Propofol Inhibits Lung Cancer A549 Cell Growth and Epithelial–Mesenchymal Transition Process by Upregulation of MicroRNA-1284

Wei-Zhen Liu and Nian Liu

Department of Anesthesia, Hunan Cancer Hospital and The Affiliated Cancer Hospital of Xiangya School of Medicine,
Central South University, Changsha, P.R. China

Propofol has been widely used in lung cancer resections. Some studies have demonstrated that the effects of propofol might be mediated by microRNAs (miRNAs). This study aimed to investigate the effects and mechanisms of propofol on lung cancer cells by regulation of miR-1284. A549 cells were treated with different concentrations of propofol, while transfected with miR-1284 inhibitor, si-FOXM1, and their negative controls. Cell viability, migration, and invasion, and the expression of miR-1284, FOXM1, and epithelial–mesenchymal transition (EMT) factors were detected by CCK-8, Transwell, qRT-PCR, and Western blot assays, respectively. In addition, the regulatory and binding relationships among propofol, miR-1284, and FOXM1 were assessed, respectively. Results showed that propofol suppressed A549 cell viability, migration, and invasion, upregulated E-cadherin, and downregulated N-cadherin, vimentin, and Snail expressions. Moreover, propofol significantly promoted the expression of miR-1284. miR-1284 suppression abolished propofol-induced decreases of cell viability, migration, and invasion, and increased FOXM1 expression and the luciferase activity of FOXM1-wt. Further, miR-1284 negatively regulated FOXM1 expression. FOXM1 knockdown reduced cell viability, migration, and invasion by propofol treatment plus miR-1284 suppression. In conclusion, our study indicated that propofol could inhibit cell viability, migration, invasion, and the EMT process in lung cancer cells by regulation of miR-1284.

Key words: Propofol; MicroRNA-1284; Cell viability; Epithelial–mesenchymal transition (EMT); Migration; Invasion

INTRODUCTION

Lung cancer is significantly malignant and a cause of cancer-related deaths, with an estimated 1.1 million deaths worldwide annually[1]. Although recent advances have been made in different therapeutic technologies of radiation therapy, antiangiogenesis therapy, and immunotherapy[2], the long-term survival rate of lung cancer remains poor. It has been reported that the 5-year survival rate of lung cancer is approximately 15% or less because of cell metastasis[3]. Thus, there is an urgent need to develop new therapies to prevent death from lung cancer cell metastasis[4]. Molecular therapies and prophylactic measures may shift the current strategies and improve outcomes.

Propofol is one of the most commonly used intravenous anesthetic agents and is widely used in various kinds of cancer resections due to its rapid onset and short duration of action[5]. Numerous studies have recently found that propofol is able to influence the cell proliferation,

migration, and invasiveness of cancers, such as ovarian cancer[6], breast cancer[7], liver cancer[8], and lung cancer[9]. For instance, Huijin et al. showed that propofol suppressed invasion of human lung cancer A549 cells by downregulating aquaporin-3 (AQP-3) and matrix metalloproteinase-9 (MMP-9)[10]. Chen et al. reported that propofol could inhibit the proliferation of lung cancer cells and promote cell apoptosis[11]. Although the function of propofol has been reported in lung cancer, the molecular mechanism of propofol-mediated lung cancer cells remains unclear.

MicroRNAs (miRNAs) are defined as a family of single-stranded, highly conserved, and small noncoding RNA molecules of approximately 22 nucleotides in length[12]. It plays an important role in the network of gene regulation. In addition, miRNAs contribute to the majority of biological processes, such as cell development, cell proliferation, migration, invasion, and apoptosis[13].

Address correspondence to Nian Liu, Department of Anesthesia, Hunan Cancer Hospital and The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, No. 283 Tongzipo Road, Yuelu District, Changsha 410013, Hunan Province, P.R. China.
E-mail: liunian0406@126.com

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Aberrant expression of miRNAs being involved in lung cancer has been well described[14]. However, to the best of our knowledge, there is no available report on the role of miR-1284 in lung cancer.

Therefore, we aimed to explore the effect of propofol on lung cancer cell viability, migration, invasion, and the epithelial–mesenchymal transition (EMT) process, as well as identify the relationship between propofol and miR-1284. Additionally, we investigated the functions of miR-1284 by regulating Forkhead box M1 (FOXM1). These results will provide possible therapeutic strategies for lung cancer.

MATERIALS AND METHODS

Cell Culture and Treatment

The human lung cancer cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 1 antibiotic–antimycotic mixture (Gibco) and 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) and maintained at 37°C in an atmosphere of humidified 5% CO_2 and 95% air. Thereafter, the cells were cultured in different concentrations (0, 5, and 10 ng/ml) of propofol (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. To induce the EMT process, cells were treated with 10 ng/ml transforming growth factor- (TGF- ; PeproTech, Rocky Hill, NJ, USA). Nontreated cells were considered as control group.

Cell Transfection

Mature miR-1284 inhibitor and inhibitor negative control (NC) were synthesized by GenePharma (Shanghai, P.R. China). FOXM1-specific small interfering RNA (siRNA) (si-FOXM1) and siRNA negative control (si-NC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For stable transfection, A549 cells were seeded in a six-well plate at 2×10^5 cells per well, and then were transiently transfected with miR-1284 inhibitor, si-FOXM1, or their NC. Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Cell Viability

Cell viability was assessed by a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA) according to standardized universal methods described previously[15]. In brief, cells were seeded in 96-well plates with 5,000 cells/well and were exposed to propofol and/or transfected with miR-1284 inhibitor, si-FOXM1, or their NC. Forty-eight hours later, 10 µl of CCK-8 was added to the cells and incubated for 1 h at 37°C in humidified 95% air and 5% CO_2. Subsequently,

absorbance at 450 nm was read by a microplate reader (Bio-Rad, Hercules, CA, USA).

Migration and Invasion Assay

Cell migration and invasion were determined using a Transwell Millicell Standing Cell Culture 24-well CM (Millipore, Billerica, MA, USA). For cell migration assay, A549 cells were suspended in 200 µl of serum-free medium and seeded on the upper compartment of Transwells, while the lower chamber contained RPMI-1640 medium with 20% FBS. After incubation at 37°C for 24 h, the medium in the upper chamber was removed. The cells on the lower side of the membrane were washed in phosphate-buffered saline (PBS) and fixed with 4% methanol (NIST, USA) for 30 min. The nontraversed cells were removed carefully from the upper surface of the filter with a cotton swab. Traversed cells on the lower side of the membrane were stained with 0.1% crystal violet (Merck, Darmstadt, Germany) for 20 min, and images were captured using an inverted microscope (Olympus, Tokyo, Japan). For the cell invasion assay, similar procedures were performed, but the inserts were coated with BD Matrigel™ Matrix (BD Biosciences, NY, USA).

Dual-Luciferase Reporter Activity Assay

For miR-1284 binding to FOXM1, the sequence of the FOXM1 3 -untranslated region (3 -UTR) containing the predicted miR-1284 binding site was then cloned into pGL2-Basic Vector (E1641; Promega, Madison, WI, USA) as FOXM1-wt vector, while mutated FOXM1 3 -UTR was used as NC (FOXM1-mt). Then each vector was cotransfected with miR-1284 inhibitor into HEK 293T cells. The cells were collected 48 h later, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) on the basis of the manufacturer's instructions.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA of the A549 cells was extracted from transfected cells by TRIzol reagent (Invitrogen). For miR-1284 detection, reverse-transcribed complementary DNA (cDNA) was synthesized with the PrimeScript RT Reagent Kit (TaKaRa, Dalian, P.R. China), and qRT-PCR was performed using the SYBR Premix ExTaq (TaKaRa) with the Stratagene Mx3000P Real-Time PCR System (Agilent Technologies, Inc., Santa Clara, CA, USA). Expression levels were normalized against the endogenous short nuclear RNA (snRNA) U6 control. For FOXM1 analyses, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega). qRT-PCR was performed with SYBR Premix ExTaq with the Stratagene Mx3000P Real-Time PCR System. Glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) was used as the internal control. The relative expression ratio of mRNA was calculated by the 2⁻ CT method. PCRs for each gene were repeated three times. Independent experiments were done in triplicate.

Western Blot

After treatment with propofol or transfection with miR-1284 inhibitor, si-FOXM1, and their NC, the protein in A549 cells was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, P.R. China) supplemented with protease inhibitors (Roche, Basle, Switzerland). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established using a Bio-Rad Bis-Tris Gel System according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Anti-FOXM1 antibody (ab175798), anti-E-cadherin antibody (ab1416), anti-N-cadherin antibody (ab18203), anti-vimentin antibody (ab92547), anti-Snail antibody (ab53519), and anti-GAPDH antibody (ab8245; Abcam, Cambridge, MA, USA) were used to incubate membranes at 4°C overnight. Following 1 h of incubation at room temperature with goat anti-rabbit secondary antibody (ab205718; 1:2,000; Abcam) and goat anti-mouse secondary antibody (ab205719; 1:50; Abcam)

tagged by horseradish peroxidase, the polyvinylidene difluoride (PVDF) membrane-carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μl of Immobilon Western Chemiluminescent HRP Substrate (Millipore) was added to cover the membrane surface. The signals were captured, and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, P.R. China).

Statistical Analysis

All experiments were performed in triplicates, and the results of multiple experiments were presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad 6.0 statistical software (GraphPad Software, San Diego, CA, USA). The p values were calculated using a one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Propofol Inhibited A549 Cell Viability, Migration, Invasion, and the EMT Process

The chemical structure of propofol is shown in Figure 1A, which contains a benzene ring and a hydroxyl. To sufficiently explore the functions of propofol in lung cancer,
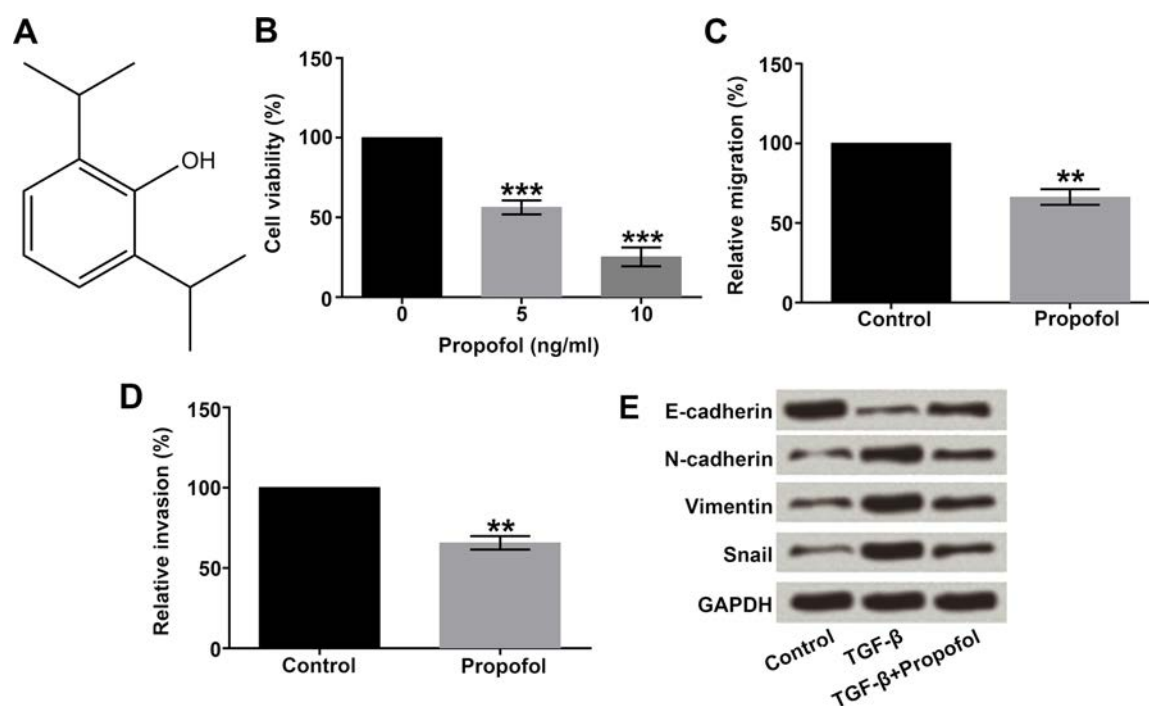

Figure 1. Effect of propofol on A549 cell viability, migration, invasion, and the EMT process. A549 cells were treated with different concentrations (0, 5, and 10 ng/ml) of propofol. (A) The chemical structure of propofol. (B) Cell viability, (C) migration, (D) invasion, and (E) the expression changes of EMT markers in A549 cells were detected by CCK-8, Transwell, and Western blot assays, respectively. EMT, epithelial–mesenchymal transition; CCK-8, cell counting kit-8. **$p < 0.01$, ***$p < 0.001$.

A549 cells were treated with different concentrations of propofol (0, 5, and 10 ng/ml), and then cell viability was measured by the CCK-8 assay. As displayed in Figure 1B, propofol at concentrations of 5 and 10 ng/ml significantly inhibited cell viability compared with the cells without propofol treatment ($p<0.001$). Subsequently, 5 ng/ml of propofol was selected for use in the further studies. Next, we detected the influences of propofol on the migration and invasion of A549 cells by using Transwell assay. Results showed that cells in the lower chamber of the Transwell were obviously reduced with propofol treatment ($p<0.01$) (Fig. 1C and D).

A recent study has demonstrated that EMT played a key role in the process of cancer metastasis[16]. Therefore, we used 10 ng/ml of TGF- to induce EMT in A549 cells, and the protein levels of EMT markers were assessed by Western blot. As shown in Figure 1E, the protein level of E-cadherin was remarkably decreased, while N-cadherin, vimentin, and Snail expressions were all increased after induction of TGF- . The results showed that these changes in the EMT marker expressions were suppressed when adding the propofol. Taken together, these results demonstrated that propofol inhibited A549 cell viability, migration, invasion, and the EMT process.

Propofol Inhibited Cell Viability, Migration, and Invasion by Upregulation of miR-1284

A previous study has demonstrated that the effects of propofol might be through the regulation of miRNAs[17]. In order to investigate the functional role of miR-1284 in propofol-treated cells, the expression level of miR-1284 was first analyzed after exposure to 5 ng/ml of propofol. As shown in Figure 2A, the results showed that the expression level of miR-1284 was significantly elevated by propofol ($p<0.01$). The results implied that propofol promoted the expression level of miR-1284 in A549 cells.

Subsequently, miR-1284 inhibitor and inhibitor NC were transfected into A549 cells, and the transfection efficiency was examined by qRT-PCR. As shown in Figure 2B, the expression level of miR-1284 was remarkably downregulated in the propofol+miR-1284 inhibitor group compared with the propofol+inhibitor NC group ($p<0.001$). This result suggested that miR-1284 inhibitor was successfully transfected into A549 cells.

Additionally, cell viability, migration, and invasion were measured by the CCK-8 and Transwell assays. The inhibitory effects of propofol on cell viability, migration,
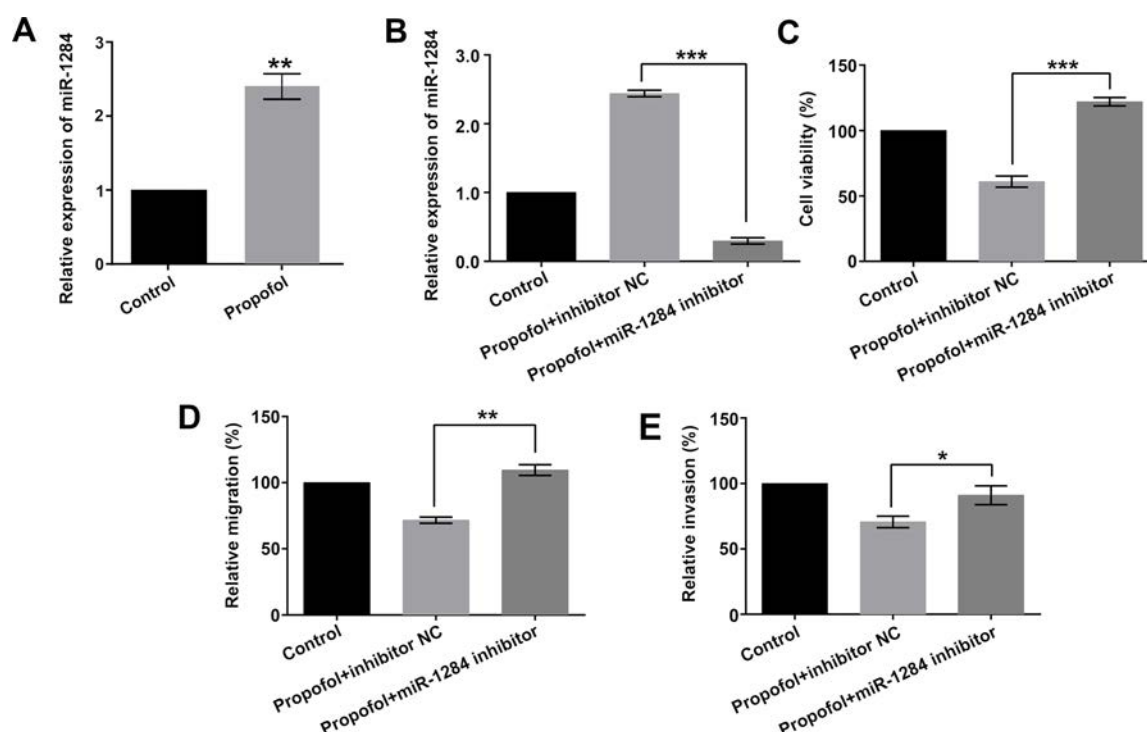

Figure 2. Effect of propofol on A549 cell viability, migration, and invasion by regulation of miR-1284. A549 cells were transfected with miR-1284 inhibitor or inhibitor NC, while being exposed to 5 ng/ml of propofol. (A) Transfection efficiency, (B) miR-1284 expression, (C) cell viability, (D) migration, and (E) invasion were detected by qRT-PCR, CCK-8, and Transwell assays, respectively. miRNA, microRNA; NC, negative control; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

and invasion were significantly reversed after addition of miR-1284 inhibitor ($p<0.001$, $p<0.01$, or $p<0.05$) (Fig. 2C–E). There results indicated that propofol inhibited cell viability, migration, and invasion by upregulation of miR-1284.

miR-1284 Negatively Regulated the Expression of FOXM1 and Inhibited Cell Viability, Migration, and Invasion by Downregulation of FOXM1

FOXM1, as an oncogene, has been found in several tumors and may be involved in cell metastasis[18]. To explore whether miR-1284 affected the expression of FOXM1, miR-1284 inhibitor and inhibitor NC were transfected into A549 cells, and then the mRNA and protein levels of FOXM1 were detected by qRT-PCR and Western blot assays. Results showed that miR-1284 inhibition upregulated the expression of FOXM1 when treated with propofol ($p<0.01$) (Fig. 3A and B). Furthermore, bioinformatics analysis confirmed that miR-1284 was predicted to reversely bind to the 3′-UTR of FOXM1 (Fig. 3C). Thereupon, Dual-Luciferase Reporter Activity Assay was used to verify the targeting relationship between miR-1284 and FOXM1. From Figure 3D, we found that miR-1284 inhibitor significantly promoted the activity of the FOXM1-wt group ($p<0.05$). Thus, we believe that miR-1284 negatively regulated the expression of FOXM1.

Further, in order to study the influences of FOXM1 on lung cancer, A549 cells were transfected with si-FOXM1 and si-NC. The transfection efficiency is shown in Figure 4A; the expression of FOXM1 was significantly deceased in the si-FOXM1 group ($p<0.01$). These results revealed that si-FOXM1 was successfully transfected into A549 cells. Subsequently, we detected cell viability, migration, and invasion to reflect the effects of FOXM1 on A549 cells. As shown in Figure 4B–D, FOXM1 knockdown inhibited cell viability, migration, and invasion with propofol exposure plus miR-1284 suppression ($p<0.01$ or $p<0.05$). Overall, these results demonstrated that miR-1284 could inhibit cell viability, migration, and invasion by downregulating FOXM1 expression.

miR-1284 Inhibited the EMT Process by Downregulation of FOXM1

To further study the roles of miR-1284 and FOXM1 in the EMT process, 10 ng/ml of TGF- was used to induce the EMT process in A549 cells. As demonstrated in Figure 5, FOXM1 and EMT markers including E-cadherin, N-cadherin, vimentin, and Snail were examined by Western blot. The expressions of FOXM1 and E-cadherin were remarkably upregulated by propofol treatment combined with miR-1284 suppression, while N-cadherin, vimentin, and Snail were significantly downregulated. FOXM1 knockdown reversed these changes. All in all,
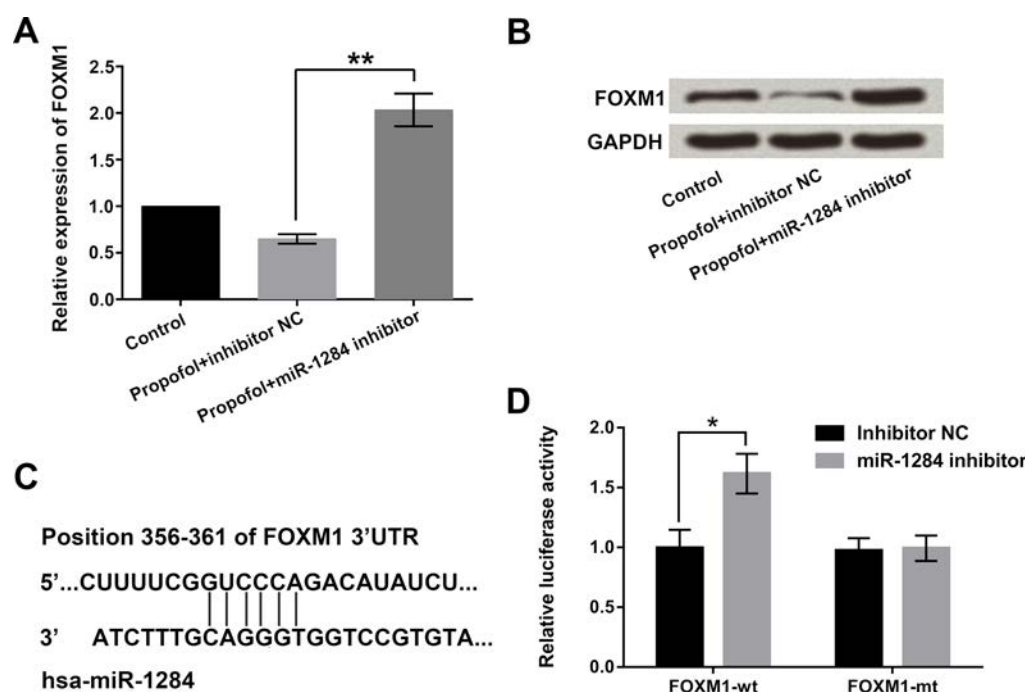

Figure 3. miR-1284 negatively regulated FOXM1 expression in A549 cells. Cells were transfected with miR-1284 inhibitor or inhibitor NC while being exposed to 5 ng/ml of propofol. The (A) mRNA and (B) protein levels of FOXM1, (C) potential binding sequences between miR-1284 and FOXM1, and (D) dual luciferase activity were assessed by qRT-PCR, Western blot, bioinformatics analysis, and dual luciferase activity, respectively. FOXM1, Forkhead box M1. *$p<0.05$, **$p<0.01$.
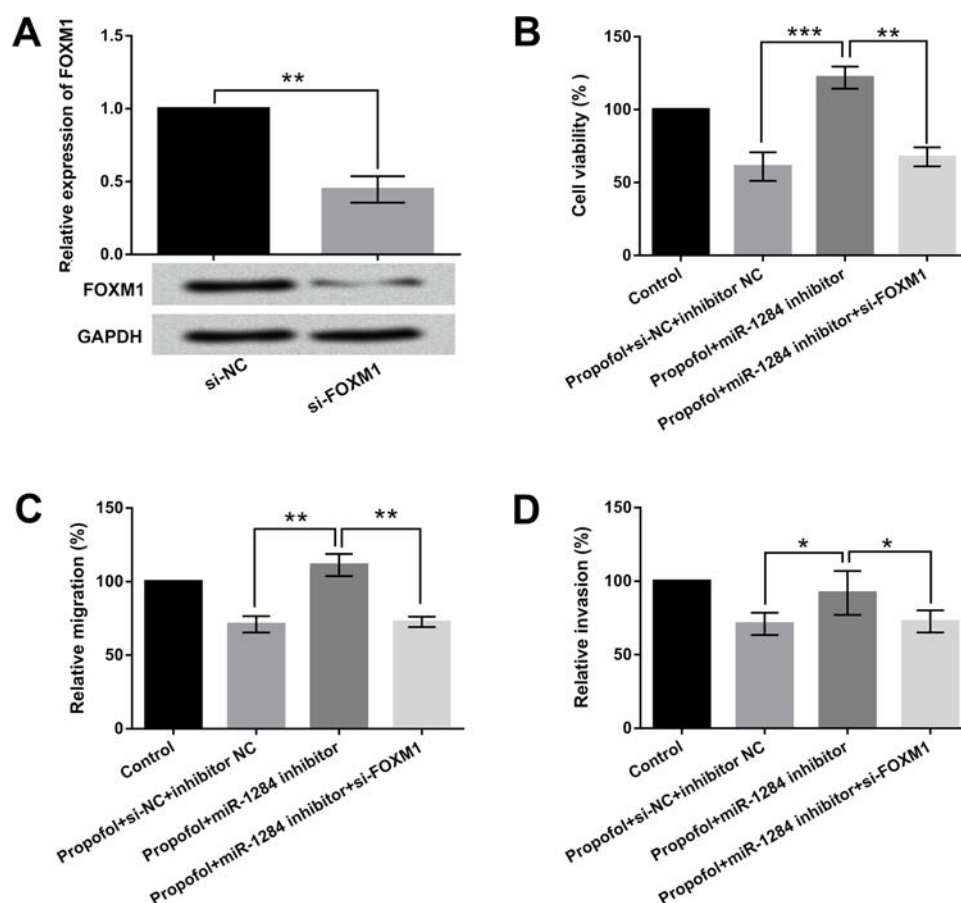
Figure 4. Effects of miR-1284 on cell viability, migration, and invasion by regulation of FOXM1 in A549 cells. Cells were cotransfected with miR-1284 inhibitor, inhibitor NC, si-FOXM1, or si-NC, while being exposed to 5 ng/ml of propofol. The (A) mRNA and protein levels of FOXM1, (B) cell viability, (C) migration, and (D) invasion were determined by qRT-PCR, Western blot, CCK-8, and Transwell assays, respectively. $*p<0.05$, $**p<0.01$, $***p<0.001$.

these expression changes implied that FOXM1 might participate in the miR-1284-mediated EMT process.

DISCUSSION

In our study, we assessed the roles and regulatory mechanisms of propofol in lung cancer A549 cells. We investigated whether propofol inhibited A549 cell viability, migration, invasion, and the EMT process. Further mechanism studies revealed that the inhibitory effects of propofol on cell viability, migration, and invasion were alleviated by suppression of miR-1284. FOXM1 was negatively regulated by miR-1284 and might be involved in the miR-1284-mediated EMT process, whereas miR-1284 decreased cell viability, migration, and invasion via downregulation of FOXM1. The results indicated that propofol might be a potential alternative for lung cancer treatment.

Propofol is a commonly used anesthetic for surgery and cancer therapy that affects cell viability, migration, and invasion of tumor cells[19], as well as exhibits similar effects as fentanyl[20]. Cui et al. and Chen et al. have reported that propofol could effect cell proliferation and apoptosis in lung cancer[9,11]. Similar to these previous studies, our study also demonstrated that propofol could suppress cell viability, migration, and invasion of lung cancer cells. Propofol may therefore be a particularly suitable anesthetic for lung cancer surgery. Although the effects of propofol have been reported in lung cancer, further investigations are still needed to discover the underlying mechanisms.

Some studies have demonstrated that the effects of propofol might be mediated by miRNAs. For example, Xu et al. showed that propofol suppressed proliferation and invasion of glioma cells by upregulating miR-218 expression[21]. Ye et al. reported that propofol suppressed proliferation and invasion of gastric cancer cells via downregulation of miR-221[22]. The tumor suppressor function of miR-1284 has been reported in gastric cancer[23]. However, to our knowledge, whether miR-1284 is involved in the effects of propofol on lung cancer has not been investigated. Our study showed that the inhibitory
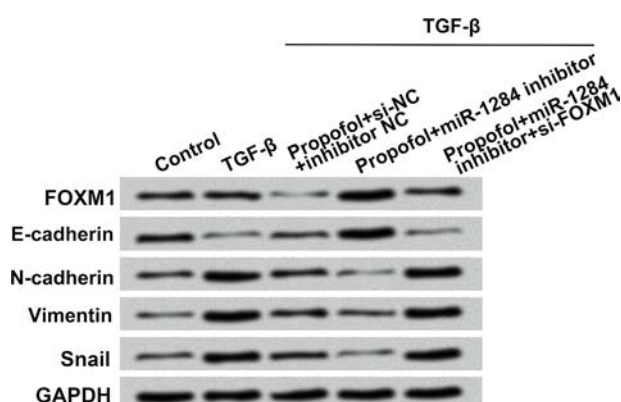
Figure 5. Effect of miR-1284 on the expressions of FOXM1 and EMT markers. A549 cells were cotransfected with miR-1284 inhibitor, inhibitor NC, si-FOXM1, or si-NC while being exposed to 5 ng/ml of propofol. The protein levels of FOXM1, E-cadherin, N-cadherin, vimentin, and Snail were detected by Western blot. GAPDH was used as internal control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

effects of propofol on cell viability, migration, and invasion were significantly reversed after the addition of miR-1284 inhibitor. These results indicated that propofol may inhibit cell viability, migration, and invasion through upregulation of miR-1284.

It is well known that lung cancer is a highly malignant carcinoma, and most of the deaths from lung cancer are caused by metastasis[24]. A recent study has demonstrated that EMT plays a key role in the process of cancer metastasis[25]. EMT is characterized by different regulations of epithelial and mesenchymal markers. The loss of the epithelial marker E-cadherin and the increases in the mesenchymal markers vimentin and N-cadherin are associated with migratory and invasive behaviors[26]. In the current study, we found that propofol remarkably increased E-cadherin expression while decreasing N-cadherin, vimentin, and Snail expressions. These data indicated that propofol could inhibit the EMT process.

Accumulating evidence has demonstrated that FOXM1 was expressed in numerous tumor cell lines and regulated the expression of genes involved in the EMT process[27]. For instance, Li et al. found that miR-134 significantly inhibited EMT of non-small cell lung carcinoma cells by targeting FOXM1[28]. Ke et al. reported that miR-149 also inhibited lung cancer cell EMT by targeting FOXM1[18]. Similarly, our study showed that FOXM1 was negatively regulated by miR-1284 and might be involved in the regulation of the EMT process. In addition, miR-1284 inhibited cell viability, migration, and invasion via downregulating FOXM1 expression. These data suggested that FOXM1 expression partially is due to miR-1284 downregulation, which might contribute to lung cancer cell metastasis.

CONCLUSION

Our study suggests that propofol suppresses cell viability, migration, and invasion in lung cancer cells by upregulation of miR-1284, which negatively regulates FOXM1 and EMT process. Our study provides basic theories of propofol for lung cancer treatment.

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