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Hypoxia-inducible factor-1α contributes to the proliferation of cholesteatoma keratinocytes through regulating endothelin converting enzyme 1 expression

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Abstract

Objective: Cholesteatoma is a hyperproliferative, pseudoneoplastic lesion of the middle ear characterized by aggressive growth and bone destruction. Hypoxia-inducible factor-1α (HIF-1α, also known as HIF1A) is a key transcription factor that enters the nucleus and upregulates many genes involved in cancer progression in the oxygen-free environment. This study is designed to explore the role and mechanism of HIF1A in the progression of cholesteatoma.

Methods: HIF1A and endothelin converting enzyme 1 (ECE1) levels were determined using real-time quantitative polymerase chain reaction. The protein levels of HIF1A, Cyclin D1, proliferating cell nuclear antigen, and ECE1 were measured using western blot. Cell viability, proliferation, and cell cycle progression were analyzed using cell counting kit-8, Colony formation, 5-ethynyl-2′-deoxyuridine, and flow cytometry assays. Binding between HIF-1α and ECE1 promoter was predicted by Jaspar and verified using Chromatin immunoprecipitation and dual-luciferase reporter assays.

Results: HIF1A and ECE1 were highly expressed in cholesteatoma patients and keratinocytes. Moreover, HIF1A knockdown might suppress the cell viability, proliferation, and cycle progression of cholesteatoma keratinocytes. Furthermore, HIF1A upregulated the transcription of ECE1 through binding to its promoter region.

Conclusion: HIF1A might expedite cholesteatoma keratinocyte proliferation partly by increasing ECE1 expression, providing a possible therapeutic target for the cholesteatoma treatment.

KEYWORDS
cholesteatoma keratinocytes, ECE1, HIF1A, proliferation

1 | INTRODUCTION

As a well-demarcated non-neoplastic cystic lesion, cholesteatoma arises from the aberrant presence and growth of the keratinized squamous epithelium in the middle ear cavity and temporal bone.[1] The wall of the capsule has a stratified squamous epithelium composed mainly of keratinized cells. Generally, the highly proliferative activity of cholesteatoma epithelial cells is locally aggressive and can be responsible for the destruction of structures within the middle ear, which results in conductive and sensorineural hearing loss, and even

vestibular dysfunction, facial nerve palsy or intracranial complications.[2,3] At present, complete surgical excision is considered the effective treatment for this disease, but a wide range of recurrence rates have been reported, ranging from 4% to 61%.[4] Hence, long-term eradication of cholesteatoma has continued to present a noteworthy challenge for otologists in recent years.[5] Despite the advances that have been made in the investigation of multiple factors and cholesteatoma keratinocyte receptors,[6] the molecular mechanisms underlying the pathogenesis of the disease have not yet been fully clarified.

Currently, the theory of the combination of retraction and proliferation has received increasing attention for cholesteatoma formation.[7] It has been stated that tympanic membrane retraction pockets are usually secondary to an Eustachian tube chronic dysfunction. Apart from that, interruption of Eustachian tube ventilation might decrease pressure and oxygenation in the middle ear cavity, which leads to hypoxia and hypercapnia in the middle ear mucosa.[8] Indeed, the hypoxic conditions in the tympanic cavity have been shown to be responsible for the eardrum retraction pockets and subsequent cholesteatoma formation.[9] Therefore, hypoxia is currently thought to be one of the major triggers for cholesteatoma progression.[10,11] Under hypoxic conditions, hypoxia-inducible factor-1α (HIF-1α, also known as HIF1A) is activated and subsequently enters the nucleus of cells, where it binds to hypoxia-responsive element (HRE) and modulates the transcription of some genes.[12] As a key transcription factor, the upregulation of HIF1A has been previously validated to support tumor progression by different mechanisms, containing proliferation, cell survival, metastasis, angiogenesis, and metabolism.[13,14] Lately, recent literature has suggested that the overexpression of HIF1A might promote cholesteatoma deterioration and bone erosion.[15] Beyond that, dysregulated HIF1A was previously validated to be closely associated with the proliferation of cholesteatoma keratinocytes.[16] Nevertheless, current studies on the expression and underlying mechanism of HIF1A in cholesteatoma keratinocyte proliferation are far from being addressed.

Herein, a public prediction server Jaspar (https://jaspar.genereg.net) predicted that HIF-1A has a number of binding sites to the endothelin converting enzyme 1 (ECE1) promoter region. Apart from that, ECE1 has been reported to participate in the proteolytic processing of endothelin precursors to biologically activate peptides.[17] Furthermore, abnormally expressed ECE1 was correlated with cell proliferation and invasiveness in several human cancers.[18,19] Of interest, an earlier study exhibited HIF binding sites at ECE1 promoter and intron regions, implying that ECE1 might be a novel target gene for HIF.[20] Therefore, this work attempted to explore whether HIF1A might control cholesteatoma keratinocyte proliferation via regulating ECE1 expression.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and cell culture

After signing written informed consent from all participants, 36 cholesteatoma tissues and matched normal posterior auricular skin or skin fragments (control group) were collected at the Changzhou No. 2 People's Hospital affiliated to Nanjing Medical University. Meanwhile, the approval of the Ethics Committee of Changzhou No. 2 People's Hospital affiliated to Nanjing Medical University for the whole research was obtained.

Human immortalized keratinocytes (HaCaT: ZQ0044) were grown in a specific medium (ZM0044, Zhong Qiao Xin Zhou Biotechnology, Shanghai, China) under standard conditions with 5% CO_2 at 37°C. Beyond that, cholesteatoma keratinocytes were isolated and characterized as described previously.[21] In brief, after extraction of cholesteatoma tissues from study subjects, tissue samples were washed with precooled PBS, followed by cutting into 1×1 mm^3 small slices. After digestion with 200 U/mL collagenase IV (Sigma Aldrich, St. Louis, MO, USA) overnight at 4°C, samples were centrifuged at 1500 rpm for 5 min. After being discarded the supernatant, Keratinocyte-serum free medium (KSFM, Invitrogen, Paisley Scotland, UK) with streptomycin/penicillin (Invitrogen) was added to these collected pellets, which then were maintained in 5% CO_2 at 37°C. Subsequently, we changed the KSFM medium every 3 days. Finally, cell cultures from Passages 3 and 4 were applied in the experiments.

2.2 | Real-time quantitative polymerase chain reaction

According to Trizol reagent (Invitrogen) and RNAiso plus (TaKaRa, Tokyo, Japan), total RNAs were extracted from cells and clinical samples, followed by the detection using NanoDrop 2000 system (NanoDrop, Wilmington, DE, USA). Subsequently, the qualified RNA samples were reverse-transcribed into cDNA with Prime Script RT Master Mix (Takara). On Thermal Cycler CFX6 System (Bio-Rad, Hercules, CA, USA), amplification reaction was conducted using SYBR Green PCR Kit (Takara). After normalizing to β-actin, the relative RNA expressions were analyzed via the $2^{-\Delta\Delta Ct}$ method. Primer sequences were presented in Table 1.

2.3 | Western blot assay

After being lysed with Radio-Immunoprecipitation Assay (RIPA) buffer (Keygen, Nanjing, China), total protein lysates from cells and clinical samples were centrifuged and collected, followed by loading on 10% sodium dodecyl sulfonate (SDS) polyacrylamide gel for separation. After transferring to nitrocellulose membranes (Millipore, Molsheim, France), primary antibodies: HIF1A (20960-1-AP, Proteintech, Wuhan, China, 1:2000), Cyclin D1 (ab16663, 1:200, Abcam, Cambridge, MA, USA), proliferating cell nuclear antigen (PCNA, ab29, 1:1000, Abcam), ECE1 (26088-1-AP, Proteintech, 1:2000), and β-actin (ab8226, Abcam) were added at 4°C. The next day, ECL reagent (Solarbio, Beijing, China) was applied to the bands after incubation with a secondary antibody at 37°C for 2 h.

2.4 | Cell transfection

In this research, short hairpin RNAs (shRNAs) targeting the HIF1A sequence (sh-HIF1A) or ECE1 sequence (sh-ECE1) were provided by

TABLE 1 Primers sequences used for PCR.

Name		Primers for PCR (5′-3′)
ECE1	Forward	GACCTGGTGGACTCGCTCTC
	Reverse	ACTGAGACACAAGCTTCGCT
FGF12	Forward	GATAGCCAGCTCCTTGATCCG
	Reverse	AAGCGCACTTTGCTGAACAC
LMNTD2	Forward	CCAGTGGGAGAAGGAGCAC
	Reverse	AGTCTCTGCCGTCACTACCT
PPHLN1	Forward	CCATTATGCGAGAGAGCGGT
	Reverse	CAGGACGCACGGACTTTCTA
ECE1 promoter P1	Forward	CTCAGACAAGATCTTGTTGTGTCGT
	Reverse	GGCAACACGGACCCCATCTCTACA
ECE1 promoter P2	Forward	GAGTCCCTAACTGAACACCTCA
	Reverse	GAAAGGAGCTGTGTGTGTGCATT
HIF1A	Forward	CGGCGCGAACGACAAGAAAA
	Reverse	GAAGTGGCAACTGATGAGCA
β-actin	Forward	CTTCGCGGGCGACGAT
	Reverse	CCACATAGGAATCCTTCTGACC

Abbreviation: ECE1, endothelin converting enzyme 1; HIF1A, hypoxia-inducible factor-1α; PCR, polymerase chain reaction.

Geneseed (Guangzhou, China) and introduced into pLKO.1 lentivirus vector (Addgene, Cambridge, MA, USA). Meanwhile, empty pLKO.1 lentivirus vector (vector) acted as the negative control (sh-NC). After being transfected into 293T cells with lentivirus packaging plasmids, the acquired cell supernatants were infected into cholesteatoma keratinocytes at 70%-80% confluence in the presence of 8 μg/mL polybrene. Finally, stable strains were selected by 5 μg/mL puromycin.

For HIF1A or ECE1 overexpression (HIF1A or pcDNA-ECE1), 6 μg of pcDNA vector specific to HIF1A or ECE1, and pcDNA empty vector were transfected into cholesteatoma keratinocytes based on Lipofectamine 3000 (Invitrogen) for 48 h.

2.5 | Counting kit assay

Cholesteatoma keratinocyte viability was assessed in this experiment. Briefly, 5×10^4 cells were cultured overnight in 96-well plates. Meanwhile, a culture system was made by mixing cell counting kit-8 (CCK8) reagent (10 μL, Dojindo, Kumamoto, Japan) with 90 μL media. At indicated time points, 100 μL this system was allowed to be added into each well for 4 h. Finally, the absorbance was recorded using a microplate reader at 450 nm.

2.6 | Cell proliferation assay

For colony formation assay, 500 transfected cholesteatoma keratinocytes were injected into 6-well plates and cultured for about 2 weeks. After being washed, samples were subjected to 4% formaldehyde fixation and crystal violet staining. When the forming colonies were macroscopically visible, colony number was counted.

For 5-ethynyl-2′-deoxyuridine (EdU) experiment, EdU working solution (20 μM, RiboBio, Guangzhou, China) was mixed with 2×10^4 cholesteatoma keratinocytes. After being cultured for 2 h, cells were fixed and then incubated with 0.5% Triton-X-100. After reaction with Apollo reaction cocktail, samples were subjected to 4',6-diamidino-2-phenylindole (DAPI) staining, followed by assessment of EdU-positive cells according to a fluorescence microscope.

2.7 | Flow cytometry for cell cycle progression

After washing, 2×10^6 cholesteatoma keratinocytes were fixed in 70% ethanol and centrifuged. After nucleic acids were stained with propidium iodide (Keygen), the sample of each group was analyzed using a flow cytometer.

2.8 | Chromatin immunoprecipitation

To verify the binding between HIF1A and ECE1, SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA) was employed for this experiment. After fixture, collection, and lysis, cholesteatoma keratinocytes were centrifuged to obtain nuclei pellets, followed by DNA digestion. For immunoprecipitation, HIF1A antibody (Novus Biologicals, CO, USA) and magnetic beads were incubated with digested chromatin for 16 h. Finally, these products were subjected to real-time quantitative polymerase chain reaction (RT-qPCR) analysis with the corresponding primers (Table 1).

2.9 | Dual-luciferase reporter assay

To analyze ECE1 promoter activity, the promoter (−1557 to −1548 bp) sequences of ECE1 containing the wild-type (WT) or mutant-type (MUT) HIF1A binding sites (RiboBio) were synthesized and inserted into Gv238 Luciferase Reporter Vector (Genechem, Shanghai, China). Then, the acquired ECE1-WT/MUT vectors were respectively transfected into cholesteatoma keratinocytes with sh-HIF1A, sh-NC, pcDNA-HIF1A, or pcDNA. 48 h later, luciferase activities were analyzed using Dual Luciferase Assay Kit (Promega, Madison, WI, USA).

2.10 | Statistical analysis

In this research, the results of statistical analyses were determined based on GraphPad Prism7 software. Meanwhile, Student's *t*-test or one-way analysis of variance with Tukey's tests were employed to compare the significance of the difference between two or multiple groups, where the significance threshold was set at a *p*-value <.05.

3 | RESULTS

3.1 | HIF1A expression was increased in cholesteatoma patients and keratinocytes

First, to investigate the functional role of HIF1A in cholesteatoma, its expression level was first detected using RT-qPCR and western blot assay. As displayed in Figure 1A,B, HIF1A mRNA level and protein level were significantly upregulated in cholesteatoma patients relative to normal skin specimens. Moreover, we further verified that the HIF1A protein level was highly expressed in cholesteatoma keratinocytes versus HaCaT cells (Figure 1C). Together, these data highlighted that HIF1A might be involved in cholesteatoma progression.

3.2 | Upregulation of HIF1A promoted cholesteatoma keratinocyte growth

Subsequently, to identify the role of HIF1A in cholesteatoma keratinocytes, gain- or loss-of-function analyses were performed *in vitro*. As presented in Figure 2A, the expression level of HIF1A was greatly upregulated in pcDNA-HIF1A-transfected cholesteatoma keratinocytes versus cells transfected with empty vector pcDNA. On the contrary, the HIF1A level was strikingly downregulated in cholesteatoma keratinocytes after sh-HIF1A introduction when compared with cells with sh-NC (Figure 2A). These data suggested that the overexpression or knockdown efficiency of HIF1A was available for the following experiments. Functionally, CCK8, colony formation, and EdU assays exhibited that cell viability and proliferation were apparently improved by HIF1A upregulation, and obviously hindered due to the silencing of HIF1A in cholesteatoma keratinocytes (Figure 2B–D). Simultaneously, flow cytometry assay revealed that the proportion of cholesteatoma keratinocytes was reduced in G0/G1-phase and increased in the S-phase caused by the overexpression of HIF1A (Figure 2E), implying that upregulated HIF1A might boost the cycle progression of cholesteatoma keratinocytes. Inversely, the deficiency of HIF1A might evidently restrain cholesteatoma keratinocyte cycle progression (Figure 2E). Meanwhile, western blot assay presented that the introduction of HIF1A elicited an obvious enhancement in Cyclin D1 (an important regulator of the cell cycle) and PCNA (proliferation marker) in cholesteatoma keratinocytes, whereas the knockdown of HIF1A might repress their protein levels (Figure 2F). Overall, these findings implied that the knockdown of HIF1A might suppress the proliferation and cycle progression of cholesteatoma keratinocytes.

3.3 | HIF1A, a transcription factor, might improve ECE1 expression

Subsequently, to screen out the candidate functional mRNAs in cholesteatoma and normal skin specimens, we downloaded the microarray expression dataset GSE102673 from Gene Expression Omnibus (GEO). Volcano plot and heatmap analysis presented that there are four aberrantly expressed mRNA (FGF22, ECE1, PPHLN1, and

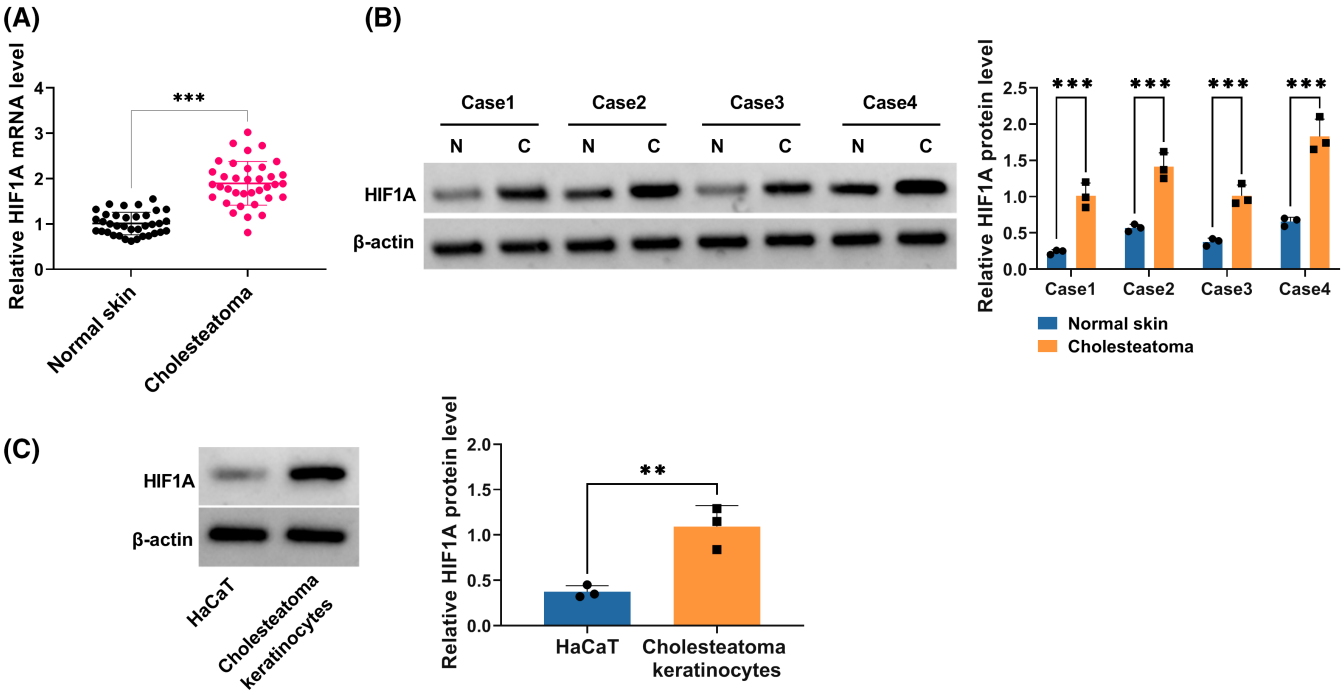

FIGURE 1 Expression pattern of hypoxia-inducible factor-1α (HIF1A) in cholesteatoma patients and keratinocytes. (A) Real-time quantitative polymerase chain reaction assay was applied to measure the expression level of HIF1A in 36 cholesteatoma and 36 normal skin specimens. (B) Western blot assay was used to examine HIF1A protein expression in randomly selected four cholesteatoma and four normal skin specimens. (C) Western blot analysis of HIF1A protein levels in HaCaT cells and cholesteatoma keratinocytes. $^{**}p < .01$, $^{***}p < .001$ (the heights are the mean value, and each small square represents the value from individual runs of the experiment that has been undertaken three times).

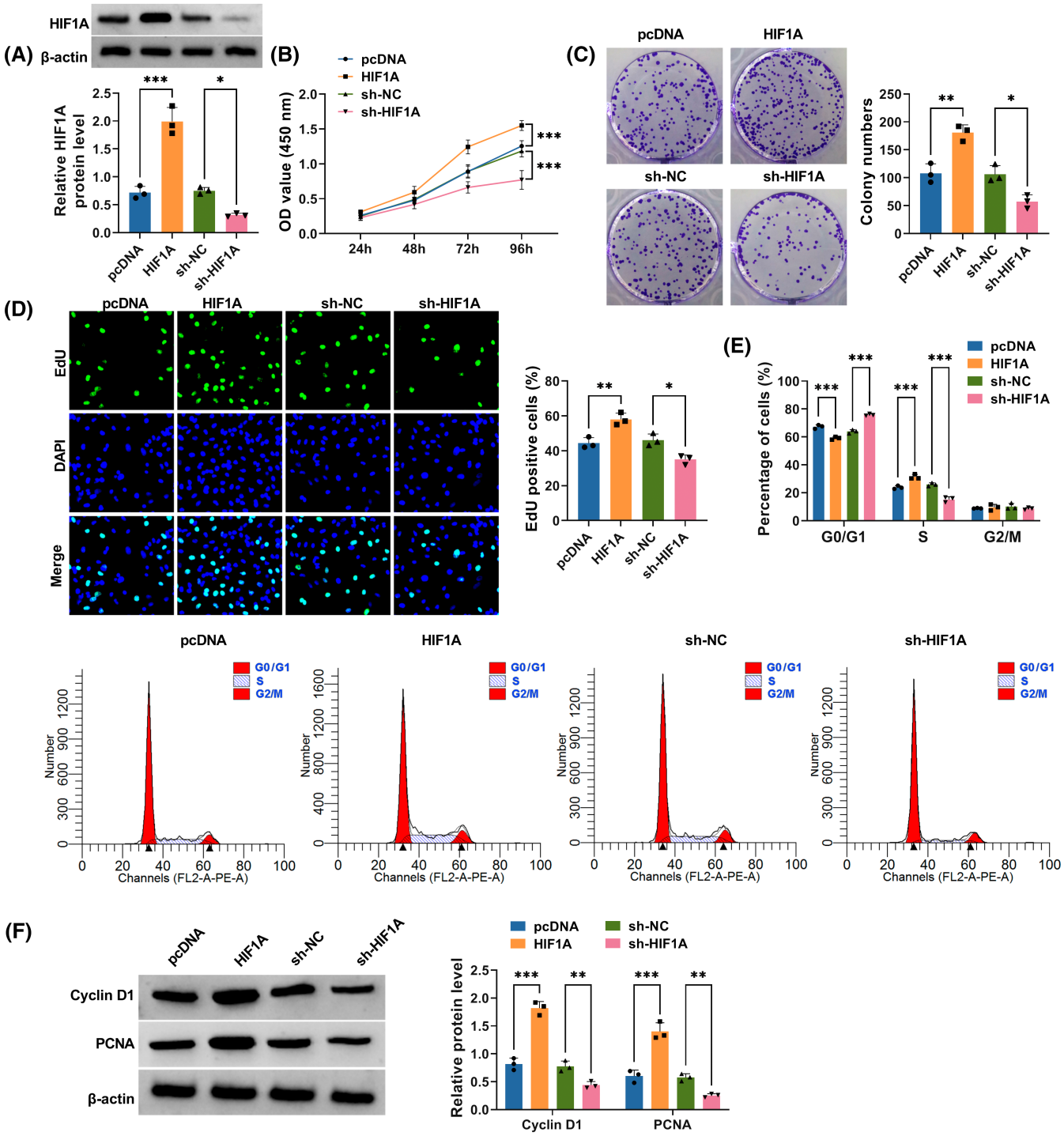

FIGURE 2 Effects of hypoxia-inducible factor-1α (HIF1A) on cholesteatoma keratinocyte growth. Keratinocytes were transfected with pcDNA, HIF1A, sh-NC, or sh-HIF1A. (A) HIF1A protein level was determined in transfected keratinocytes using western blot. (B) Cell viability was assessed using cell counting kit-8 assay in transfected cholesteatoma keratinocytes. (C,D) Cell proliferation was examined using colony formation and EdU assays in transfected cholesteatoma keratinocytes. (E) Cell cycle progression was measured using flow cytometry assay in transfected cholesteatoma keratinocytes. (F) Cyclin D1 and proliferating cell nuclear antigen protein levels were determined using western blot in transfected cholesteatoma keratinocytes. ***$p < .01$, ***$p < .001$.

LMNTD2; Figure 3A,B). For further selection, all these mRNAs were subjected to RT-qPCR analysis responding to HIF1A upregulation or knockdown. Among them, ECE1 displayed the highest fold change in cholesteatoma keratinocytes (Figure 3C,D). Hence, ECE1 was selected for further analysis in this research. Beyond that, western blot analysis showed that the ECE1 protein level was apparently improved by HIF1A overexpression and obviously reduced after sh-HIF1A transfection in cholesteatoma keratinocytes (Figure 3E). Meanwhile, a remarkable upregulation of ECE1 in cholesteatoma patients was observed (Figure 3F,G). As expected, ECE1 expression was positively

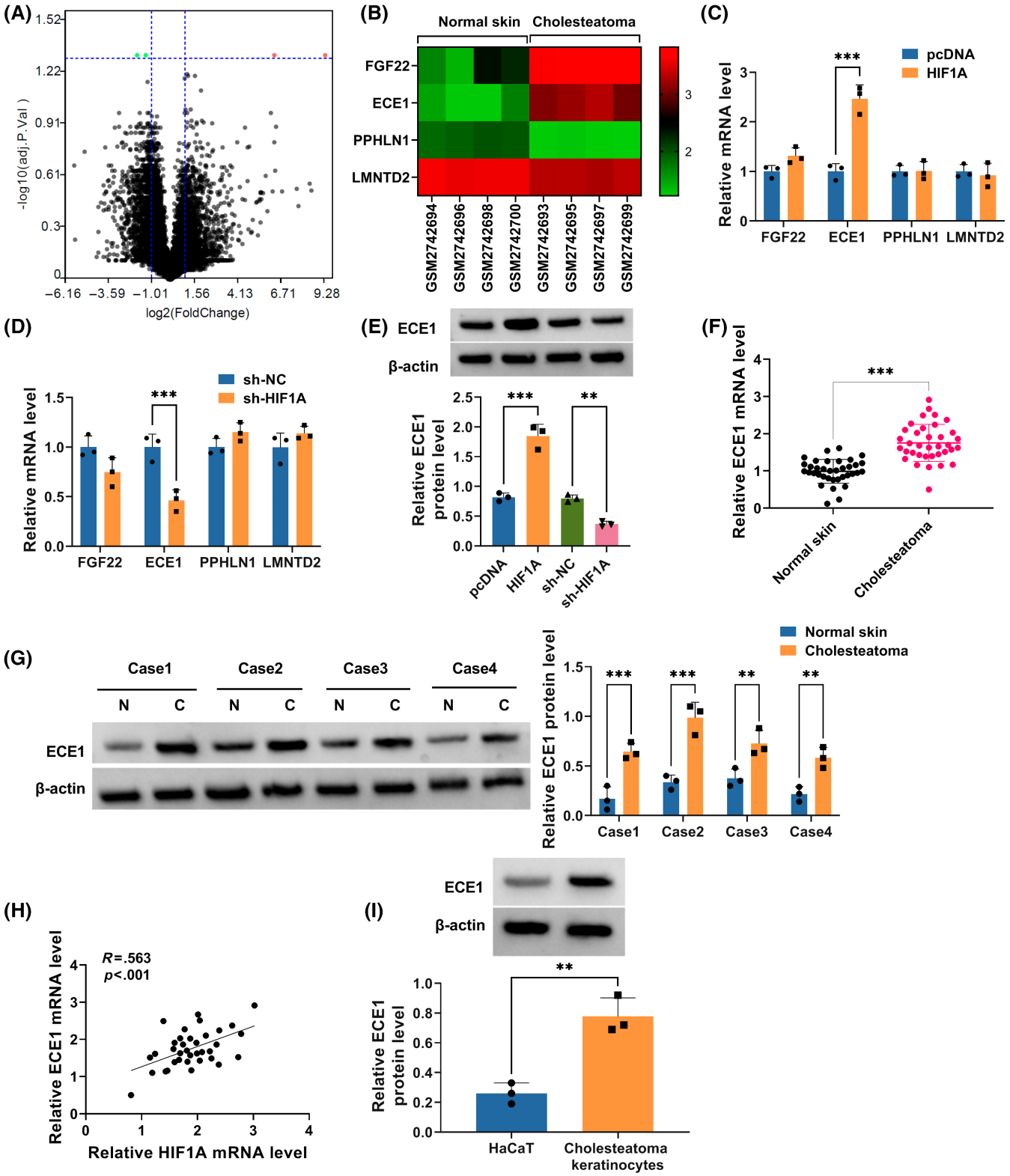

FIGURE 3 Hypoxia-inducible factor-1α (HIF1A) affected endothelin converting enzyme 1 (ECE1) expression. (A,B) GSE102673 dataset was adopted to screen differentially expressed mRNAs in cholesteatoma and normal skin specimens using a volcano plot and heat map. (C,D) RT-qPCR assay was used to assess the levels of these mRNAs in cholesteatoma keratinocytes transfected with pcDNA, HIF1A, sh-NC, or sh-HIF1A. (E) Western blot analysis of ECE1 protein level in pcDNA, HIF1A, sh-NC, or sh-HIF1A-transfected cholesteatoma keratinocytes. (F) The expression level of ECE1 was examined using real-time quantitative polymerase chain reaction assay in 36 cholesteatoma and 36 normal skin specimens. (G) ECE1 protein level was detected using western blot in four cholesteatoma and four normal skin specimens. (H) Pearson correlation analysis was applied to evaluate the expression association between ECE1 and HIF1A in cholesteatoma specimens. (I) Western blot analysis of ECE1 protein level in HaCaT cells and cholesteatoma keratinocytes. $^{**}p < .01$, $^{***}p < .001$.

associated with HIF1A level in cholesteatoma patients (Figure 3H). Moreover, we further verified that ECE1 was highly expressed in cholesteatoma keratinocytes in comparison with HaCaT cells (Figure 3I). Altogether, these results implied that HIF1A might regulate ECE1 expression in cholesteatoma keratinocytes.

3.4 | HIF1A-induced upregulation of ECE1 transcription

To better map the interaction between HIF1A and ECE1, we applied Jaspar database, we found that the promoter region of ECE1 possessed three potential HRE sites, which are the binding sites for the transcription factor HIF1A (Figure 4A,B). Then, chromatin immunoprecipitation (ChIP) assay was conducted and results exhibited that HIF1A bound to the first HRE site of the promoter of ECE1 (Figure 4C). In order to validate their interaction, a dual-luciferase reporter assay was carried out in cholesteatoma keratinocytes. As shown in Figure 4D, ECE1-WT and ECE1-MUT (mutant HRE) luciferase vectors were constructed. Subsequently, HIF1A knock-downing cholesteatoma keratinocytes exhibited an obvious reduction in luciferase activity, whereas the luciferase activity was apparently increased after overexpression of HIF1A (Figure 4E,F). However, the loss of binding sites eliminated the HIF1A promotion effect on luciferase activity, as shown in Figure 4E,F. Overall, these data illuminated that HIF1A might bind to the promoter of ECE1 and boost ECE1 transcription.

3.5 | HIF1A might facilitate cholesteatoma keratinocyte proliferation via increasing ECE1 expression

Considering the regulatory role of HIF1A in ECE1 expression in cholesteatoma keratinocytes, whether the impact of HIF1A on cell proliferation was correlative with ECE1 was further investigated. At first, western blot assay displayed that the ECE1 protein level was clearly

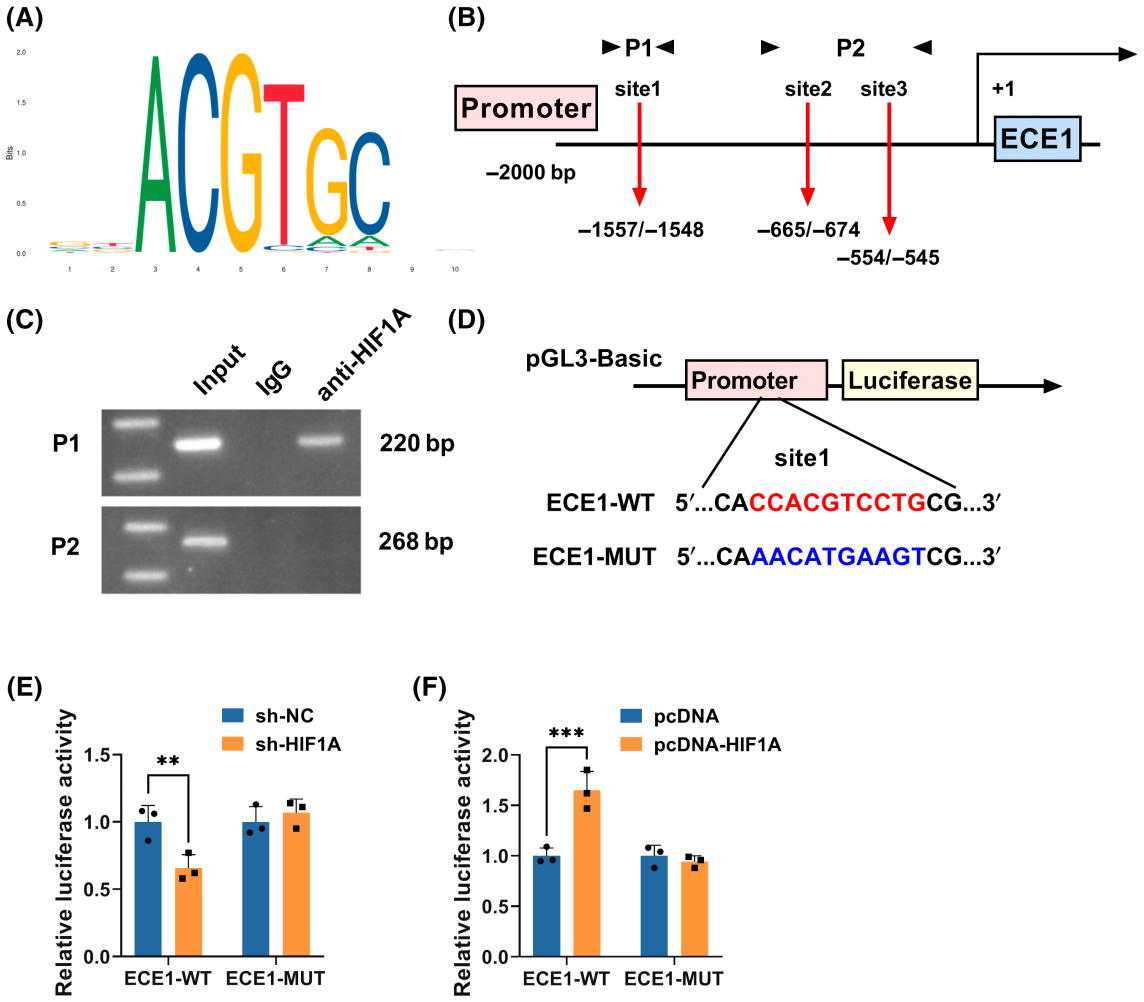

FIGURE 4 Hypoxia-inducible factor-1α (HIF1A)-induced upregulation of endothelin converting enzyme 1 (ECE1) transcription. (A) The Jaspar website predicted HIF1A to have binding sites with the ECE1 promoter sequence. (B) The promoter region of ECE1 contained three HRE (hypoxia response element) sites. (C) Chromatin immunoprecipitation assay was performed to assess the binding between HIF1A and the HREs of the ECE1 promoter. (D–F) A dual-luciferase reporter assay was performed to assess the binding between HIF1A and the HRE of the ECE1 promoter in cholesteatoma keratinocytes. $^{**}p < .01$, $^{***}p < .001$.

upregulated in pcDNA-ECE1-transfected cholesteatoma keratinocytes and was significantly decreased after sh-ECE1 transfection (Figure 5A), suggesting that the overexpression or knockdown efficiency of ECE1 is successful. Whereafter, we found that the introduction of sh-ECE1 or sh-HIF1A might greatly repress the protein level of ECE1 in cholesteatoma keratinocytes, while the overexpression of ECE1 might effectively abolish the inhibitory effect of HIF1A deficiency on ECE1 protein level in cholesteatoma keratinocytes (Figure 5B). After that, cell

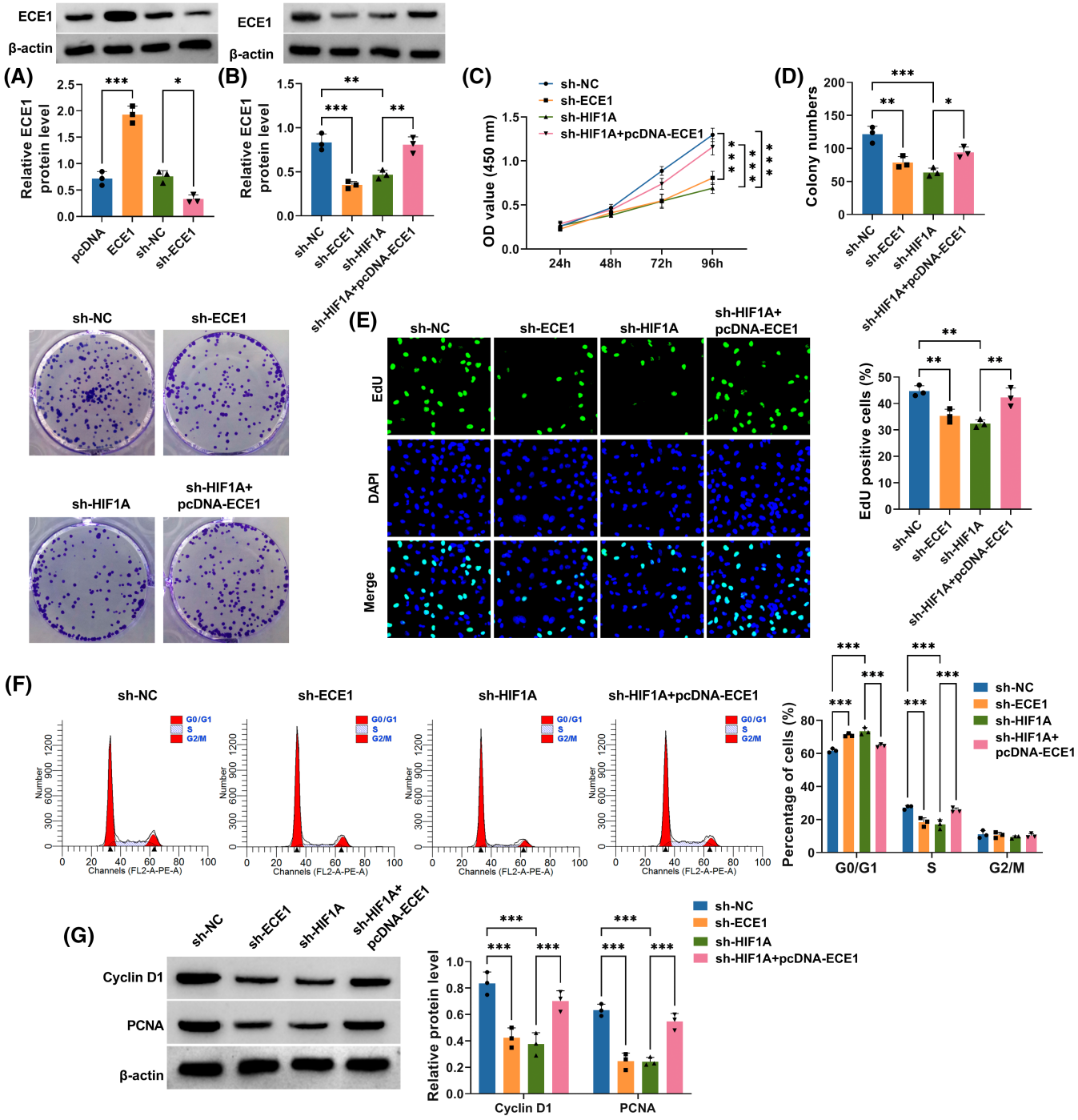

FIGURE 5 Hypoxia-inducible factor-1α (HIF1A) promoted the proliferation of cholesteatoma keratinocytes by promoting the expression of endothelin converting enzyme 1 (ECE1). (A) The overexpression or knockdown efficiency of ECE1 in cholesteatoma keratinocytes was detected using western blot. (B–G) Cholesteatoma keratinocytes were transfected with sh-NC, or sh-ECE1, sh-HIF1A, and sh-HIF1A+pcDNA-ECE1. (B) Western blot analysis of ECE1 protein level in transfected cholesteatoma keratinocytes. (C) Cell counting kit-8 assay was used to measure cell viability in transfected cholesteatoma keratinocytes. (D,E) Colony formation and 5-ethynyl-2′-deoxyuridine (EdU) assays were implemented to detect cell proliferation in transfected cholesteatoma keratinocytes. (F) Flow cytometry assay was applied to analyze cell cycle progression in transfected cholesteatoma keratinocytes. (G) Western blot analysis of cyclin D1 and PCNA protein levels in transfected cholesteatoma keratinocytes. $^{**}p < .01$, $^{***}p < .001$.

viability and cell proliferation were evidently hindered by ECE1 knockdown or HIF1A downregulation in cholesteatoma keratinocytes, whereas the cotransfection of pcDNA-ECE1 might partly overturn sh-HIF1A-mediated cell viability and cell proliferation suppression (Figure 5C–E). In parallel, ECE1 silencing or HIF1A deficiency might restrain cholesteatoma keratinocyte cycle progression; however, increased cell cycle progression due to the knockdown of HIF1A was significantly abrogated by ECE1 upregulation in cholesteatoma keratinocytes (Figure 5F). In addition, western blot assay presented that cyclin D1 and PCNA protein levels were remarkably impeded after sh-ECE1 or sh-HIF1A transfection, nevertheless, HIF1A depletion-mediated cyclin D1 and PCNA protein level decrease were partially reversed through ECE1 overexpression in cholesteatoma keratinocytes (Figure 5G). In summary, the above-mentioned results elucidated that HIF1A might boost cholesteatoma keratinocyte proliferation via increasing ECE1 expression.

4 | DISCUSSION

Recently, cholesteatoma has been recognized as hypoxic tissue resulting from under-aeration of the middle ear cleft in most cases.[16,22] In fact, it has become evidence that hypoxia represents one of the major contributing factors in the development and progression of cholesteatoma.[4] Herein, our findings provided evidence that HIF1A, as a key element in hypoxia response, is highly expressed in human cholesteatoma samples, consistent with a previous study.[23] Furthermore, previous articles have shown that the maintenance of cell differentiation, proliferation, and tissue homeostasis in cholesteatoma is attributed to interactions between stromal keratinocytes and surrounding stromal fibroblasts.[3] Meanwhile, it has been reported that hypoxia might improve cell proliferation for cholesteatoma keratinocytes. In this work, we isolated and cultured cholesteatoma keratinocytes from cholesteatoma tissues, and further validated that HIF1A expression was significantly upregulated in cholesteatoma keratinocytes. Subsequently, the influences of HIF1A on cholesteatoma keratinocyte proliferative ability were further assessed. Functional analysis exhibited that HIF1A might facilitate cholesteatoma keratinocyte viability, proliferation, and cell cycle progression. In other words, these results provided evidence that HIF1A might participate in the control of proliferation of cholesteatoma keratinocytes.

Regarding the molecular mechanism, HIF1A, an important transcription factor, might modulate the development and progression of human diseases via different downstream signaling molecules.[13] Generally, the action of HIF1A is different in response to the presence or absence of oxygen. Under conditions of normoxia, HIF1A is completely inactivated and destroyed through the ubiquitin-proteasome pathway, which is almost undetectable and has a short half-life.[24] On the contrary, when cells are exposed to hypoxia, HIF1A could escape destruction and form a complex with HIF1B entering to the nucleus of cells, which might bind to a unique and conserved sequence (HRE) in the promoter region of target genes to increase their transcription.[25–27] Indeed, many laboratory works have revealed that serval targets of HIF1A partake in supporting tumor progression via angiogenesis, proliferation, metastasis, and apoptosis.[28,29] For example, HIF1A might promote the growth and chemosensitivity of colorectal cancer via increasing the transcription of RRAGB.[30] Furthermore, HIF1A might regulate YTHDF1 transcription via directly binding to its promoter region under hypoxia, further boosting the malignant progression of hepatocellular carcinoma.[31] Herein, we observed that HIF1A and ECE1 have a positive correlation at mRNA level in cholesteatoma patients. It has been reported that hypoxia might elevate the expression of ECE1 in glioma cells.[32] Here, we first found that ECE1 content was enhanced in cholesteatoma samples and keratinocytes. Beyond that, the promoter and intron regions of ECE1 have been verified to possess HIF binding sites,[20] suggesting that ECE1 might be a new HIF-target gene. In this regard, our work displayed that HIF1A might increase the transcription of ECE1 via binding to its promoter region. As expected, HIF1A absence-cholesteatoma keratinocyte proliferation inhibition was partially overturned by ECE1 overexpression, supporting the regulatory role of the HIF1A/ECE1 axis. These findings might provide new diagnostic markers and therapeutic targets for cholesteatoma treatment, which might bring substantial clinical benefits. However, there are several limitations to this research. First, we only used a small number of clinical samples, and future studies should replicate these experiments with a larger

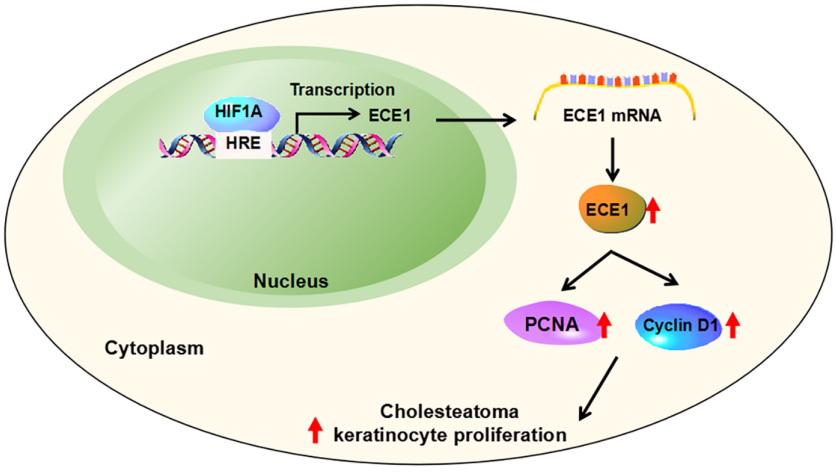

FIGURE 6 Hypoxia-inducible factor-1α (HIF1A) promoted cholesteatoma keratinocyte proliferation by increasing endothelin converting enzyme 1 (ECE1) expression. HRE, hypoxia-responsive element; PCNA, proliferating cell nuclear antigen.

sample size. Furthermore, we only concentrated on the effects of the HIF1A/ECE1 axis *in vitro*, and animal models should be considered in the following studies to validate our findings.

5 | CONCLUSION

In summary, our present results elucidated compelling evidence that HIF1A might increase ECE1 transcription through directly binding to its promoter region, thereby promoting cholesteatoma keratinocyte proliferation (Figure 6), and providing a novel avenue of therapy for cholesteatoma treatment.

CONFLICT OF INTEREST STATEMENT

All authors have no conflict of interest.

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