitargeted Therapy Modulating RTKs and the PTEN–p53–MDM2 Axis

Sabine M. Brouxhon, Stephanos Kyrkanides, Veena Raja, et al.


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