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Original Articles

MicroRNA *miR-31* targets SIRT3 to disrupt mitochondrial activity and increase oxidative stress in oral carcinoma

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ABSTRACT

MicroRNA *miR-31* is implicated in the neoplastic process of various malignancies including oral squamous cell carcinoma (OSCC). Silent information regulator 3 (Sirtuin3 or SIRT3) is a NAD-dependent deacetylase that regulates metabolic process. Suppressor role of SIRT3 has been found in neoplasms. This study investigates the disruptions of *miR-31*-SIRT3 cascade to explore their potential association with metabolic change in OSCC. We identified that *miR-31* directly targeted SIRT3 in OSCC cells, and a reverse correlation between *miR-31* expression and SIRT3 expression was noted in OSCC tumors. SIRT3 expression attenuated the *miR-31* enhanced tumor cell migration and invasion. It also reduced the tumorigenic potential of FaDu cell line. *miR-31*-SIRT3 impaired the mitochondrial membrane potential and structural integrity. The dis-regulation of this axis also contributed to the genesis of oxidative stress. In addition, *miR-31* switched tumor cells from aerobic metabolism to glycolytic metabolism. This study provides novel evidences demonstrating the presence of *miR-31*-mediated post-transcriptional regulation of SIRT3 in OSCC. The disruption of *miR-31*-SIRT3 cascade and the consequential metabolic aberrances are involved in OSCC progression.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) including oral squamous cell carcinoma (OSCC) is a prevalent malignancy worldwide. Improving the survival rate of OSCC patients is still a challenge till far, and can only be done by in-depth understanding of the pathogenesis in OSCC. MicroRNAs (miRNAs) play important roles in neoplastic process of OSCC [1–3]. Our previous studies have identified that *miR-31* is oncogenic for OSCC [3,4]. *miR-31* is up-regulated by epidermal growth factor receptor (EGFR) [5], and its upregulation modulates the hypoxic induction, and the increased gene damage and stemness by targeting factor inhibiting HIF (FIH) and other suppressors [3–7]. Moreover, *miR-31* is able to be validated as a diagnostic marker and a prediction marker for HNSCC or OSCC progression [8,9]. *miR-31* is also involved in the drug resistance or enrichment of oncogenicity in other types of malignancies [10,11].

Sirtuin (silent information regulator, SIRT) is a subclass of histone deacetylases consisting of SIRT1 to SIRT7. SIRT family members, localized in different cell compartments, regulate a wide variety of cellular physiologies [12,13]. Specifically, SIRT3 was reported as a mitochondrial deacetylase involved in metabolism and oxidation regulation [14]. Energy production in cells is mainly mediated by oxidative phosphorylation system (OXPHOS) in mitochondrial complexes [15]. As complexes I - IV are in charge of electron transfer and complex V synthesizes ATP [16], SIRT3 is able to deacetylate complex I and II to facilitate the electron transportation [17,18]. SIRT3 also contributes to the homeostasis of oxidative stress by activating manganese-dependent superoxide dismutase 2 (MnSOD2) and other genes through deacetylation when reactive oxygen species (ROS) is generated during the pathophysiological process [15,19]. Since the mitochondrial ROS conveys the intracellular homeostasis to become mitogenic, which may disrupt apoptosis, and evoke oncogenic signals, SIRT3 may be an important regulator in tumorigenesis [20–22].

SIRT3 may function as either oncogene or suppressor in different types of cancers [15]. In lung, liver, esophageal and gastric carcinomas, SIRT3 was shown to be a suppressor as it was down-regulated in

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Abbreviation

ECAR	extracellular acidification rate
EGFR	epidermal growth factor receptor
FIH	factor inhibiting HIF
HNC	head and neck cancer
HNSCC	head and neck squamous cell carcinoma
LNA	locked nucleic acid
NAM	nicotinamide
miRNA	microRNA
MnSOD2	manganese-dependent superoxide dismutase 2
MUTR	mutant reporter
NCMT	non-cancerous matched tissue
OCR	oxygen consumption rate
OE	stable overexpression cell subclone
oeSIRT3	overexpress SIRT3
OSCC	oral squamous cell carcinoma
OXPHOS	oxidative phosphorylation system
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
Scr	scramble
SDHA	dehydrogenase complex subunit A
SIRT3	silent information regulator 3
VA	vector alone
VAR	vector alone reporter
WTR	wild-type reporter

tumors, or it acted as an apoptosis inducer associated with patient's survival [23–25]. Nevertheless, the oncogenic roles of SIRT3 in breast carcinomas tend to be more contentious [15,26] and the oncogenic roles of SIRT3 in head and neck carcinogenesis are still obscure. SIRT3 downregulation was found to decrease the proliferation, and increase the sensitivity to radiation and cisplatin in OSCC cell lines [27]. However, the SIRT3 activity decreased in OSCC cell lines relative to normal oral keratinocyte [28], and the functional loss of SIRT3 increased proliferation and oxidative stress of normal oral keratinocyte [29]. Tobacco smoking, areca chewing, and alcohol use are major OSCC risk factors in Asians. Areca ingredients evoke ROS, activate downstream signals and convey SIRT3 upregulation [29,30]. Decreased SIRT3 expression was also shown in Asian OSCC [31]. Therefore, the controversies of SIRT3 role in OSCC pathogenesis remain to be clarified.

The dysfunction of mitochondria and adaptation of aerobic glycolysis for energy production are frequent events in malignancies [32,33].

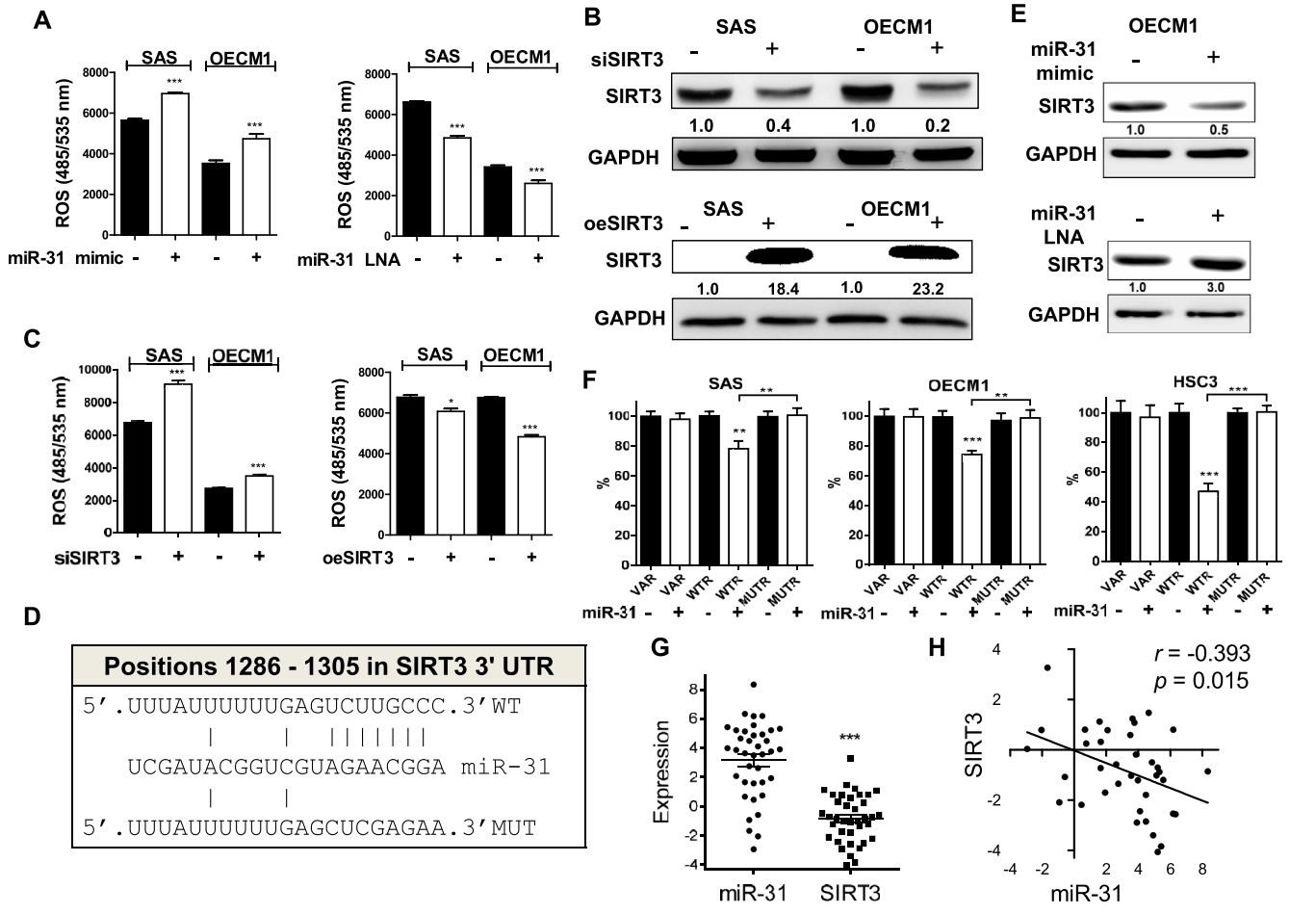

Fig. 1. *miR-31* targets SIRT3 to enhance oxidative stress in OSCC cells. (**A**, **C**) ROS analysis. (**B**, **E**) Western blot analysis for SIRT3 expression. Numbers, normalized values, oeSIRT3, overexpress SIRT3. (**D**) Complementarity between the seed sequence of *miR-31* and the targeted sequence in 3′UTR of SIRT3 predicted by TargetScan *in silico* browser. (**F**) Reporter assay. VAR, vector alone reporter; WTR, wild type reporter; MUTR, mutant reporter. (**G**) Dot plot of $-\Delta\Delta C_t$ representing *miR-31* expression and *SIRT3* mRNA expression in OSCC/NCMT tissue pairs. (**H**) The correlation analysis between *miR-31* expression and *SIRT3* mRNA expression in tumors.

Table 1
SIRT3 mRNA expression in OSCC.

n =		-ΔΔCt	p value
	43	−0.82 ± 0.27	
Age (Mean ± SE, y)	57.7 ± 1.6		
Gender (M/F)	40/3		
Buccal mucosa	17	−0.76 ± 0.55	0.93
Oral tongue and other sites	26	−0.81 ± 0.26	
Areca chewing	37	−0.74 ± 0.30	0.61
Non-areca chewing	6	−1.10 ± 0.59	
Smoking	38	−0.82 ± 0.29	0.75
Non-smoking	5	−0.64 ± 0.44	
Drinking	17	−1.79 ± 0.46	0.005
Non-drinking	26	−0.14 ± 0.26	
T1-3	18	−0.79 ± 0.40	0.99
T4	25	−0.80 ± 0.36	
N0	30	−0.90 ± 0.36	0.48
N+	13	−0.57 ± 0.32	
Stage I-III	15	−0.79 ± 0.46	0.98
Stage IV	28	−0.80 ± 0.36	
Well differentiated	17	−0.65 ± 0.49	0.69
Moderately or poorly differentiated	26	−0.89 ± 0.31	
Alive and disease-free	29	−0.63 ± 0.28	0.37
Relapse or death	14	−1.15 ± 0.59	

miR-31 targets succinate dehydrogenase complex subunit A (SDHA) mitochondria protein to elicit the glycolytic flux during metabolic reprogramming [33]. *miR-31*-FIH regulative axis also enhances glycolysis in lung cancer cells [32]. This study identifies the downregulation of SIRT3 in OSCC. Besides, we specifies the functional roles of *miR-31*-SIRT3 regulation axis in modulating the aggressiveness, ROS genesis, mitochondrial damage, and metabolic switch in OSCC.

2. Materials and methods

2.1. Cell culture and reagents

The OECM1, SAS, FaDu and HSC3 OSCC cell lines and 293T cells were cultured as previously described [2,3]. *miR-31* mimic (100 or 120 nM; PM11465), *miR-31* locked nucleic acid (LNA, 60 nM; AM12887) and miRNA control (60, 100 or 120 nM; AM17110) were purchased from Ambion (Austin, TX). The siSIRT3 (80 nM; sc-61555) oligonucleotide and scramble (Scr, 80 nM; sc-37007) control oligonucleotide were purchased from Santa Cruz Biotech (Santa Cruz, CA). Nicotinamide (NAM), a SIRT deacetylase inhibitor, and other unspecified reagents were purchased from Sigma-Aldrich (St Louise, MO).

2.2. Subjects

The OSCC tumor tissues along with paired non-cancerous matched tissues (NCMT) were used for reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. The samples were collected during 2013–2016 after obtaining written informed consent. This study was approved by the Institutional Review Board (approval number 12MMHIS177).

2.3. Quantitative reverse transcriptase polymerase chain reaction

The TaqMan miRNA assay kit (Applied Biosystems; Foster City, CA) was used to carry out qRT-PCR to analyze the expression of hsa-*miR-31,* mmu-*miR-31* and *SIRT3*, as *U6B* and *GAPDH* were used as internal control, respectively [2,3]. $-\Delta\Delta C_t$ represents the differences between an OSCC and its paired NCMT in the normalized threshold cycle number for exponential amplification.

2.4. Western blot analysis

Aliquots of cell lysates were subjected to Western blot analysis according to our protocols [2,3]. The anti-SIRT3 primary antibody (cat. no. 2627; Cell Signaling, Danvers, MA) was at 1: 200 dilution. Total OXPHOS rodent antibody cocktail (cat no. ab110413, Abcam, Cambridge, MA) at 1: 200 dilution was used to analyze respiratory chain complex proteins. Normalization of the signals to Actin or GAPDH was used to generate expression values.

2.5. Phenotypic assays

Cell viability was analyzed using trypan-blue exclusion assay. Cell migration was carried out using ORIS migration plate (Platypus Tech, Madison, WI), which measured the percentage of wound closure to represent cell mobility [4]. In addition, transwell (Corning, Acton, MA) membrane appliance with a pore of 8 μm in diameter was also used to assay cell migration. The lower surface of membrane was coated with fibronectin for chemoattraction. For invasion assay, transwell (Corning) membrane was coated with matrigel (BD Bioscience, San Jose, CA). The growth of cells was arrested with the treatment of 1 μM hydroxyurea [2,3]. For the induction of xenografic tumors, 1 or 3×10^6 FaDu cells were injected subcutaneously into the shoulders and/or flanks of athymic mice. Tumor volumes were assessed using the formula = 0.5x longest diameter x (shortest diameter)^2 [3]. After sacrificing the mice at end point, tumors were resected, measured and subjected to expression analysis. This animal study was approved by Institutional Animal Care and Use Committee of National Yang-Ming University.

2.6. Reporter construction and assay

The 3′UTR sequence of *SIRT3* was cloned into the pMIR-REPORT vector (Life Technologies, Grand Island, NY) to generate the wild-type reporter (WTR). A mutant reporter (MUTR) construct was obtained from the WTR by replacing the targeted sequence TCTTGCCC to CTC GAGAA, containing a Xho I restriction enzyme digestion site. Firefly luciferase activity after normalizing to transfection efficiency represented reporter activity. Transfectin lipid transfection reagent was purchased from BioRad Lab (Hercules, CA). The activity of vector alone reporter (VAR) was also analyzed in parallel.

2.7. Exogenous miR-31 and SIRT3 expression

The mmu-pre-*miR-31* sequence was cloned into pLAS5w.PtRFP-I2-puro vector (National RNAi Core, Academia Sinica, Taipei, Taiwan). After lentiviral infection and fluorescence sorting, stable OSCC cell subclones expressing *miR-31* were established and designated OE *miR-31*. Stable cell subclones containing empty vector only were used as controls. The coding sequence of SIRT3 was cloned into the pBabe-puro vector (Addgene, Cambridge, MA). OSCC cells were transfected with this plasmid to transiently overexpress SIRT3 (oeSIRT3). In addition, retroviral-infected OSCC cells were selected with puromycin to achieve cell subclones stably express SIRT3, designated OE SIRT3. Stable cell subclones containing empty vector alone were used as controls.

2.8. ROS analysis

Cells were challenged with 1 mM H_2O_2 at 37 °C for 20 min, then stained with 25 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, CA). The acquired values by subtracting the background fluorescence as measured in the unstained controls from the total fluorescence represented the ROS level [2].

2.9. Analysis of mitochondria structure

Cells were reacted with MitoTracker® M7511 fluorescence probe

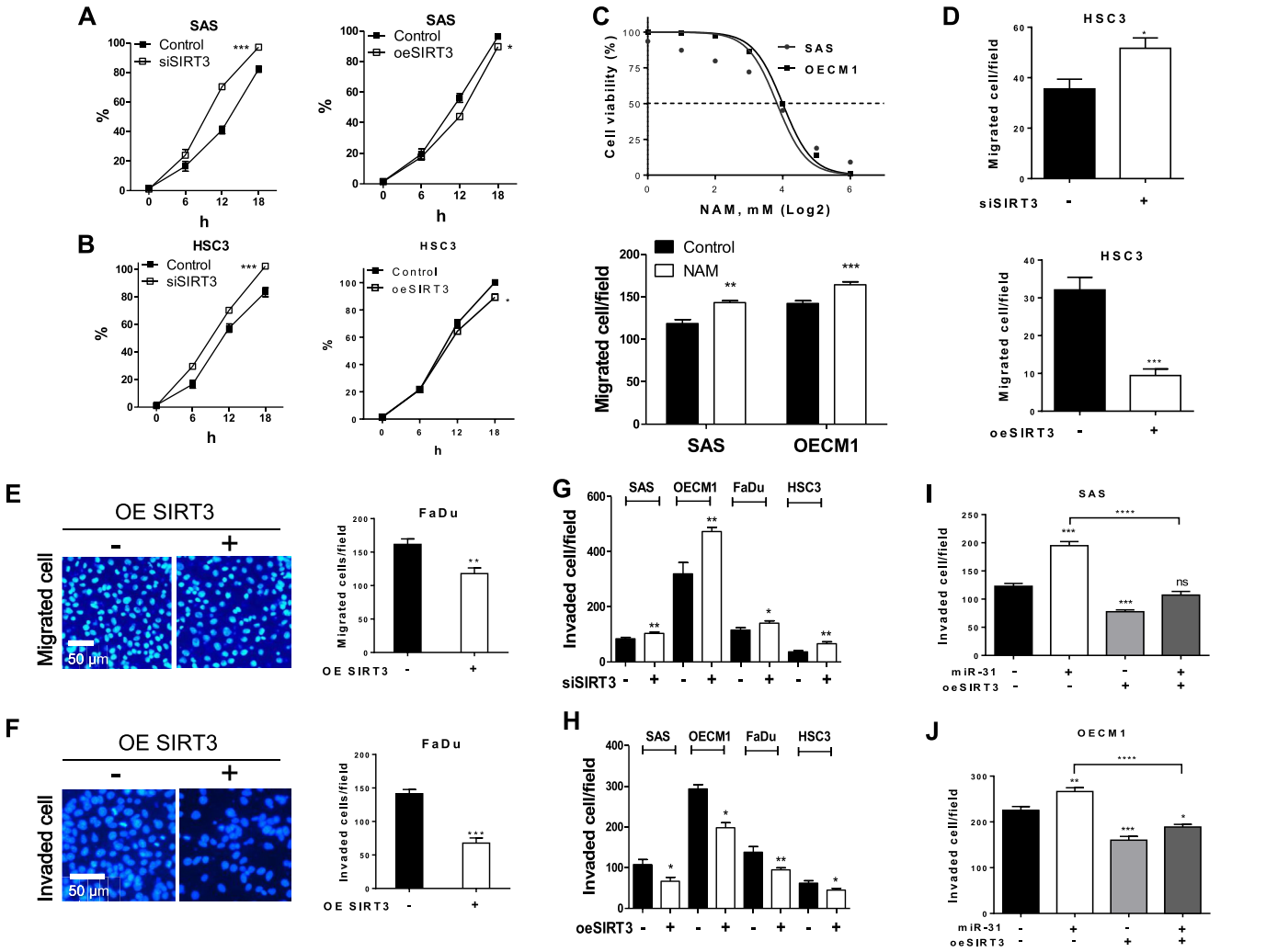

Fig. 2. SIRT3 inhibits the mobility and invasion of OSCC cells. (**A**, **B**) Wound healing assay. (**A**) SAS cells. (**B**, **D**) HSC3 cells. (**E, F**) FaDu cells. (**C - E**) Transwell migration assay. (**C**) Upper, cell viability curve achieved to define the IC_{50} of nicotinamide on SAS and OECM1 cells. Lower, the migratory induction mediated by nicotinamide in OSCC cells. NAM, nicotinamide. (**F - J**) Invasion assay. (**E**, **F**) Left, representative images of fluorescence-labelled nuclei of cells migrated to the lower surface of transwell membrane. Right, quantitation of the migrated cells. Decreased migration (in **E**) and invasion (in **F**) is noted in OE SIRT3 cell subclone of FaDu relative to controls, respectively. (**G**, **H**) Increased and decreased invasion in OSCC cells induced by knockdown and exogenous expression of SIRT3, respectively. (I, J) Rescue experiments. Reverse of *miR-31* associated invasion by exogenous SIRT3 expression in SAS cell and OECM1 cell, respectively. oeSIRT3, overexpress SIRT3; OE SIRT3, cell subclone stably expresses SIRT3.

(Invitrogen) for 30 min to label the mitochondria. The images of fluorescent mitochondria was captured by Zeiss LSM 880 confocal microscope with Airyscan (Jena, Thuringia, Germany).

2.10. Analysis of mitochondrial membrane potential change

JC-1 is a mitochondrial membrane potential indicator, which forms red fluorescent J-aggregates when reacting with negatively charged mitochondrial membrane potential. The red/green ratios of fluorescence represent the health (i.e. polarized) status of the mitochondria. The mitochondrial membrane potential was assayed using MitoProbe™ JC-1 Assay kit (Invitrogen) and the images were achieved by Zeiss LSM 880 confocal microscope.

2.11. Measurement of cellular bioenergetics

The mitochondrial functions were analyzed using Seahorse XF24 Extracellular Flux system (Seahorse Bioscience, North Billerica, MA). Cells were seeded into assay wells and grew for 12 h. The subsequent experiment procedures followed the protocols provided by the supplier. The raw readings of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were provided from the calculation software affiliated with the appliance. The calibrated basal and maximal respiration, and ATP production were achieved by normalizing the raw OCR readings with cell numbers.

2.12. Measurement of lactate and glucose

Aliquots of culture medium obtained from equivalent numbers of cells were measured for L(+)-lactate and glucose using a Lactate Colorimetric Assay Kit (Bio Vision, Milpitas, CA) and a Glucose Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI), respectively. Both assessments were conducted under the protocols supplied by manufacturers. The outputs were measured using an enzyme-linked immunosorbent assay reader. The readings were normalized with cell numbers to achieve the calibrated values.

2.13. Statistical analysis

Data were shown as Mean ± S. E. Mann–Whitney *U* test, Wilcoxon

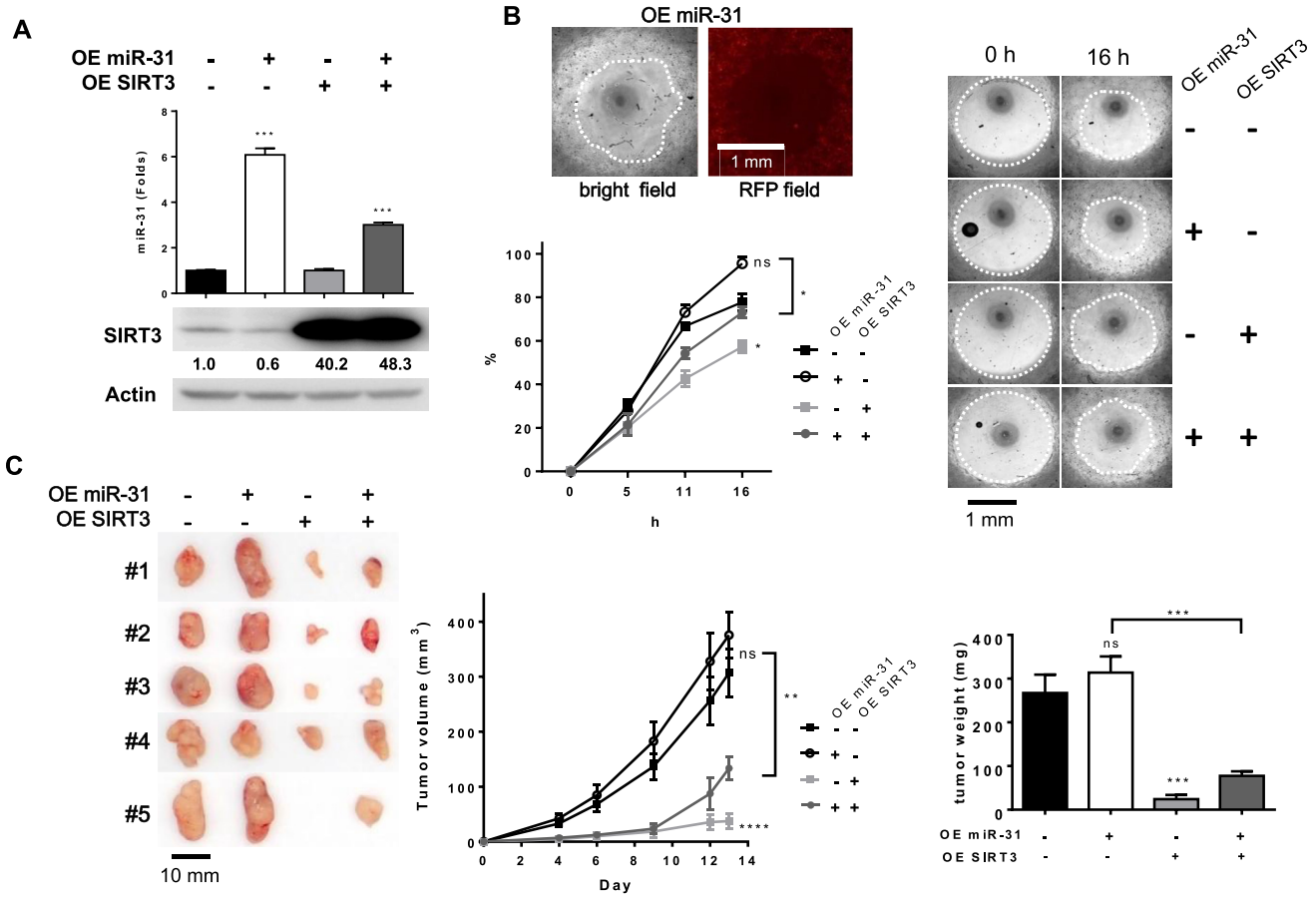

Fig. 3. *miR-31*-SIRT3 modulates the migration and tumorigenesis of FaDu cell. (**A**) qRT-PCR analysis (Upper) and Western blot analysis (Lower) illustrating the exogenous *miR-31* and SIRT3 expression in stable cell subclones. Numbers, normalize values. (**B**) Left upper, the amplified bright field and fluorescent images of OE *miR-31* cell subclone in the well for wound healing assays. Right, representative images for wound healing assays. Dot lines mark the borders of cell occupying zones. Bars, 1 mm. Left lower, quantitation for wound healing assay. (**C**) Tumorigenesis assay. Left, gross images of resected tumors. Bar, 10 mm. Middle, growth curve of xenografts. Slight increase of tumor xenografts with exogenous *miR-31* expression being drastically decreased by SIRT3 expression is noted. Right, Quantitation of the weight of tumor xenografts. OE *miR-31*, cell subclone stably expresses *miR-31*. OE SIRT3, cell subclone stably expresses SIRT3.

rank sum test, *t*-test, two-way analysis of variance and correlation analysis were used. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant.

3. Results

3.1. miR-31 targets SIRT3 and both modulate ROS in OSCC

miR-31 is oncogenic by disrupting several neoplastic processes [3,4,7]. This study investigated if *miR-31* expression can modulate oxidative stress of SAS and OECM1 cells. After challenging with H_2O_2, the treatment with *miR-31* mimic increased ROS (Fig. 1A, left), while *miR*-31 LNA decreased ROS (Fig. 1A, right) in OSCC cells. The knockdown of SIRT3 and exogenous expression of SIRT3 were established in OSCC cells (Fig. 1B). Since SIRT3 is a ROS regulator, the analysis of H_2O_2 treated cells showed that knockdown of SIRT3 increased ROS (Fig. 1C, left). In contrast, SIRT3 expression decreased ROS (Fig. 1C, right). As *SIRT3* is a predicted targeted gene of *miR-31* (Fig. 1D), SIRT3 protein expression was found decreased in the OECM1 cells with the treatment of *miR-31* mimic and increased with *miR*-31 LNA in OSCC cells (Fig. 1E). The analysis of the WTR and MUTR reporters showed that *miR-31* mimic attenuated the activity of the WTR in the OSCC cells. However, *miR-31* driven repression was reverted in the cells with the MUTR (Fig. 1F). *miR-31* expression did not affect the activity of VAR. To conclude, *miR-31* directly targets SIRT3 to repress its expression. In addition, *miR-31* may be involved in ROS regulation by affecting SIRT3 expression.

3.2. Decreased SIRT3 mRNA expression in OSCC tumors

In a total of 43 OSCC/NCMT tissue pairs, 27 (63%) showed the downregulation of *SIRT3* mRNA expression (Table 1). In 38 tissue pairs available for analysis of both *miR-31* and *SIRT3*, the conspicuous *miR-31* upregulation (87%; 33/38) being found was consistent with previous studies (Fig. 1G). In addition, a reverse correlation between *miR-31* and *SIRT3* expression was also shown (Fig. 1H). There were 23 (61%) tumors exhibiting concomitant *miR-31* upregulation and *SIRT3* downregulation. It was interesting to note that OSCC patients having drinking habits displayed a significant *SIRT3* downregulation in their tumors relative to counterpart patients (Table 1). Assessment of publicly available repositories, Gene Expression Omnibus and Oncomine, revealed the downregulation of SIRT3 expression and upregulation of *miR-31* in head and neck cancer (HNC) tumors compared with the control counterparts in six datasets (Supplementary Table S1), suggesting the presence of *miR-31* and SIRT3 aberrances across various HNC tumor subsets.

3.3. SIRT3 suppresses miR-31 associated OSCC invasion

Knockdown of SIRT3 increased the wound closure of SAS and HSC3 cells (Fig. 2A and B, left), whereas the expression of SIRT3 slightly decreased the wound closure of these cells (Fig. 3A and B, right). SAS

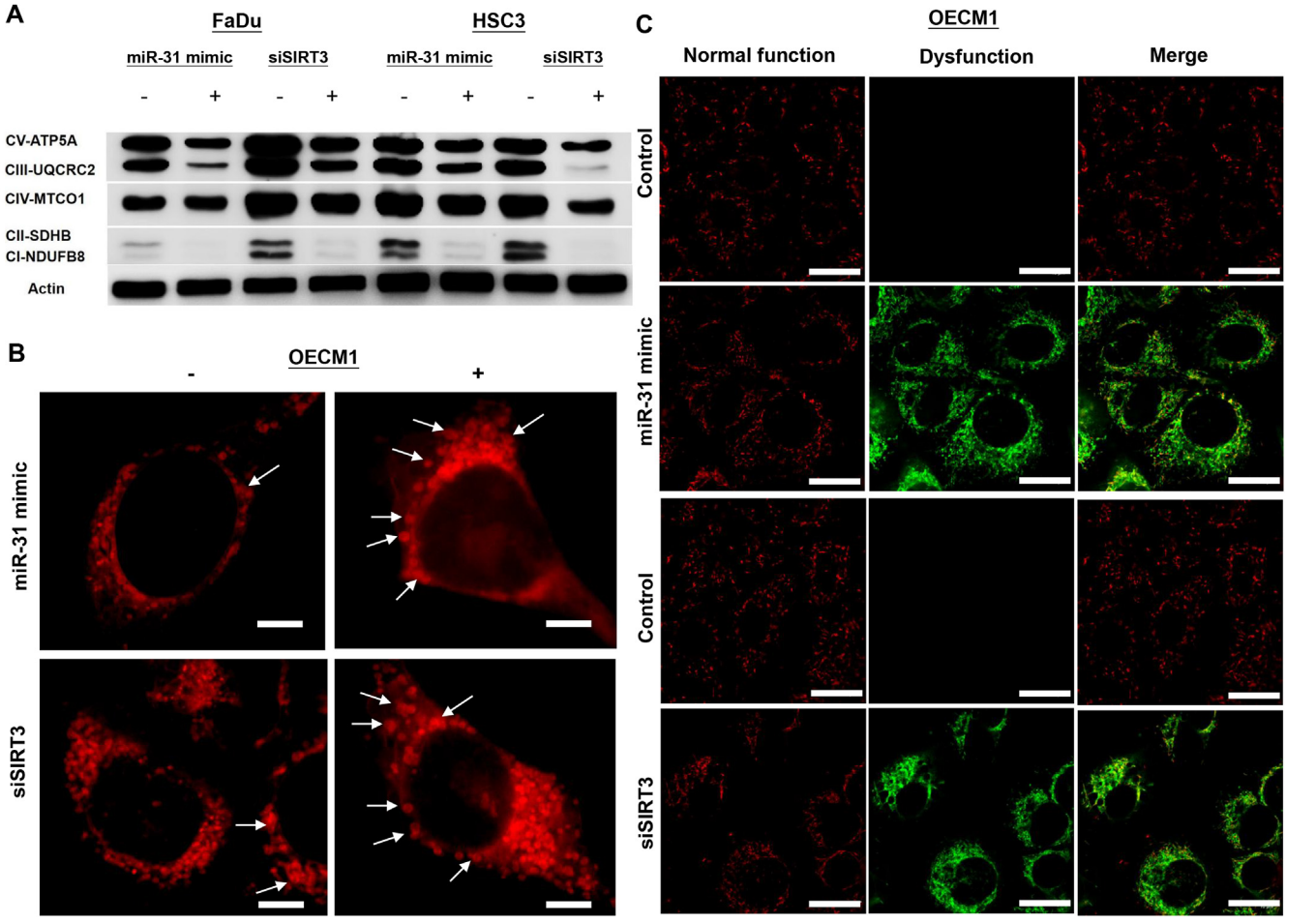

Fig. 4. *miR-31*-SIRT3 interrupts the mitochondrial integrity and functions in OSCC cells. (**A**) Western blot analysis for respiratory chain complex proteins in FaDu and HSC3 cells. Conspicuous decrease of complex proteins I and II are noted in OSCC cells following *miR-31* expression or SIRT3 knockdown. (**B**, **C**) OECM1 cells. (**B**) Immunofluorescences for MitoTracker staining. Arrows indicate representative swollen mitochondria. Bars, 5 μm. (**C**) Immunofluorescent MitoProbe staining. Red fluorescence labels polarized mitochondria (normal function); green fluorescence labels depolarized mitochondria (dysfunction). Bars, 20 μm. Increase of swollen mitochondria and dysfunctional mitochondria are seen in OECM1 cells following *miR-31* expression or SIRT3 knockdown, respectively. oeSIRT3, overexpress SIRT3.

and OECM1 cells were treated with serially-diluted NAM for 24 h to obtain an IC_{50} of 14.2 mM and 16.0 mM, respectively (Fig. 2C, left). Upon treatment with 14 mM NAM, the migration of SAS cells and OECM1 cells significantly increased (Fig. 2C, right), indicating that SIRT activity inhibited OSCC cell migration. Transwell migration assays also confirmed the inhibitory effects of SIRT3 on HSC3 migration (Fig. 2D). The transwell assays appear to yield more remarkable discrimination of HSC3 cell mobility between tested groups than the wound healing assay (Fig. 2B, D). This could be associated with the selection of migrated cell subpopulation by pore size or chemoattractant in this study. Assays being performed at different time points across experiments may also underlie the discrepancy. FaDu cell subclone stably expressed SIRT3 (OE SIRT3) was established. Relative to control, the migration (Fig. 2E) and invasion (Fig. 2F) of this cell subclone decreased. Knockdown of SIRT3 increased the invasiveness (Fig. 2G), while expression of SIRT3 decreased the invasiveness of OSCC cells (Fig. 2H). The invasion eligibility of OSCC cells mediated by *miR-31* induction was attenuated by SIRT3 expression in SAS (Fig. 2I) and OECM1 cells (Fig. 2J). The *miR-31* associated OSCC cell invasion is associated with the downregulation of SIRT3 through targeting.

3.4. SIRT3 expression decreases the tumorigenicity of FaDu cell

The xenografic growth potential of OE SIRT3 subclone of FaDu cell in athymic mice was lower than control. The increased SIRT3 expression in the tumors was confirmed by Western blot analysis (Supplementary Figs. S1A–C). The OE SIRT3 xenografts were smaller and lighter (Supplementary Fig. S1B) than control xenografts. We further established stable OE *miR-31*, OE SIRT3 and OE *miR-31*/OE SIRT3 stable cell subclones from FaDu and SAS cell lines. In FaDu cell, the increased exogenous *miR-31* and SIRT3 expression was shown in stable cell subclones. The level of exogenous SIRT3 expression was particularly enormous (Fig. 3A). All cell subclones also exhibited red fluorescence to facilitate detection. The increased migration associated with *miR-31* expression was rescued by SIRT3 expression (Fig. 3B). The modestly increased xenografic growth of FaDu cell associated with *miR-31* expression was attenuated by SIRT3 expression (Fig. 3C). The cell migration augmented by *miR-31* expression was also hindered by SIRT3 expression in the stable SAS cell subclones (Supplementary Fig. S2A) and HSC3 cells transiently overexpress *miR-31* (Supplementary Fig. S2B).

3.5. miR-31-SIRT3 disrupts mitochondrial structure and function in OSCC cells

Several results regarding to mitochondrial structure and function in OSCC cells were obtained after *miR-31* expression or SIRT3 knockdown. Analysis of respiratory chain complex revealed the decrease of complexes I - III and V protein expression to different extents (Fig. 4A). The decrease of complexes I and II proteins are particularly eminent in

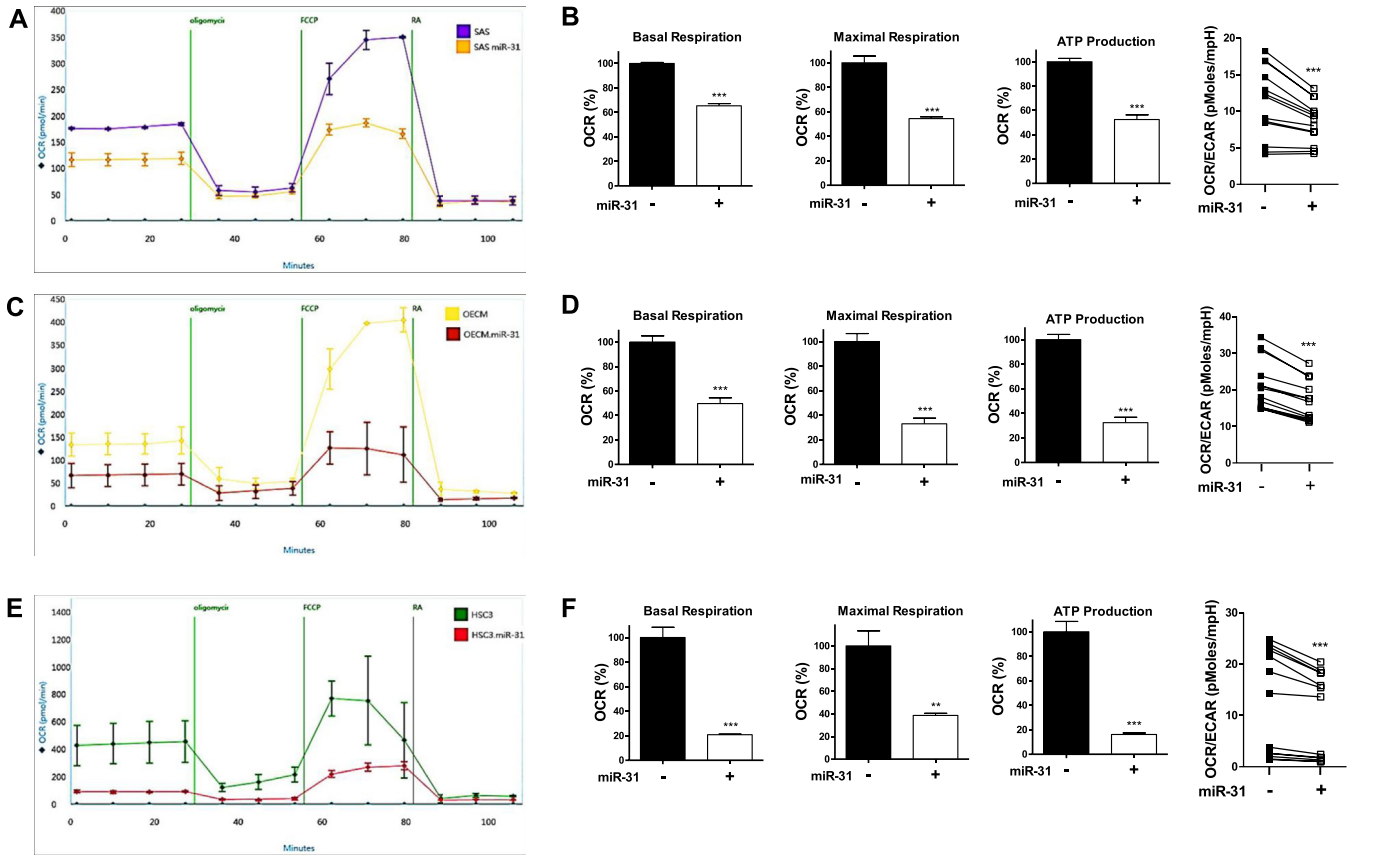

Fig. 5. *miR-31* affects oxidative phosphorylation and aerobic glycolysis in OSCC cells. (**A**, **B**) SAS cells. (**C**, **D**) OECM1 cell. (**E**, **F**) HSC3 cell. (**A**, **C**, **E**) Seahorse bioenergetic assays revealing the dynamic of respiratory status in cells. (**B**, **D**, **F**) Left, basal respiration; Left Middle, maximal respiration; Middle Right, ATP production. They show the changes of respiratory states in percentage following *miR-31* expression in comparison with controls. Right, before-after plot of OCR/ECAR ratio. Remarkable decrease of OCR/ECAR ratio is noted in OSCC cells following *miR-31* expression.

OSCC cells following *miR-31* expression or SIRT3 knockdown. MitoTracker staining displayed the enlargement of mitochondria in OSCC cells (Fig. 4B; Supplementary Fig. S3). MitoProbe assay further designated the increase of green fluorescence, which implicated the decrease of mitochondrial membrane potential and functional disturbance, in OSCC cells (Fig. 4B; Supplementary Fig. S4). The mentioned mitochondrial disruptions may underlie the increase of ROS in *miR-31* expression or SIRT3 knockdown cells following H_2O_2 challenging.

3.6. *miR-31 decreases the respiratory activity and increases lactate production in OSCC cells*

The mitochondrial functions affected by *miR-31* in OSCC cells were analyzed using Seahorse XF24 Extracellular Flux appliance according to the instructions provided. The results indicated that *miR-31* expression decreased the basal and maximal respiration, the ATP production, and the OCR/ECAR ratios in OSCC cells (Fig. 5). In addition, *miR-31* expression or SIRT3 knockdown increased the lactate production, while *miR-31* inhibition or SIRT3 expression decreased the lactate production in OSCC cells (Fig. 6A and B). Notwithstanding, *miR-31*/SIRT3 did not affect the glucose level consistently (Fig. 6C and D). In SAS and FaDu cell stable cell subclones, the increased lactate production associated with *miR-31* expression was decreased by SIRT3 expression (Fig. 6E and F). It is likely that *miR-31*-SIRT3 cascade results in the impairment of mitochondrial respiratory function, and increases the aerobic glycolytic activity of OSCC cells without changing the glucose uptake.

Additional data are presented as supplementary material.

4. Discussion

This study identifies the concordant upregulation of *miR-31* and the downregulation of *SIRT3* in the vast majority of OSCC tumors. The reverse in expression pattern together with the direct targeting of *miR-31* on the predicted 3′UTR sequences of SIRT3 transcript being identified substantiate the presence of *miR-31*-SIRT3 regulatory axis in OSCC. One previous study revealed the high SIRT3 expression in three OSCC cell lines relative to a controlled normal oral keratinocyte. Whereas, a half of OSCC tumors had low SIRT3 immunoreactivity [27]. Conspicuous downregulation of SIRT3 immunoreactivity was found in another HNSCC cohort [31]. The results of our analysis demonstrating the decrease of *SIRT3* mRNA expression were in agreement with this finding. Despite that the knockdown of SIRT3 rendered the *in vitro* growth inhibition of some HNSCC cell lines [27], the present study accomplished assays to elucidate the inhibitory effects of SIRT3 against the mobility and invasiveness in multiple OSCC cell lines. SIRT3 expression also inhibited the growth of FaDu xenografts. Apart from these clues, *miR-31*-SIRT3 axis is involved in regulating tumor cell migration, invasion and xenografic growth in our assays. Although the repression of *miR-31* on acytel-CoA oxidase 1, which impedes lipid metabolism and reinforces prostaglandin signaling, is able to enhance OSCC invasion [7], *miR-31* may also mediate tumor invasion by repressing SIRT3 in OSCC. The present study demonstrates that a miRNA is able to directly target SIRT3 to modulate a pathogenesis process. Previous studies have shown the upregulation of *miR-31* in oral premalignant disorders [6,8]. Thereby, the efficacy of early intervention using *miR*-31 LNA and SIRT3 activator should tested for abrogating OSCC pathogenesis. Since *miR-31* is involved in the pathogenesis or therapeutic resistance of various cancers [10,11], this study has validated that the

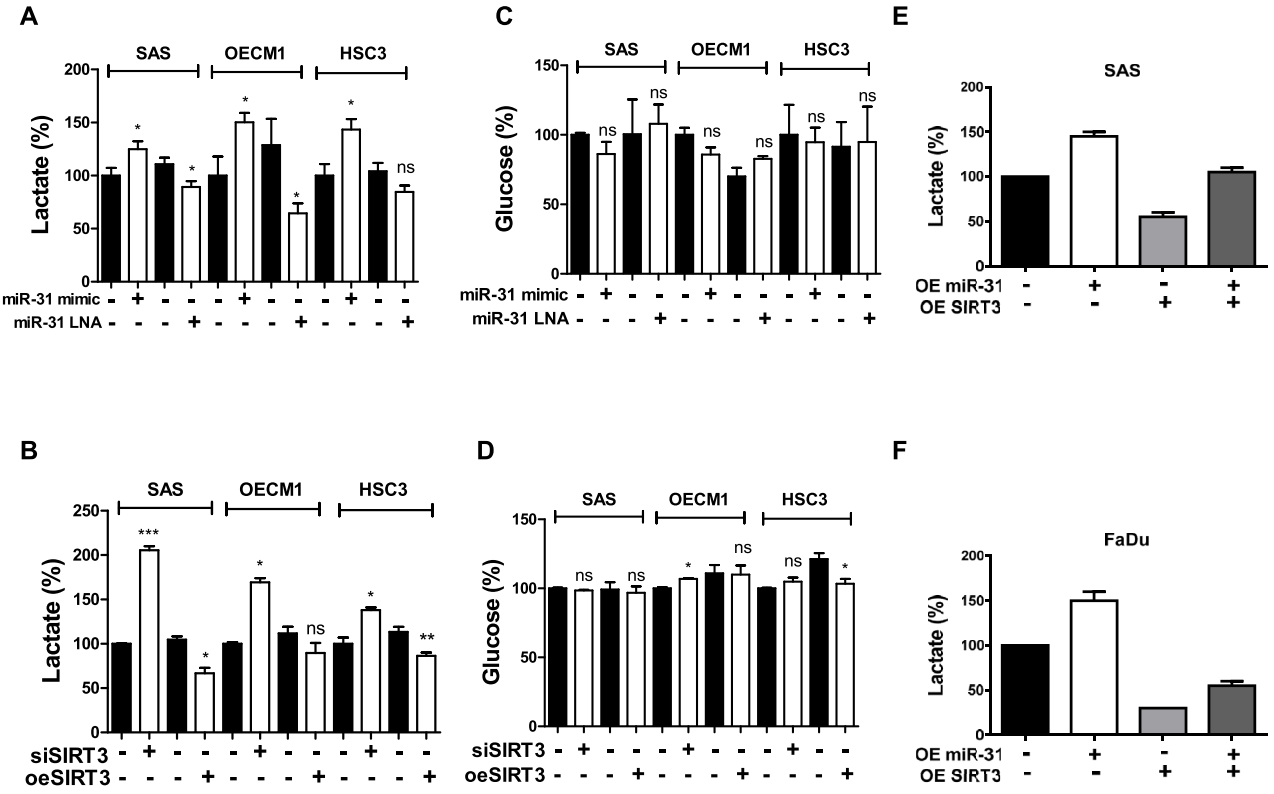

Fig. 6. Lactate and glucose in culture medium of OSCC cells as affected by *miR-31* and/or SIRT3. (**A**, **B, E, F**) The percentage of lactate concentration in test groups relative to controls. (**C**, **D**). The percentage of glucose concentration in test groups relative to control. (**A–D**) Left, SAS cell; Middle, OECM1 cell; Right, HSC3 cell. oeSIRT3, overexpress SIRT3. (**E**, **F**) SAS and FaDu cell subclones, respectively. After growing cells to around 70% confluences, the culture media are replaced. SAS cells and FaDu cells are cultured for 12 h and 24 h for medium collection, respectively. Cell numbers are counted for normalization. The analysis indicates that SIRT3 expression attenuates the *miR-31* increased lactate production. Duplicate analysis, statistic no available. OE *miR-31*, cell subclone stably expresses *miR-31*. OE SIRT3, cell subclone stably expresses SIRT3.

miR-31 modulated post-transcriptional repression could be a new mechanism underlying the silencing of SIRT3 in malignancies.

Mitochondria contributes to the majority of endogenous ROS generation, which functions as secondary messages to evoke signals for apoptosis, cellular transformation, or tumor metastasis [19,26]. The Foxo3A, IDH2 and SOD2 effectors downstream of SIRT3 play crucial roles in attenuating ROS, which protect cells from the toxicity of oxidative species [15,19,34]. This study elucidates that *miR-31*-SIRT3 cascade modulates the ROS genesis in OSCC cells. Although SIRT3 expression was shown to be responsive to areca associated oxidative stress [29], which regulated the ROS homeostasis in oral keratinocyte, we were unable to find the relevance between areca chewing and SIRT3 expression in OSCC tumors. Instead, we identified the low SIRT3 expression in tumors related to alcohol consumption. As linalool, a terpene alcohol, inhibits SIRT3 expression [35], further studies are required to understand the regulation of alcoholic ingredients on SIRT3 in modulating ROS imbalances. Since EGFR-AKT pathway increases *miR-31* expression in OSCC [5], and ethanol is able to activate EGFR in epithelial cells [36], it is likely that SIRT3 downregulation in human tumors may also be a post-transcriptional repression secondary to EGFR activation mediated by alcohol or other carcinogenic stimuli [30]. Nuclear SIRT3 was found to regulate gene expression in response to stresses [37]. Other oncogenic machineries in cells that orchestrate with *miR-31* to repress SIRT3 or relocate SIRT3 to nucleus await elucidation.

The mitochondria electron transport chain complexes drive OXPHOS flux to pyruvate for energy generation in cells. However, glycolytic metabolism plays an important role in mediating tumorigenesis as the altered expression, mutation or dysfunction of mitochondrial proteins, or enzymes occur frequently in cancer cells, which impair the oxidative phosphorylation [16]. Cancer cells may undergo aerobic glycolysis to adapt the requirements of proliferation [38]. Previous studies have specified the importance of SIRT3 in regulating metabolism since it can activate essential enzymes [13,14]. Furthermore, SIRT3 also deacetylates complex I and II to facilitate the electron transportation [17,18]. Interestingly, this study identified the attenuation of complex protein expression associating with *miR-31* upregulation or SIRT3 deficiency. The disruptive effects of *miR-31*-SIRT3 on complex I & II are particularly remarkable. The *miR-31*-SIRT3 modulated acetylation states of complex I and II proteins could be related to the structural stability of complex. Alternatively, since *miR-31* directly repressed SDHA [33], a complex II subunit, whether this *miR-31* targeting would disintegrate complex II to result in OXPHOS disturbance, needs to be addressed. Relative to normal cells, depolarization of mitochondrial membrane potential have been more widely shown in cancer cells [39]. This study identified the swelling and depolarization of mitochondria following *miR-31* expression or SIRT3 knockdown. The uncoupling of complex proteins due to deficiency in abundance would be likely to underlie such morphological or functional aberrance. As SIRT3 is crucial in the maintenance of mitochondrial function and integrity, this study also identified conspicuous metabolic switch from OCR to OEAR indicating the preference for glycolysis instead of OXPHOS upon *miR-31* expression. Since increase of lactate and the expression profile of metabolic genes could define the worst prognosis of OSCC [40,41], future use of SIRT3 stimulator or glycolytic inhibitor could improve the survival of patient with tumor harboring *miR-31* upregulation.

miR-31 plays versatile roles in tumor pathogenesis [3,4,6,7,32,33].

It has been shown to impede metabolisms [32,33], this study validated SIRT3 as a new target of *miR-31* in OSCC. *miR-31*-SIRT3 regulatory axis modulates both metabolism and aggressiveness of OSCC cells. The partial reversion of *miR-31* driven phenotypic changes modulated by means of profound SIRT3 expression may implicate the potential involvement of unspecified mechanisms. Since the *miR-31* expression is drastically upregulated in OSCC, the pathological roles of *miR-31*-SIRT3 axis and the interactive effects being unmasked in this study will facilitate the mechanistic insight and therapeutic abrogation of OSCC.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.028.

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