RacGAP1 Is a Novel Downstream Effector of E2F7-Dependent Resistance to Doxorubicin and Is Prognostic for Overall Survival in Squamous Cell Carcinoma

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Abstract

We have previously shown that E2F7 contributes to drug resistance in head and neck squamous cell carcinoma (HNSCC) cells. Considering that dysregulation of responses to chemotherapy-induced cytotoxicity is one of the major reasons for treatment failure in HNSCC, identifying the downstream effectors that regulate E2F7-dependent sensitivity to chemotherapeutic agents may have direct clinical impact. We used transcriptomic profiling to identify candidate pathways that contribute to E2F7-dependent resistance to doxorubicin. We then manipulated the expression of the candidate pathway using overexpression and knockdown in in vitro and in vivo models of SCC to demonstrate causality. In addition, we examined the expression of E2F7 and RacGAP1 in a custom tissue microarray (TMA) generated from HNSCC patient samples. Transcriptomic profiling identified RacGAP1 as a potential mediator of E2F7-dependent drug resistance. We validated E2F7-dependent upregulation of RacGAP1 in doxorubicin-insensitive SCC25 cells. Extending this, we found that selective upregulation of RacGAP1 induced doxorubicin resistance in previously sensitive KJDSV40. Similarly, stable knockdown of RacGAP1 in insensitive SCC25 cells induced sensitivity to doxorubicin in vitro and in vivo. RacGAP1 expression was validated in a TMA, and we showed that HNSCCs that overexpress RacGAP1 are associated with a poorer patient overall survival. Furthermore, E2F7-induced doxorubicin resistance was mediated via RacGAP1-dependent activation of AKT. Finally, we show that SCC cells deficient in RacGAP1 grow slower and are sensitized to the cytotoxic actions of doxorubicin in vivo. These findings identify RacGAP1 overexpression as a novel prognostic marker of survival and a potential target to sensitize SCC to doxorubicin. Mol Cancer Ther; 14(8); 1939–50. ©2015 AACR.

Introduction

Cutaneous squamous cell carcinomas (CSCC) and head and neck SCC (HNSCC) are among the most common malignancies afflicting man (1, 2). Current treatment options for advanced SCC include adjuvant chemotherapy with platinum-based drugs, such as taxanes, 5-Fluorouracil, or therapeutic antibodies against EGFR (3, 4). However, the response is generally transient and characterized by the development of drug resistance. Thus, there is a need to identify new therapeutic strategies that can bypass the emergence of a drug-resistant phenotype.

The E2F transcription factor complex comprises a family of activating (E2F1, 2, 3a) or repressive/inhibitory (E2F3b, 4, 5, 6, 7, or 8) E2Fs that regulate key cellular functions, such as transcription, differentiation, and apoptosis. In the context of keratinocytes (KC), the E2F transcription factor family has been shown to control (i) proliferation, (ii) differentiation, (iii) stress responses, and (iv) apoptosis (5–10). Consistent with their roles in KCs, dysregulation of E2F1 is a common occurrence in SCC (11, 12), and overexpression of E2Fs, such as E2F1 and E2F7, occurs in the majority of CSCCs and HNSCCCs (12–15). E2F1 and E2F7 are known to have opposing actions in the regulation of proliferation (5), differentiation (14), and apoptosis (9, 14). For example, recent reports have shown that treatment of wild-type cells with DNA-damaging agents, such as doxorubicin or etoposide, induces E2F7 protein levels and subsequent inhibition of the E2F1-mediated DNA damage response (9, 10). In the context of KCs, E2F7 was shown to causally modify responses to conventional chemotherapeutics (15) and UV responses in vitro (14). Thus, sensitivity to common cytotoxic agents and stimuli appear to be regulated by the relative ratio of E2F1 to E2F7 in the tissue. Given that both E2F1 and E2F7 are known to be overexpressed in SCC (14, 15), it is reasonable to speculate that this may also...
contribute to drug resistance in SCC. In this regard, we recently showed that the sphingosine kinase 1 (Sphk1) gene is a direct target of E2F7 in SCC (15). E2F7-dependent overexpression of Sphk1 in SCC induces increased production of the antiapoptotic phospholipid, sphingosine-1-phosphate (S1P), which in turn invokes anthracycline resistance via activation of the PI3K/AKT pathway (15). Thus, E2F dysregulation in SCC induced the activation of a Sphk1/S1P-dependent drug-resistant phenotype (15). Identification of this novel pathway was noteworthy because anthracyclines such as doxorubicin are not in clinical use for the treatment of SCC, and thus the ability to sensitize SCCs to an existing class of chemotherapeutics would be of clinical value. However, the activation of the Sphk1 pathway was clearly only part of the explanation for the anthracycline resistance observed in SCC. Thus, other pathways that control drug resistance in SCC were likely to exist.

In the present study, we used transcriptomic profiling to identify a novel druggable E2F7/RacGAP1/AKT pathway that selectively induces anthracycline resistance in SCC.

Materials and Methods

Chemicals and viability assays

The following drugs were purchased: AZA1 (Millipore), doxorubicin (Sigma Aldrich), NSC23766 (Abcam), S1P (Cayman Chemicals), Y-27632 (Sigma Aldrich), and ZVAD-fmk (Alexis Biochemicals). BGT26 was provided by Novartis, and stocks of BGT226 were prepared as described (16). ZVAD-fmk was added 30 minutes before other treatments. Cell viability was performed by trypan blue exclusion or using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Tissue culture, adenovirus infection, and transfection

Murine epidermal keratinocytes (MEK) and human epidermal keratinocytes (HEK) were isolated and cultured as described (17, 18). E2F7 KO KCs were generated by ready-to-use adenovirus harboring Cre recombinase infection of murine epidermal keratinocytes as per the manufacturer’s recommendations (MOI of 50; Vector Bioslabs). Detroit562 and SCC25 cells were obtained from the American Type Culture Collection, and cultures were not passaged for longer than 6 months. SCC15 was a kind gift from Dr. Elizabeth Musgrove (Garvan Institute, New South Wales, Australia) and was verified by short tandem repeat (STR) genotyping (12). KIDSV40 cells were maintained as described previously (12) and were verified by STR genotyping. STR-verified cells were not passaged for longer than 6 months after verification. All cell lines were regularly tested and validated to be Mycoplasma-free. Control and overexpression plasmids used for manipulating E2F7 and the siRNA used for targeting E2F7 have been described previously (19). Only genes with a fold change of 1 (in either direction) or greater and a B value of greater than 3 (exceeding the 95% probability of differential expression) were considered to be differentially expressed and further analyzed. Differentially expressed probe sets were analyzed as pair-wise contrasts. Microarray data have been uploaded to Gene Expression Omnibus under the reference: GSE58074.

Colonizing assay

Known number of SCC cells was plated and allowed to grow for 15 days. Plates were fixed and stained with Coomassie Blue and counted as previously described (20). Colonizing efficiency was expressed as the total number of colonies/total number of cells plated × 100.

DNA synthesis

DNA synthesis was measured using a colorimetric ELISA 5-bromo-2-deoxyuridine (BrdUrd) incorporation assay (Roche Diagnostics) in accordance with the manufacturer’s instructions.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology) in accordance with the manufacturer’s instructions. ChIP enrichment was determined by conducting qRT-PCR as described above. The primers used were as follows: 5'-TCTATATGACACAGGCGGGGG-3' (RacGAP1 Forward); 5'-TCCATCTTTAGGCACGTCCTT-3' (RacGAP1 Reverse).

Immunoblot

Immunoblotting was carried out according to previously published procedures (21) using the following primary antibodies: Anti-RacGAP1 (EP9018; 1:2,000; Abcam), Anti-Sphk1 (1:1,000; Sigma Aldrich), PARP (1:1,000; Cell Signaling Technology), phospho-Akt (Ser473; D9E; XP; 1:2,000; Cell Signaling Technology), Akt (1:2,000; Cell Signaling Technology), phospho-p44/42 MAPK (Erk1/2; Th202/Tyr204; E10; 1:2,000; Cell Signaling Technology), ERK 1 (C-16; 1:2,000; Santa Cruz), and β-actin (1:10,000; Sigma Aldrich).

Immunohistochemistry

Immunohistochemistry was carried out according to previously published procedures (21, 22) using the following primary antibodies: Anti-PCNA (1:3,000; Sigma Aldrich), Anti-RacGAP1 (EP9018; 1:100; Abcam), cleaved caspase-3 (Asp 175; 1:50; Cell Signaling Technology), and phospho-Akt (Ser473; D9E; XP; 1:50; Cell Signaling Technology). Secondary antibody was Starr Trek Universal HRP Detection System (Biocare Medical) followed by colorimetric immunohistochemical staining with Cardassian DAB Chromogen as per the manufacturer’s instructions (Biocare Medical).
Tissue microarrays
Generation and composition of the patient tissue microarrays (TMA) have been previously described (15). Immunohistochemistry was conducted using a Dako EnVision + System-HRP (DAB) kit in accordance with the manufacturer’s instructions (DAKO). Sections were incubated with Anti-E2F7 (1:250; Abcam) and Anti-RacGAP1 (EPR9018; 1:100; Abcam) antibodies. Staining intensity was evaluated by two pathologists in a blinded fashion using a modified quickscore method as described (23).

Determination of RhoA and Rac1 activity
RhoA and Rac1 activities were measured with RhoA/Rac1/Cdc42 activation assay combo biochem kit (Cytoskeleton) in accordance with the manufacturer’s instructions.

Animal studies
All animal experiments were approved by the Institutional Animal Ethics Committee. In vivo tumor studies used 6-week-old female nonobese diabetic/severe combined immunodeficient mice. Mice were injected subcutaneously on the flank with 2 × 10^6 cells. Groups of 6 mice received treatments (intraperitoneal injections twice/week) when tumors were around 4 mm in diameter. Animal weight and tumor growth were monitored for a period of up to 3 weeks, and animals were sacrificed when tumors reached 10 mm in diameter.

Statistical analysis
Statistical significance was calculated by a Student t test with a 95% confidence level using GraphPad Prism v5 (GraphPad software).

Results
RacGAP1 is a novel downstream effector of E2F7
We generated E2F7 knockdown (KD) murine KCs via adenovirus-mediated Cre deletion of floxed sequences in primary KCs isolated from E2F7-floxed mice (15). KD KCs were grown for 48 hours with increasing concentrations of doxorubicin (0–1 μmol/L), etoposide (0–100 μmol/L), and cisplatin (0–20 μmol/L). Figures 1A–C show that E2F7 deficiency sensitizes KCs to doxorubicin, modestly to cisplatin, but not at all to etoposide. These data suggest that E2F7-mediated doxorubicin resistance is not attributable to topoisomerase inhibition because etoposide sensitivity was not modified by E2F7. Moreover, pan-caspase inhibition significantly protected E2F7-deficient cells from doxorubicin-induced cytotoxicity (Fig. 1D), indicating that apoptotic pathways are being activated.

We undertook a screen of doxorubicin sensitivity in HEKs and 4 SCC cell lines. The KJDSV40 cell line exhibited the highest sensitivity to doxorubicin (IC50 of 0.082 μmol/L; Fig. 1E), whereas SCC25 cells displayed the least sensitivity (IC50 of 0.55 μmol/L; Fig. 1E) and HEKs displayed intermediate sensitivity (IC50 of 0.29 μmol/L; Fig. 1E). We have previously shown that the insensitive SCC25 cell line expresses high levels of E2F7, whereas the sensitive KJDSV40 cell line expresses low levels of E2F7 (15). Based on these data, we selected the sensitive KJDSV40 cell line and the insensitive SCC25 cell line for transcriptomic profiling.

Specifically, we generated a list of genes that were poorly expressed in KJDSV40 cells and highly expressed in SCC25 (Fig. 2A). We also generated a second list of genes that were differentially regulated in SCC25 cells in which E2F7 had been silenced with siRNA (Fig. 2A). We then used these two lists to identify those transcripts (referred to as List A; Supplementary Table S1) that displayed E2F7-dependent expression between the SCC25 cell lines (Fig. 2A). We also generated an additional list of transcripts for SCC25 cells or SCC25 cells in which E2F7 is silenced by siRNAs that have been treated with 1 μmol/L of doxorubicin. The transcripts that were found to be E2F7-dependent in the context of doxorubicin-treated SCC25 cells were then referred to as List B (Fig. 2A; Supplementary Table S1). By combining Lists A and B, we identified RacGAP1 as the most differentially overexpressed genes with a B value of 17 (Supplementary Table S1).

RacGAP1 (also known as MgcRacGAP and CYK4) is an evolutionarily conserved GTPase activating protein (GAP) that displays activity toward the Rho family of GTPases. The Rho family of GTPases is a subfamily of the Ras superfamily and consists of small signaling proteins: Rho (A, B, and C isoforms), Rac (1,2,3 isoforms and RhoG), and Cdc42 (Cdc42, Tc10, TCL, Chp/Wrch-2, and Wrch-1 ref. 24), which function as molecular switches between a GDP-loaded "ON" and a GTP-loaded "OFF" state (24). Thus, RacGAP1 has the potential to regulate a diverse array of cellular functions through its central role as a regulator of the activation state of the Rho family of GTPases. In particular, RacGAP1 is known to play important roles in the completion of cytokinesis (25), cell transformation, motility, migration, and metastasis (26–29). RacGAP1 is also involved in IL6-induced macrophage differentiation (30) and nuclear transport of STAT3/5 transcription factors (31). However, a role for RacGAP1 in SCC or doxorubicin sensitivity has not been shown previously.

Quantitative RT-PCR and Western blotting were used to confirm that RacGAP1 was more highly expressed in SCC25 (doxorubicin insensitive) cells than in KJDSV40 (doxorubicin sensitive) cells (Fig. 2B and C). Similarly, we showed that knockdown of E2F7 by siRNA in SCC25 cells caused a reduction in RacGAP1 mRNA (Fig. 2D) and protein level (Fig. 2E). Conversely, overexpression of E2F7 in KJDSV40 cells resulted in elevated levels of RacGAP1 transcript (Fig. 2F) as well as RacGAP1 protein (Fig. 2G). These data suggest that RacGAP1 is a downstream target of E2F7 in SCC cells. Supporting this, ChIP analysis of E2F7 binding showed that E2F7 could bind the RacGAP1 promoter, suggesting that RacGAP1 is a direct transcriptional target of E2F7 (Fig. 2H). This is the first report to show that RacGAP1 is a downstream effector of E2F7.

RacGAP1 expression is elevated in SCCs
We examined RacGAP1 expression levels by immunohistochemistry using a TMA consisting of 35 paired normal, primary tumor, and matched metastasis from HNSCC patients treated at the Princess Alexandra Hospital (PAH). The TMAs were stained for E2F7 and RacGAP1 protein expression and scored blinded by two pathologists. All matched adjacent “normal” epithelia demonstrated either negative or weak staining for RacGAP1, which was predominantly nuclear in location (Fig. 3A). Conversely, moderate to high levels of RacGAP1 expression were consistently recorded for the primary tumor (Fig. 3B) and its matched lymph node metastasis (Fig. 3C). The tumor epithelial cells showed nuclear and cytosolic expression for RacGAP1. RacGAP1 was significantly overexpressed in 73% of primary and metastatic human SCCs compared with matched adjacent normal tissue (P < 0.0001; Fig. 3D). In addition, our analyses showed that E2F7 expression is significantly upregulated in HNSCC compared with matched adjacent normal tissue (P < 0.0001; Fig. 3D). The

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Kaplan–Meier analysis revealed an inverse relationship between RacGAP1 expression levels and progression-free survival (PFS) of HNSCC patients studied over a period of 42 months whose samples were arrayed on the TMA (Fig. 3E). These data show, for the first time, that RacGAP1 is overexpressed in HNSCC and is associated with a poorer PFS.

RacGAP1 expression/activity determines sensitivity to doxorubicin

We examined whether the overexpression of RacGAP1 in the SCC cell lines was reflected in alterations of the GTP loading (activation status) of the model targets RhoA and Rac1. Specifically, RhoA GTP loading was constitutively higher in E2F7-deficient murine KCs, which express very low levels of E2F7 and RacGAP1, compared with SCC25 cells, which express high levels of E2F7 and RacGAP1 (Fig. 5A). In contrast, GTP loading of Rac1 was higher in SCC25 cells when compared with KJDSV40 cells (Fig. 5B), and the GTP loading of Rac1 was significantly reduced in SCC25 cells following RacGAP1 knockdown (Fig. 5B). Finally, knockdown of RacGAP1 in SCC25 cells resulted in an increase in the GTP loading of RhoA (Fig. 5A). These data suggest that E2F/RacGAP1-dependent resistance to doxorubicin may be mediated by activated Rac1.
or inactivated RhoA. To determine which, if any, of these possibilities may apply, we coincubated SCC25 cells with doxorubicin +/− an established selective Rho/ROCK1 inhibitor (10 μmol/L Y-27632; ref. 33), an established Rac1/Cdc42 inhibitor (10 μmol/L AZA1; ref. 34), or a Rac1-selective inhibitor (25 μmol/L NSC23766; ref. 35) for 48 hours and established viability (Fig. 5C). These experiments showed that doxorubicin resistance was unaltered following incubation with a Rho/ROCK1 inhibitor and was moderately reduced following incubation with the Rac1/cdc42 inhibitor (Fig. 5C). In contrast, treatment of SCC25 cells with the Rac1-selective inhibitor significantly increased sensitivity of SCC25 cells to doxorubicin (Fig. 5C). These results indicate a number of important points. Firstly, RhoA appears to be a preferred substrate for RacGAP1 in SCC cells. This is reflected by the high level of RhoA-GTP loading compared with Rac1-GTP loading as well as the increase in GTP loading observed following RacGAP1 knockdown in the SCC25 cells. Secondly, although Rac1-GTP loading behavior is not indicative of it being a preferred substrate of RacGAP1, it is clear that alterations in RacGAP1 activity modify Rac1-GTP loading. Thirdly, use of a Rac1-selective inhibitor phenocopies the inhibition of doxorubicin resistance observed with RacGAP1 knockdown (Fig. 4E). Finally, the preference for RhoA by RacGAP1, in SCC cells, is consistent with a previous report showing that the conventional preference for Rac1 can be switched to RhoA following phosphorylation of the Serine 387 site of RacGAP1 by Aurora B kinase (36, 37).
RacGAP1 modulates doxorubicin sensitivity via downstream activation of the PI3K/AKT pathway.

There is an existing literature showing that the PI3K/AKT pathway is an important component of RacGAP1 signaling (38). However, whether PI3K/AKT signaling lies upstream or downstream of the Rho family of GTPases remains less clear and appears to be context-specific (38). Dysregulation of the PI3K/AKT pathway is a common event in HNSCC, which can be attributed to multiple factors, such as mutations, amplifications, and signal-induced activation of the pathway (39). For example, we recently showed that E2F7 overexpression or knockdown caused an increase and decrease in p-AKT levels in SCC cells respectively (15). Since RacGAP1 is a downstream effector of E2F7 in SCC cells, we examined whether RacGAP1 could modify the PI3K/AKT signaling pathway in SCC cells. In the first instance, we noted that knockdown of RacGAP1 in SCC25 cells had no impact on the activation status of the ERK pathway (Fig. 5D). In contrast, RacGAP1 knockdown in SCC25 cells significantly reduced the level of p-AKT (Fig. 5E), whereas RacGAP1 overexpression in KIDSV40 cells increased p-AKT levels (Fig. 5F). We had previously shown that the PI3K/mTOR inhibitor, BGT226, was able to ablate AKT signaling and induce apoptosis in SCC cell lines (16). We compared the sensitivity of SCC25 cells with BGT226 in SCC25 cells or SCC25 cells in which RacGAP1 was knocked down. Figure 5G indicates that knockdown of RacGAP1 is able to reduce SCC25 cell viability to 70% that of control cells. Similarly, inhibition of PI3K activity using a dose of BGT226 known to induce maximal inhibition (16) reduced SCC25 cell viability to approximately 50% (Fig. 5G). Finally, exposure of RacGAP1-deficient SCC25 cells to BGT226 resulted in a further decrease in viability to below 20% (Fig. 5G). These data indicate that RacGAP1 participates in AKT-dependent and AKT-independent events.

We recently reported that E2F7 is able to directly activate the Sphk1/SIP axis in SCC cells, which induces doxorubicin resistance (15). It is also interesting to note that both the E2F7/RacGAP1 pathway identified in this study and the E2F7/Sphk1/SIP pathway (15) induced doxorubicin resistance and converged on the AKT pathway. Therefore, we examined whether the Sphk1 and RacGAP1 pathways may interact with one another. Figure 5H shows that knockdown of Sphk1 can induce loss of RacGAP1 mRNA, whereas knockdown of RacGAP1 induces loss of Sphk1 mRNA expression. Although the mechanism controlling this
feedback is unknown, it is clear that targeted inhibition of either the RacGAP1 pathway or the Sphk1 pathway is likely to impact one another. To illustrate this point, knockdown of RacGAP1 or Sphk1 in SCC25 cells results in reduced p-AKT levels (Fig. 5I) and increased sensitivity to doxorubicin (Fig. 5J), which can be reversed by the addition of exogenous S1P (Fig. 5I and J).

RacGAP1 suppression enhances sensitivity of SCC25 to doxorubicin in vivo

SCC25 cells were generated to stably express either vector control or RacGAP1 shRNA and inoculated into NOD/SCID mice. When tumors were around 4 mm in diameter, mice were randomized into four groups and treated with vehicle, dimethyl sulfoxide (DMSO), or 0.5 mg/kg doxorubicin by i.p. injections twice per week. RacGAP1 knockdown was confirmed by Western blotting immediately before the inoculation of SCC25 cells (Fig. 6A). Treatment with doxorubicin was well tolerated by the NOD/SCID mice, and the body weights remained stable throughout the study (Fig. 6A). RacGAP1-deficient cells showed reduced tumor growth rate (Fig. 6B). Treatment of mice bearing vector control SCC25 tumors with/without 0.5 mg/kg doxorubicin had minimal effect on tumor growth rates (Fig. 6B). However,

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Figure 5.
Alterations in RacGAP1 activity modify Rac1-GTP loading, and RacGAP1 lies upstream of AKT. Rhotekin-binding domain (RBD; A) binding and p21 activated kinase I-binding domain (PAK-PBD; B) assays were performed on the extracts from KJDSV40, SCC25, and SCC25 cells in which RacGAP1 had been silenced by shRNA, as indicated. The amount of activated or total RhoA and Rac1 was detected by immunoblotting the RBD and PAK-PBD samples and the whole cell lysate with RhoA or Rac1 antibodies. To confirm equal input, the membrane was reprobed with an actin antibody. C, SCC cells were treated with increasing doses of doxorubicin in combination with 10 μmol/L AZA1 or 10 μmol/L NSC23766 or 25 μmol/L NSC23766 for 48 hours. Viability was then assessed and plotted as percentage doxorubicin only treated. SCC25 cells were transfected with the RacGAP1 shRNA or a scrambled shRNA construct. D, after 48 hours p-ERK and total ERK protein levels and (E) p-AKT and total AKT protein levels were determined by immunoblotting. F, p-AKT and total AKT protein levels are shown for KJDSV40 cells in which RacGAP1 was overexpressed. G, RacGAP1 shRNA and control shRNA–transfected SCC25 cells were treated with BGT226 (300 nmol/L) for 48 hours and viability estimated by trypan blue exclusion and plotted as percentage control shRNA. H, SCC25 cells were transfected with Sphk1 shRNA (left) and RacGAP1 shRNA (right) as well as control shRNA. After 48 hours, RacGAP1 (left) and Sphk1 mRNA (right) levels were determined by qRT-PCR. I, SCC25 cells were transfected with RacGAP1 or Sphk1 shRNAs. Forty-eight hours after transfection, cells were treated with 1 μmol/L S1P for 24 hours. p-AKT and total AKT protein levels were then determined by immunoblotting. J, SCC25 and SCC25 in which RacGAP1 or Sphk1 had been silenced were treated with 1 μmol/L doxorubicin and 1 μmol/L S1P for 24 hours. Viability was then assessed and plotted as percentage doxorubicin-only treated. Western blot figures are representative of three independent experiments. All quantitative data presented as mean ± SEM obtained from triplicate determinations of three independent experiments for C, G, and J. Data are the mean ± SEM of duplicate determinants normalized for expression of the housekeeping gene TBP for H; n = 3. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure 6.
RacGAP1 suppression enhanced sensitivity of SCC25 to the cytotoxic actions of doxorubicin in vivo. A, on the day of subcutaneous injections, RacGAP1 deficiency was confirmed by immunoblotting using protein extracts from SCC25 cells in which RacGAP1 had stably silenced with shRNA. β-Actin is provided as a loading control. All animals were inoculated subcutaneously with 2 × 10^6 SCC25 cells expressing vector alone (scrambled shRNA) or Sphk1 shRNA and tumors allowed to establish until they reached the indicated sizes. Established tumors were treated with vehicle or 0.5 mg/kg doxorubicin twice per week. A, animal weight was determined twice per week. B, tumor volumes were monitored twice weekly. The inset includes vehicle or 0.5 mg/kg doxorubicin-treated SCC tumors harboring RacGAP1shRNA. C, after 13 days of treatment, animals were sacrificed and tumors excised. Representative results from distinct groups are shown. +, tumors formed from control shRNA-transfected SCC25 cells; −, tumors formed from RacGAP1shRNA-transfected SCC25 cells. D, immunostaining for RacGAP1, PCNA, cleaved caspase-3, and p-AKT or normal Rabbit IgG as a negative control. Representative images of at least three independent tumors are shown for each group (Bar, 100 μm). Data presented as mean ± SEM of individual measurements from 6 mice per group. E, schematic model showing how disruption of the novel E2F/RacGAP1/Rac1/AKT pathway can lead to doxorubicin resistance in SCC.
RacGAP1-deficient SCC25 tumors treated with doxorubicin started to regress by day 4 after treatment, which continued for a further 7 days at which time all mice were sacrificed due to the tumor burden in control mice. The subcutaneous tumors were excised, photographed, and examined histologically (Fig. 6C).

Immunohistochemical examination of the excised tumors showed that knockdown of RacGAP1 was maintained throughout the study (Fig. 6D). Tumors from vehicle and doxorubicin-treated control mice stained strongly for PCNA, indicating a higher proportion of proliferating cells in control tumors compared with RacGAP1-deficient tumors (treated or untreated; Fig. 6D). In contrast, doxorubicin induced higher apoptosis indices in tumors derived from SCC25/RacGAP1shRNA than in the SCC25/vector control as estimated by immunostaining for cleaved caspase-3 (Fig. 6D). Ser473 p-AKT levels in RacGAP1-deficient cells were also decreased (Fig. 6D). Collectively, these results suggest that RacGAP1 contributes to the growth of HNSCC in vivo and that targeted inhibition of RacGAP1-overexpressing tumors may sensitize them to the cytotoxic actions of doxorubicin.

Discussion

This is the first study to identify an E2F7/RacGAP1/AKT axis through which SCC cells acquire resistance to doxorubicin (Fig. 6E). Specifically, we show that (i) RacGAP1 is a novel downstream effector of E2F7, (ii) RacGAP1 is overexpressed in patient SCC and is associated with poor PFS, (iii) RacGAP1 overexpression is associated with inactivation of RhoA and activation of Rac1, (iv) pharmacologic inhibition of Rac1, but not RhoA, reinstates doxorubicin sensitivity similar to that observed following RacGAP1 knockdown in SCC cells, and (v) E2F7-dependent doxorubicin resistance is mediated via induction of RacGAP1/Rac1 and Sphk1, which in turn activates AKT-dependent and AKT-independent pathways in vitro and in vivo.

The E2F transcription factor family is involved in a diverse array of cellular functions that are controlled by the relative ratio of atypical E2F (e.g., E2F7) to activating E2F (e.g., E2F1). For example, the apoptotic actions of E2F1, in SCC cells, can be antagonized by E2F7 overexpression (14). Similarly, E2F7 inhibits doxorubicin-induced cytotoxicity by inducing the expression of Sphk1 resulting in increased levels of S1P which enhance the Ser473 p-AKT–dependent prosurvival response (15). These data are of particular relevance because we know that the majority of human SCCs express high levels of both E2F1 and E2F7. In the present study, we found that high levels of RacGAP1 in advanced SCC patients were associated with a poor PFS. Moreover, we demonstrated that forced overexpression of E2F7 was able to induce RacGAP1 overexpression and doxorubicin resistance, whereas knockdown of E2F7 reduced RacGAP1 expression and induced sensitivity to doxorubicin in vitro and in vivo. These data indicate that RacGAP1 is a direct downstream transcriptional target of E2F7. The RacGAP1 promoter contains E2F-binding sites, and E2F activation has been reported to be required for the initiation of transcription at the RacGAP1 promoter in human lymphocyte cell line (40). Consistent with this, we showed that elevated E2F7 levels in SCC are associated with increased binding of E2F7 to the RacGAP1 promoter and increased expression of RacGAP1 in SCCs. E2F7 is traditionally considered to be a transcriptional repressor; however, it has also been shown that E2F7 can function as a direct transcriptional activator of the VEGFA promoter via the formation of an E2F7–HIF1α transcriptional complex (41). Similarly, E2F7 has been shown to bind the Sphk1 promoter in SCC cells and is associated with increased Sphk1 transcription (15). The precise mechanism by which E2F7 regulates the transcription of RacGAP1 and Sphk1 is currently under examination. Regardless of the mechanism, our functional data show that E2F7 regulates doxorubicin-induced cytotoxicity via transcriptional induction of RacGAP1.

To our knowledge, this is the first report showing overexpression of RacGAP1 in HNSCC tumor samples. This is also the first report to show that overexpression of tumor-associated RacGAP1 is directly controlled by E2F7, which itself is known to be overexpressed in SCC and to induce drug resistance (15). Overexpression of RacGAP1 has been reported in high-grade meningiomas, non–small cell lung cancer, gastric cancer, hepatocellular carcinoma, breast cancer (24, 38, 42–44) as well as in the more aggressive tumor phenotypes of epithelial ovarian cancer, high-grade breast cancer, and invasive cervical cancer (45–47). However, it is unknown whether the overexpression of RacGAP1 in these tumors is linked to overexpression of E2F7. Although the overexpression of RacGAP1 is not considered to be simply a "passenger" in other cancer types (26–29), its contribution to HNSCC is unknown. Our data show that loss of RacGAP1 expression is able to reduce SCC growth in a xenotransplant model via inhibition of proliferation and increased basal apoptosis. In addition, we show that high levels of expression are associated with poor PFS of HNSCC patients. Thus, our clinical and preclinical data would suggest that E2F7–dependent overexpression of RacGAP1 is likely to be a driver of tumor growth and drug resistance in HNSCC.

The functional consequences of RacGAP1 overexpression are not reflective of a generalized loss of GTP loading of the Rho/Rac family of GTPases. Our data indicated that RacGAP1 favored the conversion of RhoGTP to RhoGDP in HNSCC cells. However, it was clear that the GTP-loading status of Rac1 was also responsive to changes in RacGAP1 expression. For example, RacGAP1 appeared to negatively regulate the GTP-loading status of Rac1 such that knockdown of RacGAP1 resulted in a reduction in the GTP-loading state of Rac1. Although this seemed counterintuitive, it has been shown that phosphorylation of RacGAP1, by Aurora B kinase, on Serine 387 shifts its GAP activity from Rac to Rho, resulting in increased GTP loading (activation) of Rac1 and reduced GTP loading of Rho (inactivation; ref. 36). Significantly, we show that incubation of doxorubicin-resistant SCC cells with a selective Rac1 inhibitor was able to reinstate doxorubicin sensitivity, whereas incubation with a RhoA/ROCK1 selective inhibitor did not. These data suggest that the resistance to doxorubicin correlates with the GTP-loaded "active" state of Rac1. This conclusion is supported by a previous study showing that the antiapoptotic protein, Bcl-2, interacts with Rac1 to protect tumor cells from the cytotoxic actions of etoposide and daunorubicin (48). Moreover, our conclusion is also consistent with a previous report suggesting that Rac1 was a potential therapeutic target in chemoradioresistant HNSCC (49). Thus, the E2F7–dependent drug-resistant phenotype we observed in SCC cells may result from an Aurora-B kinase–mediated switch of RacGAP1 activity from Rac1 to RhoA leading to Rac1–dependent activation of AKT-mediated doxorubicin resistance.

There is an existing literature on the role of PI3K/AKT in controlling RacGAP1 activity and the GTP loading of the Rho family of GTPases. In particular, it has been shown that AKT directly binds to and activates RacGAP1 activity via
phosphorylation of T249 (50). In the present study, we show that E2F7 induces RacGAP1 expression, which is associated with (i) an increase in activated Rac1, (ii) a decrease in RhoA activity, (iii) an increase in Ser473 p-AKT, and (iv) resistance to doxorubicin. These findings are consistent with the observation that PI3K/AKT signaling is among the most significantly altered canonical pathways following RacGAP1 silencing in HCC (38). Our observations also suggest that the overexpression of RacGAP1 in SCC may contribute to the activation of the AKT pathway that is seen in more than 40% of all HNSCCs. However, how RacGAP1 contributes to AKT activation remains unclear. We certainly know that overexpressing or knocking down E2F7 or RacGAP1 modifies Ser473 phosphorylation of AKT. However, we also know that E2F7 directly suppresses Spk1 expression and S1P levels in SCC, leading to increased Ser473 phosphorylation of AKT (15). Finally, we now show that Spk1 and RacGAP1 indirectly modify another cell’s expression. Thus, it is difficult to determine whether RacGAP1-dependent effects on AKT phosphorylation status are modified by RacGAP1 or indirectly via changes in Spk1/S1P. Regardless of the mechanism, our results demonstrate the existence of a novel, complex, and interdependent network between E2F7, RacGAP1, and Spk1/S1P, and the importance of such a network in chemosensitivity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Hazar-Re thinam, S. Boros, M. Dze nis, D.D.E. Dantzig
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