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

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Abstract

Background—Regulating crosstalk between anoikis and survival signaling pathways is crucial to regulating tissue processes and mitigating diseases like cancer. Previously, we showed that anoikis activates a CD95/Fas-mediated signaling pathway regulated by receptor-interacting protein (RIP), a kinase that shuttles between Fas-mediated cell death and integrin/FAK-mediated survival pathways. Since sirtuin-3 (SIRT3), an NAD-dependent deacetylase, is known to regulate cell survival, metabolism, and tumorigenesis, we hypothesized that SIRT3 might engage in crosstalk 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* we examined the roles of RIP and SIRT3 in oral squamous cell carcinoma (OSCC) anoikis resistance and tumorigenesis.

Results—RIP and SIRT3 have an opposite expression profile in OSCC cells and tissues. Stable suppression of RIP enhances SIRT3 levels, whereas, stable suppression of SIRT3 does not impact RIP levels in OSCC cells. As OSCC cells become anoikis-resistant they form multicellular aggregates or oraspheres in suspension conditions, and their expression of SIRT3 increases as their RIP expression decreases. Also, anoikis-resistant OSCC cells with higher SIRT3 and low RIP expression induce an increased tumor burden and incidence in mice unlike their adherent OSCC cell counterparts. Furthermore, stable suppression of SIRT3 inhibits anoikis resistance and reduces tumor incidence.

Conclusion—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.

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; two 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

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progression[2–5]. Although smoking, alcohol consumption, and HPV are risk factors for oral cancer, other factors contributing 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 one of the leading causes of death worldwide, and oral squamous cell carcinoma (OSCC) accounts for more than 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 showed that receptor interacting protein (RIP), shuttles between CD95/Fas death and 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 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 (SIRT1-7), the mammalian homologues of the Sir2 gene 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, one of the mitochondrial sirtuins[13–15], is overexpressed in OSCC cells and tissues compared to normal, and that downregulation of SIRT3 in OSCC cells inhibited cell growth and proliferation, and increased their sensitivity to both radiation and chemotherapy treatments[14]. In addition, by using a floor-of-mouth oral cancer murine model that mimics human OSCC[16, 17], we showed that SIRT3 downregulation reduced tumor burden *in vivo*, implicating a prosurvival role for SIRT3 in oral cancer tumorigenesis[14]. However, 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 relationship with RIP.

Materials and Methods

Cell lines and culture

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

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, CA) according to the manufacturer's instructions. Antibodies to SIRT3 (AP6242a) and RIP (610459) were from ABGENT (San Diego, CA), and Millipore (Billerica, MA), respectively. We recently reported that SIRT3 staining intensity was higher in OSCC tongue samples[14] and also the tongue accounts for approximately 30% of oral malignancies, thus 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) and 3 (strong). Each core was also evaluated for the percentage of tumor cells staining positive (staining proportion). A combined score based on the staining intensity and the percentage of cells stained was used to assign a final score. Low expression was defined as intensity 1 or 2 and <10% staining proportion; High expression was defined as intensity 2 or 3 and >10% staining proportion.

Transient transfection

HSC-3 cells at 60–70% confluency were transiently transfected with small interfering RNA (siRNA) (25 or 50 nM) against RIP or with a nontargeting control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) in serum-free medium that contained Lipofectamine Plus (Invitrogen, Carlsbad, CA). $RIP^{-/-}$ 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-shRNA (sc-44326-V), SIRT3-shRNA (sc61555-vs) or scrambled-shRNA (sc-108084) (Santa Cruz Biotechnology) lentiviral particles in 0.5 mL of serum-free media, then selected in 10 μg/ml puromycin (sc-108071; Santa Cruz Biotechnology) for an additional 10 days. Surviving cell colonies were picked and propagated before testing for RIP or SIRT3 expression using Western blot analysis. Importantly, 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 (h) lentiviral particles used in this study are from a pool of concentrated, transduction-ready viral particles containing 3 target-specific constructs that encode 19–25 nt (plus hairpin) 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 figure legends, washed once with phosphate-buffered saline, lysed in RIPA buffer (R0278, Sigma) that contained 1% protease inhibitor cocktail (P8340, Sigma), then kept on ice for 30 minutes. Lysates were adjusted for protein concentration with the BCA protein assay kit (Bio-Rad, Hercules, CA). Western blot analyses was performed with various primary antibodies and horseradish peroxidase-conjugated antirabbit or antimouse IgG 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, Billerica, MA), Cell Signaling Technology (2627, Beverly, MA) and Santa Cruz Biotechnology (7148 Santa Cruz, CA), 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 OSCC 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 OSCC cell lines examined. These OSCC cells were developed by maintaining cells under

suspension conditions on poly- HEMA coated plates (7.5 mg/ml in 95% ethanol, Sigma) 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, NCI, 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 35–60 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, mice were euthanized, tumor volumes were measured by digital caliper using the formula $a \times a \times b/2$, where a is the smaller dimension. Tumor tissues were then harvested, rinsed in PBS, and fixed overnight in 10% buffered formalin. Tissues were paraffin-embedded, sectioned, and processed for routine histopathological assessment with hematoxylin and eosin staining and for SIRT3 and RIP immunostaining.

Apoptosis cell death detection by ELISA

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

Statistical Analysis

In general, values are expressed as means ± SD. Intergroup differences were determined by two-way analysis of variance (ANOVA) and Scheffe's multiple-comparison test. Statistical significance was defined as * $p \leq 0.05$. For tissue microarray analyse, McNemar's test was used to compare the two proportions and are 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 to other sirtuins and its stable suppression reduces tumor burden in vivo, implicating SIRT3 as a prosurvival and tumor promoting factor[14]. In addition, we showed that RIP plays a critical role in OSCC cells by regulating anoikis through its shuttling between 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, sirtuin-3 (SIRT3) is known to regulate cell survival, metabolism, and tumorigenesis. Therefore, we hypothesized that SIRT3 might engage in crosstalk 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. Interestingly, we found that SIRT3 and RIP were oppositely expressed in OSCC tissues (Fig. 1A). In 86% of the samples where SIRT3 expression was high, 75% of them had low RIP expression (Fig. 1B; Table 1). Thus 64% (18/28) of the specimens showed an opposite expression pattern for SIRT3 and RIP (Fig. 1A and B, Table 1; $p \leq 0.05$).

SIRT3 and RIP are oppositely expressed in oral squamous cell carcinoma *in vitro*

Similarly, when we examined several OSCC cells (HSC-3, OSCC-3, and UM-SCC-14A) for SIRT3 and RIP expression, we again found that SIRT3 expression was opposite to that of RIP expression in these cells (Fig. 2A). Primary oral keratinocytes also showed an opposite pattern of SIRT3 and RIP expression, however, these cells generally exhibited higher levels of RIP expression compared to SIRT3 (Fig. 2A). These findings again further support the concept that RIP and SIRT3 exhibit a strong negative relationship. 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 relationship. These OSCC cell lines were chosen because they represent the extremes of SIRT3 and RIP expression among 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 OSCC 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; as described previously). Our data show 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. Interestingly, however, the oraspheres express higher levels of SIRT3 and lower levels of RIP compared to 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 OSCC cells that express higher levels of SIRT3 and lower levels of RIP induce a greater tumor burden

Furthermore, to investigate the *in vivo* relevance of our *in vitro* findings, we 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 express higher SIRT3 levels (UM-SCC-14A) or their adherent OSCC cell counterparts that express lower SIRT3 levels (Fig 4). Our data show that mice injected with anoikis-resistant orasphere-derived cells exhibit greater tumor burden compared to their adherent counterparts (Fig. 4A and Table 2). Not only did the mice injected with orasphere-derived cells develop significantly larger tumors (12 fold difference in size; $p \leq 0.027$), but they also developed more tumors (9/10), compared to (2/8) mice injected with adherent control cells (Table 2; $p \leq 0.001$) 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 to

their control counterparts (Fig. 4B). These "aggressive" tumors also revealed high levels of SIRT3 expression and low levels of RIP expression (Fig. 4B). In 80% of the samples where SIRT3 expression was high, 78% of them had low RIP expression (Fig. 4C). In contrast, the few tumors that emerged in mice injected with adherent control cells revealed a tendency towards 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 burden and a more aggressive phenotype in OSCC. In addition, these cells seem to rely, at least in part, on SIRT3 upregulation and RIP downregulation to maintain their survival and aggressive phenotype. Thus, high SIRT3 and low RIP expression may impart resistance to anoikis-mediated cell death.

SIRT3 upregulation and RIP downregulation regulate the fate of OSCC 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 (Clone2) and scrambled-shRNA (Clone 1) were examined under adherent and suspension conditions. Our data show that under suspension conditions, cells expressing SIRT3-shRNA can no longer form oraspheres, maintain their survival, or escape anoikis, unlike cells expressing scrambled-shRNA (Fig. 5A), confirming the role of SIRT3 in anoikis resistance. In addition, cells in suspension conditions stably expressing SIRT3-shRNA (oraspheres and single cells) showed increased levels of RIP expression, cleaved/active caspase-3 and higher levels of DNA fragmentation compared to cells expressing SIRT3-shRNA and grown in adherent conditions or cells expressing scrambled-shRNA and 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 show that SIRT3 and RIP are at opposite ends of anoikis resistance in OSCC cells, and SIRT3 is required for escaping anoikis and for acquisition of an anoikis-resistant phenotype. Based on these findings, we propose a model for SIRT3's role in regulating survival and anoikis resistance (Fig. 7).

Discussion

Cancers that are prone to metastases possess an anoikis-resistant phenotype, thereby acquire a more aggressive behavior, resistance to treatment, and contributing to poor survival rates. This has been the case for several cancers, such as prostate, hepatic, and oral cancer[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 found that SIRT3 downregulation decreased OSCC cell proliferation and survival, enhanced the sensitivity of OSCC radio- and chemo-resistant cells to both radio- and chemotherapeutic treatments, and reduced tumor burden *in vivo*[14]. Here, we show for the first time a link between anoikis-resistance and SIRT3 in oral cancer and its potential cross-talk with RIP, thus highlighting one important new mechanism by which SIRT3 can modulate OSCC progression.

Our data show that SIRT3 and RIP are oppositely expressed in OSCC and that OSCC cells escape anoikis by forming multicellular aggregates or oraspheres to maintain their survival compared to single cells, which undergo anoikis-mediated cell death. Interestingly, OSCC cells seem to rely, at least in part, on altering their SIRT3 and RIP levels to escape anoikis. Furthermore, OSCC cells stably expressing downregulated 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 p-Src to maintain their survival under suspension conditions. Thus, Src was found to be an essential regulator in the development of anoikis-resistance in lung adenocarcinomas[20]. Also, mammosphere cultures of breast cancer cells from pleural effusions are tumorigenic and induce tumors in SCID mice[5]. Furthermore, a recent report demonstrates that ovarian cancer spheroids use integrin- and talin-dependent activation of myosin and traction forces to promote displacement of mesothelial cells from underneath a tumor cell spheroid[21]. In summary, multiple studies support 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 show that anoikis-resistant OSCC cells are more aggressive than their anoikis-sensitive counterparts, and mice injected with these cells have shorter survival rates (17 days versus 30 days) compared to 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 showed that anoikis-resistant OSCC cells, which express increased levels SIRT3 and decreased levels of RIP, exhibit greater tumor burden compared to their adherent OSCC counterparts. These data further support our previous *in vivo* findings, whereby OSCC cells with stably suppressed levels of SIRT3 injected in the same murine floor-of-mouth model exhibited reduced tumor burden compared to controls[14]. Importantly, our current data further show that suppressing SIRT3 also blocks the acquisition of an anoikis resistant phenotype in vitro and in vivo. Thus, our current data support 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. Since this short 6-week floor-of-mouth model is not optimal for 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 ADP/ATP exchange via 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 implicates RIP in mitochondrial-mediated cell death [24]. Our data show that SIRT3 and RIP are oppositely expressed in OSCC and they regulate anoikis-resistance in OSCC cells. Since 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 two molecules may interact indirectly via other molecules in the context of regulating anoikis resistance in OSCC cells. One putative molecule would be cyclophilin-D. Interestingly, RIP seems to negatively regulate cyclophilin-D, thus inducing mitochondrial-mediated cell death. On the other hand, 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 via a common third molecule, cyclophilin-D.

Furthermore, some evidence suggests that RIP is critical for regulating ROS mechanisms[36, 37] and it is well known that SIRT3 is functionally also important in ROS regulation[15, 38, 39]. Therefore, RIP and SIRT3 may be functionally related via ROS regulation pathways in the context of anoikis resistance. However, whether this relationship is mediated via ROS modulation or indirect interactions via 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 chemo- 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 via potential negative relationship with RIP. This work further enriches our understanding of SIRT3's role in the regulation of oral cancer tumorigenesis and implicates SIRT3 as new potential therapeutic target to treat oral cancer.

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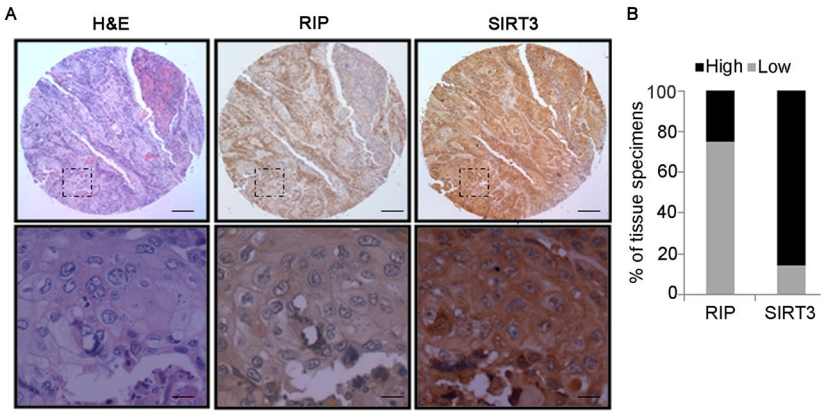

Figure 1. RIP expression shows an opposite relationship to SIRT3 expression in oral squamous cell carcinoma (OSCC)

(A) RIP and SIRT3 expression in OSCC TMA specimens are illustrated. Scale bars, 200 μm for low-magnification photographs (top) and 50 μm for high-magnification photomicrographs (bottom). (B) Percentage of OSCC tissue specimens expressing RIP and SIRT3 in tongue serial sections, determined by immunohistochemical staining. Staining intensity was graded as High or Low. McNemar's test was used to compare the two proportions and are significantly different, $P \leq .001$.

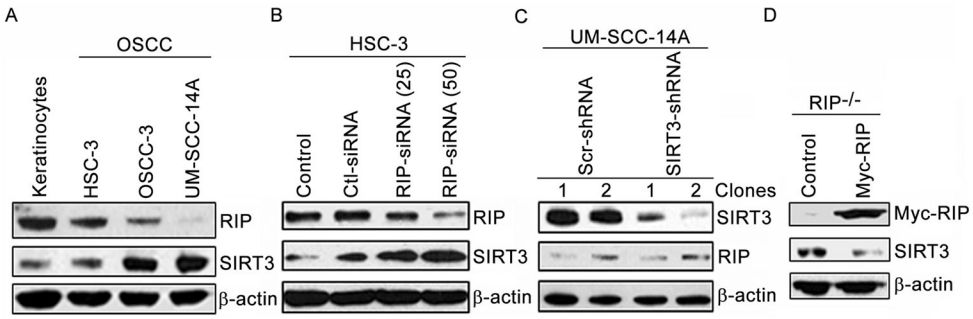

Figure 2. RIP may be an upstream negative regulator of SIRT3

(A) Immunoblots show RIP and SIRT3 levels in normal human keratinocytes and oral squamous cell carcinoma cells (OSCC) plated for one day. β-actin served as loading control. (B) Immunoblots show RIP and SIRT3 levels after transfection with control siRNA or RIP siRNA (25 or 50 nM) in HSC-3 cells for 30 h (C) Immunoblots show SIRT3 and RIP levels after stable SIRT3 suppression using lentiviral particles (scrambled controls or SIRT3-shRNA) in UM-SCC-14A cells. (D). Immunoblots show RIP and SIRT3 levels after transfection with wild-type myc-RIP in RIP null cells ($RIP^{-/-}$) for 30 h.

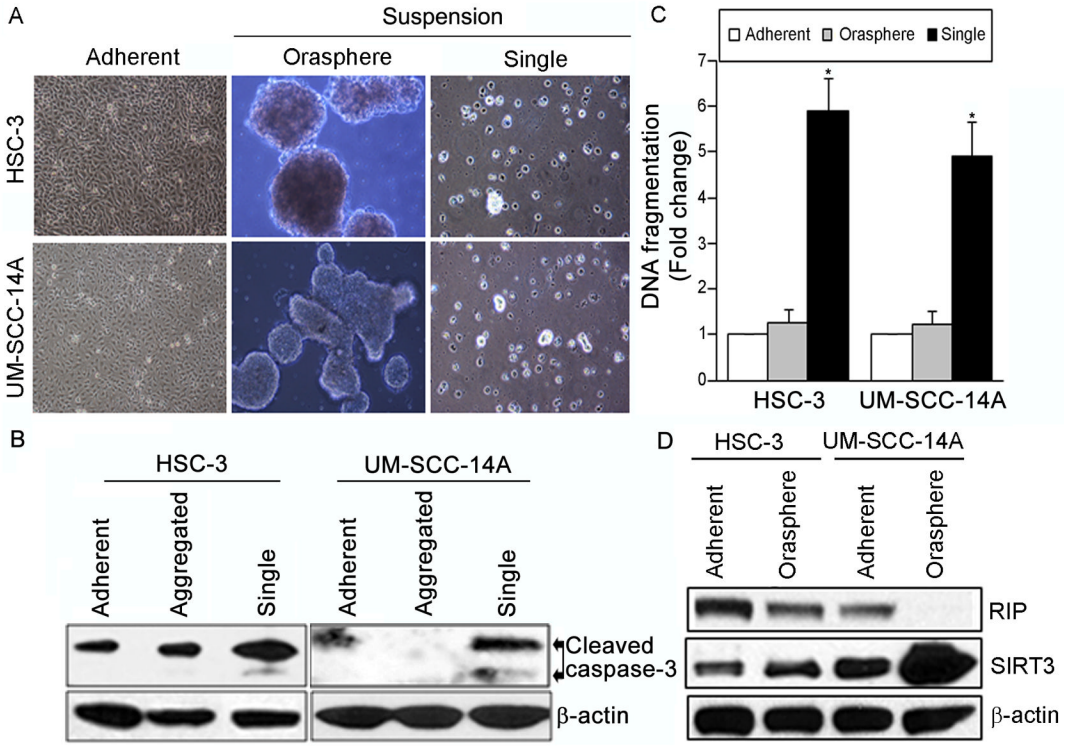

Figure 3. As OSCC cells become anoikis resistant their SIRT3 expression increases as their RIP expression decreases

(A) Phase contrast images of OSCC cells (HSC-3 and UM-SCC-14A) cultured under adherent or suspension conditions for 6 days and orasphere and single cells were separated for analysis. (B) Immunoblots showing cleaved/active caspase-3 expression, and (C) fold change of DNA fragmentation in adherent, orasphere and single cells. (D) Immunoblots showing RIP and SIRT3 expression in adherent and orasphere.

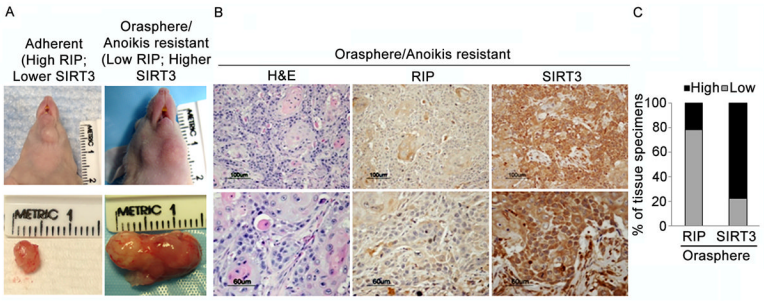

Figure 4. Anoikis resistant OSCC cells induce greater tumor burden in mice

(A) Images of tumor-bearing mice 6 weeks after injection with adherent or orasphere/ anoikis resistant UM-SCC-14A cells. Top panels show superficial tumors and lower panels show dissected tumor. (B) Representative orasphere derived tumor section stained with H&E (left) and immnostained with antibodies for RIP (middle) and SIRT3. (C) Percentage of tissue specimens expressing RIP and SIRT3 in orasphere derived tumor sections, determined by immunohistochemical staining. Staining intensity was graded as High or Low. McNemar's test was used to compare the two proportions and are significantly different, $P \leq .001$.

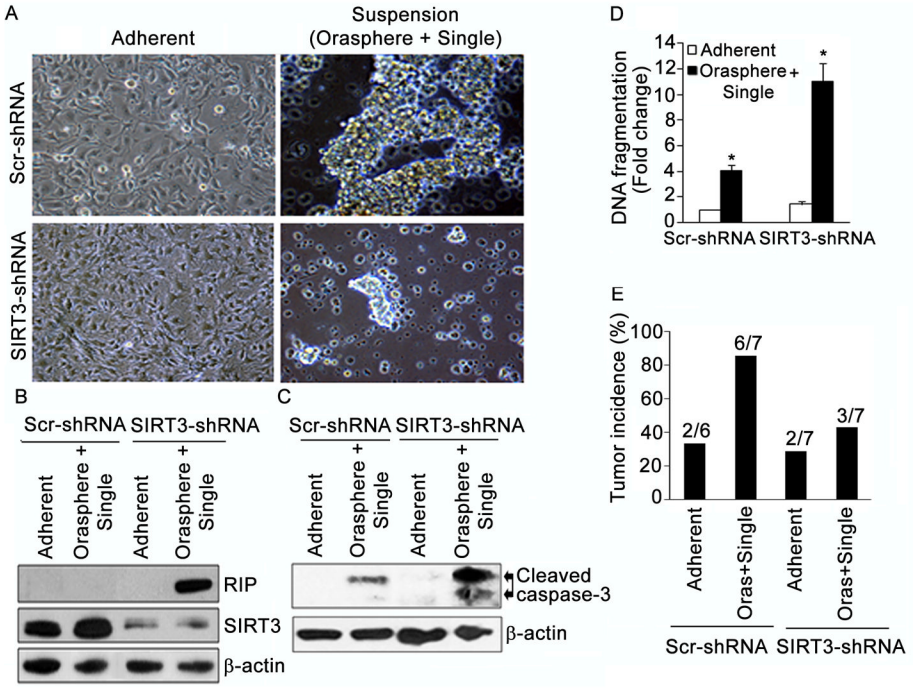

Figure 5. SIRT3 suppression blocks orasphere formation, inhibits anoikis resistance, and reduces tumor incidence *in vivo*

(A) Phase contrast images of UM-SCC-14A cells transduced with scrambled shRNA (Scr-shRNA) or SIRT3 shRNA (viral transduction and puromycin selection for 10 days) then cultured under adherent or suspension (oraspheres plus single cells) conditions for 6 days. (B&C) Immunoblots show RIP and SIRT3 (B) and cleaved/active caspase-3 (C) levels in scrambled controls or SIRT3 suppressed UM-SCC-14A cells cultured under adherent or suspension (oraspheres/anoikis-resistant) conditions for 6 days. (D) Fold change in DNA fragmentation in adherent or suspension (orasphers plus single cells) conditions for 6 days. (E) Percentage of tumor incidence in nude mice injected with SIRT3 suppressed UM-SCC-14A cells (SIRT3-shRNA) or scrambled controls (Scr-shRNA) from adherent or suspension (oraspheres plus single cells) conditions after 6 weeks. Tumor incidence is shown relative to the number of animals in each group.

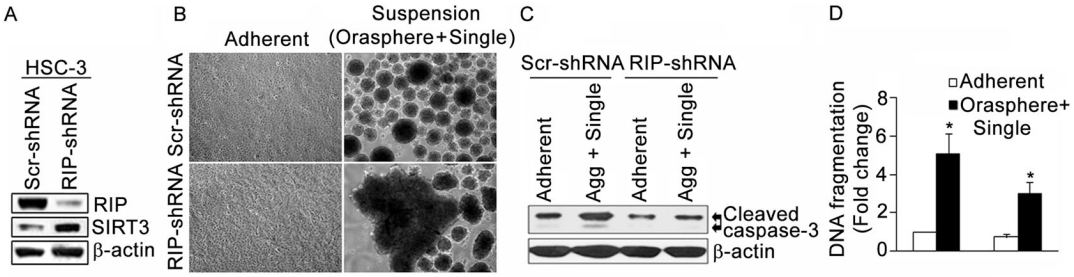

Figure 6. RIP suppression inhibits anoikis, caspase-3 activation and DNA fragmentation

(A) Immunoblots show RIP and SIRT3 levels after stable RIP suppression using lentiviral particles (scrambled controls or RIP-shRNA). (B) Phase contrast images of HSC-3 cells transduced with scrambled shRNA (Scr-shRNA) or RIP shRNA (viral transduction and puromycin selection for 10 days) then cultured under adherent or suspension (oraspheres plus single cells) conditions for 6 days. (C) Immunoblots showing cleaved/active caspase-3 expression, and (D) fold change of DNA fragmentation in adherent or suspension (orasphers plus single cells) conditions for 6 days.

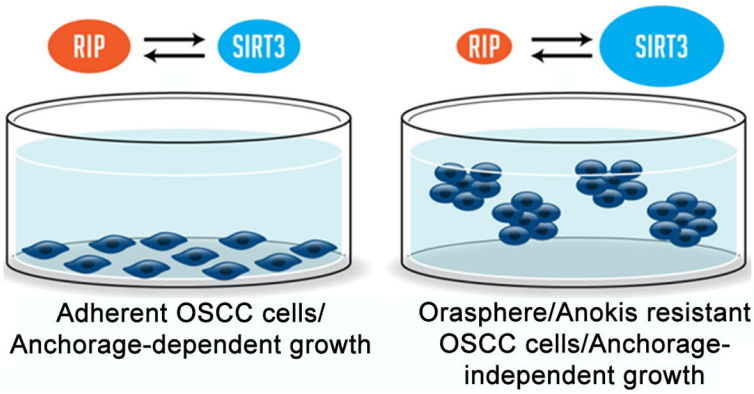

Figure 7. Working model of anoikis resistance

Anoikis-resistant cells form multicellular aggregates (oraspheres) that express higher SIRT3 levels as their RIP expression decreases thereby promoting their survival and more aggressive phenotype in OSCC development and progression.

Table 1

The expression profile of RIP and SIRT3 from 28 different tongue TMAs samples

Sample number	Section number	Sex	Age	RIP	SIRT3
1	A1	M	57	L	H
2	A6	F	35	H	H
3	A7	M	78	L	H
4	A9	F	39	L	L
5	A10	F	46	L	H
6	B3	F	57	L	H
7	B4	F	36	L	H
8	B8	F	47	H	H
9	B9	F	63	L	L
10	B10	M	56	L	L
11	C2	F	55	L	H
12	C3	M	76	L	H
13	C4	F	50	L	H
14	C6	M	55	H	H
15	C9	M	60	L	H
16	D1	M	64	L	H
17	D2	F	52	L	H
18	D3	F	50	L	H
19	D4	F	46	L	H
20	D5	F	45	L	H
21	D6	M	35	L	H
22	D7	F	46	H	H
23	D8	F	48	H	L
24	E3	M	60	H	H
25	E4	M	37	L	H
26	E6	M	60	L	H
27	E8	M	60	H	H
28	E10	M	73	L	H

Staining intensity was graded as Low (L) or High (H)

Table 2

Tumor volumes for mice injected with UM-SCC-14A

Animal	Adherent	Orasphere/Anoikis resistant
	Tumor Volume (mm^3)	Tumor Volume (mm^3)
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*	311.84*

Statistical analysis: Independent t-test with unequal variances $p \leq 0.027$