GAS5, a FoxO1-activated long noncoding RNA, promotes propofol-induced oral squamous cell carcinoma apoptosis by regulating the miR-1297-GSK3β axis

Chengshun Gao**, Chunmei Ren**, Zhongxi Liu*a,b, Li Zhangc, Ranran Tangb and Xiaojie Lia

aDepartment of Anesthesiology, the Second Affiliated Hospital & Department of Prosthodontics, College of Stomatology, Dalian Medical University, Dalian, Liao Ning, China; bNanjing Maternity and Child Health Care Hospital, Women’s Hospital of Nanjing Medical University, Nan jing, Jiangsu, China; cLaboratory of Pathogenic Biology, College of Basic Medical Science, Dalian Medical University, Dalian, Liaoning, China

ABSTRACT
Propofol, an intravenous anaesthetic agent, has been found to exhibit antitumour effects in various kinds of cancer cells. However, the potential roles and regulatory mechanisms of propofol in oral squamous cell carcinoma (OSCC) remain unknown. Herein, we found that propofol inhibits OSCC cell growth and promotes cell apoptosis in a dose- and time-dependent manner. Further mechanistic studies revealed that the long noncoding RNA GAS5 is induced by propofol in OSCC cells. Elevated GAS5 acts as a competing endogenous RNA for miR-1297 and attenuates its inhibitory effect on GSK3β, leading to GSK3β increase and Mcl1 decrease. Additionally, we found that FoxO1 binds to the promoter of GAS5, facilitating its transcription in response to propofol treatment. Thus, these results suggest that propofol exhibits antitumour effects in OSCC cells and that the FoxO1-GAS5-miR-1297-GSK3β axis plays an important role in propofol-induced OSCC cell apoptosis.

Introduction
Oral squamous cell carcinoma (OSCC) is one of the most frequent head and neck tumours and accounts for more than 90% of all oral malignancies [1,2]. Although multiple therapeutic strategies can be administered clinically to treat OSCC, the overall 5-year survival rate after diagnosis remains less than 50%, mainly owing to its metastasis or resistance to conventional therapeutic drugs [3]. Thus, in order to improve the prognosis of patients with OSCC, it is important to identify effective therapeutic drugs.

Propofol (2,6-diisopropylphenol) is extensively used as an intravenous anaesthetic agent due to its rapid induction and fast recovery from anaesthesia [4]. Accumulating evidence has indicated that propofol exerts a number of non-anaesthetic effects, including antitumour activity [5]. Recently, propofol was reported to trigger apoptosis and suppress proliferation, motility and invasion in multiple human cancers, including breast cancer, pancreatic cancer, ovarian cancer, gastric cancer, lung cancer and hepatocellular carcinoma [6-10]. However, the effect of propofol on OSCC cells remains unknown.

Long non-coding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides with no protein-coding capacity [11]. lncRNAs, initially thought to be non-functional, were recently shown to play important regulatory roles in many human diseases, especially in malignancies [12]. Growth arrest-specific 5 (GAS5) can encode ncRNAs of various sizes, including lncRNAs [13]. Emerging evidence has proven that lncRNA GAS5 is an important tumour suppressor that is downregulated in many types of cancers and involved in many cellular processes, such as cell proliferation, apoptosis and invasion [14]. Although GAS5 has been indicated to play an important role in cancer, the functions of GAS5 in response to propofol treatment are not well studied.

Here, we found that GAS5 is increased under propofol treatment in OSCC cells. Elevated GAS5 enhances propofol-induced OSCC cell apoptosis and inhibits cell proliferation. Subsequent mechanistic studies indicated that GAS5 acts as a competing endogenous RNA for miR-1297 and attenuates its inhibitory effect on GSK3β, which in turn facilitates the degradation of Mcl1 and promotes the antitumour activity of propofol. Additionally, we discovered that FoxO1 directly binds to the promoter of GAS5, facilitating its transcription in response to propofol treatment. Thus, these data indicate that the FoxO1-GAS5-miR-1297-GSK3β signalling axis plays an important role in propofol-induced OSCC cell apoptosis.

Materials and methods
Cell culture and reagents
The human OSCC cell line UM-SCC6 was provided by Dr. Thomas E. Cary (University of Michigan, Ann Arbor, MI). SCC-
090 cells were obtained from American Type Culture Collection (ATCC), SCC-090 cells were cultured with MEM containing 10% foetal bovine serum (FBS, Gibco-BRL). The medium was renewed every day, and cells were passaged before reaching confluence. The following antibodies were used: GAPDH (Santa Cruz Biotechnology, Dallas, TX; SC-25778), PARP1 (Santa Cruz Biotechnology, SC-8007), Mcl1 (Cell Signalling Tech, 94296), phospho-Mcl1 (ser159/thr163) (Cell Signalling Tech, 4579), GSK3β (Cell Signalling Tech, 12456), GSK3α (Cell Signalling Tech, 4818), and FoxO1 (Cell Signalling Tech, 2880), Bcl2 (Cell Signalling Tech, 2872), pGSK3β(ser9) (Cell Signalling Tech, 5558), pGSK3α (ser21) (Cell Signalling Tech, 4542).

**Real-time RT-PCR and RT-PCR**

Total RNA was isolated using Trizol (Invitrogen, Waltham, MA). One microgram of total RNA was used to synthesize cDNA using PrimeScript™ RT reagent kit (Takara, RR047A) according to the manufacturer’s instructions. The primers for miR-1297 were purchased from MyBioSource (MBS825976). The primers for GASS and Actin were as follows: GASS: F: 5'-ACAGGCCATTAGACAGAAGC-3' and R: 5'-TACCCAAGCAGGTCATCCA-3'; Actin: F: 5'-GACCTGACTGACTACCTCATGAAGAT-3' and R: 5'-GTCACACTTCTGATGGAGTTGAAGG-3'.

**RNA interference and virus infection**

RNA interference was performed as previously described [15]. The shRNA was purchased from Sigma, and the sequence targeting FoxO1 was 5’-TTAGACTGACTCATGAGAT-3’. GASS siRNAs were purchased from GenePharma Company (Shanghai, People’s Republic of China), and the siRNA sequences of GASS were as follows: (1) 5’-GGACCAGCTTAATGGTTCT-3’ and (2) 5’-GCAAGGCCCTACTCAAGCCA-3’.

**Introduction of microRNA mimics and inhibitors**

Mimics and inhibitors of miRNA-1297 were synthesized by the GenePharma Company (Shanghai, People’s Republic of China). For each transfection in a six-well plate, 100 nM miRNA mimics, scramble or inhibitor, or scramble was used. The transfection of melanoma cells by Oligofectamine (Invitrogen) was performed according to the manufacturer’s instructions.

**Dual-luciferase reporter assay**

The assay was performed as previously described [15–17].

**ChIP assay**

Cells were crosslinked with 1% formaldehyde for 10 min at room temperature. The ChIP assay was completed according to the manufacturer’s instructions using FoxO1 antibody and the ChIP assay kit (Millipore, Merck KGaA, Darmstadt, Germany). Anti-rabbit IgG was used as a control. The bound DNA fragments were eluted and amplified by PCR. The PCR products were separated on a 2% agarose gel by gel electrophoresis.

**Colony formation and EdU assays**

UM-SCC and SCC090 cells with knockdown of GAS5 were harvested and thoroughly pipetted to generate single-cell suspensions in complete culture media at a given concentration (such as 1 x 10^6/ml). The single-cell suspension was diluted to 500 or 1000 cells per well of a 6 well-plate and incubated at 37°C with 5% CO2 for 2 weeks. Then, the colonies were stained with 0.04% crystal violet-2% ethanol in PBS. Photographs of the stained colonies were then taken.

The EdU assay was performed with an EdU Assay Kit (Guangzhou RIBOBIO, Guangzhou, China) according to the manufacturer’s instructions. Briefly, the cells were incubated with DMEM medium containing 50 μM EdU for 2 h. The nuclei were also stained with Hoechst 33342 (Sigma, St. Louis, MO), and the images were acquired with an Olympus DP71X microscope (Olympus, Tokyo, Japan).

**Caspase-3 activity assay**

Caspase-3 activity was analyzed using the Caspase-3 Activity Assay Kit (Roche) according to the manufacturer’s instructions. Briefly, the tissue lysate was incubated with 100 μL of caspase-3 reagent at 37°C for 1–2h, and the fluorescence intensity (at 370–425 nm excitation and 490–530 nm emission wavelengths) was then measured using a Spectra MAX M5 spectrophotometer (Molecular Devices, Sunnyvale, CA).

**Statistics and data analyses**

The data are expressed as the means ± SD, and the statistical evaluation was performed using one-way analysis of variance (ANOVA). Values of p < .05 were considered statistically significant.

**Results**

**Propofol triggers OSCC cell apoptosis and inhibits cell proliferation**

To investigate the effects of propofol on OSCC cells, we first examined the influence of propofol on cell apoptosis. We treated the OSCC cell lines UM-SCC6 and SCC090 with propofol at different doses. After 48 h of treatment, cell apoptosis was analyzed. As shown in Figure 1(A–D), PARP1 cleavage and Caspase 3 activity were elevated following treatment with increasing concentrations of propofol. Subsequently, UM-SCC6 and SCC090 were treated with 20 μg/ml propofol for different times, and cell apoptosis was detected. Similarly, we found that cleaved PARP1 and the activity of Caspase 3 were increased following longer propofol treatment durations (Figure 1(E–H)).

In addition, the effects of propofol on cell proliferation were also analyzed. Colony formation and EdU assays revealed that propofol treatment significantly reduced the proliferation of UM-SCC6 and SCC090 cells (Figure 1(I–M)). Therefore, our data suggest that propofol exerts suppressive effects on OSCC cells in a dose- and time-dependent manner.
LncRNA GAS5 induced by propofol inhibits cell growth and promotes cell apoptosis in OSCC cells

To explore the underlying mechanism of the suppressive effects of propofol on OSCC cells, UM-SCC6 cells were treated with 20 μg/ml propofol for the indicated times, and lncRNA expression profiles were then obtained by RNA sequencing analysis. The sequencing results revealed more than 51 upregulated and 83 downregulated genes in UM-SCC6 cells upon propofol treatment compared with control cells (Figure 2(A,B)). Among the altered lncRNAs, we found that GAS5 was significantly increased in response to propofol treatment (Figure 2(C)). To confirm this, UM-SCC6 and SCC090 cells were treated with propofol at different doses and times. Consistent with the RNA sequencing analysis, GAS5 was dramatically upregulated upon propofol treatment in OSCC cells (Figure 2(D–G)).
Next, to investigate the role of GAS5 in propofol-induced cell decrease in proliferation and increase in cell apoptosis, we first knocked down GAS5 expression using siRNAs in UM-SCC6 and SCC090 cells. The cells were then treated with propofol as indicated. Cell proliferation was analyzed by colony formation assay, and cell apoptosis was analyzed by western blot. For (D–G), (I), and (K), the results are representative of three independent experiments, and the data are shown as the means ± s.d. Student’s t-test, *p < .05, **p < .01, ***p < .001.

As shown in Figure 2(H–M), knockdown of GAS5 partially attenuated the increased apoptosis and reduced colony formation caused by propofol treatment. Thus, these results reveal that GAS5 is an important regulator of propofol-induced OSCC cell apoptosis increase and cell proliferation decrease.
**GASS acts as a ceRNA of miR-1297 and downregulates miR-1297 expression upon propofol treatment**

GASS can act as a competing endogenous RNA (ceRNA) to regulate tumourigenesis [14]. A recent study indicated that GASS sponges miR-205 and suppresses miR-205 expression [18]. Thus, we asked whether GASS promotes propofol-induced OSCC cell apoptosis by binding microRNAs. Based on the RNA sequencing data, we found that miR-1297 was dramatically decreased in response to propofol treatment (Figure 3(A)). The bioinformatics analysis also suggested that GASS contains a potential binding region of miR-1297 (Figure 3(B)). To confirm this, we first examined the expression of miR-1297 in response to propofol treatment and found that propofol significantly reduced miR-1297 expression in OSCC cells (Figure 3(C)). However, the propofol-induced downregulation of miR-1297 was recovered by GASS knockdown in OSCC cells (Figure 3(D,E)). Then, RIP was performed on UM-SCC6 cell extracts using an antibody against Ago2 to test whether GASS could function as a miR-1297 sponge. The qRT-PCR results showed that GASS and miR-1297 were preferentially enriched in the anti-Ago2 immunoprecipitates compared with the IgG immunoprecipitates (Figure 3(F,G)). We next inserted the wild-type GASS or the mutated version downstream of the luciferase gene in the reporter plasmid for the luciferase assay (Figure 3(H)). Compared with the control group, the luciferase activity of the vector with wild-type GASS was notably inhibited by miR-1297, and the reduction was abolished when the binding site was mutated (Figure 3(I)). In contrast, the luciferase activity of the vector with GASS was notably increased in response to propofol treatment; however, the increase disappeared when miR-1297 was overexpressed (Figure 3(J)). Thus, these results indicate that GASS can act as a ceRNA of miR-1297 and inhibits its expression.

**MiR-1297 suppresses GSK3β in human OSCC cells**

Having identified that GASS promotes propofol-induced apoptosis via binding miR-1297, we next sought to investigate the downstream targets of miR-1297. Previous studies have indicated that propofol induces cell apoptosis by downregulating Bcl2 expression [19]. Thus, we asked whether the Bcl2 is also involved in propofol-induced OSCC cell apoptosis. To this end, we first detected the expression levels of Bcl2 family proteins Bcl2 and Mcl1 in response to propofol treatment. Interestingly, we found that Mcl1, but not Bcl2, was significantly decreased in UM-SCC6 cells upon propofol treatment. Additionally, phosphorylation of Mcl1 at ser 159 was increased in response to propofol treatment (Figure 4(A)). Increasing evidence has indicated that phosphorylation of Mcl1 at ser 159 plays an important role in Mcl1 stability [20,21]. To further validate that propofol affects Mcl1 protein stability, we treated the indicated cells with the protein synthesis inhibitor cycloheximide (CHX) together with or without propofol treatment. Notably, propofol decreased Mcl1 stability (Figure 4(B,C)).

Glycogen synthase kinase 3 (GSK3) is reported to phosphorylate Mcl1 at Ser159 and trigger the degradation of Mcl1 [21]. To determine whether the decrease in Mcl1 by propofol was GSK3-dependent, we investigated the expression levels of GSK3 upon propofol treatment in OSCC cells. As shown in Figure 4(D,E), the expression levels of GSK3β, not the GSK3α or pGSK3β (ser 9) and pGSK3α (ser21), were induced by propofol.

Based on the data that miR-1297 is downregulated by propofol, we thus hypothesized that GSK3β is a potential target of miR-1297. To test this hypothesis, we first searched the TargetScan database for potential targets of miR-1297. Among the candidates identified, the 3’UTR of GSK3β contains a putative region (nucleotides 4636–4643) that matched perfectly to the miR-1297 “seed” region (Figure 4(F)).

To examine whether GSK3β was indeed inhibited by miR-1297, we introduced luciferase reporter plasmids containing the wild-type 3’UTR (WT) or the matched mutant (MUT) of GSK3β into UM-SCC6 and SCC090 cells, and the cells were then treated with or without the miR-1297 mimics. We found that overexpression of miR-1297 markedly suppressed the luciferase activity of the wild-type 3’UTR of GSK3β and led to a decrease in endogenous GSK3β (Figure 4(G–I)). Furthermore, treatment with the miR-1297 inhibitor resulted in the luciferase activity of wild-type 3’UTR elevation and increased GSK3β expression (Figure 4(J–L)).

To further determine whether GASS enhances propofol-induced OSCC cell apoptosis by regulating the miR-1297-GSK3β pathway, we first assessed the effect of GASS on GSK3β and Mcl1 upon propofol treatment. We found that knockdown of GASS suppressed the propofol-induced GSK3β increase and Mcl1 decrease, which was then reversed by the miR-1297 inhibitor in OSCC cells (Figure 4(M,N)). Additionally, cell apoptosis was detected by western blotting. We found that the decreased cell apoptosis by GASS knockdown was also reversed by the miR-1297 inhibitor or GSK3β overexpression upon propofol treatment (Figure 4(N,O)).

**FoxO1 upregulates GASS expression upon propofol treatment in OSCC cells**

Forkhead box protein O1 (FoxO1), an important transcription factor, has been reported to be upregulated in response to propofol treatment [22]. A recent study indicated that FoxO1 transcriptionally upregulates GASS in human glioma stem cells [23]. Thus, we asked whether FoxO1 contributes to the propofol-induced increase in GASS in OSCC cells. To address this, we first assessed FoxO1 expression under propofol treatment in OSCC cells and found that the expression levels of FoxO1 were increased (Figure 5(A)). Subsequently, we knocked down FoxO1 expression using shRNAs in UM-SCC6 and SCC090 cells, and the expression levels of GASS were then analyzed. As shown in Figure 5(B–E), we found that knockdown of FoxO1 suppressed propofol-induced GASS expression, which then led to miR-1297 and Mcl1 increase and GSK3β decrease. In addition, we found four potential binding sites of FoxO1 on the GASS promoter, named PCR 1, 2, 3. Subsequent chromatin immunoprecipitation (ChIP) showed that the chromatin fragments corresponding to the
putative FoxO1-binding sites (PCR 3) were specifically present in anti-FoxO1 immunoprecipitates from OSCC cells, and the binding increased upon propofol treatment (Figure 5(F)).

Taken together, these data indicate that FoxO1 can bind to the promoter of GASS5, leading to its increase in response to propofol treatment.
Figure 4. miR-1297 suppresses GSK3β in human OSCC cells. (A) UM-SCC6 cells were treated with propofol for the indicated times. The expression levels of p-Mcl1 and Mcl1 were analyzed by western blot. (B, C) UM-SCC6 cells with or without knockdown of GAS5 were treated with 20 μg/ml propofol for 36 h, and the cells were incubated with 10 μM CHX for the indicated periods. Cell lysates from the indicated time points were subjected to western blot analysis with anti-Mcl1 antibody. (D, E) UM-SCC6 and SCC090 cells were treated with 20 μg/ml propofol for the indicated times. The expression levels of GSK3α and GSK3β were analyzed by western blot. (F) Schematic diagrams of luciferase reporters containing the wild-type or mutational binding site of miR-1297 on GSK3β 3’UTR, named GSK3β-3 UTR wild type (WT) or Mut. (G–I) GSK3β-3 UTR WT or Mut together with miR-1297 were transfected into UM-SCC6 and SCC090 cells. Luciferase activity was measured by luciferase assay, and the protein levels of GSK3β were analyzed by western blot. (J–L) GSK3β-3 UTR WT or Mut together with the miR-1297 inhibitor were transfected into UM-SCC6 and SCC090 cells. Luciferase activity was measured by luciferase assay, and the protein levels of GSK3β were analyzed by western blot. (M) UM-SCC6 cells with or without GAS5 knockdown were treated with 20 μg/ml propofol for the indicated times, and cell lysates were analyzed using the indicated antibodies. (N) mir-1297 inhibitor or the negative control was transfected into UM-SCC6 cells with or without GAS5 knockdown. The cells were then treated with 20 μg/ml propofol, and cell lysates were analyzed using the indicated antibodies. (O) Flag-GSK3β or the empty vector was transfected into UM-SCC6 cells with or without GAS5 knockdown, and the cells were then treated with 20 μg/ml propofol. Cell lysates were analyzed using the indicated antibodies. For G, H, I, and K, the results are representative of three independent experiments, and the data are shown as the means ± s.d. Student’s t-test, *p < .05, **p < .01, ***p < .001.
Propofol is an intravenous sedative-hypnotic agent administered to induce and maintain anaesthesia [4]. Additionally, many studies have reported that propofol exerts antitumour properties in numerous cancers [5]. However, its role in OSCC remains unknown. In this study, we found that propofol suppresses OSCC cell proliferation and induces cell apoptosis via upregulating GAS5 expression. Elevated GAS5 can bind to miR-1297, leading to decreased miR-1297 in response to propofol treatment. Subsequently, we found that GSK3β is a downstream target gene of miR-1297. Propofol promotes GSK3β expression via miR-1297 downregulation. Increased GSK3β enhances Mcl1 degradation and propofol-induced cell apoptosis. Our data also revealed that FoxO1 directly binds to the promoter of GAS5, facilitating its transcription in response to propofol treatment. Taken together, these results suggest that propofol exhibits antitumour effects in OSCC.

GAS5 is a tumour suppressor gene located at chromosome 1q25 and was first discovered in 1988 by screening highly expressed genes in growth-arrested cells [24]. Downregulation of GAS5 expression is observed in many cancers, including colorectal cancer, breast cancer, renal cell carcinoma, and lung cancer [14]. Many studies have indicated that GAS5 is induced in response to various stimuli, such as ER stress and chemotherapeutic drugs [25,26]. Consistently, our study revealed that GAS5 is increased in response to propofol treatment in OSCC cells, and elevated GAS5 enhances propofol-induced cell apoptosis. Previous studies have suggested that GAS5 can function as a competing endogenous RNA (ceRNA) to regulate signalling pathways and biological functions [27].

Bioinformatics analysis of complementary regions of GAS5 to microRNAs identified 690 candidates, among which 234 microRNAs showed statistically significant binding [28]. In the context of cancer, GAS5 has been reported to bind to miR-21, miR-221 and miR-222 [29,30]. Similarly, we found that increased GAS5 by propofol can bind to miR-1297 and inhibit its expression in OSCC cells.

Accumulating studies have revealed that miR-1297 plays an important role in cancer. MiR-1297 was reported to suppress tumourigenesis in most cancers, including lung cancer, colorectal cancer, and prostate cancer [31–33]. However, we found that GSK3β is a novel target gene of miR-1297. GSK3β and GSK3α are two isoforms of GSK3, which are encoded by separate genes. GSK3 is a serine/threonine kinase, and thus transfers a phosphate group to either the serine or threonine residues of its substrates [34]. Phosphorylation of GSK3β at ser9 and GSK3α at ser 21 render their inactive [35,36]. Interestingly, we found that phosphorylation of GSK3β at ser9 and GSK3α at ser 21 were not altered in response to propofol treatment. Only GSK3β was increased under propofol treatment in OSCC cells, which may own to the miR-1297 decrease by propofol. Thus, our data indicated that downregulation of miR-1297 by propofol enhances GSK3β expression and decreases Mcl1 protein levels.

In addition, we found that the transcription factor FoxO1 promotes GAS5 expression upon propofol treatment in OSCC cells [23]. Previous studies have indicated that the protein levels of FoxO1 were increased in response to propofol treatment [9]. FoxO1 was also reported to regulate GAS5 expression in glioma cells. Consistently, our data indicated that FoxO1 directly binds to the GAS5 promoter and facilitates its
transcription upon propofol treatment. Collectively, our study suggests that FoxO1-GAS5-miR-1297-GSK3β plays an important role in propofol-induced OSCC cell apoptosis.

**Disclosure statement**

The authors declare no competing interests.

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