A Novel E2F/Sphingosine Kinase 1 Axis Regulates Anthracycline Response in Squamous Cell Carcinoma

Mehlika Hazar-Rethinam1, Lilia Merida de Long1, Orla M. Gannon1, Eleni Topkas1, Samuel Boros2, Ana Cristina Vargas2, Marcin Dzioniś3, Pamela Mukhopadhyay1, Fiona Simpson1, Liliana Endo-Munoz2, and Nicholas A. Saunders1

Abstract

Purpose: Head and neck squamous cell carcinomas (HNSCC) are frequently drug resistant and have a mortality rate of 45%. We have previously shown that E2F7 may contribute to drug resistance in SCC cells. However, the mechanism and pathways involved remain unknown.

Experimental Design: We used transcriptomic profiling to identify candidate pathways that may contribute to E2F7-dependent resistance to anthracyclines. We then manipulated the activity/expression of the candidate pathway using overexpression, knockdown, and pharmacological inhibitors in in vitro and in vivo models of SCC to demonstrate causality. In addition, we examined the expression of E2F7 and a downstream effector in a tissue microarray (TMA) generated from HNSCC patient samples.

Results: E2F7-deficient keratinocytes were selectively sensitive to doxorubicin and this was reversed by overexpressing E2F7. Transcriptomic profiling identified Sphingosine kinase 1 (Sphk1) as a potential mediator of E2F7-dependent drug resistance. Knockdown and overexpression studies revealed that Sphk1 was a downstream target of E2F7. TMA studies showed that E2F7 overexpression correlated with Sphk1 overexpression in human HNSCC. Moreover, inhibition of Sphk1 by shRNA or the Sphk1-specific inhibitor, SKI-1 (BML-E1411), enhanced the sensitivity of SCC cells to doxorubicin in vitro and in vivo. Furthermore, E2F7-induced doxorubicin resistance was mediated via Sphk1-dependent activation of AKT in vitro and in vivo.

Conclusion: We identify a novel druggable pathway in which E2F7 directly increases the transcription and activity of the Sphk1/S1P axis resulting in activation of AKT and subsequent drug resistance. Collectively, this novel combinatorial therapy can potentially be trialed in humans using existing agents. Clin Cancer Res; 21(2); 1–11. ©2014 AACR.

Introduction

Head and neck squamous cell carcinomas (HNSCC) arise from stratified squamous epithelia of the mucosa of the upper aerodigestive tract. At present, the mainstay of treatment for advanced HNSCC is surgery and/or radiotherapy plus adjuvant chemotherapy (1). The use of adjuvant chemotherapy provides modest improvements to overall survival but are not considered curative in their own right (1). Thus, if we are to improve outcomes in patients with advanced HNSCC, we need to develop systemic therapies that target novel pathways activated in HNSCC cells.

HNSCC is a complex cancer associated with a large mutational burden (2, 3) and accompanied by dysregulation of proliferation, differentiation, and apoptosis. HNSCC is also accompanied by dysregulation of the main functions of the E2F transcription factor family (4, 5). E2F refers to a family of 10 gene products from eight members of the E2F family with the exception of E2F6 (9, 10). It has been shown that normal human and murine keratinocytes express all proteins and HDACs (7, 8). In the context of keratinocytes, it has been shown that proliferation and differentiation of keratinocytes is regulated by the opposing actions of E2F1 and E2F7 (4, 9, 11, 12). Significantly, E2F1 and E2F7 are overexpressed in patient SCCs (10) and contribute to the development of cutaneous SCC (13, 14).

In addition to the role of E2Fs in proliferation and differentiation, E2Fs are key regulators of apoptosis and stress responses (7, 8). For example, E2F1 has been shown to have potent
Translation Relevance

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent cancers diagnosed worldwide. Current chemotherapies are not considered a curative option for HNSCC. Thus, there is a need for new and selective therapies. In this regard, the E2F family of transcription factors has been shown to contribute to the development and maintenance of HNSCC. However, E2F-based therapies are currently not available. To circumvent this problem, we embarked on a transcriptomics screen to identify factors that were responsible for E2F7-dependent resistance to anthracyclines in HNSCC. The present study demonstrates that E2F7 directly controls the expression of sphingosine kinase 1 (Sphk1) resulting in increases in AKT phosphorylation, which drives drug resistance. Thus, we have identified a previously undescribed E2F7/Sphk1/AKT axis that contributes to anthracycline resistance in HNSCC. A significant implication of this finding is that combining an anthracycline with a Sphk1 inhibitor may provide a curative option for treating HNSCC.

Materials and Methods

Animal studies

All animal experiments were approved by the Institutional Animal Ethics Committee. E2F7Flox/Flox, E2F8Flox/Flox, and E2F8 KO mice have been described previously (15, 18). FVB × C57BL/6 crosses were generated in house. In vivo tumor studies used female NOD/SCID.

Reagents and viability assays

The following drugs were purchased: doxorubicin (Sigma-Aldrich), SKI-1 (BML-E1411; Enzo Life Sciences), sphingosine-1-phosphate (S1P; Cayman Chemicals). Stocks of BGT226 were prepared as described previously (19). Viability was determined using trypan blue, Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega), or Western blot analysis for cleaved caspase-3 or PARP cleavage as described previously (20, 21). Sphk1 activity and S1P levels were estimated using commercially available kits (Echelon Biosciences).

Tissue culture and adenovirus infection

Murine epidermal keratinocytes (MEK) and human epidermal keratinocytes (HEK) were isolated and cultured as described previously (22, 23). SCC25 cells were obtained from the ATCC. FaDu cells were a kind gift from Dr. Elizabeth Musgrove (Garvan Institute, New South Wales, Australia) and were verified by short tandem repeat genotyping (11). KIDSV40 cells were maintained as described previously (11). To generate E2F7 and E2F8 KD keratinocytes, we incubated MEKs with ready-to-use Ad-CMV-Cre as per the manufacturer’s recommendations (MOI of 50; Vector Biolabs).

Gene-expression studies

Total RNA was isolated, cDNA prepared, and quantitative reverse transcriptase PCR (qRT-PCR) performed as described previously (10, 24). For microarray analysis, complementary RNA was generated with the Illumina TotalPrep RNA Amplification Kit and hybridized with Illumina HumanHT-12 v4 Expression BeadChips (Illumina) as per the manufacturer’s protocol. Expression data from the microarrays were analyzed as previously described (25). The microarray data reported in this article have been deposited in the NCBI’s Gene Expression Omnibus (GEO) database under the accession number GSE58074. Chromatin immunoprecipitation (ChIP) was conducted using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology) in accordance with the manufacturer’s instructions.

shRNA studies, siRNA delivery, and transfections

Control and overexpression plasmids and siRNAs used for manipulating E2F7 have been described previously (10, 17). SureSilencing shRNA plasmids directed against Sphk1 were purchased from SuperArray Bioscience Corp. An Sphk1 expression (TrueORF Gold Clones) and control plasmids were purchased from OriGene Technologies.

Immunoblot

The following primary antibodies were used: E2F-1 (C-20) 1:1,000 (Santa Cruz Biotechnology), anti-E2F7 1:2,000 (Abcam), cleaved caspase-3 (Asp 175) 1:1,000 (Cell Signaling Technology), anti-Sphk1 1:1,000 (Sigma), 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), and β-actin 1:10,000 (Sigma-Aldrich). Where a Western blot analysis has been quantitated, results represent relative protein levels normalized to β-actin as quantified by Image J (Wayne Rasband, NIH).

Immunohistochemistry and tissue microarrays

Immunohistochemistry was conducted as described previously (20, 21). The following primary antibodies were used: PCNA 1:3,000 (Sigma-Aldrich), cleaved caspase-3 (Asp 175) 1:50 (Cell Signaling Technology), phospho-Akt (Ser473; D9E) 1:50 (Cell Signaling Technology). Secondary antibody was the Starr Trek Universal HRP Detection System followed by colorimetric immunohistochemical staining with Cardassian DAB Chromagen as per the manufacturer’s instructions (Biocare Medical). TMAs were generated using duplicate 1-mm cores of matched (i) adjacent normal tissue, (ii) primary HNSCC lesion, and (iii) matched metastatic lymph node from patients treated for HNSCC at the Princess Alexandria Hospital (PAH; Queensland, Australia). Immunohistochemistry was conducted using the Dako EnVision

Published OnlineFirst November 19, 2014; DOI: 10.1158/1078-0432.CCR-14-1962
Results

E2F7 selectively regulates cytotoxic responses to doxorubicin in keratinocytes

To examine the downstream pathways involved, we generated primary cultures of MEKs from E2F1 KO mice (15), or from E2f7Fl/fl or E2f8Fl/fl mice (18). We generated E2F7 and E2F8 knockdown (KD) MEKs via adenosine (Ad)-mediated Cre deletion of floxed sequences in primary keratinocytes isolated from E2f7- and E2f8-floxed mice. E2f1 gene-expression levels in keratinocytes isolated from conventional E2f1 KO mice were reduced by 70%, whereas E2F7 and E2F8 mRNA expression was reduced more than 90% following 48 hours infection of the cognate-floxed keratinocytes with Ad-CMV-Cre (Supplementary Fig. S1). The reduction in E2f1 expression was less than expected, but sequencing confirmed that the PCR product was E2f1. Significantly, infection with an empty adenovirus viral vector did not alter cell viability, miRNA expression, differentiation—competence or cytotoxic responses to UVB, doxorubicin, or cisplatin (Supplementary Fig. S2).

We examined the dose-dependent cytotoxic profiles of unaffected control, E2F7 KD, E2F8 KD, and E2F1 KO cells to increasing concentrations of doxorubicin (0–1 μmol/L) for 48 hours. E2F7-deficient MEKs were hypersensitive to the cytotoxic actions of doxorubicin (Fig. 1A) or another anthracycline, epirubicin (Supplementary Fig. S3A). Significantly, E2F7 deficiency only had minimal effect on cisplatin sensitivity (Supplementary Fig. S3B) and no impact on etoposide sensitivity (Supplementary Fig. S3C). E2F8 had no effect on cytotoxic responses to any of the drugs (Fig. 1A and Supplementary Fig. S3), whereas E2F1 deficiency resulted in modest protection against doxorubicin-induced cytotoxicity (Fig. 1A). To confirm that the effect of E2F7 deficiency was attributable to E2F7, we reintroduced E2F7 into E2F7-deficient keratinocytes to confirm that it suppressed doxorubicin sensitivity. Reintroduction of E2F7 into E2F7-deficient MEKs resulted in a 2.5-fold increase in E2F7 mRNA expression determined by qRT-PCR, and was sufficient to reestablish doxorubicin resistance (Fig. 1B). We then determined whether E2F7-mediated reduction in cell survival is due to activation of apoptotic pathways.

Uninfected control MEKs isolated from floxed E2F7 mice

Figure 1.

E2F7 selectively regulates sensitivity to doxorubicin in MEKs. A, dose–response curve of doxorubicin-induced cytotoxicity at 48 hours in untreated, E2F1KO, E2F7KD, E2F8KDs MEKs. B, dose–response curve of doxorubicin-induced cytotoxicity at 48 hours in E2F7KD MEKs, which were transfected with either an E2F7b overexpression plasmid, or pcDNA3.1(+) control plasmid. Viability is plotted as the percentage of control (untreated). C, activation of caspase-3 was determined by immunoblotting extracts of HEK, KJDSV40, and SCC25 cell lines. β-Actin is provided as a loading control. Densitometric analysis of E2F7 and E2F1 in KJDSV40 and SCC25 cell lines was quantified using ImageJ. Expression level was normalized against β-actin and plotted as E2F7/E2F1. F. SCC25 cells were transfected with E2F7b overexpression plasmid or pcDNA3.1(+) control plasmid. G, SCC25 cells were treated with siRNA-targeting E2F7 or a control siRNA. In both instances (F and G), cells were left for 48 hours after transfection after which viability was estimated by trypan blue exclusion. Viability was expressed as the percentage of viable cells and plotted as a percentage of untreated control. Western blot figures are representative of three independent experiments. Quantitative data represent the mean ± SEM obtained from triplicate determinations of three independent experiments for A, B, D, F, and G. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
showed a modest increase in cleaved caspase-3 levels after 48 hours treatment with 0.3 μmol/L doxorubicin (Fig. 1C). In contrast, there was a profound activation of caspase-3 when E2F7-deficient MEKs were treated with 0.3 μmol/L doxorubicin (Fig. 1C). Combined, these data identify a unique and isoform-specific function of E2F7 in modulating sensitivity to anthracyclines in keratinocytes.

Dysregulation of E2F7 expression in human SCC cell lines contributes to insensitivity to the cytotoxic action of doxorubicin

It is reasonable to speculate that the overexpression of E2F7, observed in human SCCs (10), may invoke insensitivity to doxorubicin. We screened a suite of SCC cell lines (FaDu, KJDSV40, and SCC25) as well as normal HEKs for their sensitivity to doxorubicin-induced cytotoxicity (Fig. 1D). These studies showed that KJDSV40 cell lines were the most sensitive to doxorubicin treatment with 70% reduction in cell viability at 1 μmol/L (Fig. 1D). On the other hand, SCC25 cells displayed the least sensitivity in which 80% of the cells were still viable at 1 μmol/L (Fig. 1D). Examination of E2F1 and E2F7 protein expression levels demonstrated that insensitive SCC25 cells had high levels of E2F7 to E2F1, whereas sensitive KJDSV40 cells had low levels of E2F7 relative to E2F1 (Fig. 1E). Overall, the insensitive SCC25 cells had 10-fold greater E2F7 expression relative to E2F1 than did sensitive KJDSV40 cells. It was not possible to generate values for the HEKs due to their low levels of expression of E2F1 and E2F7.

We next sought to determine whether selective upregulation or reduction of E2F7 expression by expression plasmid or siRNA (validation of siRNA is shown in Supplementary Fig. S4A), respectively, would change the dose–response profile of KJDSV40 and SCC25 cells to doxorubicin. Results showed that previously sensitive KJDSV40 cells became resistant to doxorubicin compared with vector only control cells when transfected with E2F7 expression plasmid (Fig. 1F). In contrast, silencing of E2F7 in insensitive SCC25 cells resulted in a 2.2-fold reduction in E2F7 mRNA expression determined by qRT-PCR and could enhance doxorubicin-induced cytotoxicity compared with control siRNA-transfected SCC25 cells (Fig. 1G). These data unequivocally demonstrate that resistance to doxorubicin is E2F7-dependent in SCC cells.

Sphingosine kinase 1 is a downstream effector of E2F7-mediated suppression of doxorubicin-induced cytotoxicity

To identify the downstream effectors of E2F7 in SCC cells, we generated transcriptomic profiles for differentially expressed transcripts between the KJDSV40 cells (sensitive and low E2F7/E2F1 ratio) and SCC25 cells (insensitive and high E2F7/E2F1 ratio). We also generated a list of upregulated genes between SCC25 cells and SCC25 cells in which E2F7 had been silenced with siRNA. This latter list identified E2F7-modulated transcripts, which were then cross-referenced against the list of genes identified as downregulated in KJDSV40 cells compared with SCC25 cells. A detailed explanation of this analysis is being prepared for publication elsewhere (Hazar-Rethinam and colleagues; unpublished data). By selecting for genes with a B value greater than 3 (exceeding the 95% confidence interval) and a fold change greater than 1, we were able to identify four genes (Sphk1, RACCAP1, CD44, and RRIP) that were differentially upregulated.

Of the transcripts identified in our screen, Sphk1 was the most significantly overexpressed. Sphk1 is a kinase responsible for the conversion of sphingosine to S1P (27–29). Interrogation of publicly available microarray data indicates statistically significant increases in Sphk1 expression in breast, colon, lung, ovary, prostate, melanoma, stomach, uterus as well as squamous cell carcinoma and its precursor actinic keratosis (28). Importantly, Sphk1 has been shown to modulate proliferation, differentiation, and apoptosis in keratinocytes (30). Facchinetti and colleagues (30) recently reported that Sphk1 is overexpressed in malignant oral epithelia compared with nonmalignant tissue, and the expression of Sphk1 was correlated with poor prognosis, shorter patient survival and loss of p21 expression in HNSCC. Of relevance to the present study, Bonhoure and colleagues (31) reported that forced expression of Sphk1 led to resistance to doxorubicin- and etoposide-induced cell death in HL-60 leukemia cells, and degradation of Sphk1 resulted in induction of apoptosis in MCF7 breast cancer cells treated with doxorubicin (32). Thus, there is sufficient evidence to speculate that a novel E2F7/Sphk1/S1P axis may exist in SCC cells that regulates doxorubicin sensitivity.

Quantitative RT-PCR was used to confirm that Sphk1 was more highly expressed in SCC25 cells than in KJDSV40 cells (Fig. 2A). Consistent with this, we show that transfection of SCC25 cells with siRNA directed against E2F7 resulted in profound inhibition of Sphk1 expression (Fig. 2A), whereas E2F1 mRNA expression was derepressed (Supplementary Fig. S4B). Similarly, Sphk1 activity was significantly elevated in SCC25 cells compared with KJDSV40 (Fig. 2B). Moreover, we showed that KD of E2F7, by siRNA, in SCC25 cells reduced Sphk1 protein expression (Fig. 2C), whereas transient overexpression of E2F7 in KJDSV40 cells resulted in an increase in Sphk1 protein levels (Fig. 2C). These data suggested that Sphk1 may be a downstream effector of E2F7-induced resistance to doxorubicin. As shown in Fig. 2D (left and center), E2F7 overexpression did not protect from Sphk1 KD or SKI-1–enhanced cytotoxicity in KJDSV40 cells. Similarly, Sphk1 overexpression in SCC25 cells overrides doxorubicin sensitivity induced by E2F7 siRNA (Fig. 2D, right). These data unequivocally demonstrate that Sphk1 is the downstream effector of E2F7-dependent sensitivity of SCC cells to doxorubicin. It remains unclear whether Sphk1 is a direct or indirect target of E2F7. In this regard, ChIP assays showed that E2F7 could bind the Sphk1 and E2F1 promoters in SCC25 cells compared with low levels of binding in KJDSV40 cells, indicating that the Sphk1 and E2F1 promoters are direct binding targets of E2F7 (Fig. 2E).

Next, we sought to determine whether there was evidence that Sphk1 was overexpressed in primary human SCC tumors. We have generated tissue microarrays (TMA) comprising duplicates of normal, primary tumor, and matched metastasis from HNSCC patients treated at the PAH. The TMAs were stained for E2F7 and Sphk1 protein expression by immunohistochemistry and scored by two pathologists. Figure 2F shows that Sphk1 and E2F7 are overexpressed in HNSCC compared with matched adjacent normal tissue. Figure 2F also shows that primary tumor and metastatic tumor do not differ significantly in the levels of E2F7 or Sphk1.

Sphk1 inhibition sensitizes SCC cells to doxorubicin-induced cytotoxicity

To determine whether Sphk1 contributes to doxorubicin sensitivity, we studied the effects of silencing Sphk1 in insensitive SCC25 cells. Sphk1 gene silencing was achieved via shRNA and caused a marked decrease in Sphk1 protein level (Fig. 3A), Sphk1 enzyme activity (Fig. 3B), and S1P (a product of Sphk1) measured...
in cell lysates (Fig. 3C), and significantly enhanced sensitivity of SCC25 cells to doxorubicin (Fig. 3D). Conversely, overexpression of Sphk1 in insensitive KJDSV40 cells resulted in increases in Sphk1 protein level (Fig. 3E), enzyme activity (Fig. 3F), S1P production (Fig. 3G), and reduced sensitivity to doxorubicin compared with vector control (Fig. 3H). S1P is the product of Sphk1-catalyzed phosphorylation of sphingosine, and has been shown to mediate the antiapoptotic effects of Sphk1 (27, 29). Consistent with this, we found that treatment of KJDSV40 cells with 1 μmol/L S1P reduced cytotoxicity by 2.6-fold (Fig. 3I).

**Figure 2.** Sphk1 is a downstream effector of E2F7 and is elevated in expression in SCCs. A, RNA was extracted from KJDSV40, SCC25, and SCC25 cells in which E2F7 was silenced with siRNA. Quantitative RT-PCR was used to determine the expression of Sphk1 transcripts. Data, the mean ± SEM of duplicate determinants normalized for expression of the housekeeping gene TBP; n = 3. B, Sphk1 activity is shown for the KJDSV40 and SCC25 cell lines. Data, the mean ± SEM obtained from triplicate determinations of three independent experiments. C, top, SCC25 cells were transfected with siRNA-targeting E2F7 or a control siRNA. Bottom, Sphk1 protein levels are shown for KJDSV40 cells in which E2F7 was overexpressed. Immunoblot was used to determine Sphk1 levels 48 hours after transfection. β-Actin is provided as a loading control. Western blot figure is representative of three independent experiments. D, left, empty vector, E2F7 expression plasmid, E2F7 expression plasmid, and Sphk1 shRNA plasmids transfected KJDSV40 cells were exposed to 1 μmol/L doxorubicin. Center, empty vector–transfected, E2F7 expression plasmid–transfected, E2F7 expression plasmid–transfected and 10 μmol/L SKI-I–treated KJDSV40 cells were exposed to 1 μmol/L doxorubicin. Right, control siRNA, E2F7 siRNA, E2F7 siRNA, and Sphk1 expression plasmid–transfected SCC25 cells were exposed to 1 μmol/L doxorubicin. Viability was assessed 48 hours after treatment and expressed in arbitrary units. E, quantitative determinations of E2F7 binding to the E2F1 and Sphk1 promoters. ChIPs were performed using an E2F7 antibody or nonimmune IgG as control in KJDSV40 and SCC25 cell lines. Each ChIP and quantitative RT-PCR were repeated, respectively, three and two times. SDs refer to the three independent experiments. F, quantitation of E2F7- and Sphk1-staining intensity in matched samples of primary tumor, its matched normal squamous epithelium and lymph node metastasis (n = 37). Tissue sections were scored using a modified quickscore method to determine the percentage of cells stained (0%–100%) and the intensity of staining (1+ to 3+). Data, the mean ± SEM. **, P < 0.01 and ****, P < 0.0001.
OF6 Clin Cancer Res; 21(2) January 15, 2015

Clinical Cancer Research

Combined, these data indicate that sensitivity to doxorubicin is mediated via a novel E2F7/Sphk1/S1P axis in SCCs.

Knockdown of Sphk1 sensitizes resistant SCC cells to doxorubicin-induced cytotoxicity in vivo

Our data suggest that inhibition of Sphk1 activity, in combination with doxorubicin, may be a viable therapeutic strategy for treating SCC. To answer this question, SCC25 cells were constructed to stably express either vector control or Sphk1 shRNA and inoculated in NOD/SCID mice. When tumors were approximately 3 mm in diameter, mice were randomized into four groups and treated with vehicle DMSO or 0.5 mg/kg doxorubicin by i.p. injections twice per week (Fig. 4A). Treatment of mice bearing vector control SCC25 tumors with/without 0.5 mg/kg doxorubicin had minimal effect on body weight (Fig. 4B) or tumor growth rates (Fig. 4C). KD of Sphk1 in SCC25 cells did not affect tumor growth in vivo. In contrast, Sphk1-deficient SCC25 tumors treated with doxorubicin started to regress by day 7 after treatment (Fig. 4C) with no effect on body weight (Fig. 4B). Strikingly, on day 13 after treatment there was a complete loss of tumors in doxorubicin-treated mice inoculated with SCC25/Sphk1shRNA cells (Fig. 4C).

We next examined whether we could achieve similar tumor regression when tumors are larger at the commencement of therapy. Because the tumors derived from Sphk1-deficient SCC25 cells treated with vehicle (Fig. 4C and D, blue triangle) were similar in growth rate and size to tumors in mice bearing vector control-transfected SCC25 tumors that had been treated with 0.5 mg/kg doxorubicin (Fig. 4C and D, red square), we started to treat these mice when their tumors reached around 0.5 cm³ with 0.5 mg/kg doxorubicin (Fig. 4C and D, red square). As shown in Fig. 4D, doxorubicin treatment dramatically reduced the tumor volume showing profound regression 1 week after doxorubicin was started in the Sphk1-deficient group of animals as compared with those inoculated with control vector (Fig. 4D). All mice were sacrificed at day 28 after treatment when the tumor burden in the control mice reached the ethically approved maximum size. Upon autopsy, the mice inoculated with the Sphk1-deficient SCC cells (Fig. 4C and D, green triangle) only contained a fragile cluster of cellular material that could not be harvested for histopathology.

The Sphk1-specific inhibitor, SK1-I (BML-EI411), sensitizes SCC cells to doxorubicin in vitro and in vivo

SK1-I is a water-soluble sphingosine analogue with a Ki value of approximately 10 μmol/L, which potently inhibits Sphk1 activity (33). Importantly, SK1-I does not significantly inhibit SPHK2, PKA, AKT1, ERK1, EGFR, or CDK2 (33). We treated SCC25 cells with increasing doses of SK1-I for 48 hours and then measured Sphk1 enzyme activity. As anticipated, SK1-I significantly reduced Sphk1 activity in a dose-dependent manner, indicating inhibition...
of Sphk1 activity (Fig. 5A). Moreover, we confirmed that the inhibition was not due to the loss of Sphk1 protein expression (Fig. 5B).

Next, we investigated whether Sphk1-specific inhibition can enhance the cytotoxic effects of doxorubicin in insensitive SCC25. After 48 hours incubation with SK1-I alone, the viability of control HEK (Supplementary Fig. S5A) and resistant SCC25 cells did not change (Fig. 5C). However, treatment of doxorubicin-resistant SCC25 cells with 1 µmol/L doxorubicin with increasing doses of SK1-I resulted in profound and dose-dependent loss of cell viability (Fig. 5C). Predictably, SK1-I did not enhance doxorubicin sensitivity in KJSV40 cells (Supplementary Fig. S5B). In contrast with SCC25 cells, the addition of increasing doses of SK1-I to doxorubicin, in HEKs, did not change the cytotoxicity obtained with doxorubicin alone (Supplementary Fig. S5A). Next, we examined whether cell death effects of SK1-I and doxorubicin were mediated via apoptosis. Consistent with an apoptotic reaction, we observed increases in cleaved caspase-3 and cleaved PARP1 in response to doxorubicin + SK1-I (Fig. 5D).

We inoculated NOD/SCID mice with SCC25 cells and allowed tumors to establish subcutaneously. When tumors were around 4 to 5 mm in diameter, mice were randomized into six groups and treated with DMSO, 0.5 mg/kg doxorubicin, 5 mg/kg SK1-I, 10 mg/kg SK1-I, 5 mg/kg SK1-I + 0.5 mg/kg doxorubicin, or 10 mg/kg SK1-I + 0.5 mg/kg doxorubicin by i.p. injection twice per week. Treatment with 5 and 10 mg/kg SK1-I was well tolerated by the NOD/SCID mice, and the body weights remained stable (Fig. 5E). Tumors in animals treated with 5 and 10 mg/kg doses of SK1-I alone showed modest, yet significant, decreases in tumor growth rate (Fig. 5G). Doxorubicin treatment alone did not affect the tumor size (Fig. 5F and G). However, in sharp contrast with doxorubicin treatment alone, treatment with 10 mg/kg SK1-I + 0.5 mg/kg doxorubicin as well as 5 mg/kg SK1-I + 0.5 mg/kg doxorubicin resulted in profound regression of explanted tumors (Fig. 5F and G). Thirteen days after treatment, animals had to be sacrificed due to the tumor burden in control mice. Tumors were excised, photographed, and histologically examined (Fig. 5H).

The benefit of combining SK1-I with doxorubicin was not restricted to the SCC25 cell line. Specifically, we show that the FaDu cell line displays intermediate sensitivity to doxorubicin in vitro and in vivo and are completely insensitive to SK1-I in vitro and moderately so in vivo (Supplementary Fig. S6). However, combining doxorubicin + SK1-I in vitro or in vivo induces profound cytotoxicity (Supplementary Fig. S6A and S6B).

Sphk1/SIP has been shown to exert its proapoptotic activity via signaling through a family of SIP receptors linked to the PI3K/AKT pathway (34, 35). Consistent with this, we found that SK1-I could reduce phospho-AKT (p-AKT; Ser473) in a dose-dependent manner (Fig. 6A). Previous reports have shown that p-AKT is a downstream effector of the prosurvival effects of increased Sphk1 activity and SIP production (36). Moreover, it is established that the PI3K/AKT pathway is frequently dysregulated via mutations in PI3K family members, gene amplifications, or pathway activation in HNSCC (37). Thus, we examined whether E2F7 induced
prosurvival responses were mediated via increased Sphk1/SIP and subsequent AKT phosphorylation. Transient overexpression of E2F7 in KDSV40 cells results in an increase in Sphk1 protein levels (Fig. 2C, bottom) and an increase in the p-AKT relative to total AKT (Fig. 2C, top) and a reduced p-AKT/total AKT ratio (Fig. 2C). Furthermore, transient overexpression of Sphk1 in KDSV40 cells or KD of Sphk1 with shRNA in SCC25 cells resulted in increased and decreased p-AKT, respectively (Fig. 2D and E). These data indicate that the changes in AKT activity lie downstream of Sphk1, which in turn is downstream of E2F7. These data would predict that the profound tumor regression observed as a result of a doxorubicin/Sphk1-1 combination could be recapitulated using doxorubicin + AKT inhibitor.

We have previously shown that the mTOR/P38 inhibitor, BGT226, is able to reduce tumor growth rates in mice transplanted with SCC cells (19). In the present study, mice were injected with SCC25 cells, and when the tumors were between 4 and 5 mm in diameter, we treated them with (i) vehicle, (ii) 0.5 mg/kg doxorubicin i.p. twice weekly, (iii) 10 mg/kg BGT226 i.p. twice weekly, or (iv) 10 mg/kg BGT226 + 0.5 mg/kg doxorubicin i.p. twice weekly. Tumor growth in the vehicle control (DMSO) and doxorubicin-treated mice was unchanged, whereas those mice treated with BGT226 alone displayed a modest reduction in tumor growth rate (Fig. 6F). Mice treated with the doxorubicin/BGT226 combination displayed significant regression of the tumor mass (Fig. 6F).

We next examined levels of PCNA, cleaved caspase-3, and p-AKT levels within the tumors resected from mice treated with 10 mg/kg SK1-I or 10 mg/kg BGT226, alone or in combination with 0.5 mg/kg doxorubicin. Immunohistochemical examinations showed that combination treatment inhibited intratumoral proliferation (at either dose) as measured by the levels of PCNA staining (Fig. 6G). SK1-I or BGT226 treatment markedly elevated the number of apoptotic cells induced by doxorubicin treatment as shown by examination of apoptotic indices of tumors by immunohistochemical staining with antibody against cleaved caspase-3 compared with drug alone treated tumors (Fig. 6G). Consistent with p-AKT lying downstream of Sphk1, we found significant inhibition of p-AKT (Ser473) in tumors treated with SK1-I or BGT226 (Fig. 6G).

**Discussion**

In the present study, we provide, *in vitro, in vivo*, and patient data that identify a novel E2F7/Sphk1/SIP/AKT axis that regulates sensitivity to anthracyclines in SCC. Specifically, we show that (i) E2F7 selectively modulates sensitivity to doxorubicin in keratinocytes and SCC, (ii) that E2F7-dependent doxorubicin resistance is mediated via induction of Sphk1, which in turn activates AKT, and (iii) that pharmacological inhibition of Sphk1 or AKT sensitizes SCC cells to the cytotoxic actions of doxorubicin *in vitro* and *in vivo*. Combined, these findings highlight a novel mechanism through which SCC cells acquire resistance to anthracyclines.
Overall, these data relating to the mechanisms regulating E2F control of apoptosis are complex. For example, E2F1 is known to be induced by cytotoxic stimuli and DNA damage (7, 8). This induction can occur at the level of posttranslational modification and protein stabilization and/or can occur through increased E2F1 transcription (16, 38). The main outcome of the increased E2F activity is mediated via ARF-stimulated inhibition of MDM2 resulting in increased p53-dependent apoptosis. However, complicating this is the observation that E2F1 can recognize double-strand breaks induced by UV and recruit NER machinery to the DNA break (16). In this way, E2F1 has been proposed to display antiapoptotic actions (16). This latter pathway has been demonstrated to exist in normal MEKs (39). Further complicating this is the observation that E2F1 can recognize double-strand breaks induced by UV and recruit NER machinery to the DNA break (16). In this way, E2F1 has been proposed to display antiapoptotic actions (16). This latter pathway has been demonstrated to exist in normal MEKs (39). Regardless of the underlying mechanism, it is clear that the effects of E2F7 were independent of the topoisomerase inhibitory actions of doxorubicin. We also show that regulation of doxorubicin sensitivity in SCC cells is E2F isoform-dependent—specific because the other inhibitory E2F, E2F8, did not modify the sensitivity of keratinocytes to doxorubicin or any other drug studied. In addition, we show that E2F7 suppresses doxorubicin sensitivity via increases in the expression of Sphk1 resulting in increased levels of S1P, which in turn enhance the Ser473 p-AKT–dependent prosurvival response. The E2F dependence of S1P/AKT-mediated drug resistance has not been described before and has significant pathologic and clinical implications in SCC.

The relevance of an E2F/Sphk1/S1P/AKT axis in SCC is highlighted by a number of independent observations. In particular, E2F1 (5, 11), E2F7 (10), Sphk1 (30), S1P (41), PI3K, and Akt (37) are all increased in SCC. Part of these increases may be explained by activation of signaling pathways that regulate their activity/expression such as MAPK-mediated activation of Akt and/or Sphk1 (35, 37) or disrupted Rb activity mediated via p16 deletion or cyclin D amplification for E2F1/E2F7 (7, 8). However, PI3K/Akt is commonly mutated or amplified in SCC (2, 3, 37). Regardless of the underlying mechanism, it is clear that the individual members of the E2F/Sphk1/S1P/AKT axis are all present in SCC.
In particular, ceramide and sphingosine have been shown to be responsible for catalyzing the conversion of sphingosine to S1P, which is also responsible for changing the physiology of the cell from proapoptotic to antiapoptotic. Many of the antiapoptotic effects of S1P are mediated via a family of G protein coupled S1P receptors, which in turn activate PI3K/AKT pathway (27–29, 43). Interestingly, the use of an AKT inhibitor was able to induce modest levels of cell death and reduced tumor growth in vivo. Given that Sphk1 inhibitors profoundly inhibited p-AKT (Ser473), these data would suggest that some of the cytotoxic effects observed for BGT226 alone may be mediated via non-AKT targets. Finally, doxorubicin alone displayed no measurable anticancer activity in our xenotransplant model. These data suggest that the cytotoxicity observed with the SKI-1 or BGT226 plus doxorubicin combination reflects an unidentified synthetic lethal reaction. The clinical potential for this novel combination (e.g., Sphk1 or AKT inhibitor combined with an anthracycline) is highlighted by the profound tumor regression observed in this study.

A previous report had shown that the activation of AKT in ovarian and breast cancer suppressed E2F1-induced apoptosis and was associated with a poor prognosis and chemoresistance (44). Similarly, Reimer and colleagues (45) reported that poor prognosis and chemoresistance of ovarian tumors were associated with a high E2F7/E2F1 ratio. We now provide an integrated model in which E2F7 is causally linked to the overexpression of Sphk1, the activation of the AKT pathway and doxorubicin resistance. This is definitively shown by our observation that Sphk1, S1P, and p-AKT (Ser473) are all directly modulated by E2F7. Second, Sphk1 inhibition or overexpression directly effects the Ser473 phosphorylation of AKT. Finally, inhibition of Sphk1 or AKT sensitizes SCC cells in vitro to the cytotoxic effects of doxorubicin. The observation that antiapoptotic effects of S1P are mediated via the PI3K/AKT pathway has been previously reported (35, 36). What is new in our study is that the dysregulation of the E2F pathway, in SCC, directly activates the Sphk1/S1P/PI3K/AKT pathway resulting in selective resistance to doxorubicin. This is an advance that can be immediately translated to a clinical trial with existing pharmacological agents.

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

**Authors’ Contributions**
Conception and design: M. Hazar-Rethinam, N.A. Saunders
Development of methodology: M. Hazar-Rethinam
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Hazar-Rethinam, A.C. Vargas, M. Dzienis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Hazar-Rethinam, A.C. Vargas, P. Mukhopadhyay, F. Simpson, N.A. Saunders
Writing, review, and/or revision of the manuscript: M. Hazar-Rethinam, O.M. Gannon, M. Dzienis, F. Simpson, N.A. Saunders
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Hazar-Rethinam, L. Merida de Long, E. Topkas, S. Boros, M. Dzienis, N.A. Saunders
Study supervision: F. Simpson, L. Endo-Munoz, N.A. Saunders

**Acknowledgments**
The authors acknowledge the generous gift of E2f7flhfl and E2f7flwfl mice from Professor Gustavo Leone, The Ohio State University. The authors acknowledge the generous donations of tissue samples from the patients without which this project could not happen.

**Grant Support**
N.A. Saunders and L. Endo-Munoz are supported by grants from the Australian NHMRC (#APP1049182) and the Cancer Council Queensland (#APP1025479). N.A. Saunders is supported by a Senior Research Fellowship awarded by the Cancer Council Queensland. O.M. Gannon is supported by a grant from the Wesley Medical Research Institute. M. Hazar-Rethinam and E. Topkas are supported by an Australian Postgraduate Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 4, 2014; revised October 17, 2014; accepted November 11, 2014; published OnlineFirst November 19, 2014.

**References**


A Novel E2F/Sphingosine Kinase 1 Axis Regulates Anthracycline Response in Squamous Cell Carcinoma


Clin Cancer Res  Published OnlineFirst November 19, 2014.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-1962

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/11/20/1078-0432.CCR-14-1962.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.