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Original Article

Ornithine decarboxylase antizyme 1 upregulate *LOR* to promote differentiation of SCC15 cells by binding CBP/p300 in promoter region

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Abstract: Ornithine decarboxylase antizyme 1 (OAZ1) participates in multiple biological processes, such as cell proliferation, differentiation, apoptosis *etc*. In SCC15 cells, OAZ1 can efficiently upregulate loricrin (*LOR*), the epithelial differentiation marker gene, by promoting the degradation of Smad nuclear interacting protein 1 (SNIP1). However, the underlying mechanisms have not yet been identified. To explore the mechanisms of LOR induction by OAZ1, SCC15-OAZ1 cell which *OAZ1* stably expressed was constructed. And SNIP1 specific shRNA was used to transfected SCC15 cells. The results showed that OAZ1 can upregulate *LOR* to promote the degradation of SNIP1. Morphological observation revealed that overexpression *OAZ1* and SNIP1 knockdown would increase epithelial island formation. Moreover, ChIP analyses showed that CBP/p300 was increased in *LOR* promoter region in SCC15-OAZ1 cells or SNIP1-shRNA-transfected SCC15 cells. These results showed that OAZ1 can enhance terminal differentiation of SCC15 cells. Over-expression OAZ1 promotes the degradation of SNIP1, knockdown of SNIP1 increase the binding of CBP/p300 in *LOR* promoter region, and then induce transcription of *LOR*.

Keywords: OAZ1, tongue squamous cancer cells, SNIP1, LOR, CBP/p300

Introduction

Tongue squamous cell carcinoma (TSCC) is one of the most common malignant oral tumors [1, 2]. It's known as high malignant, fast growth, high invasiveness, early metastasis but poor prognosis [3-5]. Previous studies showed that well-differentiated tongue squamous cell carcinoma had a better prognosis than low-differentiated tongue squamous cell carcinoma [6, 7]. In several human oral cancer cell lines, expression of *OAZ1* was down-regulated, and ectopic expression of *OAZ1* in these cancer cells can promote cell differentiation and apoptosis [8-12]. Our previous experiments demonstrated that OAZ1 can efficiently enhance transcription of loricrin (*LOR*), the epithelial differentiation marker gene, by degradation of Smad nuclear interacting protein 1 (SNIP1, a CBP/p300 repressor protein) in human malignant oral cancer cell line SCC15 [13].

CBP (CREB-binding protein, CREBBP) and p300 are members of the histone acetyltransferase family of transcriptional co-activators of various sequence-specific DNA-binding transcriptional factors and involved in DNA repair, cell growth, differentiation, and apoptosis [14-16]. CBP/p300 play an important role in transcription of *LOR* [17]. SNIP1 has been reported to competitively inhibit recruitment of CBP/p300 in the promoter region of target gene, which leading to inhibit the gene transcription [18, 19]. OAZ1 can promote degradation of SNIP1, release of CBP/p300, which result in increasing target gene expression [20]. A thorough understanding of the mechanism of LOR upregulated by OAZ1 awaits additional investigation.

Table 1. Primer sequences of Real-time RT-PCR

Primers		Sequence (5'-3')	Product length/bp
OAZ1 (NM_004152)	-F	TCTCCCTCCACTGCTGTAGTAACC	198
	-R	GTTGAGAATCCTCGTCTTGTCGTT	
LOR (NM_000427)	-F	GCACCGATGGGCTTAGAG	130
	-R	AGAAACCAAAGAGGCTAAACAG	
GAPDH (NM_002046)	-F	CGCTGAGTACGTCGTGGAGTC	172
	-R	GCTGATGATCTTGAGGCTGTTGTC	

In the present study, the lentiviral vector, containing the *OAZ1* gene, was constructed and transfected into human tongue cancer cell line, SCC15. The results showed that OAZ1 promoted the degradation of SNIP1, and increased the binding of CBP/p300 in *LOR* promoter region. *OAZ1* expression also induced epithelial islands formation. Silencing of *SNIP1* increased CBP/p300 to bind to *LOR* promoter region, and up-regulated *LOR* induced more epithelial islands formation. Our results demonstrate up-regulated *OAZ1* promote degradation of SNIP1, enhance CBP/p300 bind to *LOR* promoter region, and increased *LOR* induce SCC15 differentiation.

Materials and methods

Cell culture and transfection

The human oral cancer cell line, SCC15, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was cultured in Dulbecco's modified Eagles medium/F12 medium supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) in a humidified 37°C incubator with 5% CO_2. The SCC15 cells were transfected with Lentiviral vector containing OAZ1 in 6-well plates, G418 (1,000 μg/ml) and flow cytometry were used to screen the stably transfected clones.

RNA interference

RNA interference was performed by using small hairpin RNA (shRNA). ShRNA targeting SNIP1 were designed and synthesized by GeneChem (Shanghai, China). Cells were transfected with 4 μg shRNA/well in 6-well plates, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. The shRNAs used in this study were: sh-SNIP1, 5'-TCGATGTATGTACATGACT-3'; sh-NC, 5'-TTCTCCGAACGTGTCACGT-3'. Sh-NC was used as the negative control.

Western blot

Protein lysates were obtained using whole-cell protein extraction buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS, and 1% protease inhibitor cocktail). Equal amounts of total protein were separated by SDS-PAGE electrophoresis and transferred to a polyvinylidene fluoride membrane. The membranes were blocked and probed overnight at 4°C with the specific antibodies. Applied antibodies included antibodies against OAZ1, SNIP1, CBP, p300 (all from Abcam, Hong Kong, China); against LOR (Santa Cruz, CA, USA); against GAPDH (Cell Signaling Technology, Shanghai, China). Immunoreactive protein bands were visualized using horseradish peroxidase-conjugated secondary antibodies and an ECL detection system (Advansta, USA). The protein bands of interest were analyzed using FluorChem 8900.

Immunofluroscence

The cells were cultured on 6-well chamber slides and fixed for 30 min in 4% paraformaldehyde/PBS at room temperature. After three rinses with PBS, the cells were treated with 0.2% Triton X-100 in PBS for 15 min and blocked by 1% bovine serum albumin/PBS for 30 min at room temperature. Cells were incubated with anti-LOR (Santa Cruz, CA, USA) antibody overnight at 4°C. Cells were then washed three times with PBS and incubated with anti-rabbit antibodies Alexa fluor 594 conjugated (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature, washed with PBS, and stained 5 min with DAPI. Slides were then washed with PBS and imaged with an Olympus BX40 fluorescence microscope.

Morphological observation

Cells (1×10^3/well) were plated in 100×20 mm plates and observed under an inverted microscope after 1 week.

Chromatin immunoprecipitation (ChIP)

ChIP was performed with Express Enzymatic Kit and Enzymatic Shearing Kit according to the manufacturer's protocol. Briefly, 10 million cells

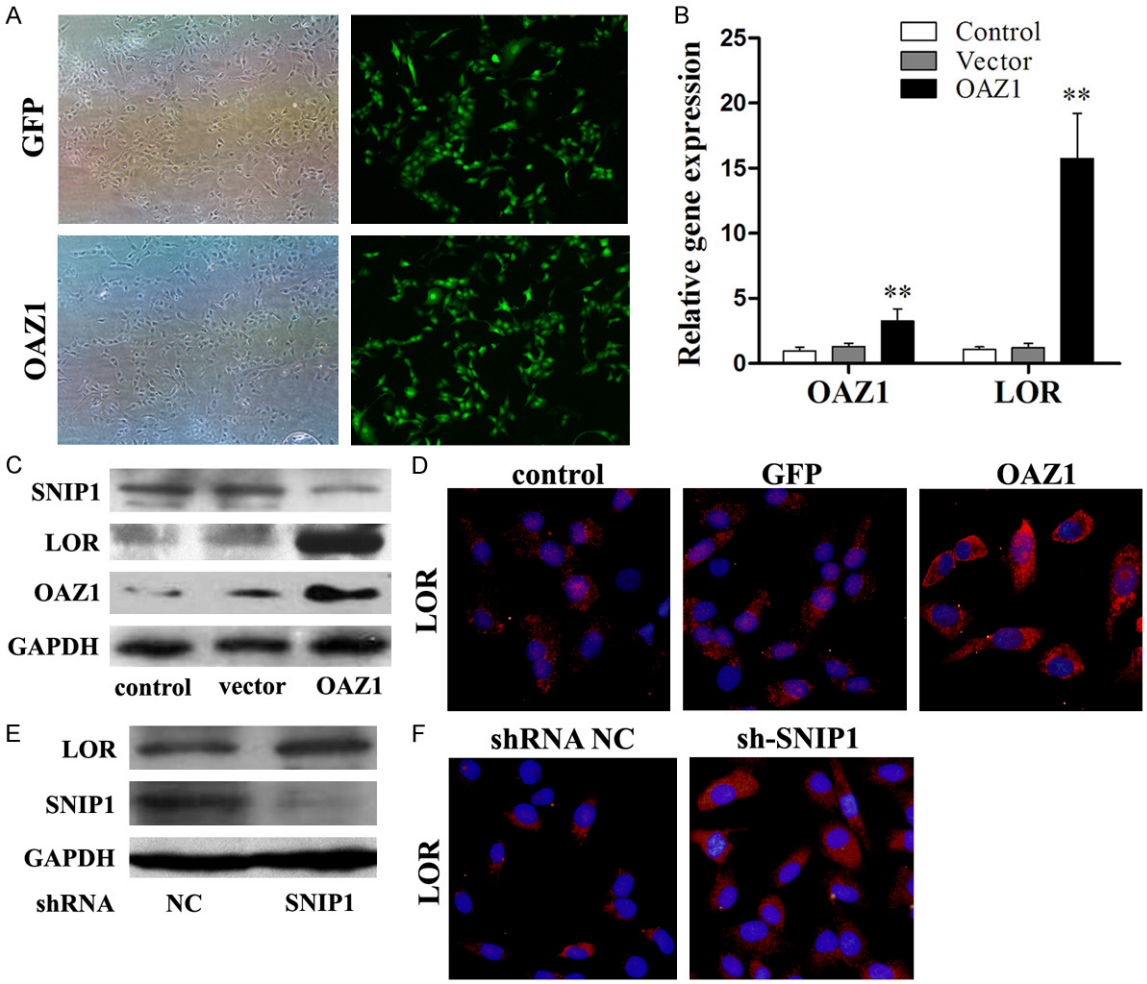

Figure 1. OAZ1 induce LOR expression by promoting SNIP1 degradation. A. GFP expression detected by florescent microscope (100×) in SCC15 cells transfected with GFP or OAZ1. B. Q-PCR analysis of OAZ1 and LOR gene expression in SCC15 cells transfected with GFP or OAZ1. **indicates $P<0.01$ relative to SCC15 (Control) or SCC15/GFP (Vector), n=6. C. Western blot analysis of OAZ1, LOR and SNIP1 in SCC15 cells transfected with GFP or OAZ1. D. Immunohistochemical analysis of LOR immunoreactivity (red) and DAPI (blue) in SCC15 cells transfected with GFP or OAZ1. E. Western blot analysis of LOR and SNIP1 in SCC15 cells transfected with shRNA-NC or shRNA-SNIP1. F. Immunohistochemical analysis of LOR immunoreactivity (red) and DAPI (blue) in SCC15 cells transfected with shRNA-NC or shRNA-SNIP1.

were fixed with 1% formaldehyde for 15 min, quenched with 0.125 M Glycine, washed with cold PBS, and lysed in cell Lysis Buffer (supplemented protease inhibitor cocktail and PMSF). The Sheared Chromatins were prepared by Enzymatic Shearing, and the sheared chromatin samples were cleared by centrifugation at 12,000 RCF for 15 min. The supernatants were incubated overnight at 4°C with antibody-conjugated protein G-Dynal beads dynabeads. The beads were then washed sequentially one time with ChIP Buffer 1 and then two times with ChIP Buffer 2. The protein-DNA complexes were eluted with Elution Buffer AM2 and then incubated at 65°C for 6 h for cross-link reversal. The immunoprecipitated DNA was purified by phenol/chloroform extraction and ethanol precipitation after Proteinase K treatment. Binding of factors were determined by End Point PCR with designed primer sets (*LOR-F:* 5'-TGCAATCACAGGGAGG-3'; *LOR-R:* 5'-CCAAAGCACCAGGAAGG-3').

Quantitative real-time PCR analysis

Total RNA was extracted from human cells using RNAiso Plus kit (Takara Bio, Inc., Shiga, Japan) and Full-length cDNA was synthesized by PrimeScript RT reagent Kit (Takara Bio, Inc.,

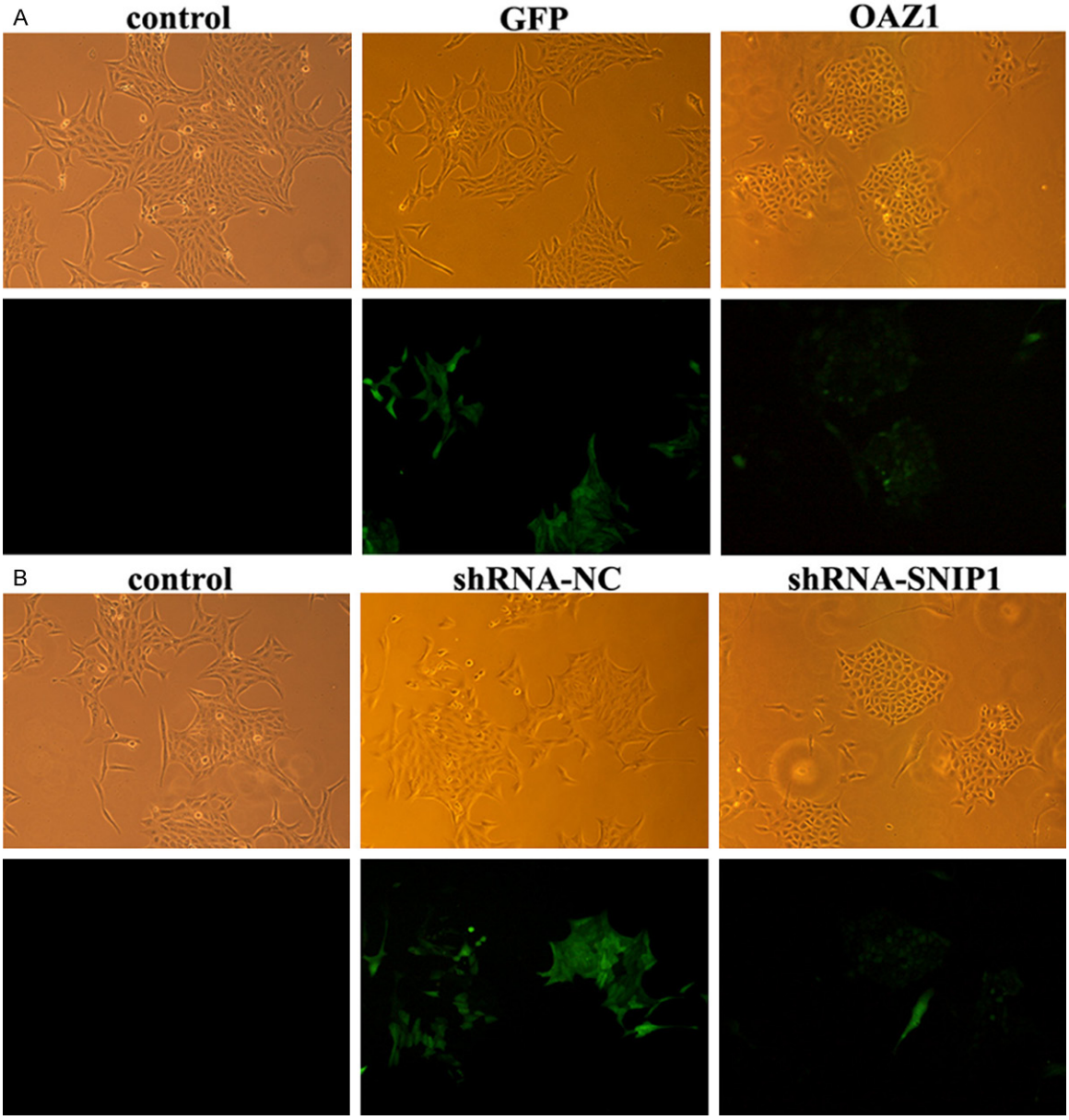

Figure 2. OAZ1 and SNIP1 affect cell morphology in SCC15 cells. A. Forced expression OAZ1 leads to epithelial islands formation. B. Knockdown of SNIP1 induces epithelial islands formation.

Shiga, Japan). Real-Time RT-PCR was carried out in the Applied Biosystems PRISM 7300 qPCR using SYBR-Green I (Takara Bio, Inc., Shiga, Japan). Primer sequences are listed in **Table 1**.

Statistical analysis

SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as means ± standard deviation. One-way analysis of variance was used and $P<0.05$ was considered to indicate a statistically significant difference.

Results

OAZ1 promote SNIP1 degradation to upregulate LOR expression

OAZ1 can induce transcription of the epithelial differentiation marker gene *LOR* by promoting the degradation of SNIP1 in SCC15 cells [13]. We first established a SCC15 cell-line, stably expressing OAZ1 (SCC15/OAZ1) (**Figure 1A**). OAZ1 overexpression resulted in up-regulation of *LOR* and promoted SNIP1 degradation, which suggest that OAZ1 may increase *LOR* expression through degradation of SNIP1 (**Figure**

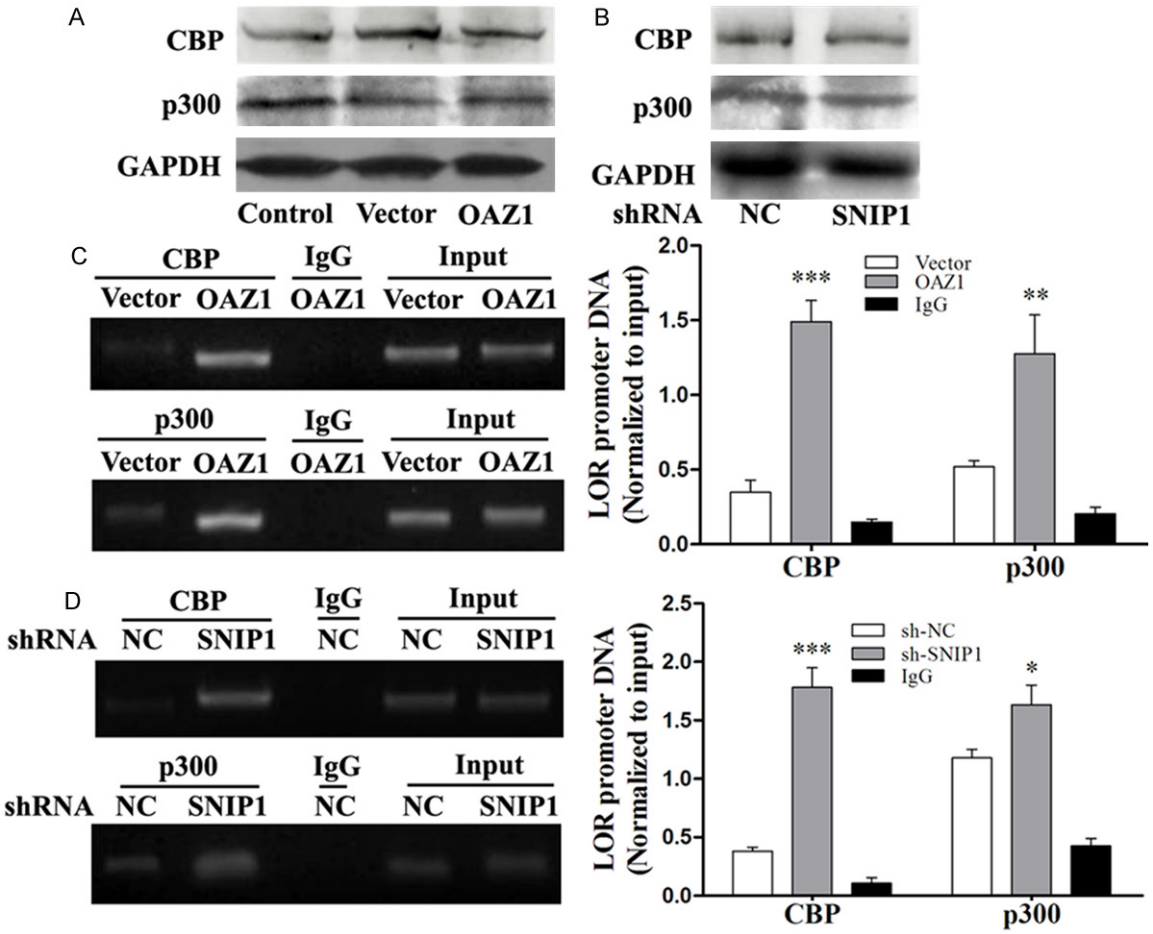

Figure 3. OAZ1 promote the binding of CBP/p300 in LOR promoter region by inducing degradation of SNIP1. A. Western-blotting analysis of CBP, p300 in control and OAZ1-overexpressed SCC15 cells. B. Western-blotting analysis of CBP, p300 in SNIP1 silencing group and (shRNA-NC) control. C. ChIP-PCR analysis of LOR promoter region with CBP and p300 antibodies in SCC15 cell transfected with GFP or OAZ1. **indicates $P<0.01$ relative to SCC15/GFP (Vector), ***indicates relative $P<0.001$ versus to SCC15/GFP (Vector), n=3. D. ChIP-PCR analysis of LOR chromatin from in SCC15 cell transduced with (shRNA-NC) control or shRNA-SNIP1. *indicates $P<0.05$ relative to SCC15/sh-NC, ***indicates relative $P<0.001$ versus to SCC15/sh-NC, n=3.

1B-D). To further confirm this hypothesis, SNIP1 specific shRNA were used to transfect SCC15 cells. We found that knockdown of SNIP1 could increase *LOR* expression (**Figure 1E**, **1F**). Overall these findings suggest that OAZ1 induces LOR by promoting SNIP1 degradation.

OAZ1 induces morphological change result from SNIP1 degradation

Epithelial island is a marker of terminal differentiation of epithelial cells [10]. Morphological observation showed an increased epithelial island formation in *OAZ1*-transfected SCC15 cells, whereas epithelial islands were not observed in cells transfected with vector control (**Figure 2A**). Expression of *LOR* in SCC15 cells was increased by inhibition of *SNIP1*. To clarify a potential role of SNIP1 in epithelial island formation during terminal differentiation of epithelial cells, we analysed the morphological change in SNIP1-shRNA-transfected SCC15 cells. We found that epithelial island was induced when the inhibition of SNIP1 was performed (**Figure 2B**). These findings imply OAZ1 can induce terminal differentiation of epithelial cells by promoting SNIP1 degradation in SCC15 cells.

CBP/p300 is promoted to LOR promoter region by OAZ1 inducing SNIP1 degradation

Since CBP/p300 plays an important role in transcriptional regulation of *LOR* [17]; and SNIP1 has been reported to competitively

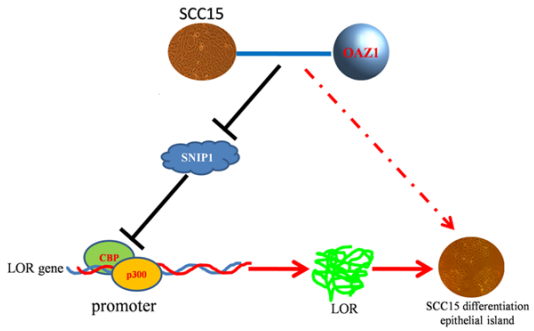

Figure 4. Reciprocal antagonism between OAZ1, SNIP1, CBP/p300 which regulate SCC15 cell fate during differentiation. In the undifferentiated state of SCC15 cells, SNIP1 represses terminal differentiation by directly suppressing CBP/p300 binding site in LOR gene promoter region. Inhibition of SNIP1 releases binding site to trigger SCC15 differentiation.

inhibit recruitment of CBP/p300 co-activators to promoter region [18, 19]. We speculated OAZ1 might enhance *LOR* expression by promoting CBP/p300 to bind to LOR promoter region. For further investigation, we performed chromatin immunoprecipitation (ChIP) assays and detected CBP/p300 protein level. ChIP analysis results showed that CBP/p300 was increased at *LOR* promoter region in OAZ1-transfected SCC15 cells (**Figure 3C**). However, CBP/p300 showed stable expression on protein level (**Figure 3A**). To further clarify this hypothesis, we detected the chromatin-related CBP/p300 at the *LOR* promoter region of SNIP1-shRNA transfected SCC15 cells. Knockdown of SNIP1 caused an increase of CBP/p300 at the examined region, but had little impact on CBP/p300 protein expression (**Figure 3B**, **3D**). Taken together, these results suggested overexpression of OAZ1 or SNIP1 knockdown caused up-regulation of *LOR*, which primarily correlated with an increased CBP/p300 at *LOR* promoter region.

Discussion

Ornithine decarboxylase antizyme 1 (OAZ1) is known as an anti-ontogene and it can affect differentiation and apoptosis of multiple cancer cell lines [21]. In several human oral cancer cell lines, ectopic expression of *OAZ1* induce cell differentiation and increase epithelial islands formation [8, 10]. The mechanism employed for cell differentiation by OAZ1 remains unclear and further exploration may help with understanding.

LOR is the major component of cell membrane in terminal differentiated keratinocytes and as a marker gene during epithelial differentiation [22, 23]. Recent reports demonstrate that OAZ1 induce transcription of *LOR* by promoting SNIP1 degradation with inducing epithelial islands formation of SCC15, human malignant oral cancer cell line [13]. Here we report knockdown of SNIP1 cause the formation of epithelial islands as well. Therefore, OAZ1 may induce terminal differentiation by promoting SNIP1 degradation.

LOR promoter locates on the region between -150 to +9 [17]. In undifferentiated cells, *LOR* expression has been suppressed by Jun B, Sp3, and KSR-1 compound binding to promoter, which prevents Sp1, c-Jun and CBP/p300 binding in the same region. On the contrary, Sp3, KSR-1, and CREB proteins level become lower in differentiated cells; Sp1 and c-Jun are increased concurrently; and then CBP/p300 is recruited. The interaction between Sp1, c-Jun and CBP/p300 results in basal transcription machinery assembling and *LOR* transcription proceeding [17]. The SNIP1 inhibits gene transcription by competing binding to the CBP/p300 transcriptional co-activators [18, 19]. OAZ1, Smad1 and HsN3 form a ternary complex, and transport into nucleus, where SNIP1, the repressor of CBP/p300, is recruited to Smad1/HsN3/OAZ1 complex and degraded, all of which lead CBP/p300 to release [20]. The present study showed that OAZ1 promote CBP/p300 bind to *LOR* promoter region. And knockdown of SNIP1 caused an increase of CBP/p300 binding to the promoter. However, CBP/p300 express stably in OAZ1-transfected or SNIP1-shRNA-transfected SCC15 cells. These results indicate overexpression OAZ1 or SNIP1 knockdown may increase free state level of CBP/p300 in nucleus. Thus, we presume that OAZ1 up-regulate *LOR* gene expression by releasing CBP/p300, which results from OAZ1 induce SNIP1 degradation.

In conclusion, OAZ1, as a regulator of cell differentiation, triggers terminal differentiation by promoting SNIP1 degradation in SCC15 cells. Further exploration showed the mechanism of LOR gene up-regulation by OAZ1: degradation of SNIP1 by OAZ1 increases the level of free CBP/p300 protein, which is recruited to *LOR* promoter, and then induces transcription of *LOR* (**Figure 4**). Mining the pathway regulated

by OAZ1 will reveal factors that control cell differentiation during tumorigenesis and thus may constitute novel cancer therapeutic targets, especially in human oral cancer.

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Disclosure of conflict of interest

None.

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