Original Research

MicroRNA-892a regulates laryngocarcinoma cell proliferation via Dicer

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Impact statement

This work expanded the knowledge of the molecular mechanisms underlying LC progression by exploring the role of miR-892a in the viability of TU212 and M4E cells. The results showed that miR-892a, which exhibited elevated expression in LC cells and tissue specimens of patients with LC, exerted an inhibitory effect on Dicer expression, whereas silencing of miR-892a in TU212 and M4E cells hindered cell proliferation and growth and promoted apoptosis. Furthermore, miR-892a was demonstrated to directly target Dicer 3′-UTR and inhibit its expression. These findings demonstrated that miR-892a acted as an LC oncogene via its action on Dicer, which further confirmed that miR-892a can serve as a diagnostic indicator or promising agent for LC treatment.

Abstract

MicroRNA (miR) plays a critical role in the progression of multiple malignancies. Nevertheless, knowledge of the role it plays in laryngeal cancer is limited. In this study, we explored the role of miR-892a in laryngeal cancer cell proliferation and apoptosis. miR-892a expression was increased in 17 laryngeal cancer samples and cells compared with that in healthy tissues, laryngeal cancer normal surrounding tissues, and the NP69 human nasopharyngeal epithelial cell line. Conversely, Dicer expression was downregulated in human laryngeal cancer samples as well as in the laryngeal cancer cell lines. CCK-8 assays and colony formation assay confirmed that depleted miR-892a expression damaged the proliferation and growth of TU212 and M4E cells. Annexin V/PI flow cytometry displayed that miR-892a inhibition led to increased apoptosis of TU212 and M4E cells. By conducting bioinformatic analysis and dual-luciferase reporter assay, it was revealed that that miR-892a targets Dicer 3′-UTR for silencing. Dicer expression inhibition offsets the effect of miR-892a on the growth and apoptosis of laryngeal cancer cells. Dicer overexpression displayed similar phenotype with miR-892a inhibition on the properties of laryngeal cancer cells. Results of *in vivo* experiments further confirmed that miR-892a silencing suppressed tumor growth in a mouse model. Hence, the results of this study provide new ideas about the biological and molecular mechanisms behind laryngeal cancer progression, thereby obtaining novel laryngeal cancer treatments.

Keywords: Laryngeal cancer, miR-892a, Dicer, apoptosis, 3′-UTR

***Experimental Biology and Medicine* 2020; 245: 1222–1232. DOI: 10.1177/1535370220934036**

Introduction

Laryngeal cancer (LC) is one of the most prevalent malignancies occurring in the neck and head.[1] The incidence of LC ranks 2nd among neck and head cancers and 11th among all cancers.[2] However, the incidence rate of LC has been increasing progressively in China, especially in the northeast regions.[3] Although there has been remarkable progress in chemotherapy and radiotherapy as well as surgical treatments to improve the five-year survival rate of patients with LC, a novel diagnostic and therapeutic approach for LC is still required owing to its potentially high morbidity and incommensurably low cure rate.[4,5] Thus, exploration of novel therapeutic targets for LC treatment is needed.

MicroRNA (miR), a series of small non-coding RNAs, was first recognized in 1993.[6] Increasing evidence has indicated that irregular miR expression is closely related to the development of various cancers. The fact that miRNAs can act as oncogenes or tumor suppressors, facilitating or suppressing cancer, makes them an important target for molecular treatment for cancer.[7] In LC, miRNAs play an essential role in carcinogenesis through post-translational regulation of cell proliferation, metastasis, invasion, and apoptosis-associated oncogenes;[8,9] for example, miR-16 was reported to be overexpressed in LC samples. After miR-16 inhibition, the adhesive force of Hep-2 cells increased, and cell migration was repressed.[10] Liu *et al.* found that miR-21 expression in

LC samples was augmented and was capable of acting on the BTG2 gene, a well-known pan-cell cycle regulator and tumor inhibitor. Therefore, ectopic expression of miR-21 resulted in the suppression of tumor cell growth activity.[11] MiR-892a regulated the proliferation and invasion of hepatocellular carcinoma cells and colorectal cancer cells via targeting CD226 and PPP2R2A, respectively.[12,13] However, its role in LC development and progression is still poorly investigated.

In this study, microarray analysis was performed to identify differentially expressing miRs in the LC samples and normal laryngeal tissue specimens; a series of experiments was performed to investigate the role of miR in LC progression. Furthermore, whether miR expression affected the biological processes of LC cells was also investigated.

Materials and methods

Subjects

Seventeen patients with LC ($n = 17$; aged 35–70 years; average, 55 years) were included in this study. Clinicopathological data were collected from medical records. Adjacent normal tissues ($n = 9$) measuring at least 2 cm in size from regions affected by LC were collected. All subjects were from the Second Hospital of Hebei Medical University between June 2016 and December 2018. This study has been approved by the Ethics Committee of the Second Hospital of Hebei Medical University. Patients and doctors provided signed informed consent concerning sample use. The diagnosis of LC was completed by three pathologists separately. The clinical features of patients are included in Table 1.

Table 1. Relationship between miR-892a and clinicopathological features.

Features	Number	miR-892a level	*P*
Sex			
Male	12	4.42 ± 0.15	0.089
Female	5	4.25 ± 0.23	
Age, y			
≥65	11	4.19 ± 0.05	0.101
<65	6	4.56 ± 0.19	
N stage			
N0	6	3.89 ± 0.23	0.067
N1, N2, N3	11	4.67 ± 0.52	
T stage			
T1-2	8	4.91 ± 0.67	0.093
T3-4	9	4.14 ± 0.25	
Lymph node metastasis			
Negative	10	4.55 ± 0.52	0.099
Positive	7	4.22 ± 0.33	
Differentiation			
Well	11	4.11 ± 0.19	0.103
Moderately/poorly	6	4.67 ± 0.67	
Clinical stage			
I–II	6	4.29 ± 0.51	0.062
III–IV	11	4.39 ± 0.91	

Ethics statement

All experiments concerning patients were performed according to the Animal Management Regulations of the Chinese Health Ministry (55, 2001), and the animal experiments were performed according to standardized operating procedures agreed by the Animal Use and Care Committee at the Second Hospital of Hebei Medical University.

MicroRNA microarray

Total RNA from LC samples and adjacent normal tissue was extracted using TRIzol. RNA quality was evaluated using Agilent 2100 bioanalyzer (Agilent Technologies) prior to microarray experiment. All the samples had rRNA Ratio (28 s/18 s) over 1.8 and RNA Integrity Number (RIN) over 8.0. Data were analyzed by miRNA QC Tool software (Affymetrix). MicroRNA with $P < 0.05$ and 1.5-fold difference was considered as dysregulated microRNA in LC tissue.

Cell culture

Human LC TU212, M4E, M2E, and Hep-2 cells as well as NP69 human nasopharyngeal epithelial cells provided by the Shanghai Biological Science Institutes, Chinese Academy of Sciences were cultivated in RPMI 1640 medium supplemented by 10% FBS, 0.1 g/mL streptomycin, and 100 U/mL penicillin (Gibco) with 5% CO_2 atmosphere at 37°C.

MiR-892a mimic/inhibitor synthesis

The miR-892a mimic and inhibitor as well as their negative controls (NCs) provided by RiboBio (Guangzhou, China) were diluted with 0.9% NaCl to 10 g/L for subsequent experiments.

Cell transfection

To overexpress Dicer, full frame of Dicer sequence was inserted into pcDNA3.1 (referred as Dicer). pcDNA3.1 empty vector was used as the negative control (NC) for Dicer overexpression. miR-892a mimics, inhibitors, and the corresponding NC were synthesized and transfected into cells and then were harvested after 48 h transfection. The lipofectamine 2000 reagent (Invitrogen) was used for cell transfection.

RNA interference

The short hairpin RNA silencing Dicer was prepared with vector pLKO.1 puro and oligonucleotides targeting the following Dicer sequence: 5′-ATT AAC AGT CTT GAT TCG TGC-3′.

CCK-8 assay

Cells were inoculated in 96-well plates (5000 cells/well), and cell proliferation was evaluated via the CCK-8 assay. Briefly, following stimulation, the CCK-8 solution was transferred into the medium, which was then cultivated at 37°C for 60 min in a damp atmosphere with 5% CO_2.

A_{450} was determined using a microplate reader (Bio-Rad, Hercules, CA).

Colony generation assay

Cells were transfected with diverse vectors, resuspended for two days in DMEM containing 10% FBS and 0.4% top agar (8 mm) and then added into 12-well plates with 0.5% bottom agar (500 μL). After 10 days, three areas of each plate were randomly picked for colony quantification.

Annexin V/PI flow cytometry

Cell apoptosis was evaluated using an Annexin V-FITC/PI kit after trypsinization. The apoptosis proportion was calculated using FACSCalibur FC (Beckman Coulter).

Western blot

RIPA buffer added to the protease inhibition cocktail (Roche) was applied to lyse the cells. The proteins were quantified using the BCA Quantitation Kit. The proteins were isolated on 10% SDS-PAGE gels and were then added on to PVDF membranes (0.45 μm), which were blocked for 1 h in PBST with BSA (5%) at RT and cultivated for 60 min at 4°C with Bax, Bcl-2, Dicer, and GAPDH antibodies. The membranes were subsequently cultivated for 60 min at RT with secondary antibodies. Immunoreactivity was assessed using a Maximum Sensitivity Substrate Kit (Thermo). The experimental bands from three Western blots were quantified by Image J software and analyzed for statistical significance.

RNA extraction and RT-qPCR

Total RNA was extracted using TRIzol. Transcription levels were investigated using a PCR system (Roche). RT-qPCR (0.02 mL) was performed using the SYBR Green PCR Master Mix. For quantification through GAPDH normalization, $2^{-\Delta\Delta CT}$ method was used, and this was related to the average of the control samples.

TargetScan prediction

The targets of miR-1225 were identified through the prediction algorithm TargetScan; predictions are categorized on the website (http://www.targetscan.org) based on their efficiency. Furthermore, they are ranked likewise by their conserved targeting possibility.[14]

Dual-luciferase reporter assay

Dicer 3′-UTR was amplified before being fused with the GV126 luciferase gene. Its binding site and miR-892a were destroyed through oriented mutation. Thymidine kinase promoter (TaKaRa, Japan) and plasmids with renilla luciferase were applied to ameliorate the transfection level. The cells were subjected to miR-892a or the NC mimic containing luciferase reporter vectors, and DLRA was performed.

In vivo tumorigenesis experiment

Tumor generation was measured via an *in vivo* experiment. BALB/c nude mice were subcutaneously inoculated with M4E-NC inhibitor (NC inhibitor) and M4E-miR-892a inhibitor cells (2×10^6 cells/0.2 mL). Tumor size was determined every five days following inoculation; four weeks later, the mice were humanely euthanized; the tumors were removed and were assessed for volume according to the following formula: $A \times B^2/2$ (A: largest diameter; B: perpendicular diameter to A).

Statistical analysis

The results were expressed as mean ± SD, and ANOVA or *t*-tests were performed to analyze significance, with P values of <0.05 considered to be statistically significant. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL).

Results

MiR-892a is upregulated in LC specimens from patients and LC cell lines

The miRNA microarray analysis revealed that compared with the specimens from the healthy controls, miR-892a was significantly upregulated in the LC tissues of three patients (Figure 1(a)). To confirm these data, the miR-892a expression was likewise assessed in 17 LC and 9 adjacent healthy laryngeal samples (Figure 1(b)). Furthermore, RT-qPCR data showed that miR-892a expression was significantly increased in the LC cell lines (TU212, M4E, M2E, and Hep-2 cells) compared with that in the NP69 cells, especially for the TU212 and M4E cells (Figure 1(c)). Our data suggested that compared with normal tissues or cells, LC tissues or cells had upregulated miR-892a expression.

MiR-892a inhibition repressed proliferation and triggered apoptosis of TU212 and M4E cells

The role of miR-892a in the viability of TU212 and M4E cells was explored. In these two cell types, a noticeable gradual decrease in the miR-892a level was observed after transfection with different dose of miR-892a inhibitor (Figure 2(a) and (b)). A CCK-8 assay was conducted to demonstrate the proliferation rate of TU212 and M4E cells in a dose-dependent manner, caused by a significant decrease in different level of miR-892a inhibition during one to four days after transfection compared with that of the NC inhibitor group (Figure 2(c) and (d)). Colony formation assay also showed that the growth of TU212 and M4E cells was also significantly suppressed by the miR-892a inhibitor (Figure 2(e) and (f)). Because the miR-892a inhibitor reduced the proliferation and growth of TU212 and M4E cells, miR-892a might play a regulatory role in the apoptosis of these two cell types. Compared with the NC inhibitor group, an obvious increment of apoptotic cells with transfection of the miR-892a inhibitor was observed (Figure 3(a) and (b)). The role of miR-892a dosage involved in anti-apoptotic Bcl-2 and pro-apoptotic Bax expressions was thoroughly explored. The Bcl-2 mRNA and protein

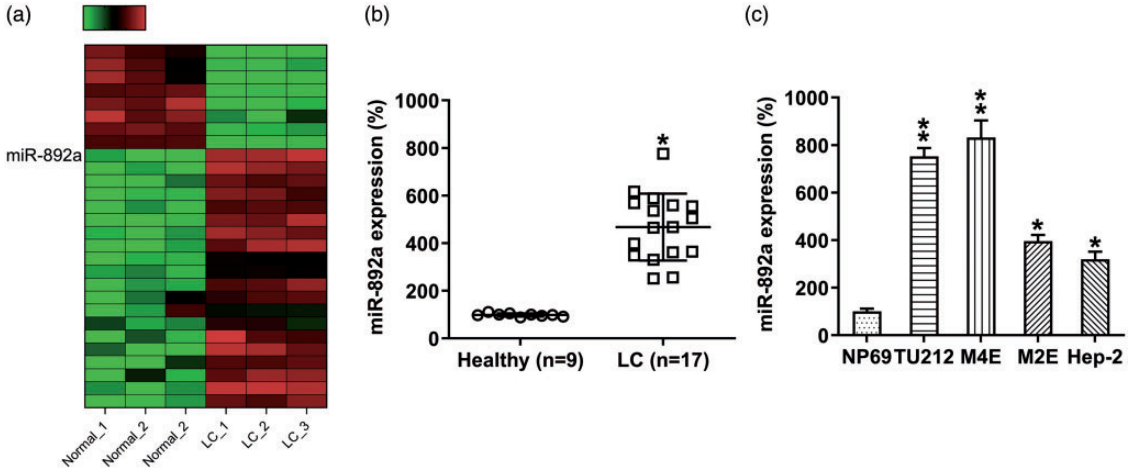

Figure 1. Expression of miR-892a in LC specimens and cells. (a) Microarray of differentially expressed miRs in the specimens of three patients with LC and three normal healthy tissues. (b) Decreased miR-892a expression of LC samples ($n = 17$) versus that of normal laryngeal tissue ($n = 9$). (c) The miR-892a expression of LC and NP69 cells was observed via RT-qPCR ($n = 3$). Data are shown as average ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

expression were damaged by the miR-892a inhibitor (Figure 3(c), (e), (f) and (h)), whereas Bax expression was promoted compared with that of the NC inhibitor group in a dose-dependent manner (Figure 3(d), (e), (g) and (h)).

Dicer is a target of miR-892a

Bioinformatic analysis showed that miR-892a targeted Dicer (Figure 4(a)). DLRA was performed to assess the direct link of Dicer 3′-UTR and miR-892a. The findings show that transfection with a miR-892a mimic reduced luciferase activity (LA), fused with Dicer 3′-UTR, by 65% compared with the negative controls (Figure 4(b)). We examined the expression of Dicer in the LC specimens and cell lines. We found that miR-892a was significantly reduced in the LC specimens and cell lines compared with that in the normal healthy group and NP69 cells, and there was a significant negative correlation between miR-892a and Dicer mRNA expression in human laryngocarcinoma tissues. (Figure 4(c) to (e)). Via WB and RT-qPCR, Dicer expression was tested in the TU212 and M4E cells subjected to different dosage of miR-892a mimic/inhibitor or their NCs. The mRNA and protein expression of Dicer was significantly reduced after miR-892a mimic transfection in a dose-dependent manner (Figure 4(f), (g), (j) and (k)), while Dicer expression was clearly elevated due to miR-892a inhibition (Figure 4(h), (i), (l) and (m)). The data indicated that miR-892a targeted Dicer 3′-UTR.

Dicer inactivation offsets the effect of miR-892a on LC cell proliferation and apoptosis

To further determine whether Dicer was involved in miR-892a-mediated LC properties, Dicer expression was silenced in the TU212 and M4E cells subjected to miR-892a inhibition. The knockdown of Dicer was confirmed by RT-qPCR and WB (Figure 5(a) to (d)). By performing the colony formation assay, Dicer silencing restored the growth rate of TU212 and M4E cells that had been suppressed by the miR-89a inhibitor (Figure 5(e) and (f)). FC was conducted to evaluate apoptosis in the TU212 and M4E cells. Dicer silencing brought about a remarkable reduction in the number of apoptotic cells subjected to miR-892a inhibition (Figure 5(g) and (h)). In other words, silencing of Dicer expression reversed the growth and apoptosis of TU212 and M4E cells that had been inhibited by miR-892a inhibition.

To evaluate the role of Dicer on the LC properties, Dicer itself was overexpressed in the TU212 and M4E cells. The RT-qPCR and WB data revealed that Dicer expression was upregulated after transfection (Figure 6(a) to (d)). Colony formation assay showed that Dicer overexpression repressed the growth rate of LC cell lines (Figure 6(e) and (f)). Flow cytometry data clearly displayed that Dicer overexpression resulted in the increased apoptotic rate of LC cells (Figure 6(g) and (h)).

MiR-892a inhibition suppressed LC tumor occurrence in mice

The mice were subcutaneously injected with the adenoviral-miR-892a inhibitor-transfected M4E cells used for investigating the role of miR-892a inhibition in xenograft LC tumor occurrence. miR-892a and Dicer expression in the tumor samples was examined; expression of miR-892a was downregulated, whereas that of Dicer was upregulated in the inoculated mice (Figure 7(a)). The mice were humanely destroyed four weeks later, and the tumors were excised and weighed. The findings showed that the tumors in the miR-892a silencing group had a slower growth rate and a notably reduced average weight and volume than those in the control group (Figure 7(b) and (c)).

Discussion

Tumorigenesis, inducing epigenetic alterations in tumor occurrence, is a synergetic course associated with many tumor inhibitor genes and oncogenes. Although LC has been studied in many previous studies, the molecular mechanisms underlying LC progression still need to be

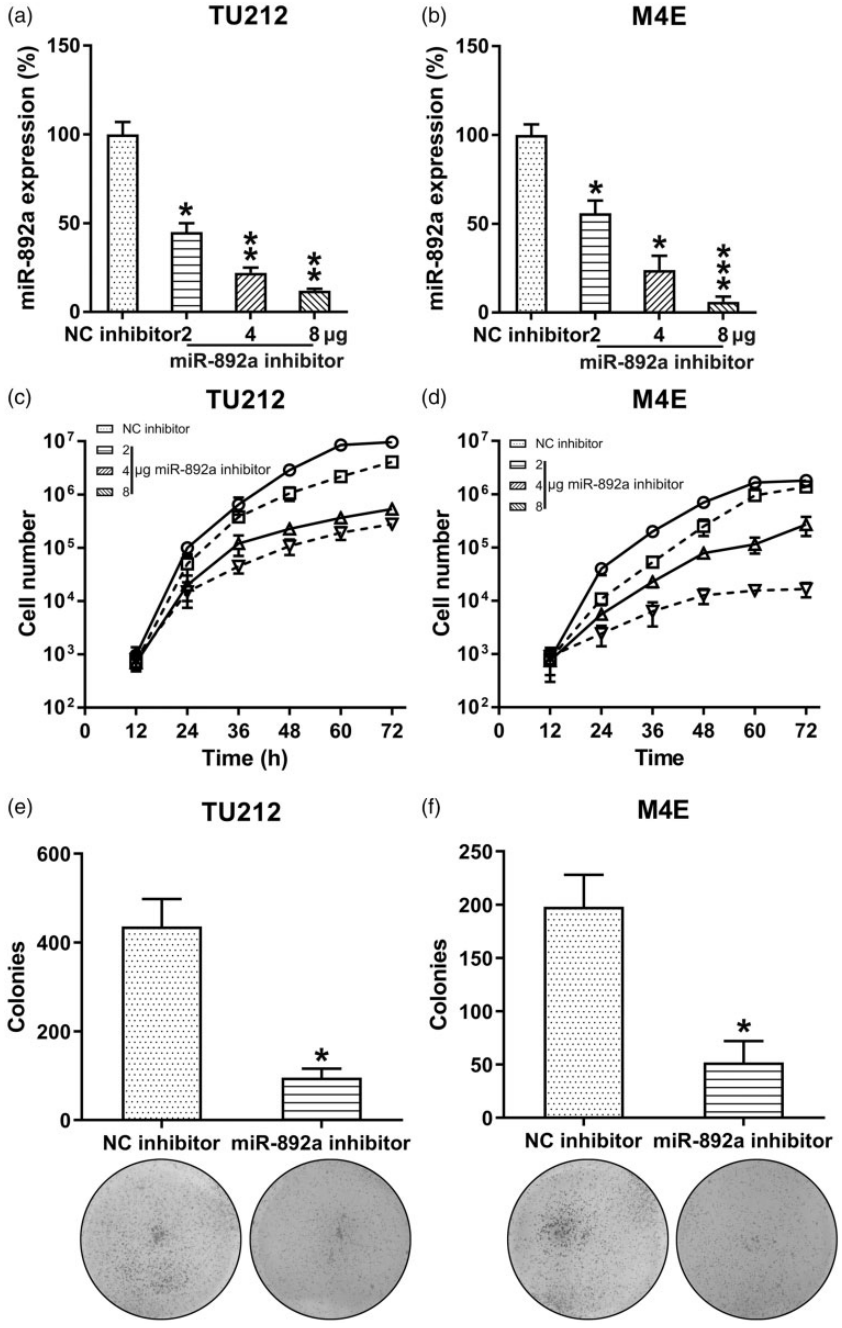

Figure 2. The part of miR-892a expression in LC cell growth and proliferation. TU212 and M4E cells were transfected with NC inhibitor or different dose of miR-892a inhibitor (2, 4, and 8 μg) for 48 h. (a, b) miR-892a expression in TU212 and M4E cells subjected to miR-892a or NC inhibitor was determined through RT-qPCR. (c, d) Proliferation of TU212 and M4E cells was determined at 12, 24, 36, 48, 60, and 72 h after transfection via the CCK-8 assay. (e, f) Soft agar colony formation assay with TU212 and M4E cells subjected to miR-892a or NC inhibitor indicated the colony number in all groups. $n = 3$, Data are displayed as average ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

investigated further. In the current study, miR-892a, which exhibited elevated expression in LC cells and tissue specimens of patients with LC, exerted an inhibitory effect on Dicer expression. Silencing of miR-892a in TU212 and M4E cells hindered cell proliferation and growth and promoted apoptosis. Furthermore, miR-892a directly associates with Dicer 3′-UTR and inhibits its expression. Dicer knockdown expression reversed LC cell suppression resulting from miR-892a downregulation. The *in vivo* study showed that miR-892a silencing reduced LC tumor formation in the mouse model. The findings strongly exhibited that miR-892a acted as an LC oncogene via its action on Dicer and confirmed its use as a diagnostic indicator or promising agent for LC treatment.

Apoptosis, identified as programmed cell death, retains the balance of cell survival and cell death. Apoptosis dysfunction leads to serious consequences; nevertheless, aberrantly augmented apoptosis promotes the development of conditions relevant to degeneration. Previous studies have demonstrated that Dicer depletion or downregulation is

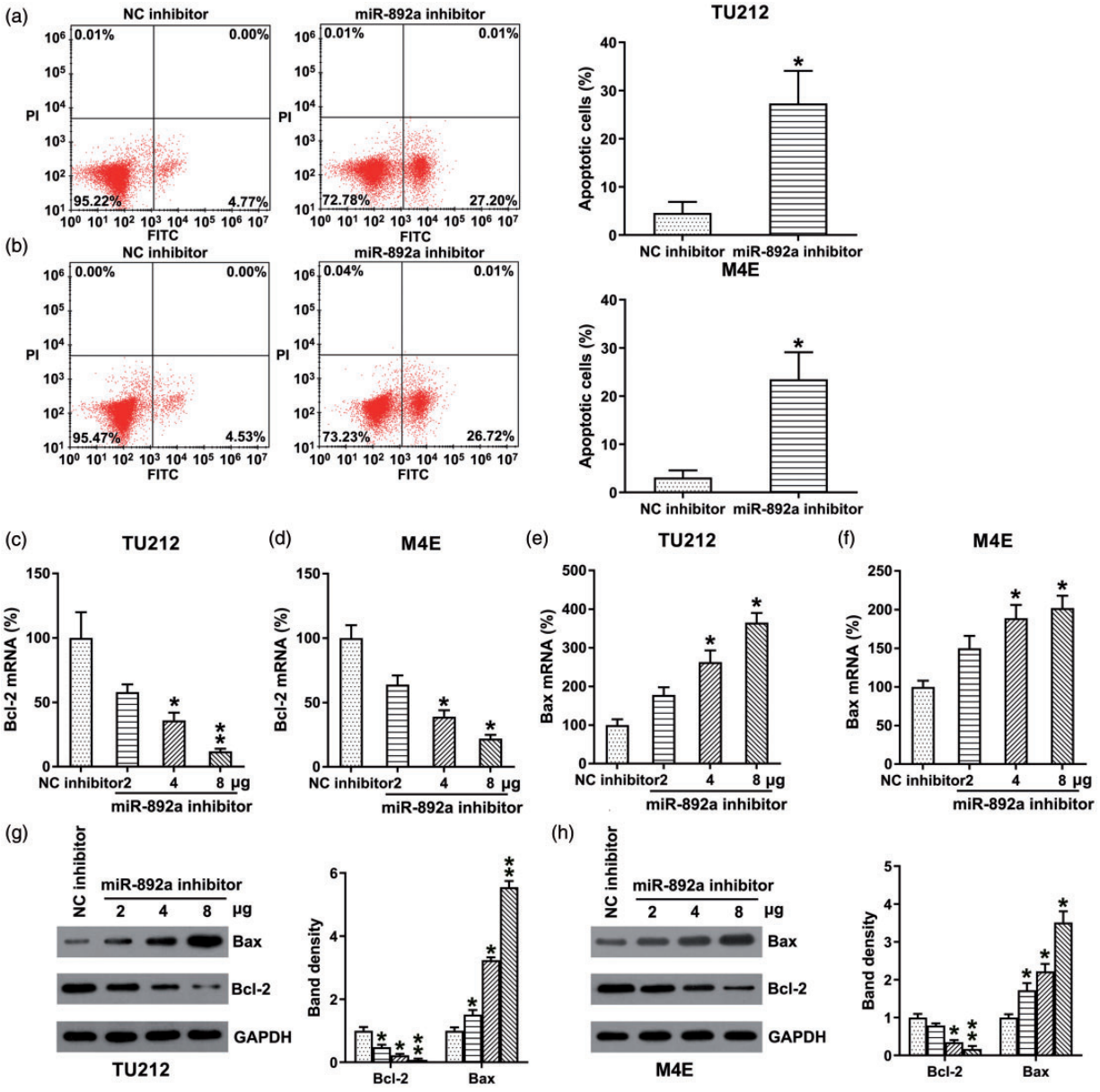

Figure 3. Inhibited miR-892a expression triggered TU212 cell and M4E cell apoptosis. TU212 and M4E cells were transfected with NC inhibitor or different dose of miR-892a inhibitor (2, 4, and 8 μg) for 48 h. (a, b) Annexin V/PI FC was conducted to assess the number of early apoptotic cells, which were presented in the right quadrant of the plots. The apoptotic rates of the TU212 and M4E cells were indicated in the right panel. (c–h) miR-892a inhibition expression modulated the expression of Bcl-2 and Bax mRNA and protein in the TU212 and M4E cells via RT-qPCR and WB. $n = 3$, Data are displayed as average ± SD. $^*P < 0.05$. (A color version of this figure is available in the online journal.)

essential to inhibit apoptosis.[15,16] Dicer acts as the ribonuclease III for mature functional miRNA preparation, thereby assuming a critical role in modulating cell development. Dicer 1 expression in the granulosa follicle and oocyte cells of mice was detected.[17] Dicers (1 and 2) could convert long double-stranded RNA or miRNA precursors into miRNA and small interference RNA or mature effector interference RNA molecule pathways, respectively.[18] Sky *et al.* suggested that Dicer was able to be downregulated by beta-catenin to promote ovarian cancer metastasis.[19] Furthermore, Wang *et al.*[20] showed that decreasing Dicer dramatically suppressed cisplatin-triggered apoptosis in ovarian cancer cells and reduced the protein levels associated with the apoptosis pathways, such as P73, P63, P53, and caspases 3 and 9. Dicer triggers paclitaxel chemosensitization and inhibits cancer stemness in breast cancer through AXL suppression.[21] In bladder cancer cells, Dicer ablation facilitates an intrusive and mesenchymal phenotype, higher matrix metalloproteinase-2 levels, and reduced pivotal miRNA levels.[22] However, Cao *et al.*[23] suggested that upregulated Dicer expression was related to unsatisfactory prognosis in laryngeal squamous cell carcinoma. Kaplan–Meier's survival analysis indicated a close relevance of Dicer expression to the survival of patients with laryngeal squamous cell carcinoma. However, in the present study, we observed downregulation of the Dicer

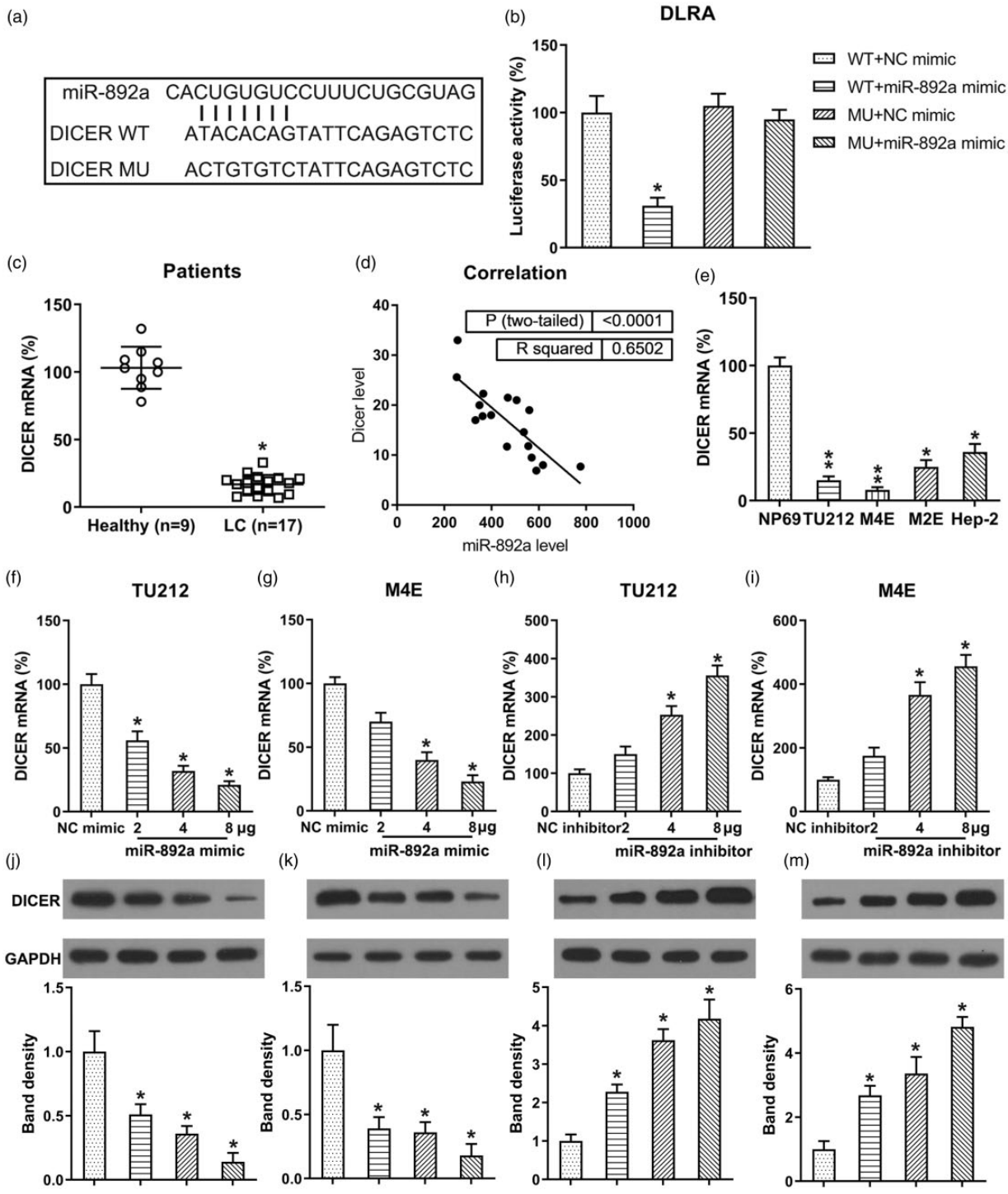

Figure 4. MiR-892a directly targeted Dicer. (a) Graphical illustration of the conserved miR-892a binding motif at Dicer 3′-UTR. The miR-892a seed region-complementary sequences and relevant Dicer 3′-UTR of the three species are conserved. (b) The LA of luciferase reporter constructs with WT or MU of Dicer 3′-UTR following miR-892a mimic transfection. The firefly LA was standardized with renilla as the reference. Upregulated miR-892a dramatically reduced relative LA in WT 3′-UTR but not in MU Dicer mRNA 3′-UTR. (c) Dicer mRNA expression in the LC samples ($n = 17$) versus that in normal laryngeal tissue ($n = 9$). (d) Significant correlation was observed between miR-892a and Dicer expression in human laryngocarcinoma tissues ($n=17$). (e) miR-892a expression in human LC and NP69 cells was observed via RT-qPCR. TU212 and M4E cells were transfected with NC mimic/inhibitor or different dose of miR-892a mimic/inhibitor (2, 4, and 8 μg) for 48 h. (f, g, h, i) RT-qPCR and (j, k, l, m) WB were used for demonstrating Dicer expression in TU212 and M4E cells. $n = 3$, Data are displayed as average ± SD. $^*P < 0.05$.

expression in the LC cell lines, whereas the transfection of miR-892a inhibitor further increased Dicer expression, suggesting that Dicer expression is positively correlated with that of miR-892a. Furthermore, silencing of Dicer led to a reduction in the apoptotic cell number, therefore it restored cell growth of TU212 and M4E cells. These observations revealed that miR-892a inhibited proliferation and induced apoptosis of LC cells via interacting with miR-892a.

Briefly, this work was directed toward exploring the role of miR-892a in the viability of TU212 and M4E cells and

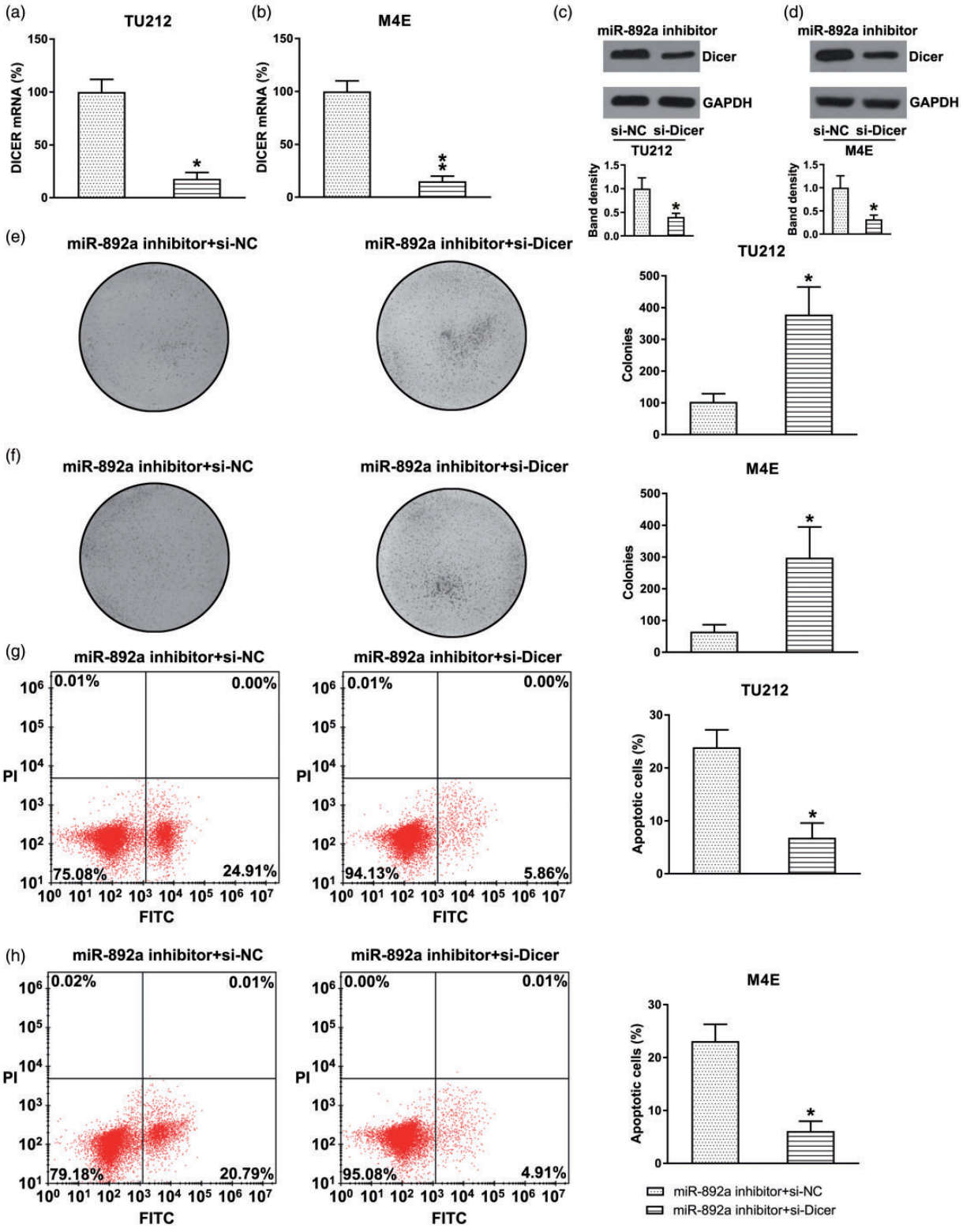

Figure 5. Inhibited Dicer expression reversed the suppressive function of miR-892a inhibition in LC cell characteristics. TU212 and M4E cells were co-transfected with miR-892a inhibitor and siNC (or siDicer) for 48 h. (a–d) The Dicer mRNA and protein expression were examined in TU212 and M4E cells with different transfection. (e, f) Silencing of Dicer promoted the growth of the LC cells by colony formation assay. (g, h) Annexin V/PI FC was used for assessing apoptosis of TU212 and M4E cells differentially transfected at two days. $n = 3$, Data were displayed as average ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

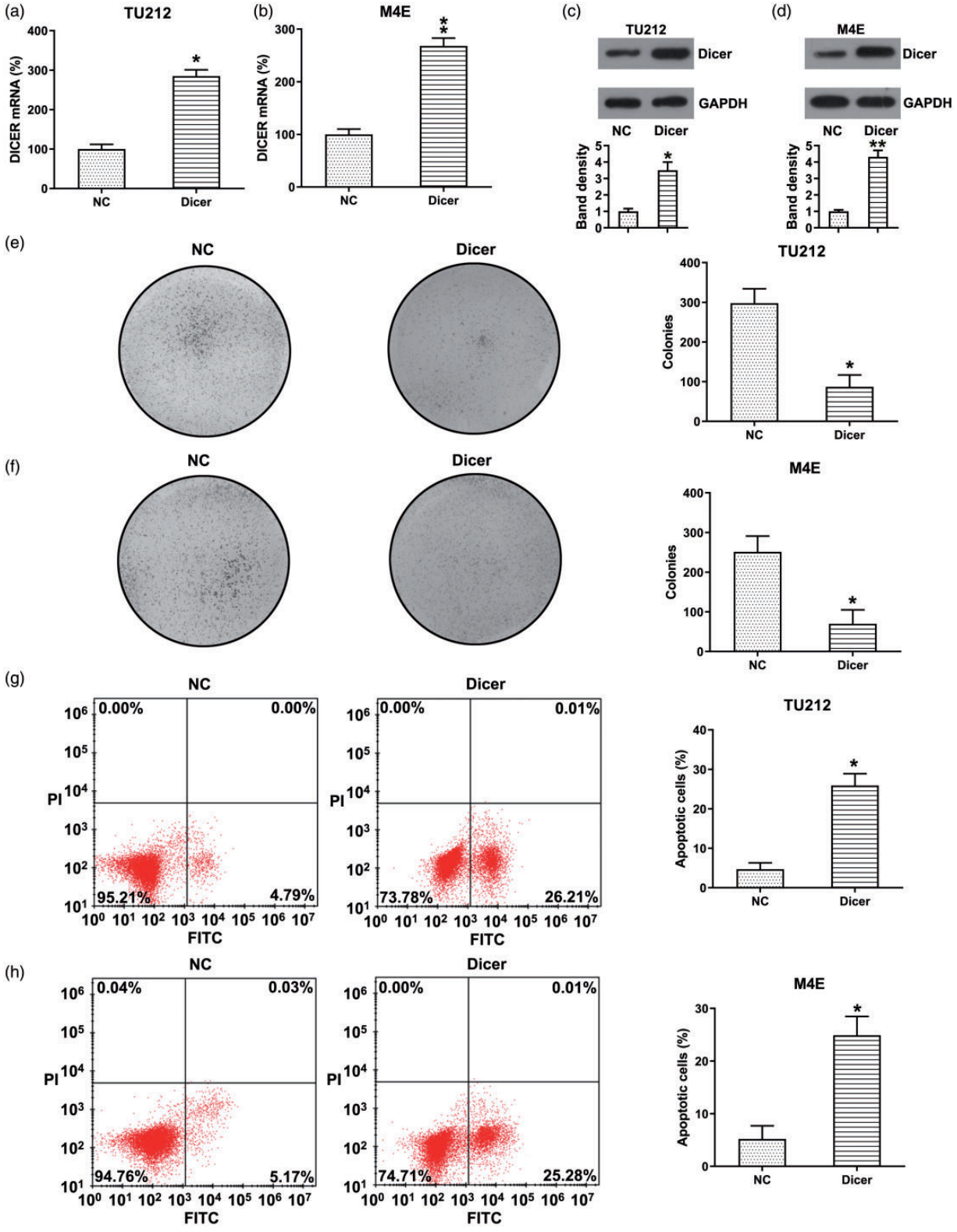

Figure 6. Effect of overexpression of Dicer on the LC cell characteristics. TU212 and M4E cells were transfected with pcDNA3.1-Dicer or pcDNA3.1 for 48 h. (a–d) The Dicer mRNA and protein expression were examined in TU212 and M4E cells with different transfection. (e, f) Overexpression of Dicer inhibited the growth of the LC cells by colony formation assay. (g, h) Annexin V/PI FC was used for assessing apoptosis of TU212 and M4E cells at two days after transfection with pcDNA3.1-Dicer. $n = 3$, Data were displayed as average ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

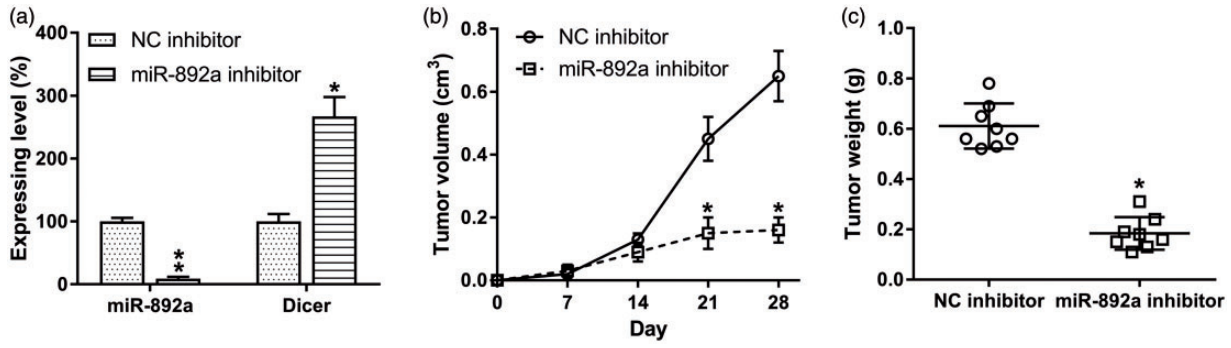

Figure 7. MiR-892a inhibition suppresses xenograft LC occurrence. M4E cells were subjected to adenoviral-miR-892a/NC inhibitor for one day and introduced into the right back flanks of the BALB/c nude mice through subcutaneous injection (aged 42 days). (a) The miR-892a and Dicer mRNA expression in the LC samples was detected through RT-qPCR. (b, c) Mice were humanely destroyed, and the tumor blocks were taken out and weighed four weeks after inoculation. $n = 8$, Data are displayed as average ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

showed the potential role of the miR-892a–Dicer axis during LC development. Therefore, miR-892a can serve as a diagnostic and prognostic biomarker for LC.

Authors' contributions: DJH and XO designed experiments; WJX, SCG and ZHH carried out experiments, analyzed experimental results. DJH wrote the manuscript, XO revise the manuscript. All authors approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Natural Science Foundation of Hebei Province (No. H2016206535).

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(Received February 11, 2020, Accepted May 21, 2020)