MicroRNA-135a acts as a putative tumor suppressor by directly targeting very low density lipoprotein receptor in human gallbladder cancer

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The precise functions and mechanisms of microRNAs (miR) in gallbladder cancer (GBC) remain elusive. In this study, we found that miR-135a-5p expression is often dampened and correlated with neoplasm histologic grade in GBC. MicroRNA-135a-5p introduction clearly inhibited GBC cell proliferation *in vitro* and *in vivo*. Moreover, very low density lipoprotein receptor (VLDLR), which is often upregulated in GBC tissues, was identified as a direct functional target of miR-135a-5p. Furthermore, the p38 MAPK pathway was proven to be involved in miR-135a-VLDLR downstream signaling. Together, these results suggested that the miR-135a–VLDLR–p38 axis may contribute to GBC cell proliferation.

Gallbladder cancer (GBC) is one of the most prevalent aggressive malignant neoplasms with poor prognosis. In Shanghai, GBC is the tenth most common cancer and the patients' average survival is approximately 9 months,[1] with many diagnosed patients unavailable for radical operation. Despite its high morbidity and poor prognosis, the key factors and molecular mechanisms of GBC remain largely unknown.

MicroRNAs (miRNAs) are a class of small, single-stranded, non-coding RNA molecules that serve gene silencing through binding to the 3′-UTR of target genes at the post-transcriptional stage,[2,3] leading to mRNA degradation or suppression of protein translation, and thus participate in various biological processes.[3–6] It has been predicted that miRNAs could regulate approximately 60% of human genes, including many oncogenes and tumor suppressor genes. In the past decade, many studies have indicated that miRNAs can play critical roles in many cancers.[7–10] However, the roles of miRNAs in GBC development and progression remain to be comprehensively elucidated.

In this study, we found that miRNA-135a (miR-135a) was significantly downregulated in GBC tissues compared with non-cancerous tissues and ectopic mir-135a expression inhibited the proliferation of GBC cells *in vitro* and *in vivo*. Moreover, miR-135a exerted this function through directly targeting very low density lipoprotein receptor (*VLDLR*), thus leading to the activation of the p38 MAPK pathway.

Materials and Methods

Clinical specimens. Gallbladder cancer tissues and matched adjacent non-tumorous gallbladder tissues (2 cm from the tumor) were obtained from surgical specimens removed from GBC patients at Huashan Hospital (Fudan University, Shanghai, China). Each case was confirmed and diagnosed using pathological examination. Informed consent was obtained from each patient who provided specimens, and the research protocol was approved by the Ethics Committee of Huashan Hospital (ethical permit no. 2012-108).

Cell cultures and reagents. HEK293T (ATCC, Manassas, VA, USA), GBC-SD (Chinese Cell Bank, Shanghai, China), EH-GB1 (a gift from Professor Qijun Qian, The Second Military Medical University, Shanghai, China), and SGC-996 (a gift from Professor Yaoqin Yang, Tongji University, Shanghai, China) cells were cultured in DMEM (Gibco, Carlsbad, California,

USA), supplemented with 10% FBS (Gibco), 100 IU/mL penicillin G, and 100 μg/mL streptomycin sulfate (Sigma-Aldrich, St. Louis, Missouri, USA), in an incubator at 37°C with 5% CO_2 and a humidified atmosphere. The p38 inhibitor SB 202190 (Sigma–Aldrich) was dissolved in DMSO (Sigma–Aldrich) and applied at a final concentration of 1.0 μg/mL.

Extraction of RNA, reverse transcription, and quantitative real-time PCR. Total RNA was extracted from tissues or cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Complementary DNA was synthesized with the Prime-Script RT reagent Kit (TaKaRa, Dalian, China). Quantitative real-time PCR analyses were carried out with SYBR Premix Ex Taq II (TaKaRa). The primers used are listed in Table S1. *Taq*Man microRNA assays (Applied Biosystems, Foster City, California, USA) were used to quantify the expression levels of miR-135a-5p and miR-135a-3p.

Oligonucleotide transfection. MicroRNA mimics were synthesized by Genepharma (Genepharm, Shanghai, China). Small interfering RNAs targeting *VLDLR* and specificity protein 3 (*SP3*) were synthesized by RiboBio (RiboBio, Guangzhou, China). The sequences are listed in Table S1. Cells were transfected with oligonucleotides using Lipofectamine RNAiMAX reagent (Invitrogen) at a final concentration of 50 nM and collected for assays at 48 h post-transfection.

Cell proliferation and colony formation assays. Cell proliferation was measured with a Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) following the manufacturer's instructions and cell numbers were reflected by the optical density at 450 nm. For colony formation assay, 1×10^3 cells were plated in each well of a 6-well plate and incubated at 37°C for 2 weeks. Cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet (Sigma–Aldrich). Megascopic cell colonies were counted and analyzed.

***In vivo* tumor formation assay.** The GBC-SD cells stably expressing miR-135a or vector control were collected and suspended in serum-free DMEM. Each mouse (male BALB/c-nu /nu, 6 weeks old) was injected s.c. in the lower back with 2.5×10^6 GBC-SD cells in 200 μL DMEM. The mice were killed after 6 weeks and examined for the growth of s.c. tumors. Mice were housed and manipulated following the protocols approved by the Shanghai Medical Experimental Animal Care Commission.

Vector constructs. The human pri-mir-135a sequence was amplified from normal human genomic DNA and cloned into the lentivirus expression vector pWPXL (a generous gift from Dr. Didier Trono, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) to generate pWPXL–mir-135a. The 3′-UTR of *VLDLR* was amplified and inserted into the downstream of the stop codon of Renilla luciferase in psiCHECK2 vector (Promega, Fitchburg, Wisconsin, USA). The ORF of VLDLR was amplified and cloned into another lentiviral vector, pLVX-IRES-Neo (Clontech, Mountain View, California, USA), to generate pLVX-VLDLR. The primers used are listed in Table S1.

Lentivirus production and transduction. Lentivirus particles were harvested 48 h after pWPXL-mir-135a (or pLVX-VLDLR) transfection with the packaging plasmid psPAX2 and VSV-G envelope plasmid pMD2.G (a gift from Didier Trono) into HEK293T cells by using Lipofectamine 2000 reagent (Invitrogen). Both GBC-SD and EH-GB1 cells were infected with recombinant lentivirus plus 6 μg/mL polybrene (Sigma–Aldrich).

Luciferase assay. HEK293T cells were cultured in 96-well plates and cotransfected with 20 ng psiCHECK-2-VLDLR-3′-

UTR and 5 pmol miR-135a-5p mimic or negative control. After 48 h of incubation, the firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega).

Cell cycle analysis. Cells were collected and fixed in 75% ethanol at −20°C overnight. The fixed cells were washed three times with PBS and stained with 25 μg/mL propidium iodide (Sigma–Aldrich), 10 μg/mL RNase A (Sigma–Aldrich), 0.05 mM EDTA, and 0.2% Triton X-100 in PBS for 30 min. DNA content was analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). The results were analyzed using ModFit software (BD Biosciences).

Western blot analysis. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, California, USA). Then the membrane was blocked with 5% non-fat milk and incubated with rabbit anti-VLDLR antibody (1:400; Abgent), rabbit anti-SP3 antibody (Abgent, San Diego, California, USA) (1:400), mouse anti-GAPDH antibody (1:3000; Sigma-Aldrich), mouse anti-c-Myc antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit anti-p44/42 MAPK (Erk1/2) (1:1000; Cell Signaling Technology [CST], Danvers, Massachusetts, USA), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (1:1000; CST), mouse anti-SAPK/JNK (56G8) antibody (1:1000; CST), rabbit anti-phospho-SAPK/JNK (Thr183 /Tyr185) (81E11) antibody (1:1000; CST), rabbit anti-p38 MAPK (D13E1) antibody (1:1000; CST), or rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (12F8) antibody (1:1000; CST). The proteins were detected with enhanced chemiluminescence reagents (Thermo Scientific, Waltham, Massachusetts, USA).

Statistical analysis. Data were presented as the mean ± SEM from at least three independent experiments. Unless otherwise noted, Student's *t*-test and one-way ANOVA were used to compare the differences between two groups and more than two groups, respectively. The clinical significance of miR-135a was analyzed by the way of Fisher's exact test. $P < 0.05$ was set as the level of statistical significance. Statistical analyses were carried out with GraphPad Prism 5 (GraphPad Software, Inc. San Diego, California).

Results

Functional screen of miRNAs and miR-135a-5p is often downregulated in GBC. We identified 23 downregulated miRNAs based on the miRNA chip results of four paired GBC and paracancerous tissues (fold-change >10; Fig. 1a, Table 1) and investigate the roles of these 23 miRNAs on GBC cell proliferation using the CCK-8 assay according to the procedure (Fig. S1). Among them, miR-135a-5p and miR-26a were considered to significantly affect GBC cell proliferation (fold-change <0.8 /fold-change >1.2, $P < 0.05$; Fig 1b). By real-time PCR, we found that miR-135a-5p was significantly downregulated in GBC tissues (Fig. 1c), whereas the expression of miR-135a-3p had no significant difference between GBC tissues and their paracancerous tissues (Figs 1d, S2a). In this study, we used miR-135a-5p for further investigations.

MicrRNA-135a-5p inhibits GBC cell proliferation *in vitro* and *in vivo*. MicrRNA-135a-5p mimic transfection could inhibit the proliferation and colony formation ability of GBC-SD and EH-GB1 cells (Fig. S2b,c), and arrest the cells in G_1/S phase (Fig. S3a). Caspase-Glo 3/7 Assay (Promega) showed that miR-135a-5p could slightly upregulate caspase 3/7 activity (Fig. S3b), whereas SA-β-gal staining showed that miR-135a-5p had no obvious influence on GBC cellular senescence (Fig. S3c).
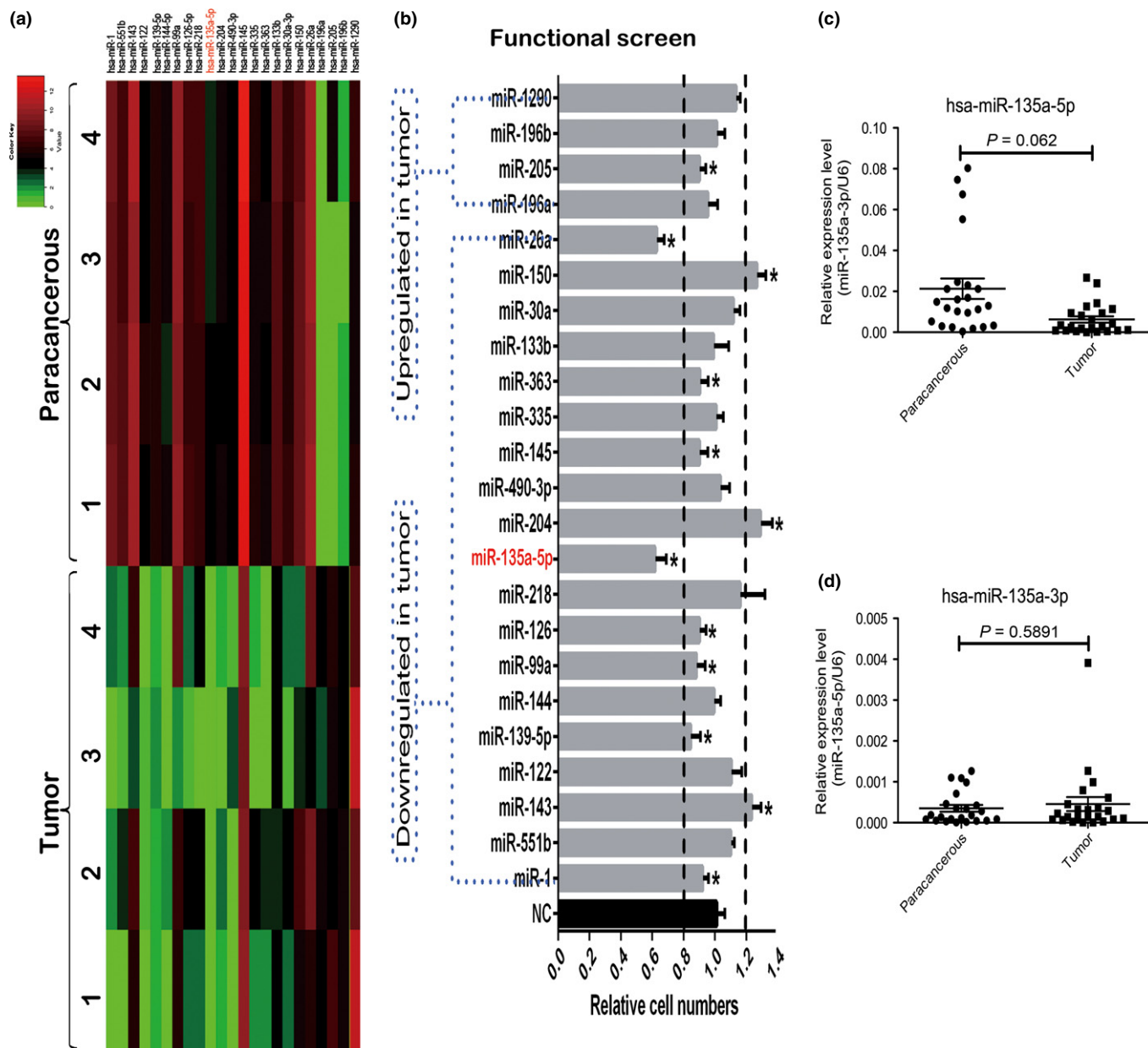
Fig. 1. Functional screen of microRNAs (miRNAs); miR-135a-5p is often downregulated in gallbladder cancer (GBC). (a) Heatmap of miRNA microarrays resulted in four pairs of GBC tissues and paracancerous tissues. (b) Functional screen of miRNAs in which the expression level changed above 10-fold in chip results. *P < 0.05 (miRNA vs negative control). (c) miR-135a-5p expression level in GBC tissues and paracancerous tissues. (d) miR-135a-3p expression level in GBC tissues and paracancerous tissues.

We then established stable GBC-SD and EH-GB1 cells overexpressing miR-135a via lentivirus (Fig. S2d), and found that the proliferation rates of lenti-miR-135a GBC cells were significantly decreased compared with those cells infected with lenti-GFP (Fig. 2a,b). The cells stably expressing miR-135a or GFP were s.c. injected into nude mice. After 6 weeks, the mice were killed and the xenografts were evaluated. The tumors derived from the miR-135a cells were much smaller than those from the GFP cells (Fig. 2c). In sum, miR-135a-5p could inhibit the proliferation of GBC cells both *in vitro* and *in vivo*.

Very low density lipoprotein receptor a direct downstream target of miR-135a. Potential targets of miR-135a-5p were predicted by TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de), and mRNA microarray assays were carried out in EH-GB1 cells transfected with miR-135a-

5p mimic. Six genes were considered as the candidate target gene (Fig. 3a). In GBC-SD cells, miR-135a-5p mimic could dampen the mRNA levels of *SP3* and *VLDLR*, which have inhibitory effects on cancer cell proliferation (Fig. 3b).[11,12] Very low density lipoprotein receptor siRNAs could clearly inhibit the proliferation of both EH-GB1 and GBC-SD cells, yet *SP3* siRNAs did not affect GBC-SD cell proliferation (Fig. S4), indicating that *VLDLR* was responsible for the effect of miR-135a-5p on GBC cell proliferation.

TargetScan suggested one highly conserved miR-135a binding site in the *VLDLR* 3'-UTR region, then we constructed the *VLDLR* 3'-UTR with a wild-type or mutant binding site into the downstream of the firefly luciferase gene (Fig. 3c). MicroRNA-135a-5p could significantly decrease the luciferase activity of wild-type *VLDLR* 3'-UTR, whereas the mutant

Table 1. MicroRNA (miR) microarray chip results of four paired gallbladder cancer and paracancerous tissues, showing significant difference

MicroRNA	Upregulated ∕downregulated	Fold change	P-value
hsa-miR-1	Down	170.63847	0.000795000
hsa-miR-551b	Down	59.522526	0.003004470
has-miR-143	Down	25.309427	0.010694763
hsa-miR-122	Down	24.126710	0.000008650
hsa-miR-139-5p	Down	20.263464	0.000019100
hsa-miR-144	Down	18.984410	0.001211098
hsa-miR-99a	Down	17.650175	0.046102613
hsa-miR-126	Down	16.482191	0.013887554
hsa-miR-218	Down	15.803111	0.029852837
hsa-miR-135a-5p	Down	14.394995	0.000002190
hsa-miR-204	Down	13.278212	0.005123142
hsa-miR-490-3p	Down	12.573485	0.009996969
hsa-miR-145	Down	12.058656	0.000540000
hsa-miR-335	Down	11.556526	0.040737227
hsa-miR-363	Down	11.283744	0.024815170
hsa-miR-133b	Down	11.189270	0.000307000
hsa-miR-30a	Down	10.997444	0.018918402
hsa-miR-150	Down	10.542399	0.035363853
hsa-miR-26a	Down	10.136220	0.033066160
hsa-miR-196a	Up	20.916237	0.002211535
hsa-miR-205	Up	19.793240	0.039628908
hsa-miR-196b	Up	14.974539	0.000166000
hsa-miR-1290	Up	14.189396	0.030898692

VLDLR 3′-UTR was not affected (Fig. 3d), suggesting the direct binding of miR-135a-5p to *VLDLR* 3′-UTR.

The mRNA and protein level of VLDLR was found to be upregulated in GBC tissues compared to the paired paracancerous tissues (Figs 3g,S2d). Furthermore, in the 23 pairs of GBC and paracancerous tissues, the *VLDLR* mRNA level and miR-135a-5p level were negatively correlated, which fits the exponential equation (one phase decay) (Fig. 3e). MicroRNA-135a-5p mimic could also decrease the endogenous VLDLR protein level in GBC cells (Fig. 3f). Taken together, these results indicated that *VLDLR* is a direct downstream target of miR-135a-5p in GBC cells.

Reintroduction of VLDLR abrogates miR-135a-5p-induced suppression of GBC cell proliferation. To further address the critical role of *VLDLR* in miR-135a-5p-induced suppression of GBC cell proliferation, we constructed a lentiviral expression vector of *VLDLR* ORF without the 3′-UTR, and established the stable GBC cells using this vector. The infection of lentivirus carrying *VLDLR*-ORF antagonized the inhibition of miR-135a-5p on GBC cells proliferation (Fig 4a,b), with VLDLR protein recovery in Lenti-miR-135a cells (Fig 4c). Furthermore, it was shown that enforced expression of VLDLR could counteract the miR-135a-5p-induced G_1/S arrest (Fig. S3a). These results suggested that *VLDLR* is a functional target of miR-135a-5p in GBC cells.

MicroRNA-135a-5p–VLDLR axis exerts its function through activation of p38 MAPK pathway. The molecular mechanisms responsible for the effect of miR-135a-5p and *VLDLR* on GBC cell proliferation remain unclear. The Kyoto Encyclopedia of Genes and Genomes analysis based on mRNA microarray assays showed that the MAPK signal pathway might be involved in the inhibition of miR-135a-5p-induced GBC cell proliferation (Table S2). The protein levels of ERK 1/2,

SAPK/JNK, p38, and their phosphorylation levels were detected in the GBC cells infected with GFP lentivirus, miR-135a lentivirus, or both. In both GBC-SD and EH-GB1 cells, VLDLR could reduce the phosphorylation level of p38 (Figs 4c,S4c), and we also found that *VLDLR*-siRNA could induce phosphorylation of p38 (Fig. S4c). After pretreatment of p38 inhibitor, the miR-135a-induced inhibitory effect on GBC cell proliferation was abrogated (Fig. 5a,b), and the enhanced phosphorylation level of p38 by miR-135a-5p was diminished (Fig. 5c). Furthermore, the miR-135a-5p-induced G_1/S arrest could also be abolished by the p38 inhibitor (Fig. 5d), and it was not surprising to find the restoration of suppressed cyclin D1 expression (Fig. 5c), yet the treatment of miR-135a-5p mimic or p38 inhibitor did not significantly affect the protein level of c-MYC in GBC cells (Fig. 5c). Taken together, these results implied that the p38 MAPK pathway is involved in the miR-135a-5p–*VLDLR* axis on GBC cell proliferation.

Expression level of miR-135a-5p correlated with neoplasm histologic grade. To further determine the clinical significance of miR-135a-5p in GBC, we analyzed its expression level with real-time PCR in 23 primary GBC patients. Based on the overall expression level of miR-135a-5p, we divided GBC specimens into two groups (greater than or equal to the median, and less than the median). We found that the expression level of miR-135-5p was correlated with the neoplasm histologic grade (Table 2).

Discussion

In this study, we found that miR-135a-5p is downregulated in GBC tissues and inhibits the proliferation of GBC cells, and the expression of miR-135a-5p is correlated with neoplasm histologic grade of GBC tissue (Figs 1,2, Table 2). Furthermore, we identified *VLDLR* as a direct and functional target gene of miR-135a-5p in GBC tissues (Figs 3,4). In addition, the p38 MAPK pathway is involved in the inhibitory effects of miR-135a-5p on GBC cell proliferation (Fig. 5).

In the last decade, many studies have shown possible links between miR-135a and tumor biological behavior. In classic Hodgkin's lymphoma and gastric cancer, miR-135a can function as a tumor suppressor through the targeting of *JAK2* to repress STAT3 activation, reduce *cyclin D1* and *Bcl-xL* expression, and inhibit tumor cell proliferation.[13,14] It was also revealed that miR-135a inhibits cancer cell proliferation by targeting *c-MYC* in renal cell carcinoma and functions as a selective killer of malignant glioma,[15,16] yet we found that miR-135a-5p mimic transfection does not affect c-MYC protein levels in GBC cells (Fig. 5c). Enforced miR-135a expression sensitized lung cancer cells to cisplatin-induced apoptosis by targeting *MCL1*, and paclitaxel resistance of non-small cell lung carcinoma cell is associated with upregulation of miR-135a.[17,18] These results suggest that miR-135a could act as a predictor of treatment in some cancers. For the first time, we have shown that miR-135a-5p could inhibit the proliferation of GBC cells.

To explore the molecular mechanism of the miR-135a-5p-induced inhibition effect on the proliferation in GBC cells, we identified that *VLDLR*, the direct target gene of miR-135a-5p, contributes to the inhibitory function of miR-135a-5p in GBC (Figs 3,4). *VLDLR* is a member of the low-density lipoprotein receptor superfamily and is bound with a variety of different ligands, including Mr-40 000 receptor-associated protein, apolipoprotein E, and some serine proteinase/serpin
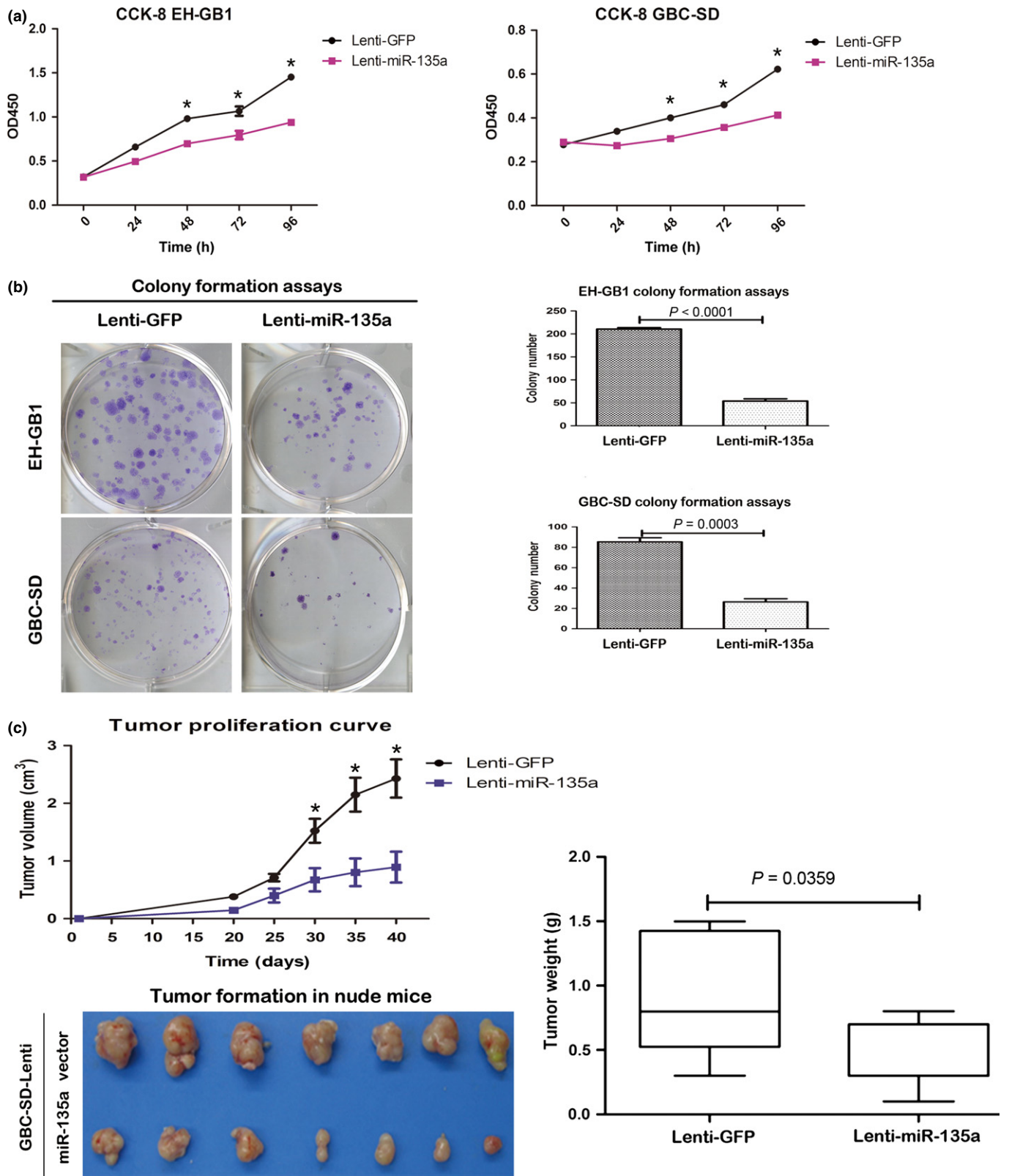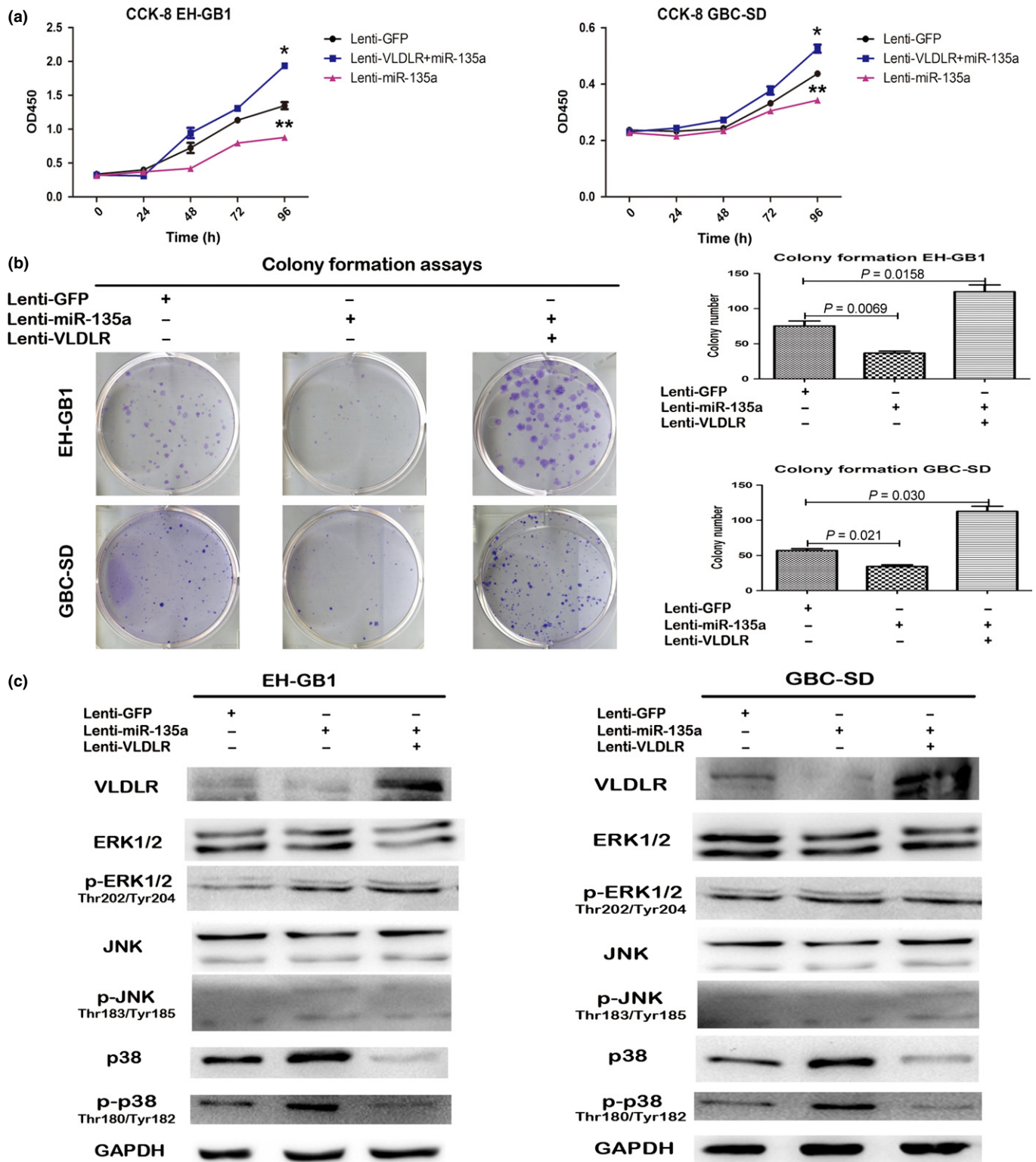
Fig. 2. MicroRNA-135a (miR-135a) inhibited gallbladder cancer (GBC) cell proliferation *in vitro* and *in vivo*. (a,b) Cell counting (CCK-8) and colony formation assays of GBC-SD and EH-GB1 cells stably expressing miR-135a or GFP control. *P < 0.05 (Lenti-GFP *vs* Lenti-miR-135a). (c) Tumor formation in nude mice with GBC-SD cells stably expressing miR-135a or GFP control and the tumor weight. *P < 0.05 (Lenti-GFP *vs* Lenti-miR-135a).
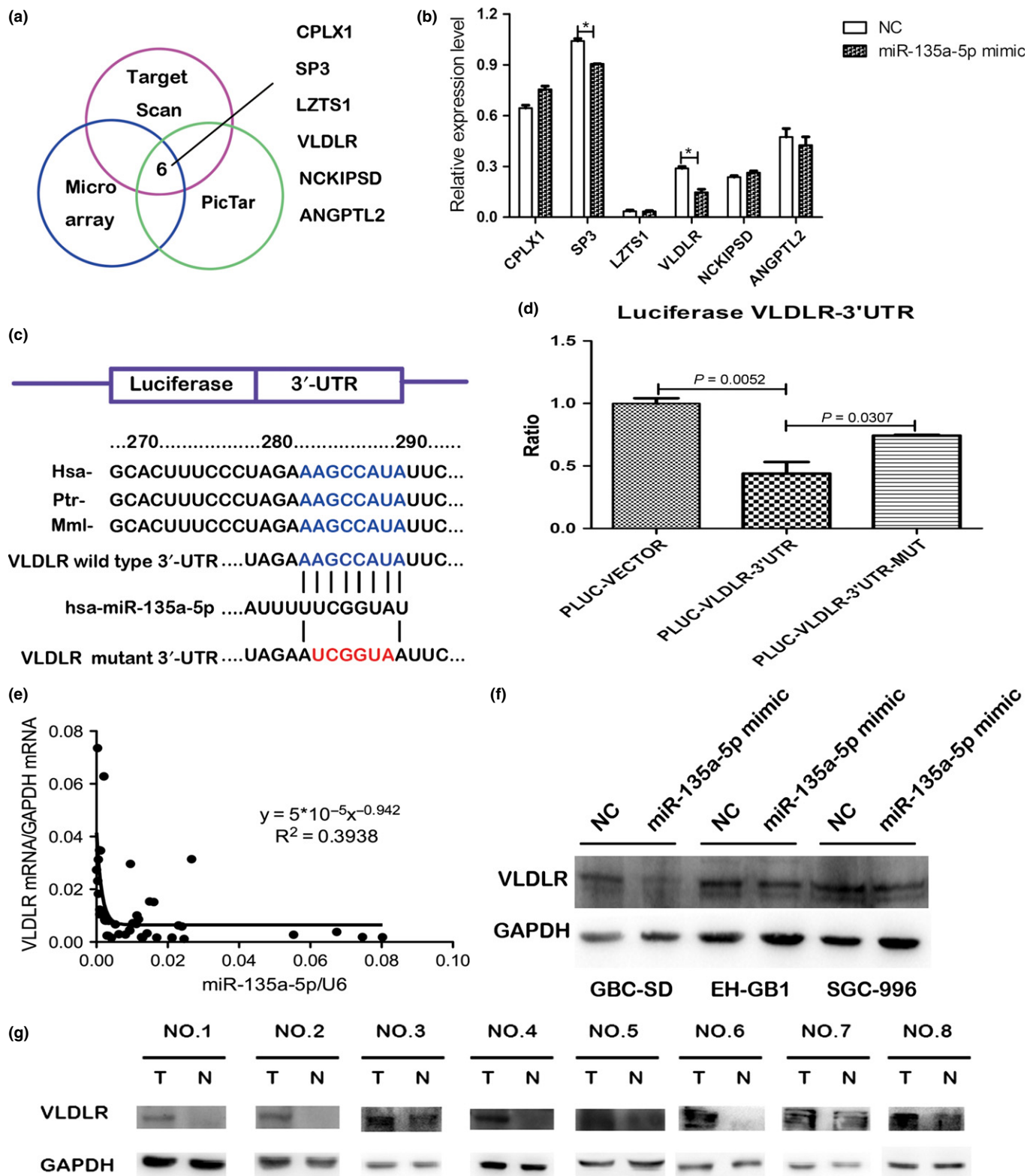
Fig. 3. MicroRNA-135a (miR-135a) downregulates very low density lipoprotein receptor (VLDLR) expression by directly targeting its 3′-UTR. (a) Search for the identification of potential genes combining microarray assays, TargetScan prediction, and PicTar prediction. (b) Potential target genes' mRNA levels in GBC-SD cells transfected with miR-135a-5p mimic or negative control (NC). (c) Putative binding site for miR-135a-5p within human (Has), chimpanzee (Ptr), and rhesus (Mml) VLDLR 3′-UTR. Binding site sequences are indicated with blue, the sequences of miR-135a-5p binding site in the wild-type (indicated with blue), and mutant (indicated with red) *VLDLR* 3′-UTR. (d) Relative luciferase activity analyses. The pLUC, pLUC-wild-type–VLDLR–3′-UTR, or pLUC-mutant–VLDLR–3′-UTR was transfected into HEK-293T cells with pWPXL or pWPXL-miR-135a. Renilla luciferase vector was used as an internal control. Representative experiments are shown with the mean ± SD. (e) Regression and correlation analysis of miR-135a-5p and *VLDLR* mRNA in tissues. (f) Protein levels of VLDLR were determined by Western blot assays in GBC-SD, EH-GB1, and SGC-996 cells transfected with miR-135a-5p mimic or NC. (g) The VLDLR protein level was often downregulated in tumor tissue (T). (N means non-tumor tissue).
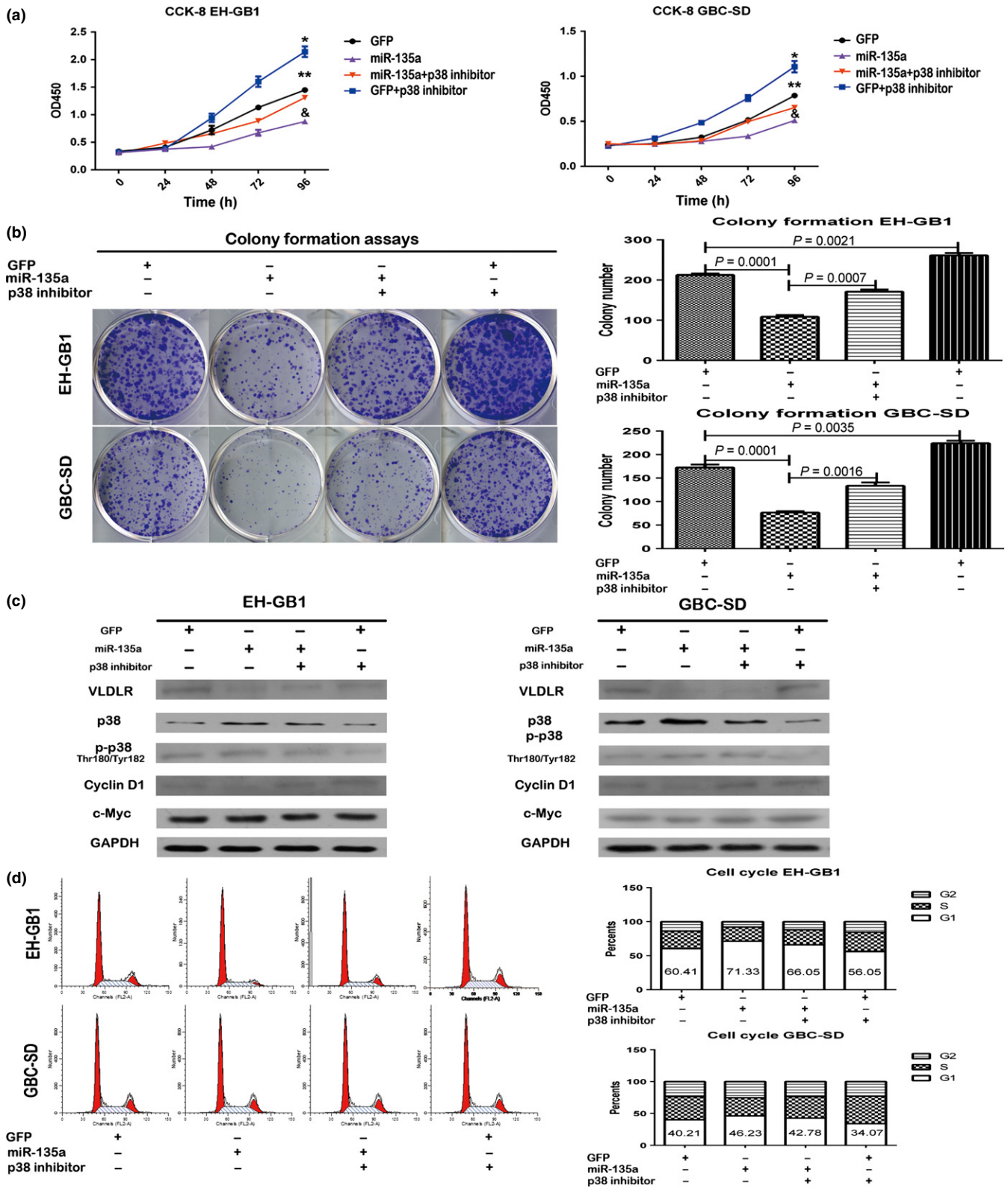
Fig. 4. Reintroduction of very low density lipoprotein receptor (VLDLR) abrogated microRNA-135a (miR-135a)-induced inhibition of proliferation in gallbladder cancer (GBC) cells. (a,b) Cell counting (CCK-8) and colony formation assays of GBC-SD and EH-GB1 cells stably expressing miR-135a or lenti-GFP were infected with lenti-VLDLR or corresponding lenti-GFP. *$P < 0.05$ (Lenti-GFP vs Lenti-miR-135a); **$P < 0.05$ (Lenti-GFP vs Lenti-VLDR+miR-135a). (c) Protein levels of VLDLR, ERK(1/2), SAPK/JNK, and p38 and their phosphorylation (p−) levels were detected by Western blot assays after GBC-SD and EH-GB1 cells stably expressing miR-135a or lenti-GFP were infected with lenti-VLDLR or corresponding lenti-GFP.

complexes.[19,20] *VLDLR* has been reported to be involved in the pathogenesis of gastric cancer, breast cancer, and other cancers, with involvement in cancer cell growth.[13,20] In this

study, we found that the mRNA and protein levels of VLDLR were often upregulated in GBC tissues (Fig. S2). Furthermore, knockdown of VLDLR with siRNA can inhibit proliferation of

Fig. 5. Involvement of p38 in the inhibition of very low density lipoprotein receptor (VLDLR) in gallbladder cancer (GBC) cell proliferation. (a,b) Cell counting (CCK-8) and colony formation assays of GBC-SD and EH-GB1 cells stably expressing miR-135a, pretreated with p38 inhibitor and stably expressing GFP, or pretreated with DMSO and stably expressing GFP or miR-135a. *$P < 0.05$ (Lenti-GFP pretreated with DMSO *vs* Lenti-miR-135a pretreated with DMSO); **$P < 0.05$ (Lenti-GFP pretreated by DMSO *vs* Lenti-VLDLR+miR-135a pretreated with p38 inhibitor); & $P < 0.05$ (Lenti-GFP pretreated by DMSO *vs* Lenti-GFP pretreated with p38 inhibitor). (c) Protein levels of VLDLR, cyclin D1, and p38 and their phosphorylation (p−) levels were detected by Western blot assays in GBC-SD and EH-GB1 cells stably expressing miR-135a, pretreated with p38 inhibitor and stably expressing GFP, or pretreated with DMSO and stably expressing GFP or miR-135a. (d) Cell cycle assays in GBC-SD and EH-GB1 cells stably expressing miR-135a, pretreated with p38 inhibitor and stably expressing GFP, or pretreated with DMSO and stably expressing GFP or miR-135a.

Table 2. Clinical significance of microRNA-135a-5p (miR-135a-5p) in patients with gallbladder cancer (n = 23)

		miR-135a-5p high†	miR-135a-5p low‡	Total	*P*-value
Age, years	>55	6	6	11	P = 1.0000
	≤55	6	5	12	
	Total	12	11	23	
Sex	Male	9	7	16	P = 0.6668
	Female	3	4	7	
	Total	12	11	23	
Grade	I + II	9	3	12	P = 0.0391
	III + IV	3	8	11	
	Total	12	11	23	
CA199	High	8	4	12	P = 0.2203
	Low	4	7	11	
	Total	12	11	23	

CA199, a tumor marker often up-regulated in GBC patient. †Greater than or equal to the median. ‡Less than the median.

GBC cells, whereas enforced VLDLR expression promotes GBC cell proliferation. In GBC samples (Figs 4,S4), the expression level of miR-135a-5p and *VLDLR* mRNA are inversely correlated (Fig. 3e), suggesting the contribution of *VLDLR* to the miR-135a-5p-mediated effect.

It has been reported that VLDLR regulates the expression of the components of the FGFR signaling pathway through MAPK.[21] We found that miR-135a or VLDLR introduction interfered with the p38 MAPK pathway in GBC cells, and p38 inhibitor could interrupt the inhibitory effect of miR-135a-5p on GBC cells, such as cell proliferation and cell cycle distribution (Fig. 5). As the p38 inhibitor does not influence the VLDLR protein level (Fig. 5c), this may suggest that the p38 signaling is downstream of VLDLR.

For the first time, we report that miR-135a-5p is often downregulated in GBC tissues and is associated with the neoplasm histologic grade. Overexpression of miR-135a-5p can disturb the proliferation and cell cycle distribution of GBC cells, with the consequent downregulation of VLDLR by directly targeting its 3′-UTR, thus activating the p38 MAPK pathway.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials and methods.

Table S1. Primers and sequences used in this study.

Table S2. Kyoto Encyclopedia of Genes and Genomes analysis for mRNA microarray chip: results of EH-GB1 transfected with microRNA-135a mimic compared with EH-GB1 transfected with negative control (NC).

Fig. S1. Procedure of functional screen.

Fig. S2. MicroRNA-135a-5p (miR-135a-5p) level is much higher than miR-135a-3p in tissues and gallbladder cancer (GBC) cells. The miR-135a-5p mimic inhibited GBC cell proliferation *in vitro* and very low density lipoprotein receptor (VLDLR) mRNA levels were higher in GBC tumor.

Fig. S3. MicroRNA-135a induced cell cycle arrest of gallbladder cancer cells at G_1/S transition.

Fig. S4. Influences of very low density lipoprotein receptor (VLDLR) or specificity protein 3 (SP3) silencing on proliferation of gallbladder cancer cells.