MiR-195-5p Reduces Esophageal Cancer Cell Proliferation Through The IGF-1R/AKT Axis

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Abstract

Background

Esophageal cancer (ECa) remains a major cause of mortality across the globe. The expression of MiR-195-5p is altered in a plethora of tumors, but its role in ECa development and progression are undefined.

Result

Here, we show that miR-195-5p is downregulated in ECa and associated with poor survival in ECa. Function assays indicated that MiR-195-5p inhibited ECa progression. Mechanistically, we identified IGF-1R as a downstream target of miR-195-5p, and miR-195-5p/IGFR axis caused a loss of GLUT1 expression, reduced glucose uptake, reduced lactate production, and low levels of ATP production.

Conclusion

Collectively, miR-195-5p as a Eca suppressor impaired glycolysis. This highlighting miR-195-5p as a novel target for much needed anti-ECa therapeutics.

Introduction

Compared with other regions, the incidence of esophageal cancer in Chinese men is particularly high and poses a global threat to human health [1]. Advances in diagnosis and treatment have improved ECa treatment, the efficiency of ECa treatment and subsequent patient prognosis are still poor[2]. Therefore, ECA remains a global health burden and urgently needs new diagnoses and effective treatment strategies [3]. It is necessary to further understand the molecular regulators of ECa tumorigenesis.

MicroRNAs (miRNAs) are non-coding RNAs that range in size from 20–24 nucleotides. MiRNAs regulate gene expression in all mammalian cell-types [4–6]. The dysregulation of miRNAs leads to a plethora of human pathologies, including carcinogenesis and tumorigenesis, amongst other disease states [7–9]. Notable examples include miR-154 in ECa cells and tissues [10] and miR-139-3p that is suppressed in glioblastoma due to its ability to slow cell growth and metastasis [11]. Emerging evidence links other miRNAs in ECa[12, 13]. Tang and colleagues revealed that miR-204-5p suppresses ECa growth through its effects on IL-11[14], whilst Li and coworkers highlighted the tumor suppressor functions of miR-671-5p through its ability to suppress FGF2 expression and activity [15]. MiR-195 is a known tumor-suppressor in lung cancer [16], prostate cancer[17]and cervical cancer[18]. However, the mechanism(s) mediating the involvement of miR-195-5p in ECa development remains undefined.

Aerobic glycolysis occurs in an array of cancer cell types and endows tumor cells with the ability to process glucose through glycolytic enzymes in the absence of oxygen produced from mitochondrial respiration [19, 20]. To maintain rapid cancer cell growth, glucose transporters (GLUTs) and glycolytic

enzyme activity are required, and are frequently over-expressed in tumor cells. IGF-1R phosphorylates and activates AKT as a result of insulin stimulation, leading to the up-regulation of GLUTs and their transport to the cell-surface [21–23]. MiRNAs targeting the IGF-1R-AKT-GLUT1 signaling axis are promising anti-cancer agents. Here, we assessed the effects of miR-195-5p on tumor cell proliferation and invasion via the IGF-1R-AKT axis in ECa cells, in an attempt to identify this signaling axis as a novel target for much needed-ECa therapeutics.

Materials And Methods

Tissue specimens

A total of 121 pairs of ECa tumor and normal para-cancerous tissues were obtained from patients undergoing surgery at the Fujian Provincial Hospital. Enrolled patients had not undergone preoperative systemic or local anti-tumor therapy. Following collection, pathological examination was used to confirm ECa diagnosis, and samples were snap frozen prior to storage at -80 °C. All patients supplied informed consent to participate, and the Ethics Committee of the Fujian Provincial Hospital approved all human studies, the Ethics number was NO. FJPH-2013.06.05. All experiments were performed in accordance with the relevant guidelines and regulations.

Cell culture

Human endothelial HET-1A cells and ECa cells including EC9706 and TE-1 were grown in DMEM + 10% FBS at 37°C. ECa ECA109 and TE-8 cells were grown in RPMI-1640 + 10% FBS at 37°C. HEK293 cells were grown in MEM + 10% FBS at 37°C. For overexpression studies, TE-1 cells were transfected with miR-195-5p mimics or miR-195-5p negative controls (NC; 40 nM) for 24 h. For suppression studies, TE-8 cells were transfected with miR-574-3p inhibitors or miR-195-5p inhibitor NCs.

Reporter assays

Potential miR-195-5p targets were identified using the TargetScan database, which highlighted IGF-1R as a potential target. Based on this prediction, pmiR-RB-report plasmids (RiboBio Inc) were cloned to contain either a mutant or wild-type version of the 3’-UTR of IGF-1R. Plasmids were then co-transfected with appropriate miRNA mimics, inhibitors, or controls for 48 h. Commercial kits (Promega) were used to analyze luciferase activity. Firefly activity was normalized to Renilla.

Western blotting

Cell lines or tissues were lysed in RIPA (Beyotime) buffer and protein content of the lysates was assessed via BCA assays (Thermo Fisher). Proteins (~20 ng) were resolved by SDS-PAGE electrophoresis and transferred. PVDF membranes were blocked in 5% milk and probed overnight with antibodies against IGF-1R (1:1000, 9750S, CST), p-AKT (Ser473, 1:1000, 9271S; CST), and total-AKT (1:1000, 9272S, CST), GLUT1 (1:1000, sc-377228; Santa Cruz) at 4˚C. Blots were then labeled for 1 h with anti-mouse or anti-

rabbit HRP-secondary antibodies. Proteins were visualized using the ECL system on a ChemiDoc Imaging Platform (Bio-Rad).

Real-time PCR

RNA was isolated from cell lines or paraffin-embedded tissue using a miRNeasy FFPE kits (Qiagen) or miRNeasy Mini kits (Qiagen). cDNA was generated using a miScript II RT kit (Qiagen) from 1 μg of input RNA or miRNA. RT-PCR parameters were as follows: 37°C for 60 min, 95°C for 5 min. The miScript SYBR Green PCR kit was used for qRT-PCR. Reactions were performed as follows: 95°C for 15 min; 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s on a 7500 Fast Real-Time PCR platform. Values were normalized to GAPDH and U6 and quantified. All reactions were performed in triplicate.

MiR-195-5p Forward 5’-ACACTCCAGCTGGGTGTCAGTTTGTCAAAT-3’;

MiR-195-5p Reverse 5’-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT-3’,

U6 Forward 5’-TGCGGGTGCTCGCTTCGCAGC-3’;

U6 Reverse 5’-CCAGTGCAGGGTCCGAGGT-3’,

IGF-1R Forward5’-GAGGTGGGCTCGGGAGAAGAT-3’;

IGF-1R Reverse5’-TTCACCACACCCTTGGGCAAC-3’,

GAPDH Forward 5’-TTGGTATCGTGGAAGGACTCA-3’;

GAPDH Reverse 5’-TGTCATCATATTTGGCAGGTT-3’.

Cell Proliferation Assays

CCK-8 assays were used to assess ECa and non-ECa cell proliferation. Cells were co-transfected as described in 96-well plates (1,000 cells/well) for 24 h, and 10 μl of CCK-8 was added to the wells in normal culture conditions for 3 h. Absorbances at 450 nm were read on a Bio-Rad microplate reader.

Colony Formation Assays

Treated cells (1×10^3 per well) were seeded into 10 cm culture dishes for 2 weeks. Cell colonies were stained in crystal violet (1%) and colony numbers were counted.

Transwell assay

For migration/invasion assays, 12-well plates containing Transwell inserts (Corning, MA, USA) with 8 μm pore sizes were used. For migration assays, ~100,000 cells were resuspended in serum-free media and added to the upper regions of the Transwell chambers for 8 h at 37 °C. Cells in the lower chambers were then removed and methanol fixed prior to staining using 0.04% crystal violet. Cells were then quantified.

For invasion assays, the protocols were identical to those above, but the transwell inserts had first been coated using Matrigel (BD Biosciences, MA, USA), and plates were instead incubated for 24 h.

Glucose uptake, lactate, and ATP assays

Colorimetric assays were performed to assay glucose uptake. Briefly, cells (1×10^4 per 96 wells) were glucose starved in Krebs-solution plus 2% BSA for 40 min. Cells were then treated with 2-DG (10 mM) for 20 min and color changes were assessed on a plate reader. For the assessment of lactate and ATP production, cells (5×10^5) were homogenized in commercial assay buffers at 4°C. Soluble fractions were assayed using Lactate Assay Kit II reagent or ATP colometric assays.

Statistical analysis

Data are shown and the mean ± SD analyzed via SPSS 17.0. Inter-group differences were assessed via a student's t-test. Multiple groups were compared using a one-way ANOVA analysis of variance. Kaplan Meier (KM) curves were plotted for survival analysis, and groups were compared via log-rank assessments (n=3 for all). P-values < 0.05 were deemed significant differences

Results

MiR-195-5p is suppressed and correlated with poor survival in ECa

We first investigated miR-195-5p levels in ECa *vs.* non-cancer cells and tissues via qRT-PCR analysis. MiR-195-5p expression was found to be lower in ECa tissues compared to healthy tissues (Fig. 1A) with 96/121(79.34%) of ECa samples showing lower levels of the miRNA (Fig. 1B). In terms of cancer stage, miR-195-5p levels were lower in T3 + T4 tumor depths compared to T1 + T2 tissues (Fig. 1C), and in TNM3 + 4 stage samples compared to TNM1 + 2 (Fig. 1D). MiR-195-5p levels were also lower in samples showing + ve lymph node metastasis compared to non-metastatic samples (Fig. 1E). RT-PCR analysis further showed that miR-195-5p was downregulated in ECa cell lines vs. non-ECa HECC cells. Amongst the cell-types, miR-195-5p showed the lowest levels of expression in TE-1 cells, and the highest levels of expression in TE-8 cells (Fig. 1F). These data confirmed that miR-195-5p expression is suppressed in ECa tissues and cells. Following miR-195-5p expression were divided into high- and low-group according to the median levels measured in ECa tissues. Table 1 shows that low levels of miRNA-195-5p positively correlated with several clinical features such as advanced TNM staging (P = 0.004), invasion depth (P = 0.008) and lymph node metastasis (P < 0.001). KM curves suggested that low levels miR-195-5p were associated with poor survival (Fig. 1G and H). These data confirmed that miRNA-195-5p was suppressed and correlated with poor survival in ECa.

Table 1
The correlation between clinicopathological parameters and miR-195-5p expression in human esophageal cancer

Clinical features	Total	miR-195-5p		*p*-vaule
		High	Low	
		(N = 61)	(N = 60)	
Age (years)				0.210
< 60	36	15	21	
≥ 60	85	46	39	
Gender				0.792
Male	68	35	33	
Female	53	26	27	
Differentiation grade				0.521
Well	41	19	22	
Moderate + Poor	80	42	38	
TNM stage				**0.004**
I + II	76	46	30	
III	45	15	30	
Depth of invasion				**0.008**
T1 + T2	57	36	21	
T3 + T4	64	25	39	
Lymph node metastasis				**< 0.001**
No	80	53	27	
Yes	41	8	33	
Pearson chi-square test was used for comparison between subgroups.				

MiR-195-5p inhibits the metastatic phenotypes of ECa cells in vitro

We next explored the mechanistic effects of miR-195-5p in ECa cells. RT-PCR analysis revealed that miR-195-5p expression increased in TE-1 cells transfected with miRNA mimics, whilst miR-195-5p levels were lower in TE-8 cells expressing miR-195-5p inhibitors (Fig. 2A). CCK-8 assays showed that the miR-195-5p mimics reduced TE-1 cell proliferation, whilst miR-195-5p inhibitors increased TE-8 cell growth (Fig. 2B-C). MiR-195-5p mimics also led to a loss of colony counts, whilst miR-195-5p silencing increased colony

numbers (Fig. 2D). We further investigated the effects of miR-195-5p on ECa cell migration and invasion in Transwell assays. TE-1 migration was suppressed following miR-195-5p overexpression, whilst the migration and invasiveness of TE-8 cells increased in the presence of miR-195-5p inhibitors (Fig. 2E-F).

Mir-195-5p Suppresses Igf-1r Expression In Eca

To further investigate the regulatory mechanisms of miR-195-5p in ECa cells, TargetScan analysis was performed to predict cellular targets, identifying IGF-1R as a novel candidate (Fig. 3A). Dual-luciferase reporter assays showed that the transfection of miR-195-5p mimics led to a loss of luciferase activity in cell expressing IGF1R-WT reporters, but no differences were observed in cells expressing mutant IGF1R (Fig. 3B). IGF1R expression was also suppressed by miR-195-5p mimics in TE-1 cells, but increased following miR-195-5p silencing in TE-8 cells (Fig. 3C-D). GEPIA (gene expression profile Ingenuity analysis) data further indicated that IGF-1R is overexpressed in ECa tissues compared to non-ECa tissues (Fold change > 1.5, $P < 0.05$, Fig. 3E) which was experimentally confirmed at the mRNA level via RT-PCR analysis (Fig. 3F). These data revealed the negative correlation between miR-195-5p and IGF-1R in ECa tissue (Fig. 3G), highlighting the miR-195-5p/IGF-1R axis as a novel therapeutic strategy in ECa.

MiR-195-5p regulates glycolysis in ECa cells via the IGF-1R/AKT/GLUT1 pathway

IGF-1R activates AKT and GLUT1 expression, inducing cancer cell glycolysis. We thus investigated AKT signaling and GLUT1 expression in ECa cells. We found that miR-195-5p overexpression led to a loss of p-AKT (S473) and GLUT1 expression in TE-1 cells, whilst miR-195-5p silencing enhanced their levels in TE-8 cells (Fig. 4A). This revealed miR-195-5p is a negative regulator of the IGF-1R-AKT-GLUT1 axis in ECa cells. As GLUT1 regulates glucose uptake, we next investigated the effects of miR-195-5p on ECa cell glycolysis. We found that miR-195-5p mimics causes a decrease in TE-1 cellular glucose uptake (Fig. 4B), subsequently leading to a loss of lactate in ECa cells relative to control cells (Fig. 4C). The metabolic processing of glucose leads to ATP production, and as such, we investigated the effects of miR-195-5p on ATP levels. We found that the miRNA mimics decreased ATP levels in TE-1 cells (Fig. 4D), consistent with that observed for glucose uptake and lactate production. In contrast, in cells expressing miR-195-5p inhibitors, the depletion of miR-195-5p increased glucose uptake and lactate production (Fig. 4B-C). Similarly, miR-195-5p inhibitors enhanced ATP production (Fig. 4D). Collectively, these data highlight miR-195-5p as a negative regulator of glycolysis in ECa cells.

MiR-195-5p reduces ECa cell proliferation, invasion and glycolysis through IGF-1R

We next examined whether miR-195-5p influences glycolysis through its targeting and suppression of IGF-1R in ECa cells. To achieve this, we overexpressed IGF-1R in TE-1 miR-195-5p mimic cell lines, or silenced IGF-1R in TE-8 miR-195-5p inhibitor cell lines (Fig. 5A and Figure S1). CCK-8 assays showed that miR-195-5p mimics inhibited TE-1 cell proliferation, whilst IGF-1R overexpression attenuated these effects. Moreover, miR-195-5p inhibitors led to enhanced TE-8 cell proliferation, which was alleviated through IGF-1R silencing (Fig. 5B). Transwell assays showed that the loss of ECa cell migration and

invasion induced by miR-195-5p mimics increased following IGF-1R overexpression. The enhanced levels of cell migration and invasion induced by miR-195-5p inhibitors also decreased in IGF-1R silenced cells (Fig. 5C-D). Furthermore, as shown in Fig. 5E-G, miR-195-5p mimics led to a loss of glucose uptake, decreased lactate production and impaired ATP generation, all of which were reversed by IGF-1R overexpression in miR-195-5p-transfected cells. MiR-195-5p inhibitors also led to enhanced glucose uptake, lactate production and ATP generation; effects that were alleviated through IGF-1R silencing (Fig. 5E-G). These data collectively highlight the role of aerobic glycolysis in the regulation of the proliferation and invasion of ECa cells via miR-195-5p.

Discussion

In this study, lower levels of miR-195-5p expression were observed in ECa tumors and cells, which was associated with advantaged TNM staging ($P = 0.004$), invasion depth ($P = 0.008$), metastasis ($P < 0.001$), and poor survival. Moreover, miR-195-5p inhibited the proliferation and metastatic phenotypes of ECa cells through its ability to target IGF-1R and inhibit its expression, the loss of IGF-1R activity led to a concomitant decrease in pAKT and GLUT1 expression. The loss of miR-195-5p also led to a loss of glucose uptake, lactate production, and ATP production through the IGF-1R/AKT/GLUT1 axis. These findings collectively highlight miR-195-5p is a key regulator of ECa tumorigenesis.

Glycolysis is enhanced in tumor vs. healthy tissue, due to metabolic reprogramming[24, 25]. This endows tumor cells with energy reserves that promote cell proliferation[26]. Glycolysis can be targeted to inhibit tumor progression. MiRNAs target components of glycolysis including GLUT1, G6PD, and LDHA[27–30] providing a mechanism of cancer cell targeting[31]. We show that miR-195-5p inhibits glycolysis and suppresses ECa cell proliferation through IGF-1R. This reveals new roles for this important miRNA in both cancer cell proliferation and survival.

IGF-1R is upregulated in an array of human cancers, including HCC, renal cell carcinoma and gastric cancer[32–34]. IGF-1R silencing leads to a ~ 50% decrease in the size of mouse embryos[35]. IGF-1R interacts with IGF-1 to regulate tumorigenesis, proliferation and metastasis through PI3K/AKT and MAPK/ERK[36, 37]. IGF-1R also activates PI3K/AKT to regulate cancer cell proliferation and invasiveness[38–41]. Conversely the inhibition of IGF-1R functionality represents a therapeutic avenue for anti-cancer strategies. Indeed, drugs targeting IGF-1R are currently in clinical trials for a multitude of human cancers[42].

AKT or protein kinase B is activated through phosphorylation on its hydrophobic motif (Ser473) and activation loop (Thr308) in response to growth factors including IGF[43]. P-AKT is the active form of AKT that is known to phosphorylate and regulate an array of cell activities[44]. Zhang and colleagues showed that AKT is upregulated in cancer and associated with tumor aggressiveness and poor prognosis in HCC patients[45]. In addition, the overexpression of pAKT is associated with a poor prognosis many important human carcinomas[46–49]. The inhibition of AKT is a known and well-characterized target for cancer therapy. Studies have reported that PI3K/AKT signaling regulates GLUT1 trafficking and activity[50, 51].

PI3K/AKT inhibition leads to a loss of GLUT1 expression, and decreased glucose metabolism, suppressing the proliferation of colorectal cancer cells[52]. In this study, we found that the overexpression of miR-195-5p decreased IGF-1R expression in ECa cells, thereby suppressing AKT phosphorylation and GLUT1 expression. This led to a loss of glucose uptake, decreased lactate production, and reduced ATP levels in the presence of high levels of miR-195-5p, highlighting miR-195-5p as a novel negative regulator of glycolysis.

Collectively, these data highlight miR-195-5p as a tumor suppressor that is downregulated in ECa cells to impair glycolysis. This highlights miR-195-5p as a novel target for much needed anti-ECa therapeutic interventions. These findings now warrant further investigation in *in vivo* models of ECa.

Declarations

Ethics approval and consent to participate

All patients supplied informed consent to participate, and the Ethics Committee of the Fujian Provincial Hospital approved all human studies, the Ethics number was NO. FJPH-2013.06.05.

Consent for publication

N/A

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

None to declare.

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Authors' contributions

Zhao-xian Lin and Li-huan Zhu wrote the main manuscript text and All authors prepared figures and tables. All authors reviewed the manuscript.

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Figures

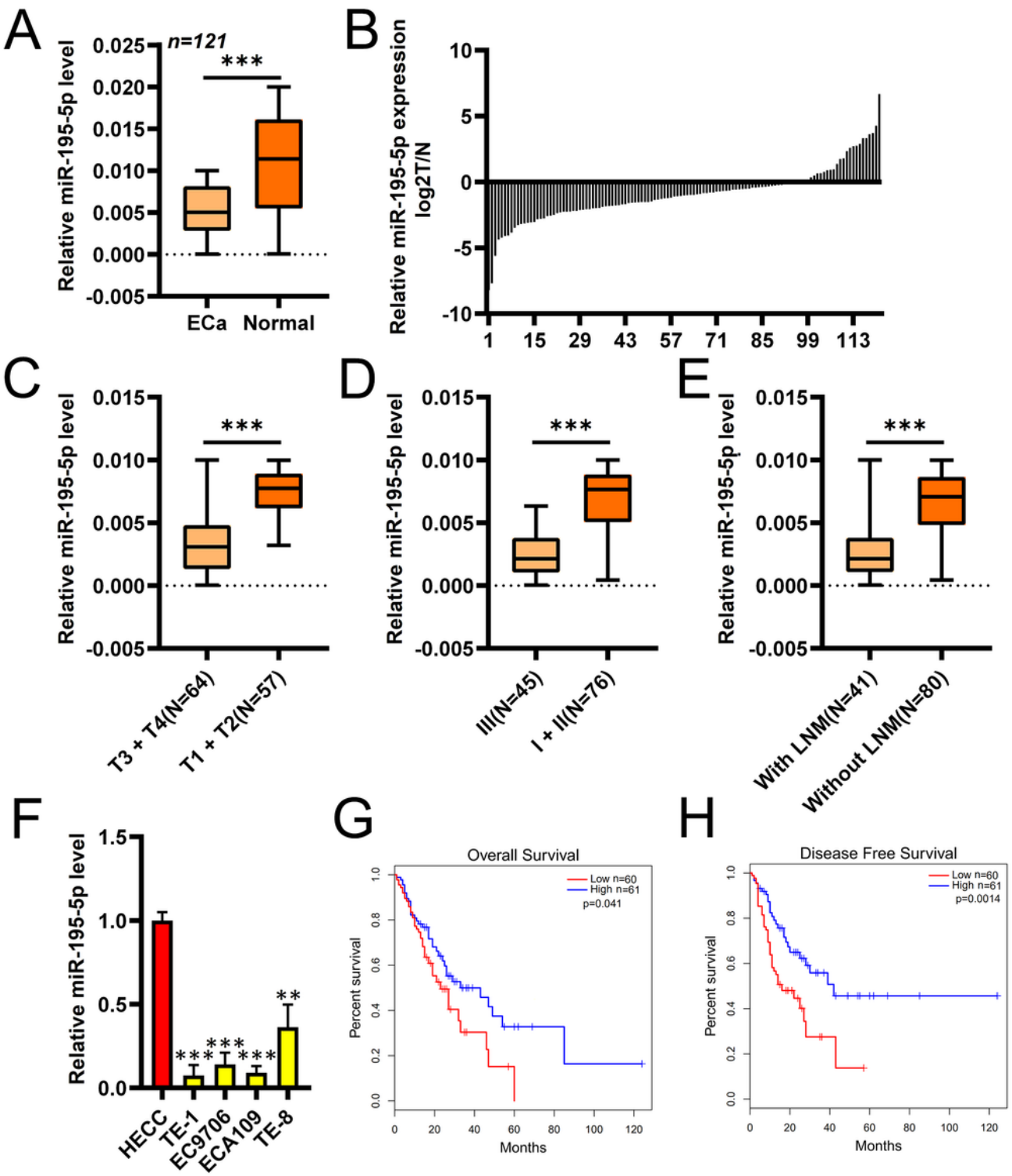

Figure 1

MiR-195-5p is suppressed in esophageal cancer tissues and cells. (A-B) RT-PCR showing miR-195-5p levels in 121 ECa tissues vs. healthy tissue. (C-E) Loss of miR-195-5p correlates with T3 + T4 invasion depth, TNM3 + 4 stage, and metastasis. (F) MiR-195-5p levels are reduced in the indicated ECa cell lines compared to HECC cells. (G-H) Overall survival (OS) and Disease-free survival (DFS) in ECa patients. Kaplan Meier (KM) curves were plotted for survival analysis. *P < 0.05. **P < 0.01. **P < 0.001

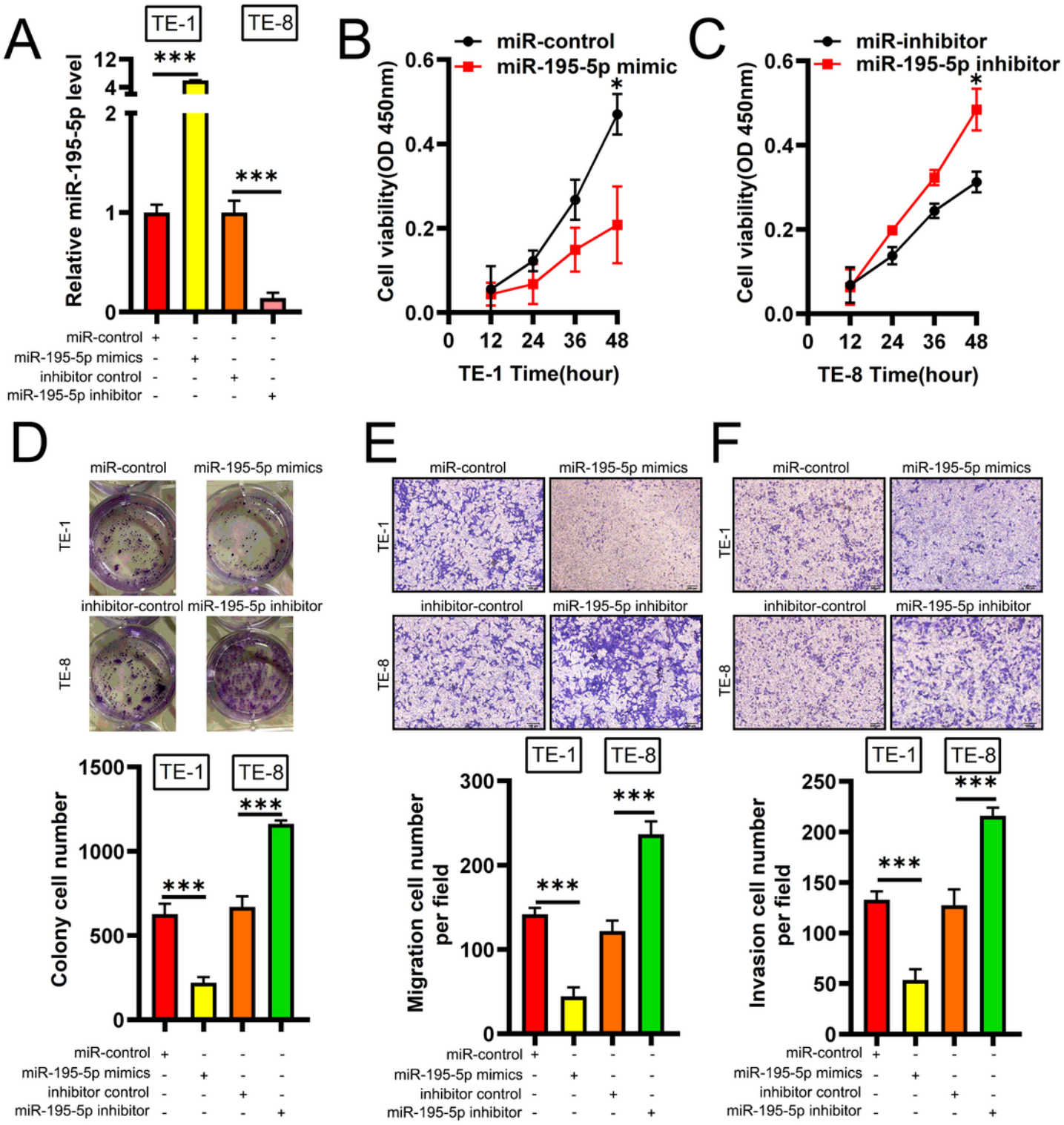

Figure 2

MiR-195-5p inhibits the metastatic phenotypes of ECa cells. (A) miR-195-5p levels in TE-1 and TE-8 cells was performed by RT-PCR. (B-C), CCK-8 assays in miR-195-5p mimic or inhibitor in indicated ECa cells. (D) Clone formation assays in miR-195-5p mimic or inhibitor cells. Upper panel: Representative images of colony formation assays. (E-F) Transwell assays in miR-195-5p mimic or inhibitor in indicated ECa cells. Upper panel: Representative images of Transwell assays. (n = 3). P-values as in Fig 1.

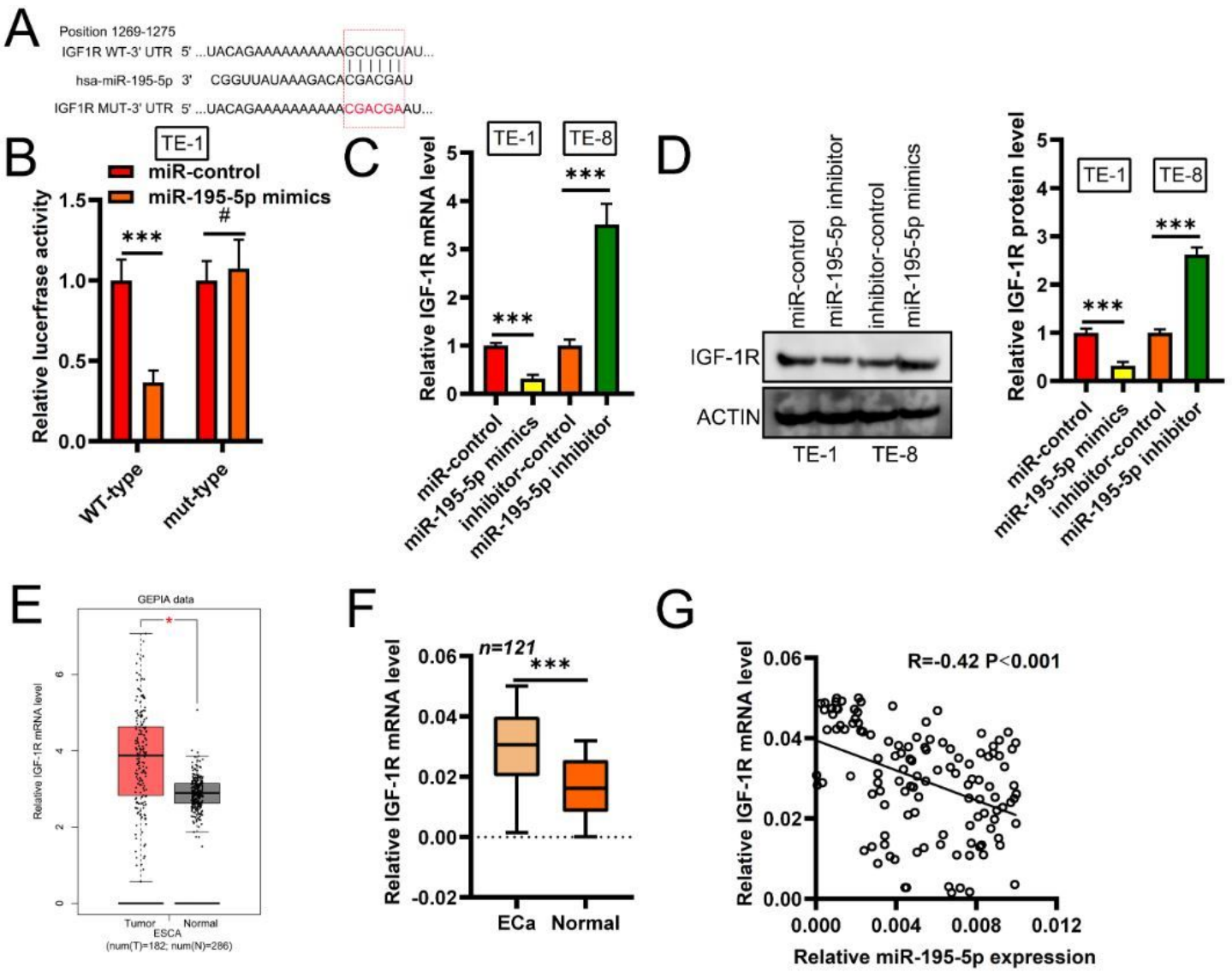

Figure 3

MiR-195-5p suppresses IGF-1R expression in ECa. (A) Bioinformatics predicting miR-195-5p targeting of IGF-1R. (B) Reporter assays showing the relationship between miR-195-5p and IGF-1R in TE-1. (C- D). WB and RT-PCR analysis of IGF-1R in miR-195-5p mimic or inhibitor cells. (E) GEPIA analysis showing higher IGF-1R levels in ECa tissues. (F) RT-PCR showing increased IGF-1R mRNA levels in ECa. (G) Correlation of miR-195-5p and IGF-1R. (n = 3) P-values as in Fig 1.

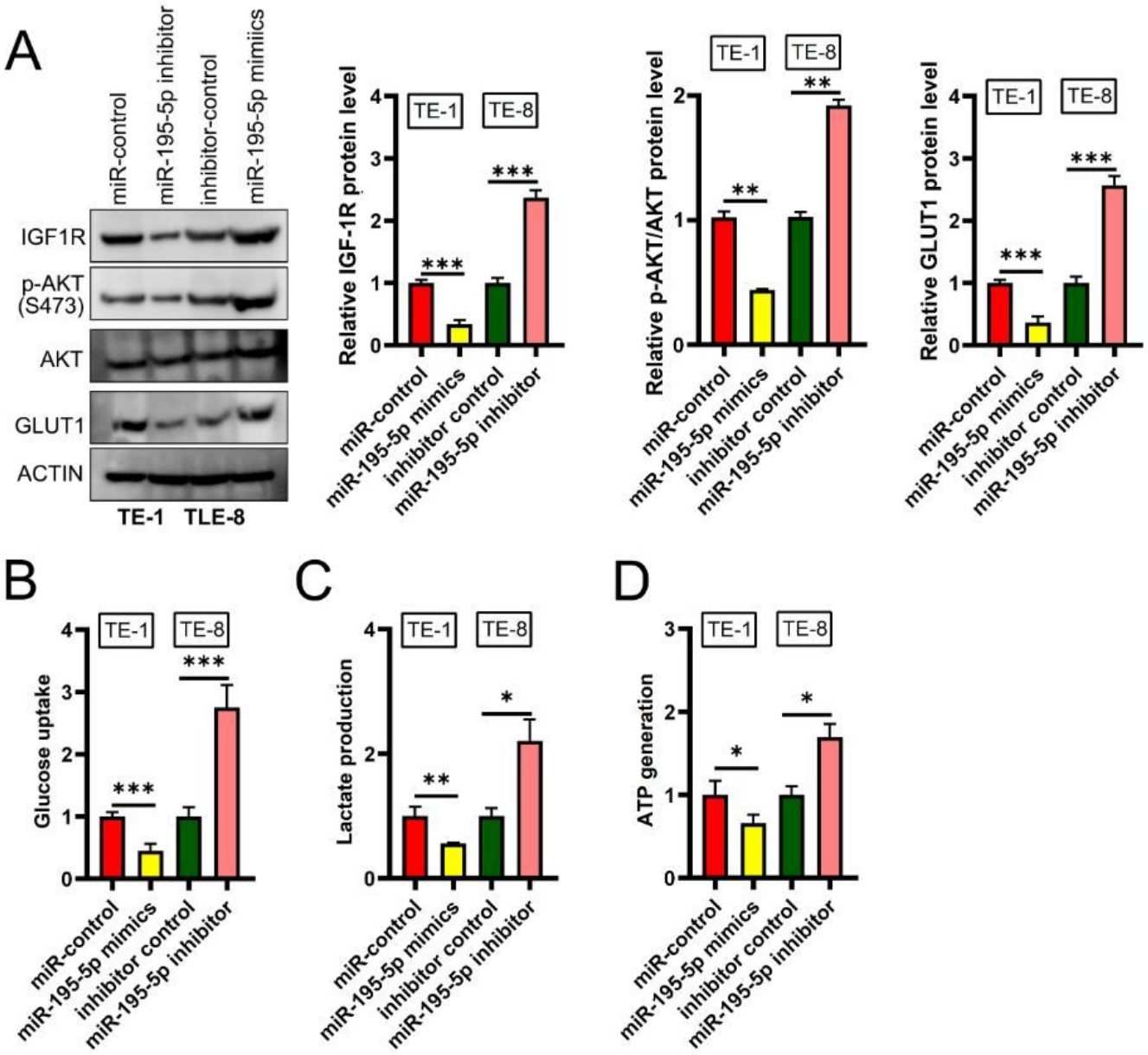

Figure 4

MiR-195-5p controls ECa cell glycolysis via the IGF-1R/AKT/GLUT1 axis. (A) IGF-1R, AKT and GLUT1 expression in mimic or inhibitor in indicated ECa cells. (B-C) Rates of glucose uptake and lactate production in mimic or inhibitor in indicated ECa cells. (D) ATP levels in mimic or inhibitor in indicated ECa cells (n = 3). P-values as in Fig.1.

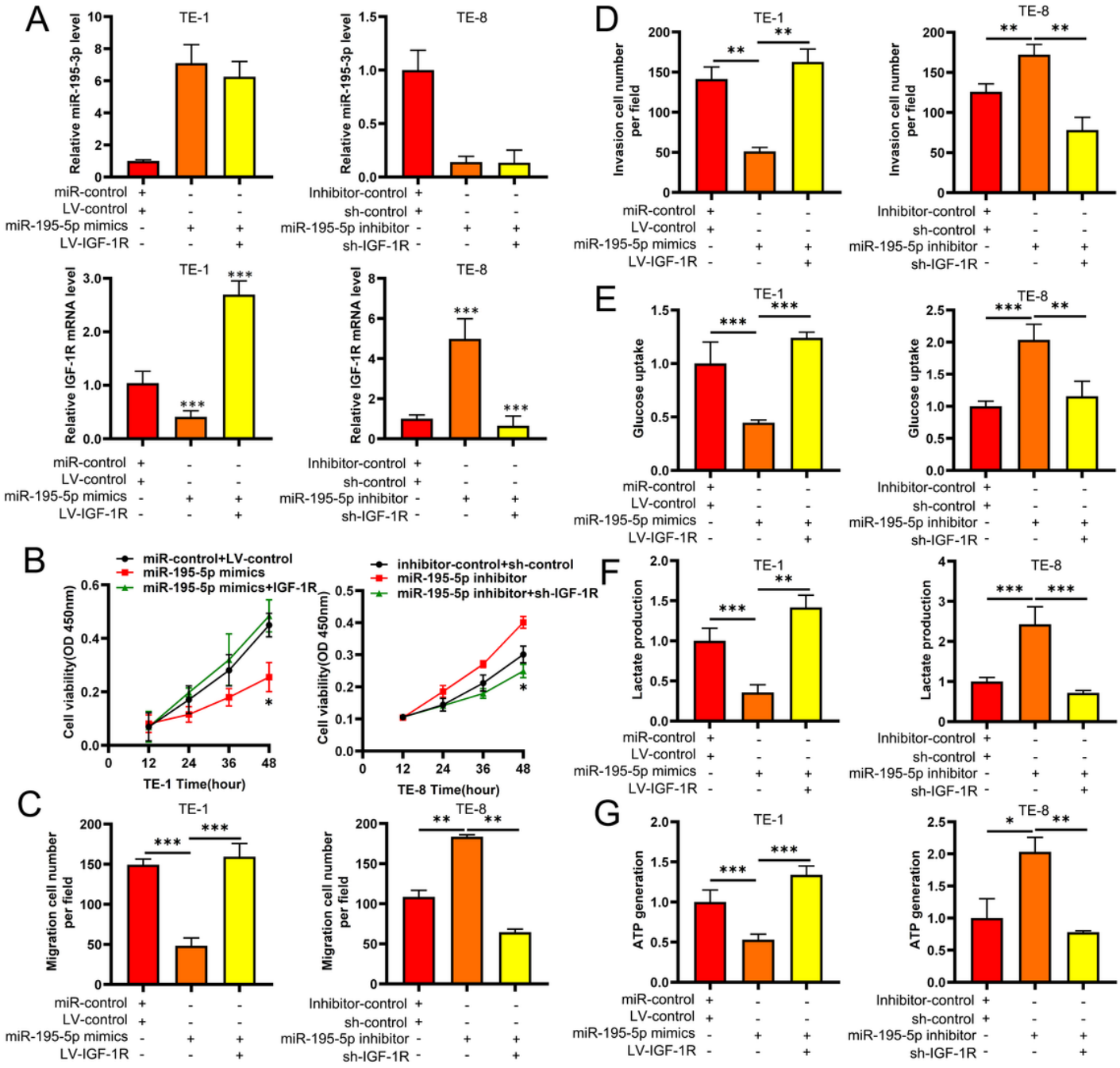

Figure 5

MiR-195-5p suppresses glycolysis via IGF-1R in ECa cells. (A) miR-195-5p and IGF-1R expression via RT-PCR. (B) CCK-8 assays in the indicated ECa cell lines. (D-E) Migration and invasion Transwell assays. (F-G) Cells expressing inhibitors or mimics were assessed for glucose uptake, the production of lactate and ATP levels. (n = 3). P-values as in Fig 1.

Supplementary Files

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