Original Article

Receptor-Interacting Protein (RIP) and Sirtuin-3 (SIRT3) are on Opposite Sides of Anoikis and Tumorigenesis

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BACKGROUND: Regulating cross-talk between anoikis and survival signaling pathways is crucial to regulating tissue processes and mitigating diseases like cancer. Previously, the authors demonstrated that anoikis activates a signaling pathway involving the CD95/Fas-mediated signaling pathway that is regulated by receptor-interacting protein (RIP), a kinase that shuttles between Fas-mediated cell death and integrin/focal adhesion kinase (FAK)-mediated survival pathways. Because it is known that sirtuin-3 (SIRT3), a nicotinamide adenine dinucleotide-dependent deacetylase, regulates cell survival, metabolism, and tumorigenesis, the authors hypothesized that SIRT3 may engage in cross-talk with Fas/RIP/integrin/FAK survival-death pathways in cancer cell systems. **METHODS:** Using immunohistochemical staining, immunoblotting, human tissue microarrays, and overexpression and suppression approaches in vitro and in vivo, the roles of RIP and SIRT3 were examined in oral squamous cell carcinoma (OSCC) anoikis resistance and tumorigenesis. **RESULTS:** RIP and SIRT3 had opposite expression profiles in OSCC cells and tissues. Stable suppression of RIP enhanced SIRT3 levels, whereas stable suppression of SIRT3 did not impact RIP levels in OSCC cells. The authors observed that, as OSCC cells became anoikis-resistant, they formed multicellular aggregates or oraspheres in suspension conditions, and their expression of SIRT3 increased as their RIP expression decreased. Also, anoikis-resistant OSCC cells with higher SIRT3 and low RIP expression induced an increased tumor burden and incidence in mice, unlike their adherent OSCC cell counterparts. Furthermore, stable suppression of SIRT3 inhibited anoikis resistance and reduced tumor incidence. **CONCLUSIONS:** The current results indicted that RIP is a likely upstream, negative regulator of SIRT3 in anoikis resistance, and an anoikis-resistant orasphere phenotype defined by higher SIRT3 and low RIP expression contributes to a more aggressive phenotype in OSCC development. ***Cancer*** **2012;118:5800-10.** © *2012 American Cancer Society.*

KEYWORDS: sirtuin-3, receptor-interacting protein, anoikis resistance, tumorigenesis, oral squamous cell carcinoma, orasphere.

INTRODUCTION

Anoikis—apoptotic cell death triggered by loss of extracellular matrix (ECM) contacts—is dysregulated in many chronic debilitating and fatal diseases. Cancer cells evade apoptosis and possess self-sufficiency in growth signals: 2 important hallmarks of cancer cells.[1] Thus, cancer cells can evade apoptosis by escaping anoikis and becoming anoikis-resistant. Anoikis resistance or anchorage-independent growth contributes to cancer development and progression.[2-5] Although smoking, alcohol consumption, and human papillomavirus (HPV) are risk factors for oral cancer, other factors that contribute to tumorigenicity are poorly studied. One such factor, anoikis resistance, induces more aggressive tumors in oral squamous cell carcinoma (OSCC).[6-8]

Oral cancer is 1 of the leading causes of death worldwide, and OSCC accounts for >90% of oral malignancies,[9] yet survival rates for oral cancer have not improved in decades. These disheartening statistics underscore the need to examine its pathogenesis and to identify novel biomarkers and modes of therapy.

We recently demonstrated that receptor interacting protein (RIP), shuttles between the CD95/Fas death and focal adhesion kinase (FAK) survival signaling pathways to mediate anoikis in OSCC cells.[10] Hence, under anoikis conditions, FAK and RIP dissociate, leading to the association of RIP with Fas (tumor necrosis factor receptor superfamily, member 6) and the formation of the death-inducing signaling complex, thus enhancing apoptosis. These findings support the development of therapeutics that can target RIP as a switch to control cell death or survival pathways to ultimately regulate normal tissue processes and tumorigenesis in cancer patients.

Sirtuins (SIRT1-SIRT7), the mammalian homologues of the Sir2 gene (nicotinamide adenine dinucleotide-dependent deacetylase) in yeast, have an emerging role in regulating cellular processes and functions, including cell survival, apoptosis, oxidative stress, development, metabolism, and aging.[11,12] We recently reported that SIRT3, 1 of the mitochondrial sirtuins,[13-15] is overexpressed in OSCC cells and tissues compared with normal samples and that the

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down-regulation of SIRT3 in OSCC cells inhibited cell growth and proliferation and increased the sensitivity of those cells to both radiation therapy and chemotherapy.[14] In addition, by using a floor-of-mouth oral cancer murine model that mimics human OSCC,[16,17] we demonstrated that SIRT3 down-regulation reduced tumor burden in vivo, implicating a prosurvival role for SIRT3 in oral cancer tumorigenesis.[14] However, to our knowledge, the role of SIRT3 in anoikis resistance has not been investigated. Here, we reported for the first time a role for SIRT3 in mediating anoikis resistance in oral cancer and its potential negative relation with RIP.

MATERIALS AND METHODS

Cell Lines and Culture

The human OSCC cell line HSC-3 was kindly provided by Randy Kramer (University of California, San Francisco, Calif). The human OSCC cell line UM-SCC-14A was a gift from Tom Carey (University of Michigan, Ann Arbor, Mich). The poorly differentiated aggressive tongue SCC cell line OSCC-3 was gift from Mark Lingen (University of Chicago, Chicago, Ill). OSCC cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Primary human oral keratinoctyes (ScienCell, Carlsbad, Calif) were maintained in oral keratinocyte medium (OKM; ScienCell, Carlsbad, Calif). $RIP^{-/-}$ mouse embryonic fibroblasts were kindly provided by Philip Leder and Michelle Kellinger (Harvard Medical School, Boston, Mass).

Tissue Microarrays

Immunohistochemical analyses were performed to determine the expression of SIRT3 and RIP in human OSCC tissues using OSCC tissue microarrays (OR601 and HN241; US Biomax, Inc., Rockville, Md) and the Histostatin Kit (95-6143; Zymed Laboratories, South San Francisco, Calif) according to the manufacturer's instructions. Antibodies to SIRT3 (AP6242a) and RIP (610,459) were obtained from ABGENT (San Diego, Calif), and Millipore (Billerica, Mass), respectively. We recently reported that SIRT3 staining intensity was higher in OSCC tongue samples[14]; and, because the tongue accounts for approximately 30% of oral malignancies, we specifically examined tongue samples in the current study. Staining intensities for RIP and SIRT3 were graded in a blinded manner by a pathologist. For RIP and SIRT3 assessment, the staining intensity and the percentage of tumor cells stained were analyzed. Staining intensity was scored as 1 (weak), 2 (moderate), or 3 (strong). Each core also was evaluated for the percentage of tumor cells that were stained positive (the *staining proportion*). A combined score based on the staining intensity and the staining proportion was used to assign a final score. Low expression was defined as an intensity of 1 or 2 and a staining proportion $<10\%$, and high expression was defined as an intensity 2 or 3 and a staining proportion $>10\%$.

Transient Transfection

HSC-3 cells at 60% to 70% confluence were transiently transfected with small interfering RNA (siRNA) (25 nM or 50 nM) against RIP or with a nontargeting control siRNA (Santa Cruz Biotechnology, Santa Cruz, Calif) in serum-free medium that contained Lipofectamine Plus (Invitrogen, Carlsbad, Calif). $RIP^{-/-}$ mouse embryonic fibroblasts were transiently transfected with *myc*-tagged wild-type RIP or control vector, as described previously.[10] Transfection efficiency was assessed by Western blot analysis.

Stable Transfection

HSC-3 and UM-SCC-14A cells were transduced with either RIP short hairpin RNA (RIP-shRNA) (sc44326-V), SIRT3-shRNA (sc61555-vs; human), or scrambled-shRNA (sc-108084; human; Santa Cruz Biotechnology) lentiviral particles in 0.5 mL serum-free media and then were selected in 10 μg/mL puromycin (sc-108071; Santa Cruz Biotechnology) for an additional 10 days. Surviving cell colonies were picked and propagated before they were tested for RIP or SIRT3 expression using Western blot analysis. It is noteworthy that, to avoid off-target effects, SIRT3 lentiviral particles are designed by blasting the mRNA accession number of SIRT3 against all the known mRNA accession numbers of the same species to minimize/ensure that there are no off-target effects. In addition, to further minimize off-target effects, the SIRT3-shRNA lentiviral particles used in this study are from a pool of concentrated, transduction-ready viral particles containing 3 target-specific constructs that encode 19 to 25 nucleotides of shRNA designed to knock down gene expression. Similar shRNA design strategies were used for RIP shRNA lentiviral particle production.

Immunoblot Analysis

To evaluate the expression levels of RIP and SIRT3, cells were treated as described above or in the figure legends, washed once with phosphate-buffered saline, lysed in radioimmunoprecipitation assay (RIPA) buffer (R0278; Sigma Chemical Company, St. Louis, Mo) that contained 1% protease inhibitor cocktail (P8340; Sigma Chemical Company), then kept on ice for 30 minutes. Lysates were adjusted for protein concentration with the BCA protein

assay kit (Bio-Rad, Hercules, Calif). Western blot analyses were performed with various primary antibodies and horseradish peroxidase-conjugated antirabbit or antimouse immunoglobulin G antibodies, and blots were developed with the ECL-Plus detection system (Pierce, Rockford, Ill). Antibodies for RIP, SIRT3, and cleaved caspase 3 were from Millipore (610459), Cell Signaling Technology (2627; Beverly, Mass), and Santa Cruz Biotechnology (7148), respectively. To demonstrate equal protein loading, membranes were stripped and reprobed with an anti-β-actin antibody (sc-1615; Santa Cruz Biotechnology).

Anoikis-Resistant and Control Adherent Oral Squamous Cell Carcinoma Cells

Anoikis-resistant oraspheres (UM-SCC-14A and HSC-3) and adherent (UM-SCC-14A and HSC-3) OSCC cells were prepared as previously reported.[4] These OSCC cell lines were selected because they represent the extremes of RIP and SIRT3 expression among the OSCC cell lines that we examined. These OSCC cells were developed by maintaining cells under suspension conditions on polyhydroxyethylmethacrylate poly-HEMA)-coated plates (7.5 mg/mL in 95% ethanol; Sigma Chemical Company) for 6 days, where they survive anchorage withdrawal by forming multicellular aggregate oraspheres. Adherent control cells were maintained in culture medium as described previously.

Immunodeficient Mouse Tumor Model

To examine the effects of anoikis-resistance and adherent OSCC cells in vivo, UM-SCC-14A and SIRT3-suppressed UM-SCC-14A cells and controls were grown in anoikis-resistant and adherent conditions as described above. Four-week-old athymic nude mice (NCr-nu/nu strain; National Cancer Institute, Frederick, Md) were anesthetized by intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine. We used a murine floor-of-mouth model, which we previously optimized to produce 4-mm to 5-mm tumors, corresponding to a palpable tumor volume of 35 to 60 mm^3, within approximately 2 to 4 weeks after injection of tumor cells.[14,16] Anoikis-resistant and adherent control UM-SCC-14A cells were prepared to a final concentration of 1.0×10^6/0.05 mL and injected submucosally into the floor of the mouth as described previously.[14] Six weeks after injection, the mice were killed, and tumor volumes were measured by digital caliper using the formula $a \times a \times b/2$, in which a is the smaller dimension. Tumor tissues were then harvested, rinsed in phosphate-buffered saline, and fixed overnight in 10% buffered formalin. Tissues were paraffin-embedded, sectioned, and processed for routine histopathologic assessment with hematoxylin and eosin staining and for SIRT3 and RIP immunostaining.

Apoptosis Cell Death Detection by Enzyme-Linked Immunosorbent Assay

Apoptosis was measured in vitro using a DNA-fragmentation enzyme-linked immunosorbent assay according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, Ind).

Statistical Analysis

In general, values are expressed as means ± standard deviations. Intergroup differences were determined by using a 2-way analysis of variance and a Scheffe multiple-comparison test. Statistical significance was defined as $P \leq .05$. For tissue microarray analyses, the McNemar test was used to compare the 2 proportions, and the difference was considered significantly different when $P \leq .001$. For the in vivo studies, independent t tests with unequal variances were used. All experiments were repeated at least 3 times.

RESULTS

SIRT3 and RIP Are Oppositely Expressed in Oral Squamous Cell Carcinoma In Vivo

We recently reported that SIRT3 is overexpressed in OSCC in vivo and in vitro compared with other sirtuins and that its stable suppression reduces tumor burden in vivo, implicating SIRT3 as a prosurvival and tumor-promoting factor.[14] In addition, we demonstrated that RIP plays a critical role in OSCC cells by regulating anoikis through its shuttling between the CD95/Fas death and FAK survival signaling pathways, thus demonstrating that RIP acts as a switch between life and death signals in OSCC cells.[10] Also, it is known that SIRT3 regulates cell survival, metabolism, and tumorigenesis. Therefore, we hypothesized that SIRT3 may engage in cross-talk with RIP to regulate anoikis resistance and tumorigenesis in OSCC cells. To test this hypothesis, we first evaluated the native expression levels of SIRT3 and RIP in serial sections of OSCC tissue microarrays (TMAs). Our data included 28 OSCC tongue samples in which SIRT3 and RIP expression were evaluated and assessed as low (L) or high (H) (Table 1). SIRT3 and RIP staining intensity data from Table 1 are illustrated in Figure 1B. We already knew that SIRT3 expression levels were elevated in human OSCC[14]; however, the relative expression level of RIP in these same tissues was not known. It is noteworthy that observed opposite expression of SIRT3 and RIP in OSCC tissues (Fig. 1A). In 86% of the samples in which SIRT3 expression was high, 75% had low RIP expression

Table 1. The Expression Profile of Receptor-Interacting Protein and Sirtuin-3 From 28 Different Tongue Tissue Microarray Samples

				Staining Intensity[a]	
Sample No.	Section No.	Sex	Age, y	RIP	SIRT3
1	A1	Man	57	Low	High
2	A6	Woman	35	High	High
3	A7	Man	78	Low	High
4	A9	Woman	39	Low	Low
5	A10	Woman	46	Low	High
6	B3	Woman	57	Low	High
7	B4	Woman	36	Low	High
8	B8	Woman	47	High	High
9	B9	Woman	63	Low	Low
10	B10	Man	56	Low	Low
11	C2	Woman	55	Low	High
12	C3	Man	76	Low	High
13	C4	Woman	50	Low	High
14	C6	Man	55	High	High
15	C9	Man	60	Low	High
16	D1	Man	64	Low	High
17	D2	Woman	52	Low	High
18	D3	Woman	50	Low	High
19	D4	Woman	46	Low	High
20	D5	Woman	45	Low	High
21	D6	Man	35	Low	High
22	D7	Woman	46	High	High
23	D8	Woman	48	High	Low
24	E3	Man	60	High	High
25	E4	Man	37	Low	High
26	E6	Man	60	Low	High
27	E8	Man	60	High	High
28	E10	Man	73	Low	High

Abbreviations: RIP, receptor-interacting protein; SIRT3, sirtuin-3.
[a] Staining intensity was graded as low or high.

(Fig. 1B, Table 1). Thus, 18 of 28 specimens (64%) had opposite expression patterns for SIRT3 and RIP ($P \leq .05$) (Fig. 1A,B, Table 1).

SIRT3 and RIP Have Opposite Expression Patterns in Oral Squamous Cell Carcinoma In Vitro

Similarly, when we examined several OSCC cell lines (HSC-3, OSCC-3, and UM-SCC-14A) for SIRT3 and RIP expression, we again noted that SIRT3 expression levels were the opposite of RIP expression levels in these cells (Fig. 2A). Primary oral keratinocytes also had opposing SIRT3 and RIP expression patterns; however, those cells generally exhibited higher levels of RIP expression versus SIRT3 expression (Fig. 2A). These findings again further support the concept that RIP and SIRT3 exhibit a strong negative correlation. To test this further, OSCC cell lines (HSC-3 and UM-SCC-14A) were selected for further experiments to determine whether RIP or SIRT3 regulate each other and to examine the hierarchy of this potential negative correlation. These OSCC cell lines were chosen because they represent the extremes of SIRT3 and RIP expression among the OSCC cell lines examined. Transient suppression of RIP effectively increased SIRT3 expression levels (Fig. 2B). However, stable suppression of SIRT3 failed to alter RIP expression levels (Fig. 2C). Furthermore, reconstitution of RIP in RIP null cells down-regulated SIRT3 expression (Fig. 2D). This indicated that SIRT3 was responsive to changes in RIP levels but not the contrary, suggesting that RIP is a potential upstream regulator of SIRT3.

Anoikis-Resistant Oral Squamous Cell Carcinoma Oraspheres Express Higher Levels of SIRT3 and Lower Levels of RIP

To examine the anoikis-resistant phenotype of OSCC cells, an orasphere culture assay was used to study the SIRT3 and RIP expression profile of OSCC cells in vitro. We specifically developed anoikis-resistant cells and their counterpart adherent controls (HSC-3 and UM-SCC-14A cells), as described previously. Our data indicated that anoikis-resistant OSCC cells in oraspheres maintain cell survival and exhibit low levels of cleaved/active caspase 3 and DNA fragmentation or apoptosis (Fig. 3A-C). In contrast, the single-cell counterpart of suspension cultures undergoes apoptosis and exhibits high levels of cleaved/active caspase 3 and DNA fragmentation (Fig. 3A-C). The adherent counterpart control cells, like the cells in oraspheres, also have low levels of cleaved/active caspase 3 and DNA fragmentation. It is noteworthy, however, that the oraspheres express higher levels of SIRT3 and lower levels of RIP compared with the adherent cells (Fig. 3D), suggesting that OSCC cells escape anoikis-mediated cell death in part by relying on SIRT3 survival signaling and suppression of RIP death signaling pathways.

Anoikis-Resistant, Orasphere-Derived Oral Squamous Cell Carcinoma Cells That Express Higher Levels of SIRT3 and Lower Levels of RIP Induce a Greater Tumor Burden

To investigate the in vivo relevance of our in vitro findings, we also used a murine floor-of-mouth model that mimics human OSCC.[14,16,17] Mice were injected with anoikis-resistant, orasphere-derived OSCC cells that expressed higher SIRT3 levels (UM-SCC-14A cells) or their adherent OSCC cell counterparts, which expressed lower SIRT3 levels (Fig. 4). Our data indicated that mice injected with anoikis-resistant, orasphere-derived cells exhibit greater tumor burden compared with their adherent counterparts (Fig. 4A, Table 2). Not only did the mice injected with orasphere-derived cells develop significantly larger tumors (12-fold difference in size; $P \leq .027$), but they also developed more tumors (9 of 10 mice developed

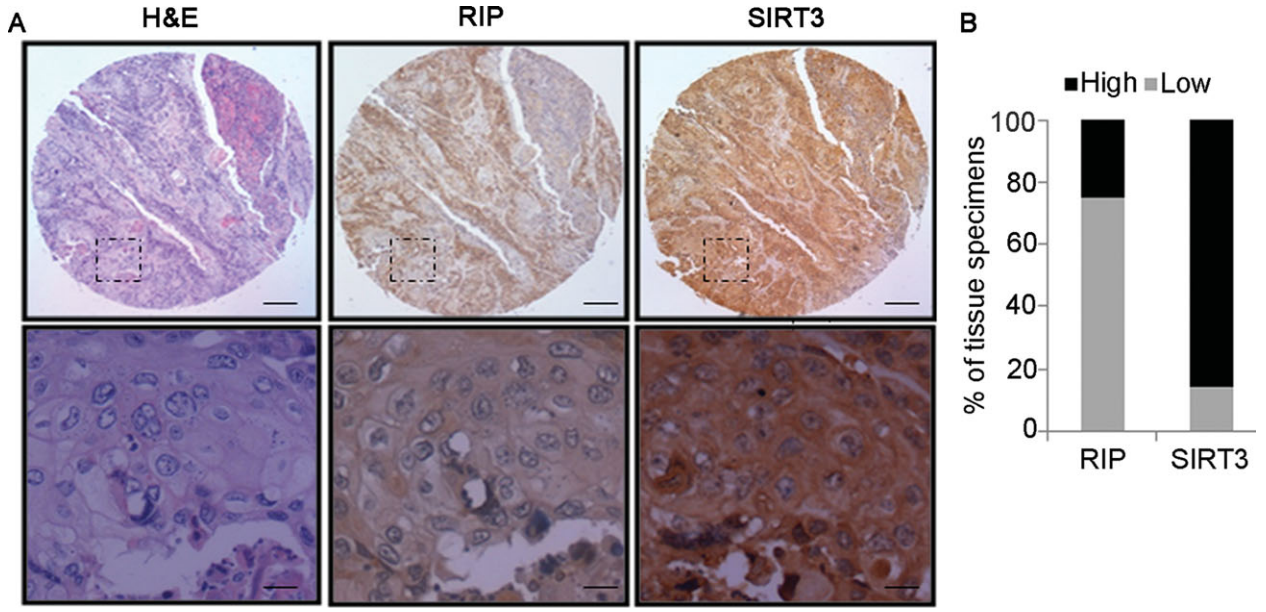

Figure 1. Receptor-interacting protein (RIP) expression has an opposite relation to sirtuin-3 (SIRT3) expression in oral squamous cell carcinoma (OSCC). (A) RIP and SIRT3 expression in OSCC tumor microarray specimens are illustrated. H&E indicates hematoxylin and eosin staining. Scale bars = 200 μm in low-magnification photomicrographs (*Top*), 50 μm in high-magnification photomicrographs (*Bottom*). (B) This chart illustrates the percentage of OSCC serial tongue tissue sections that expressed RIP and SIRT3 according to immunohistochemical staining. Staining intensity was graded as either high or low. The McNemar test was used to compare the 2 proportions, which differed significantly ($P \leq .001$).

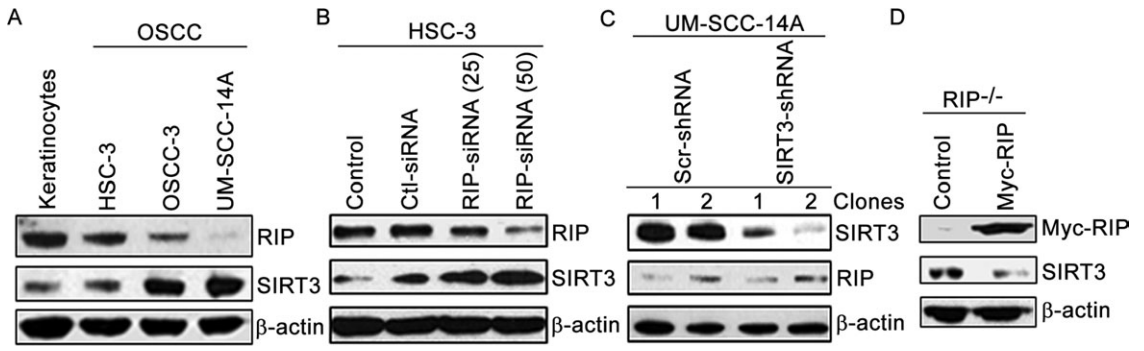

Figure 2. Receptor-interacting protein (RIP) may be an upstream, negative regulator of sirtuin-3 (SIRT3). (A) Immunoblots reveal RIP and SIRT3 levels in normal human keratinocytes and in oral squamous cell carcinoma (OSCC) cells (HSC-3, OSCC-3, and UM-SCC-14A) that were plated for 1 day. β-Actin served as a loading control. (B) Immunoblots reveal RIP and SIRT3 levels after transfection with control small interfering RNA (Ctl-siRNA) or with RIP-siRNA (25 nM or 50 nM) in HSC-3 cells for 30 hour. (C) Immunoblots reveal SIRT3 and RIP levels after stable SIRT3 suppression using lentiviral particles (scrambled controls [Scr-shRNA] or SIRT3-shRNA) in UM-SCC-14A cells. (D) Immunoblots reveal RIP and SIRT3 levels after transfection with wild-type, v-*myc* myelocytomatosis viral oncogene homolog (Myc)-tagged RIP (Myc-RIP) in RIP null cells ($RIP^{-/-}$) for 30 hours.

tumors) compared with 2 of 8 mice that were injected with adherent control cells (Table 2) ($P \leq .001$) Histologic examination of tumors dissected from mice injected with orasphere-derived cells revealed a highly disorganized histologic pattern of invasive epithelial islands with keratin pearl formation, nuclear pleomorphism, hyperchromatism, and increased nuclear-to-cytoplasmic ratios compared with their control counterparts (Fig. 4B). These "aggressive" tumors also had high levels of SIRT3 expression and low levels of RIP expression (Fig. 4B). In 80% of the samples with high SIRT3 expression, 78% had low RIP expression (Fig. 4C). In contrast, the few tumors that emerged in mice that were injected with adherent control cells revealed a tendency toward a regularly organized histologic pattern of epithelial islands and a relatively lower level of SIRT3 expression (data not shown). These data support our in vitro findings and those of others[6,7] indicating that anoikis-resistant cells induce a greater tumor

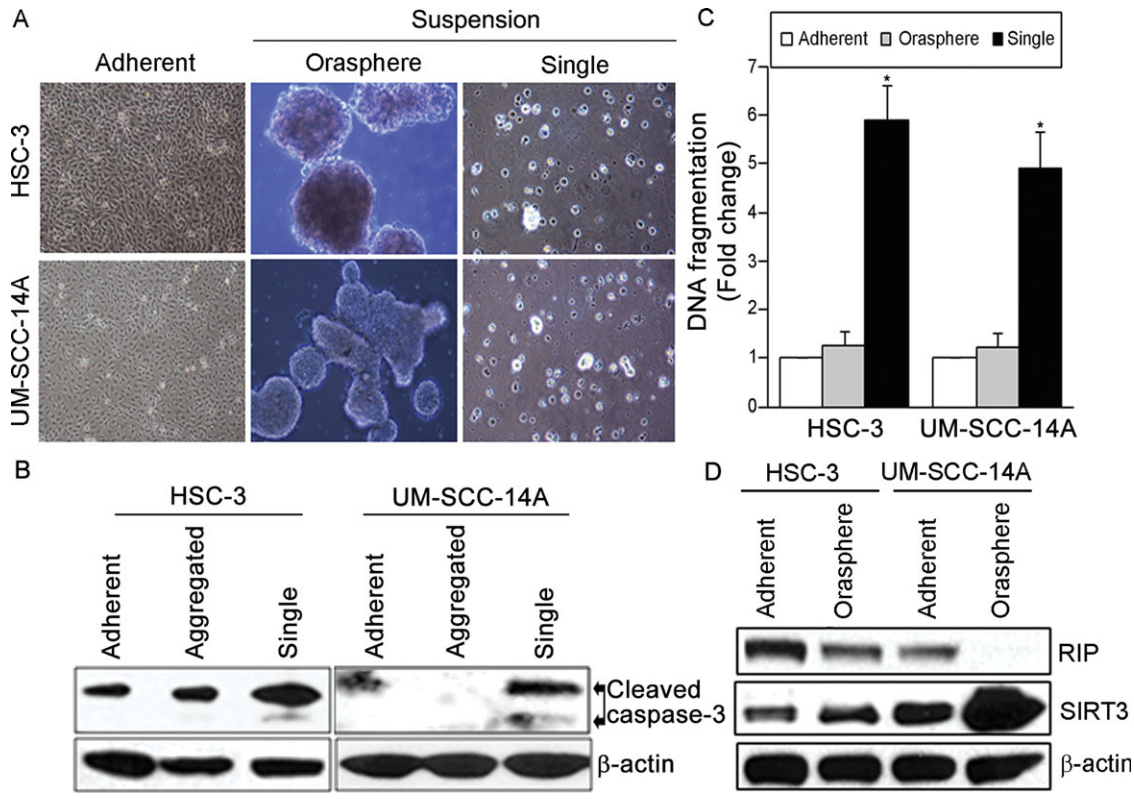

Figure 3. Oral squamous cell carcinoma (OSCC) cells, as they become anoikis resistant, have increased sirtuin-3 (SIRT3) expression as their receptor-interacting protein (RIP) expression decreases. (A) These are phase-contrast images of OSCC cells (HSC-3 and UM-SCC-14A) that were cultured under adherent conditions or suspension conditions for 6 days, when oraspheres and single cells were separated for analysis. (B,C) Immunoblots reveal (B) cleaved/active caspase 3 expression and (C) the fold change in DNA fragmentation for adherent cells, oraspheres, and single cells. An asterisk indicates $P \leq .05$. (D) Immunoblots reveal RIP and SIRT3 expression in adherent cells and oraspheres. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

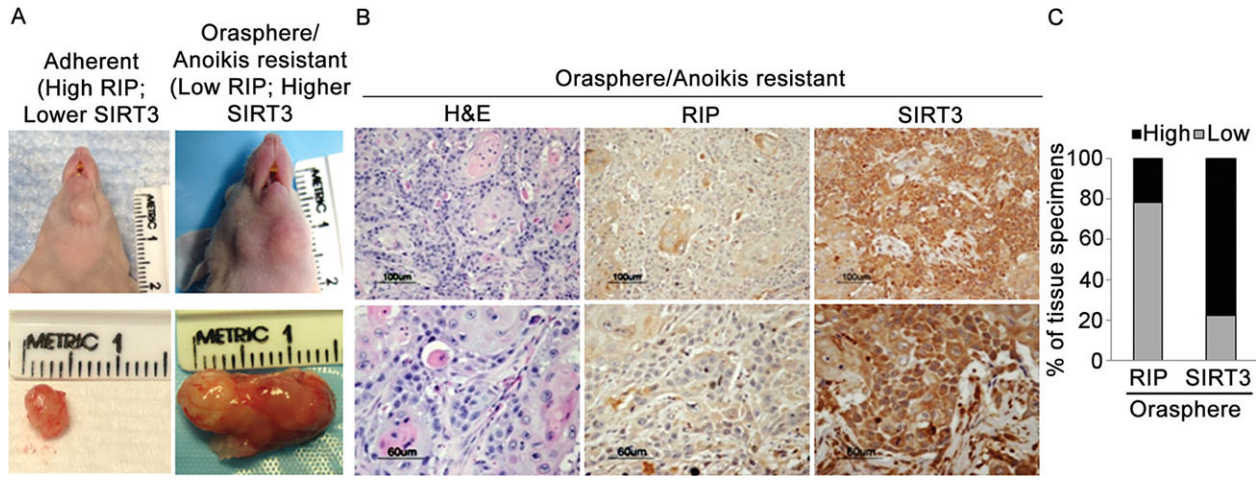

Figure 4. Anoikis-resistant oral squamous cell carcinoma (OSCC) cells induce greater tumor burden in mice. (A) These images show tumor-bearing mice 6 weeks after injection with adherent or orasphere/anoikis-resistant UM-SCC-14A cells. Shown are (*Top*) a superficial tumor and (*Bottom*) a dissected tumor. (B) Representative orasphere-derived tumor sections were stained with hematoxylin and eosin (H&E) and were immunostained with antibodies for RIP and SIRT3. (C) This chart illustrates the percentage of tissue specimens that expressed RIP and SIRT3 in orasphere-derived tumor sections, as determined by immunohistochemical staining. Staining intensity was graded as either high or low. The McNemar test was used to compare the 2 proportions, which differed significantly ($P \leq .001$).

Table 2. Tumor Volumes for Mice Injected With UM-SCC-14A Human Oral Squamous Cell Carcinoma Cells

	Tumor Volume, mm^3	
Animal No.	Adherent	Orasphere/ Anoikis Resistant
1	31.20	518.9
2	19.64	444.36
3	No tumor	22.86
4	No tumor	864
5	No tumor	243.3
6	No tumor	252
7	No tumor	215.66
8	No tumor	138.16
9	—	83.49
10	—	No tumor
Mean volume	25.42[a]	311.84[a]

[a] Statistical analysis: Independent *t* test with unequal variances $P \leq .027$

burden and a more aggressive phenotype in OSCC. In addition, these cells seem to rely, at least in part, on SIRT3 up-regulation and RIP down-regulation to maintain their survival and aggressive phenotype. Thus, high SIRT3 expression and low RIP expression may impart resistance to anoikis-mediated cell death.

SIRT3 Up-Regulation and RIP Down-Regulation Regulate the Fate of Oral Squamous Cell Carcinoma Cells in Anoikis Conditions

To further validate the importance of SIRT3 and RIP in regulating the fate of OSCC cells under suspension/anoikis conditions, stable cell clones of SIRT3-shRNA (Clone 2) and scrambled-shRNA (Clone 1) were examined under adherent and suspension conditions. Our data indicate that, under suspension conditions, cells that express SIRT3-shRNA can no longer form oraspheres, maintain their survival, or escape anoikis, unlike cells that express scrambled-shRNA (Fig. 5A), confirming the role of SIRT3 in anoikis resistance. In addition, cells in suspension conditions that stably express SIRT3-shRNA (oraspheres and single cells) had increased levels of RIP expression and cleaved/active caspase 3 and had higher levels of DNA fragmentation compared with cells that expressed SIRT3-shRNA and were grown in adherent conditions or cells that expressed scrambled-shRNA and were grown in adherent or suspension conditions (Fig. 5B-D). Furthermore, stable suppression of SIRT3 inhibits anoikis resistance and reduces tumor incidence in vivo (Fig. 5E). The higher RIP expression present in the cells transduced with SIRT3-shRNA in suspension conditions likely represents the single-cell fraction present in this mixed culture system. Furthermore, RIP overexpression promotes anoikis,[10] and RIP suppression inhibits anoikis, caspase 3 activation, and DNA fragmentation (Fig. 6A-D). These data demonstrate that SIRT3 and RIP are at opposite ends of anoikis resistance in OSCC cells and that SIRT3 is required for escaping anoikis and for acquisition of an anoikis-resistant phenotype. On the basis of these findings, we propose a model for the role of SIRT3 in regulating survival and anoikis resistance (Fig. 7).

DISCUSSION

Cancers that are prone to metastases possess an anoikis-resistant phenotype, thereby acquiring more aggressive behavior and resistance to treatment, and contributing to poor survival rates. This reportedly is the case for several cancers, such as prostate, hepatic, and oral cancers.[6,18,19] Thus, understanding the molecular mechanisms underlying an anoikis-resistant phenotype will help identify new potential therapeutic targets to treat aggressive cancer.

Our previous report that RIP can shuttle between survival and death-signaling pathways under anoikis conditions in oral cancer[10] demonstrates the important role of RIP in controlling the fate of OSCC cells under anoikis conditions. In addition, our recent finding that OSCC cells rely on SIRT3 to maintain their survival and aggressive behavior also suggests an important role for SIRT3 in OSCC tumorigenesis. Specifically, we observed that SIRT3 down-regulation decreased OSCC cell proliferation and survival, enhanced the sensitivity of OSCC radioresistant and chemoresistant cells to both radiotherapeutic and chemotherapeutic treatments, and reduced tumor burden in vivo.[14] In the current study, we demonstrate for the first time a link between anoikis resistance and SIRT3 in oral cancer and its potential cross-talk with RIP, thus highlighting 1 important new mechanism by which SIRT3 can modulate OSCC progression.

Our data demonstrate that SIRT3 and RIP have opposite expression levels in OSCC and that OSCC cells escape anoikis by forming multicellular aggregates or oraspheres to maintain their survival compared with single cells, which undergo anoikis-mediated cell death. It is noteworthy that OSCC cells appear to rely, at least in part, on altering their SIRT3 and RIP levels to escape anoikis. Furthermore, OSCC cells that stably expressed down-regulated levels of SIRT3 failed to form oraspheres and, thus, experienced significantly more anoikis, suggesting a critical role for SIRT3 in regulating anoikis resistance in OSCC cells. In agreement with our findings, lung adenocarcinoma cells form spheroids or aggregates that express E-cadherin and phosphorylated Src (p-Src) to maintain their survival under suspension conditions. Thus, it was reported that Src is an essential regulator in the development of anoikis resistance in lung

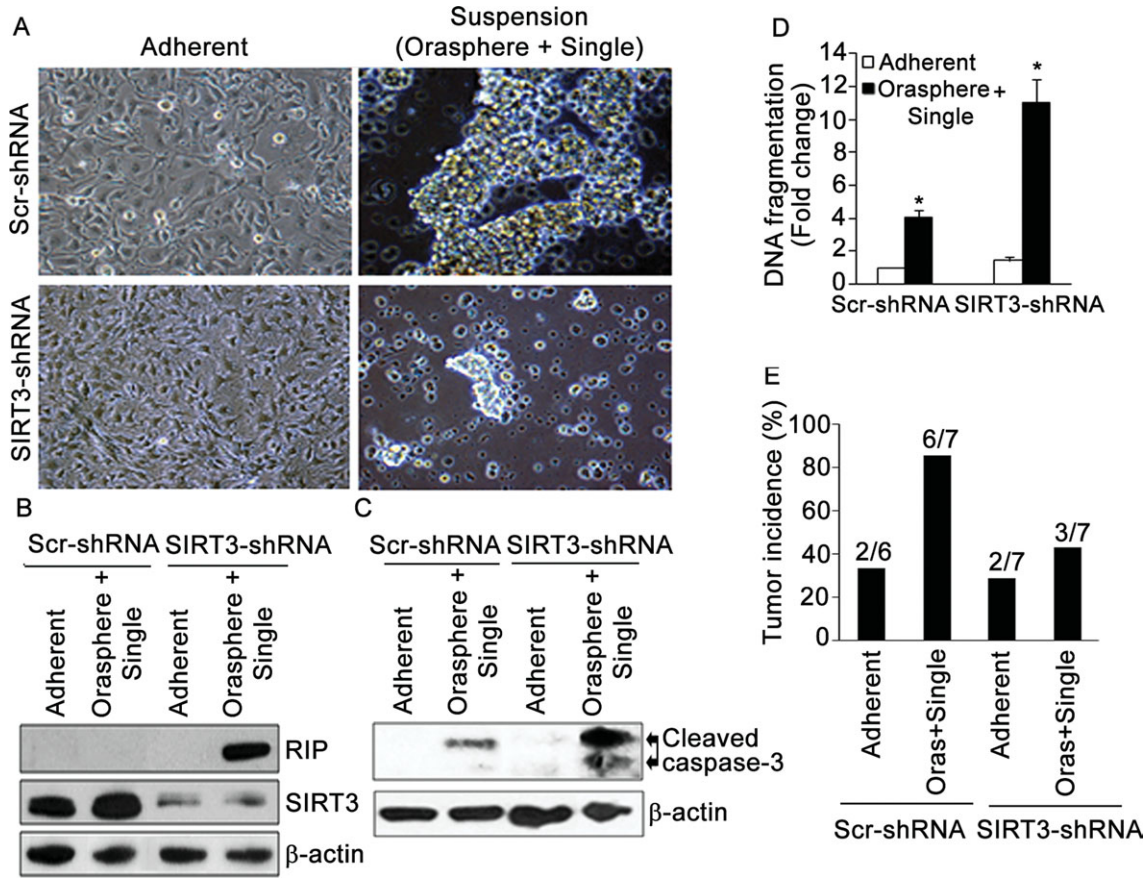

Figure 5. Sirtuin-3 (SIRT3) suppression blocks orasphere formation, inhibits anoikis resistance, and reduces tumor incidence in vivo. (A) These are phase-contrast images of UM-SCC-14A cells that were transduced with scrambled short hairpin RNA (Scr-shRNA) or with SIRT3-shRNA (viral transduction and puromycin selection for 10 days) then cultured under adherent conditions or suspension conditions (oraspheres plus single cells) for 6 days. (B,C) Immunoblots reveal (B) receptor-interacting protein (RIP) and SIRT3 levels and (C) and cleaved/active caspase 3 levels in scrambled controls or SIRT3-suppressed UM-SCC-14A cells that were cultured under adherent conditions or suspension conditions (oraspheres/anoikis-resistant) for 6 days. (D) The fold change in DNA fragmentation is illustrated under adherent conditions or suspension conditions (oraspheres plus single cells) for 6 days. An asterisk indicates $P \leq .05$. (E) The percentage of tumor incidence is illustrated in nude mice that were injected with SIRT3-suppressed UM-SCC-14A cells (SIRT3-shRNA) or scrambled controls (Scr-shRNA) under adherent conditions or suspension conditions (oraspheres [Oras] plus single cells) after 6 weeks. Tumor incidence is indicated relative to the number of animals in each group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

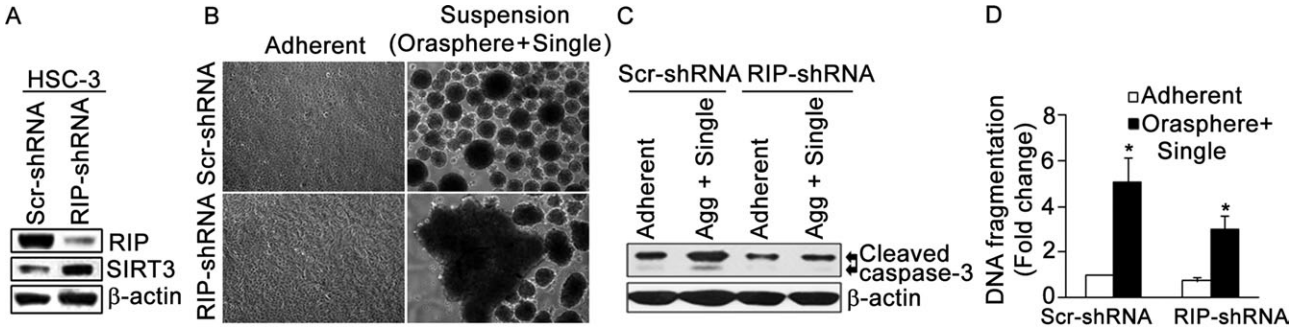

Figure 6. Receptor-interacting protein (RIP) suppression inhibits anoikis, caspase-3 activation, and DNA fragmentation. (A) Immunoblots reveal RIP and sirtuin-3 (SIRT3) levels after stable RIP suppression using lentiviral particles (scrambled short hairpin RNA [Scr-shRNA] or RIP-shRNA). (B) These are phase-contrast images of HSC-3 cells that were transduced with Scr-shRNA or RIP-shRNA (viral transduction and puromycin selection for 10 days) then cultured under adherent conditions or suspension conditions (oraspheres plus single cells) for 6 days. (C,D) Immunoblots reveal (C) cleaved/active caspase 3 expression and (D) the fold change in DNA fragmentation under adherent conditions or suspension conditions (oraspheres plus single cells) for 6 days. Agg indicates aggregates. An asterisk indicates $P \leq .05$.

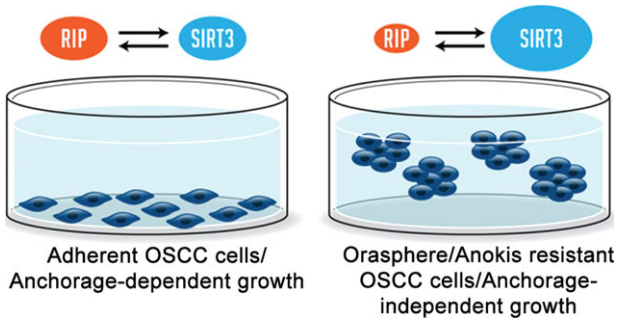

Figure 7. A working model of anoikis resistance is illustrated. Anoikis-resistant cells form multicellular aggregates (oraspheres) that express higher sirtuin-3 (SIRT3) levels as their receptor-interacting protein (RIP) expression decreases, thereby promoting their survival and more aggressive phenotype in oral squamous cell carcinoma (OSCC) development and progression.

adenocarcinomas.[20] It also was reported that mammosphere cultures of breast cancer cells from pleural effusions were tumorigenic and induced tumors in severe combined-immunodeficiency (SCID) mice.[5] Furthermore, a recent report demonstrated that ovarian cancer spheroids used integrin-dependent and talin-dependent activation of myosin and traction forces to promote the displacement of mesothelial cells from underneath a tumor cell spheroid.[21] In summary, multiple studies have supported the concept that spheroid formation promotes cancer cell survival and tumorigenesis.

Limited studies have examined the role of anoikis-resistance in OSCC progression and aggressive behavior using in vivo models. One study used a tongue oral cancer mouse model to demonstrate that anoikis-resistant OSCC cells were more aggressive than their anoikis-sensitive counterparts, and mice injected with these cells had shorter survival (17 days vs 30 days) compared with mice injected with anoikis-sensitive OSCC cells.[6] In our current study, we used a different in vivo model, a murine floor-of-mouth model that mimics human OSCC,[16,17] and demonstrated that anoikis-resistant OSCC cells, which express increased levels SIRT3 and decreased levels of RIP, exhibit greater tumor burden compared with their adherent OSCC counterparts. These data further support our previous in vivo findings, in which OSCC cells with stably suppressed levels of SIRT3 injected in the same murine floor-of-mouth model exhibited reduced tumor burden compared with controls.[14] It is noteworthy that our current data indicate that suppressing SIRT3 also blocks the acquisition of an anoikis-resistant phenotype in vitro and in vivo. Thus, our current data support the finding that SIRT3 and RIP are oppositely expressed in OSCC anoikis-resistant cells, and this enables these cells to escape anoikis and take on a more aggressive phenotype. Because this short, 6-week, floor-of-mouth model is not optimal for the examination of oral metastases, other animal models of metastases are needed to examine this process specifically in future studies.

RIP can localize to the cytoplasm[22,23] or the mitochondria.[24,25] In addition, although there is debate about the subcellular localization of SIRT3, most reports support a mitochondrial localization for SIRT3.[26-34] In the mitochondria, RIP inhibits adenosine diphosphate/adenosine triphosphate (ADP/ATP) exchange by modulating adenine nucleotide translocate (ANT). This modulation results in the loss of ANT and cyclophilin-D interactions, reduction in ATP levels, and induction of cell death, and it implicates RIP in mitochondrial-mediated cell death.[24] Our data demonstrate that SIRT3 and RIP are oppositely expressed in OSCC and that they regulate anoikis-resistance in OSCC cells. Because SIRT3, a mitochondrial sirtuin, can localize to the mitochondrial matrix, and RIP can localize to the mitochondrial outer membrane,[24,25] this suggests that these 2 molecules may interact indirectly through other molecules in the context of regulating anoikis resistance in OSCC cells. One putative molecule would be cyclophilin-D. It is noteworthy that RIP appears to negatively regulate cyclophilin-D, thus inducing mitochondrial-mediated cell death. Conversely, SIRT3 interacts and deacetylates the regulatory component of mitochondrial permeability transition pore (mPTP), cyclophilin-D, thus preventing mitochondrial dysfunction and cardiac hypertrophy.[35] This supports the idea that RIP and SIRT3 play opposite roles in regulating cell death and survival through a common third molecule, cyclophilin-D.

Furthermore, some evidence suggests that RIP is critical for regulating reactive oxygen species (ROS) mechanisms,[36,37] and it is well known that SIRT3 also is functionally important in ROS regulation.[15,38,39] Therefore, RIP and SIRT3 may be functionally related through ROS regulation pathways in the context of anoikis resistance. However, whether this relation is mediated by ROS modulation or by indirect interactions through a third molecule between SIRT3 and RIP, such as cyclophilin-D, is an area that is currently under investigation by our group.

There is also a debate in the literature regarding the role of SIRT3 in cancer.[15] Although some reports support a prosurvival role for SIRT3 in cancer,[14,15,40-43] others support a tumor suppressor role for SIRT3.[44-46] Our previous and current studies demonstrate that SIRT3 plays a prosurvival role in oral cancer; that SIRT3 assists in

chemoresistance and radioresistance; and that, by promoting anoikis resistance, SIRT3 mediates a more aggressive tumorigenic phenotype.

In summary, our studies reveal for the first time a novel role for SIRT3 as a modulator of anoikis resistance in oral cancer through a potential negative relation with RIP. This work further enriches our understanding of the role of SIRT3 in the regulation of oral cancer tumorigenesis and implicates SIRT3 as new potential therapeutic target to treat oral cancer.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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