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Original Article

KDM6A regulates cell proliferation, invasion and apoptosis by transcriptional inhibits *p16^{ink4a}*

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Abstract: Lysine demethylase 6A (KDM6A) plays a crucial function in several cancers. However, there is little known about the function of KDM6A in gastric cancer. Here, we found that KDM6A expression level was obviously higher in gastric tissues and cells, compared with normal tissues and normal gastric mucosal cells. In addition, the expression of KDM6A was correlated with tumor size, metastasis, tumor stage and poor prognosis. Cell apoptosis assay suggested that KDM6A suppressed cell apoptosis under challenged with or without VP-16 (etoposide). Colony formation assay and CCK-8 assay suggested that knockdown of KDM6A suppressed cell proliferation, and cell cycle progression assay demonstrated that knockdown of KDM6A arrested cell at G_0/G_1 phase. On the contrary, over expression of KDM6A promoted cell proliferation through facilitated cell G_1/S phase transition. To further investigate the mechanism KDM6A contributed of the cell cycle progression, western blot, ChIP assay and luciferase reporter assay suggested that KDM6A transcriptional inhibited *p16^{ink4a}*. Furthermore, KDM6A improved the ability of CTC-141 and MKN45 cells metastasis and anchorage-independent growth. These data demonstrated that KDM6A might serve as an oncogene, promote gastric cancer cells proliferation through transcriptional inhibition of *p16^{ink4a}*, KDM6A also suppressed cell apoptosis, but the detail mechanism was unknown.

Keywords: KDM6A, proliferation, apoptosis, metastasis, p16^{ink14}

Introduction

Gastric cancer (GC) has become the fourth common cancer worldwide [1, 2]. It is crucial to identify the oncogenes which facilitate gastric cancer proliferation and metastasis. These oncogenes may help us to improve the early detection of gastric cancer and provide novel therapy targets. Post-translational modifications of amino acids in histone tails and nucleic acids in DNA are important events that regulate multiple cells process [3]. For example, lysine methylation and acetylation of histone 3 lysine 4 (H3K4) are closely correlated with gene transcriptional activation [4]. Abnormal epigenetic modifications are associated with numerous human diseases, such as cancer, metabolic disorders and ammatory disorders [5].

KDM5A, also known as RBP2, belongs to the KDM5 family of demethylases [6]. All family members containsa Jumonji C (JmjC) domain and catalyzes histone H3 lysine 4 (H3K4) demethylation [7, 8]. KDM5A has been found that isoverexpressed in gastric carcinoma and plays crucial function in senescence [9]. KDM6A is also a demethylase, however, the function of KDM6A in gastric cancer is little known.

P16^{ink4a}, which serves as a tumor suppressor, belongs to CDK inhibitor (CKI) proteins. It binds to CDK4 and suppresses CDK4-cyclin D complex to phosphorylate retinoblastoma protein, thereby arrests cell at G_1 checkpoint [10, 11].

Here, we found that KDM6A was up-regulated in gastric cancer, and its expression was correlated with tumor size, metastasis and poor prognosis. Moreover, KDM6A promoted cell proliferation and suppressed cell apoptosis. In addition, gastric cancer cell metastasis was also regulated by KDM6A.

Materials and methods

Gastric cancer tissue samples

The 67 pairs of gastric cancer tissues and adjacent normal tissue samples were collected from Ningbo First Hospital during 2014-2016.

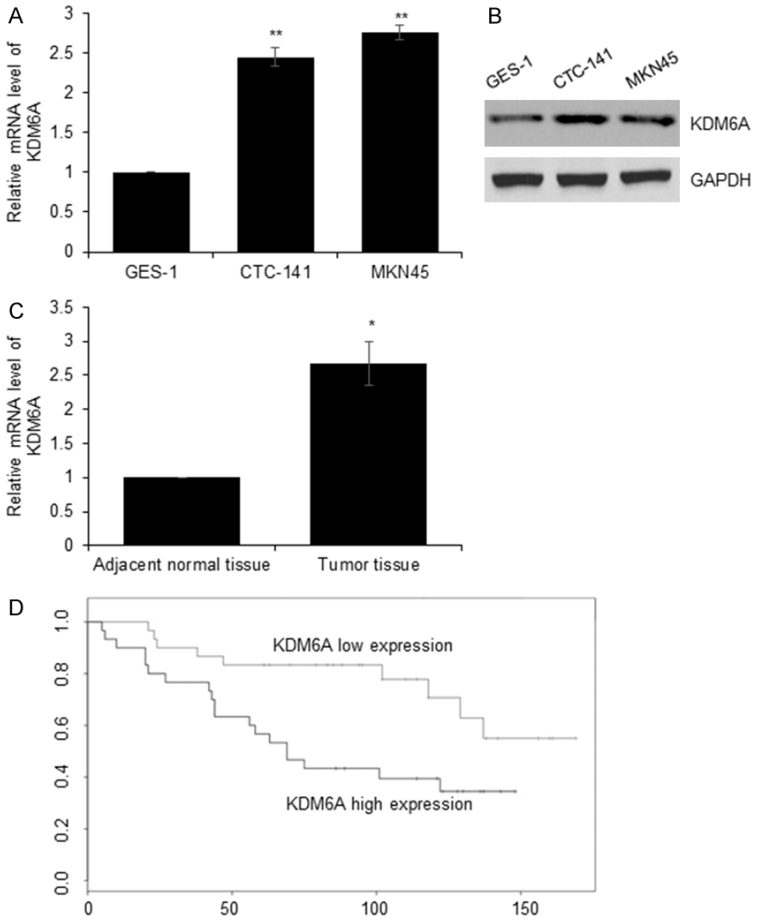

Figure 1. KDM6A is up-regulated in gastric cancer tissue and cell lines. A: qRT-PCR was performed to measure the mRNA level of KDM6A in gastric cancer cell lines, CTC-141 and MKN45, compared with GES-1 (***P*<0.01). B: Western blot was performed to measure the protein level of KDM6A in gastric cancer cell lines, CTC-141 and MKN45, compared with GES-1. C: Tissue KDM6A was detected in 67 pairs of gastric cancer patients by qRT-PCR (**P*<0.05). D: Survival curve was drawn by Kaplan-Meier method.

The experiments about tissue samples were approved by the Clinical Research Ethics Committee of Ningbo First Hospital, and all participants had been provided informed consent.

Cell culture

The human GC cell lines CTC-141, MKN45 and human normal gastric mucosal cell line GES-1 were purchased from ATCC. Cells were incubated in RPMI-1640 medium which contained with 10% fetal bovine serum (FBS) (HyClone, USA) in at 37°C with 5% CO_2.

RNA isolation and qRT-PCR

The total RNA was isolated from tissue samples and the cell lines using NucleoZOL reagent (MN, Germany) and TRIzol reagent (Invitrogen, USA), respectively. Reverse transcription reactions were conducted by Omniscript RT kit (Qiagen). The qRT-PCR was performed with special primers. The primers for *KDM6A* forward 5'-TGAATGAACTTGAGGCAATGAC3' and reverse 5'-AGCGACTACCCACTTTAGAC3'; *CCND1* forward 5'-TGGCCTCTAAGATGAAGGAG-3', and reverse 5'-GAACTTCACATCTGTGGCAC-3'; *CCNE1* forward 5'-TGATTATGAAGGCCCTTAAGTG-3', and reverse 5'-ACCATAAGGAAATTCAAGGCAG-3'; *p15*^{ink4b} forward 5'-GTGGGAAAGAAGGGAAGAG-3', and reverse 5'-ATCATCATGACCTGGATCG-3'; *p16*^{ink4a} forward 5'-CGGAGGAAGAAAGAGGAG-3', and reverse 5'-TCATCATGACCTGGATCG-3'; *GAPDH* forward 5'-GAGAAGTATGACAACAGCCTC-3', reverse 5'-ATGGACTGTGGTCATGAGTC-3'. GAPDH was used as an internal control. All the experiments were independently repeated at least three times.

Westernblot

After transfection, cells were lysed in RIPA buffer (50 mM Tris-HCl PH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 mM deoxycholic acid) containing with phosphatase inhibitors and cocktail of protease inhibitors. The equal amount protein sample (40 μg) was separated by 10% SDS-PAGE gel and transferred onto NC membrane (Millipore, USA). Membranes were blocked with 5% non-fat milk in TBST solution and incubated with indicated primary antibodies at 4°C overnight. Next, Membranes were incubated with secondary antibodies at room temperature for 1 h. Finally, the blots were visualized by ECL Kit (Thermo Scientific). The following antibodies were used: KDM6A, cyclin-D1, cyclin-E1 (abcam, USA); p15^{ink4b}, p16^{ink4a} and GAPDH (CST, USA).

Table 1. Clinic pathologic variables in 67 gastric carcinoma patients

Variables	No. (n=67)	KDM6A protein expression		*P* value
		Low (n=27)	High (n=40)	
Gender				
Male	34	17	17	0.100
Female	33	10	23	
Age				
<50	28	13	15	0.386
≥50	39	14	25	
Tumor size (diameter)				
Small (≤3 cm)	31	19	12	<0.001
Large (≥3 cm)	36	8	28	
Metastasis				
Yes	31	17	14	0.024
No	36	10	26	
Pathological stage				
I-II	22	14	8	0.006
III-IV	45	13	32	
Differentiation				
Well/moderate	39	18	21	0.249
Poor	28	9	19	
Drink status				
Never	32	12	20	0.655
Yes	35	15	20	

CCK-8 assay

Using Lipo2000 transfection reagent (Thermo Fisher, USA), cells were transfected with scrambled siRNA (SCR) or KDM6A siRNA or vector or KDM6A plasmid, after transfection for 48 h, 3 × 10^3 cell were placed in each well in 96-well plates, respectively. Cell growth rate was measured by Cell Counting Kit-8 (CCK-8) (YS-bio, China) according the manufacturer's protocol. 10 μl CCK-8 solution was added in each 100 μl RPMI-1640 medium, and incubated at 37°C for 1 h. The absorbance was measured at 450 nm wavelength. All the experiments were repeated at least three times independently.

Cell cycle progression analysis

Using Lipo2000 transfection reagent (ThermoFisher, USA), cells were transfected with SCR or KDM6A siRNA or vector or KDM6A plasmid, after transfection 48 h, cells were digested by 0.5% trypsin. The collected cells were washed by PBS solution and fixed with 70% ethanol at 4°C overnight. Next, cells were washed with PBS solution and stained by PI at 4°C for 15-20 min avoiding light. Finally, cell cycle progression was analyzed through flow cytometer (BD FACS Calibur, USA). All the experiments were repeated at least three times independently.

Matrigel invasion assay

After transfection for 48 h, the collected cells were suspended in RPMI-1640 medium without serum. 3 × 10^4 cells were placed in 300 μl RPMI-1640 medium without serum in the upper transwell chamber (Corning, USA), the lower well was filled with RPMI-1640 medium which contained 10% FBS. Cells were incubated at 37°C with 5% CO_2 for 24-h and stained with 0.1% crystal violate for visualization, and were counted under microscope. All the experiments were independently repeated at least three times.

Statistical analysis

GraphPad Prism 5 was used to perform all the statistical calculations. The values were analyzed by one-way ANOVA following by Tukey's test. All the results were reported as mean ± SD. $P<0.05$ was considered as statistically significant. All experiments were repeated at least three times.

Result

KDM6A is up-regulated in gastric cancer tissue and cell lines

Previous reports indicated that KDM6A was high expressed in multiple cancer [12-14]. In order to find out whether KDM6A was up-regulated in gastric cancer, we examined the mRNA level and protein level of KDM6A in gastric cancer cell lines, CTC-141, MKN45 and normal bronchial epithelial cell line (GES-1). The results indicated that KDM6A was up-regulated in gastric cancer cell lines, compared with normal bronchial epithelial cell line (GES-1) (**Figure 1A** and **1B**). Next, we collected 67 pairs of adjacent normal tissues and gastric cancer tissues and extracted total RNA from these tissues. The qRT-PCR was used to detect the mRNA level of KDM6A in the tissues, we found that

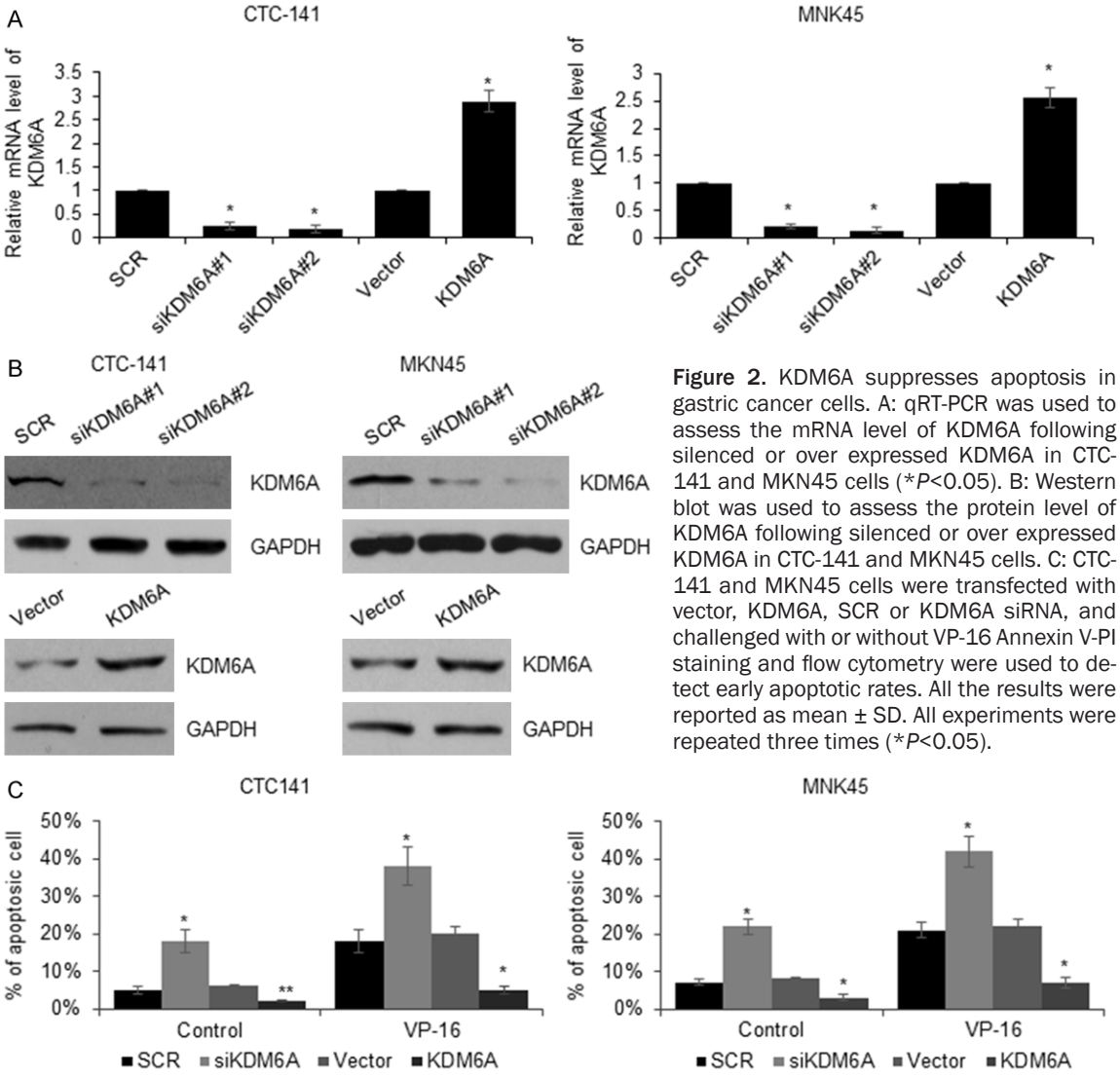

Figure 2. KDM6A suppresses apoptosis in gastric cancer cells. A: qRT-PCR was used to assess the mRNA level of KDM6A following silenced or over expressed KDM6A in CTC-141 and MKN45 cells (*P<0.05). B: Western blot was used to assess the protein level of KDM6A following silenced or over expressed KDM6A in CTC-141 and MKN45 cells. C: CTC-141 and MKN45 cells were transfected with vector, KDM6A, SCR or KDM6A siRNA, and challenged with or without VP-16 Annexin V-PI staining and flow cytometry were used to detect early apoptotic rates. All the results were reported as mean ± SD. All experiments were repeated three times (*P<0.05).

KDM6A expression was also obviously higher in gastric cancer tissues than that of adjacent normal tissues. Moreover, we found that high expression of KDM6A were significantly associated with the tumor stage, tumor size and metastasis (**Table 1**). Survival curve revealed that the patients who have high expression of KDM6A had a poor prognosis (hazard ratio: 1.357; P=0.027, **Figure 1D**).

KDM6A suppresses apoptosis in gastric cancer cells

To determine the function of KDM6A in gastric cancers, we modulated KDM6A expression through two individual KDM6A siRNAs and KDM6A expression plasmid in two gastric cancer cell lines, respectively. QRT-PCR and western blot were used to determine the KDM6A expression, the result showed that KDM6A was dramatically down-regulated in the KDM6A siRNA transfected cells, moreover, the siKDM6A#2 was more efficiency, so we used siKDM6A#2 for subsequent experiments (**Figure 2A** and **2B**). Next, we performed cell apoptosis analysis under indicated experimental conditions. The results indicated that down-regulation of KDM6A facilitated apoptosis in CTC-141 and MKN45 cells under challenging with or without VP-16, however, over expression of KDM6A suppressed cell apoptosis (**Figure 2C**).

KDM6A promotes the proliferation and cell cycle progression

To study the effect of KDM6A on cell proliferation, we performed colony formation assay in CTC-141 and MKN45 cells, the result indicated

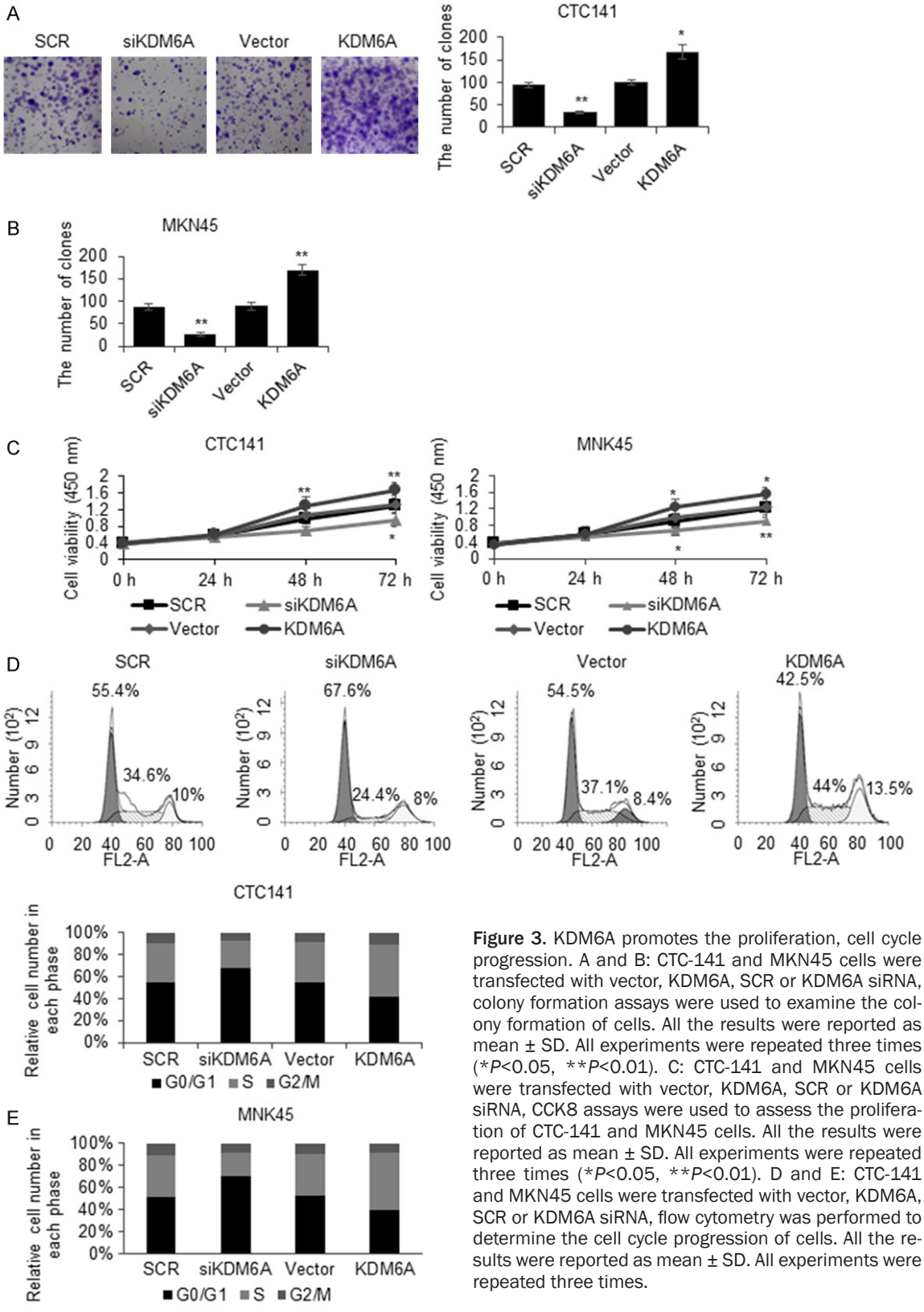

Figure 3. KDM6A promotes the proliferation, cell cycle progression. A and B: CTC-141 and MKN45 cells were transfected with vector, KDM6A, SCR or KDM6A siRNA, colony formation assays were used to examine the colony formation of cells. All the results were reported as mean ± SD. All experiments were repeated three times (*P<0.05, **P<0.01). C: CTC-141 and MKN45 cells were transfected with vector, KDM6A, SCR or KDM6A siRNA, CCK8 assays were used to assess the proliferation of CTC-141 and MKN45 cells. All the results were reported as mean ± SD. All experiments were repeated three times (*P<0.05, **P<0.01). D and E: CTC-141 and MKN45 cells were transfected with vector, KDM6A, SCR or KDM6A siRNA, flow cytometry was performed to determine the cell cycle progression of cells. All the results were reported as mean ± SD. All experiments were repeated three times.

that while knockdown KDM6A, the number of clones were significantly decreased compared with control group, but while over expressed KDM6A, the number of clones were significant-

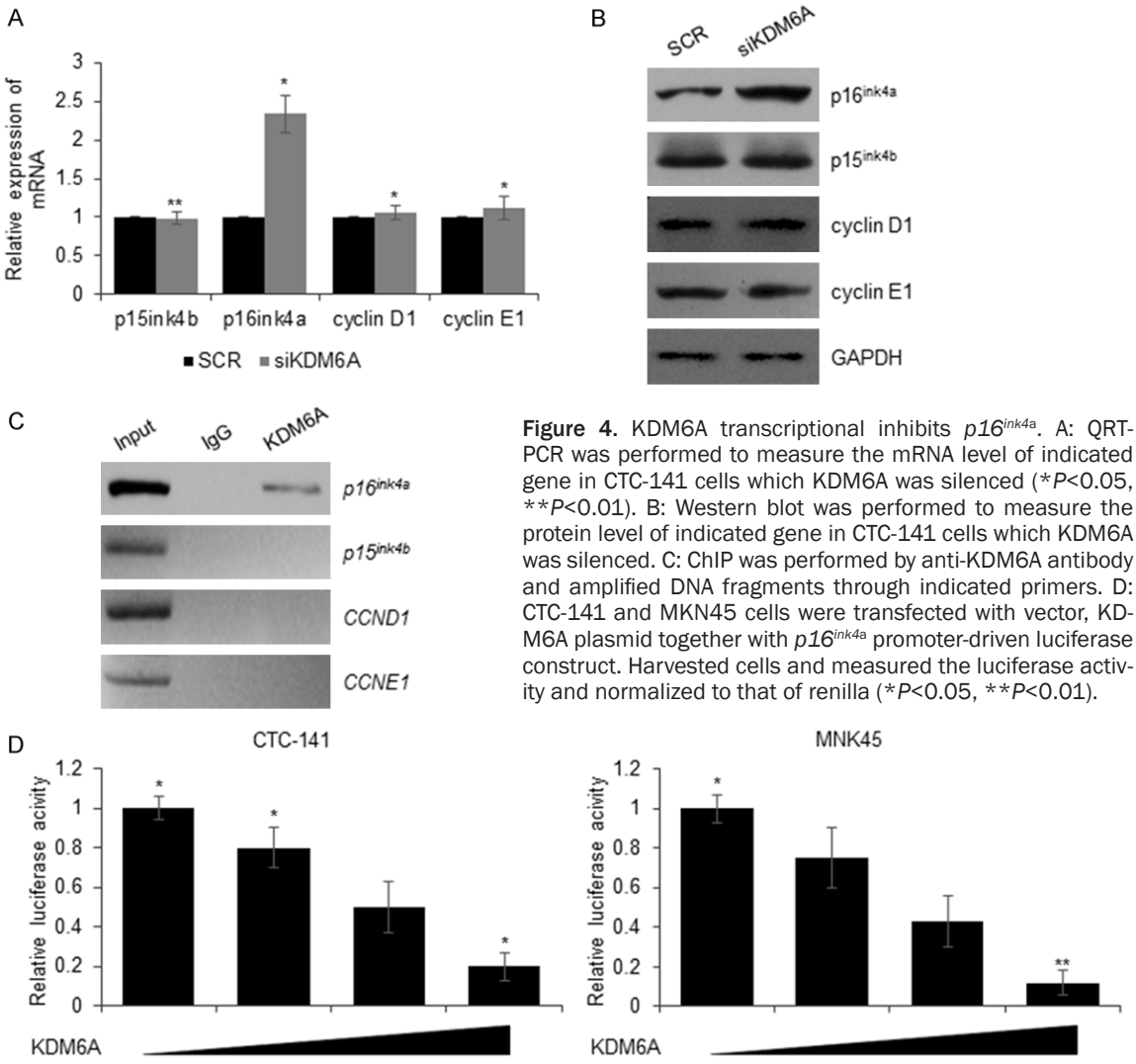

Figure 4. KDM6A transcriptional inhibits *p16*^{ink4a}. A: QRT-PCR was performed to measure the mRNA level of indicated gene in CTC-141 cells which KDM6A was silenced (**P*<0.05, ***P*<0.01). B: Western blot was performed to measure the protein level of indicated gene in CTC-141 cells which KDM6A was silenced. C: ChIP was performed by anti-KDM6A antibody and amplified DNA fragments through indicated primers. D: CTC-141 and MKN45 cells were transfected with vector, KDM6A plasmid together with *p16*^{ink4a} promoter-driven luciferase construct. Harvested cells and measured the luciferase activity and normalized to that of renilla (**P*<0.05, ***P*<0.01).

ly increased compared with control group (**Figure 3A** and **3B**). In addition, CCK-8 assay also confirmed that KDM6A-depletion would inhibit cancer cell growth and ectopic expression of KDM6A promote cancer cell growth (**Figure 3C**). To further explore the mechanism of KDM6A in regulating cancer cell proliferation, we used flow cytometry to detect the cell cycle progression, we found that KDM6A knockdown caused the cell to arrest at G_0/G_1 phase in both CTC-141 and MKN45 cells, and ectopic expression of KDM6A facilitated cell G_1/S phase transition (**Figure 3D** and **3E**).

KDM6A transcriptional inhibits p16^{ink4a}

Our previous work found that KDM6A promoted gastric cancer cell proliferation through facilitating cell G_1/S phase transition, but the molecular mechanism was still unknown. Cell cycle progression was regulated by numerous proteins, including cyclin-dependent kinase (CDK), such as cyclin E1 [15], cyclin D1 [16] and CDK inhibitor (CKI), such as p15^{ink4b} and p16^{ink4a} [17]. Next, we performed qRT-PCR and western blot to determine whether KDM6A regulated those protein. The result demonstrated that the mRNA and protein level of p16^{ink4a} was increased when knockdown of KDM6A in CTC-141 and MKN45 cells. However, cyclin D1, cyclin E1 and p15^{ink4b} were not regulated by KDM6A. While over-expressed KDM6A, p16^{ink4a} were reduced in mRNA and protein level (**Figure 4A** and **4B**). These results revealed that KDM6A transcriptional regulated *p16*^{ink4a}. Next, we performed ChIP assay by anti-KDM6A antibody, and amplified the DNA fragment by special premiers. The result showed that *p16*^{ink4a}

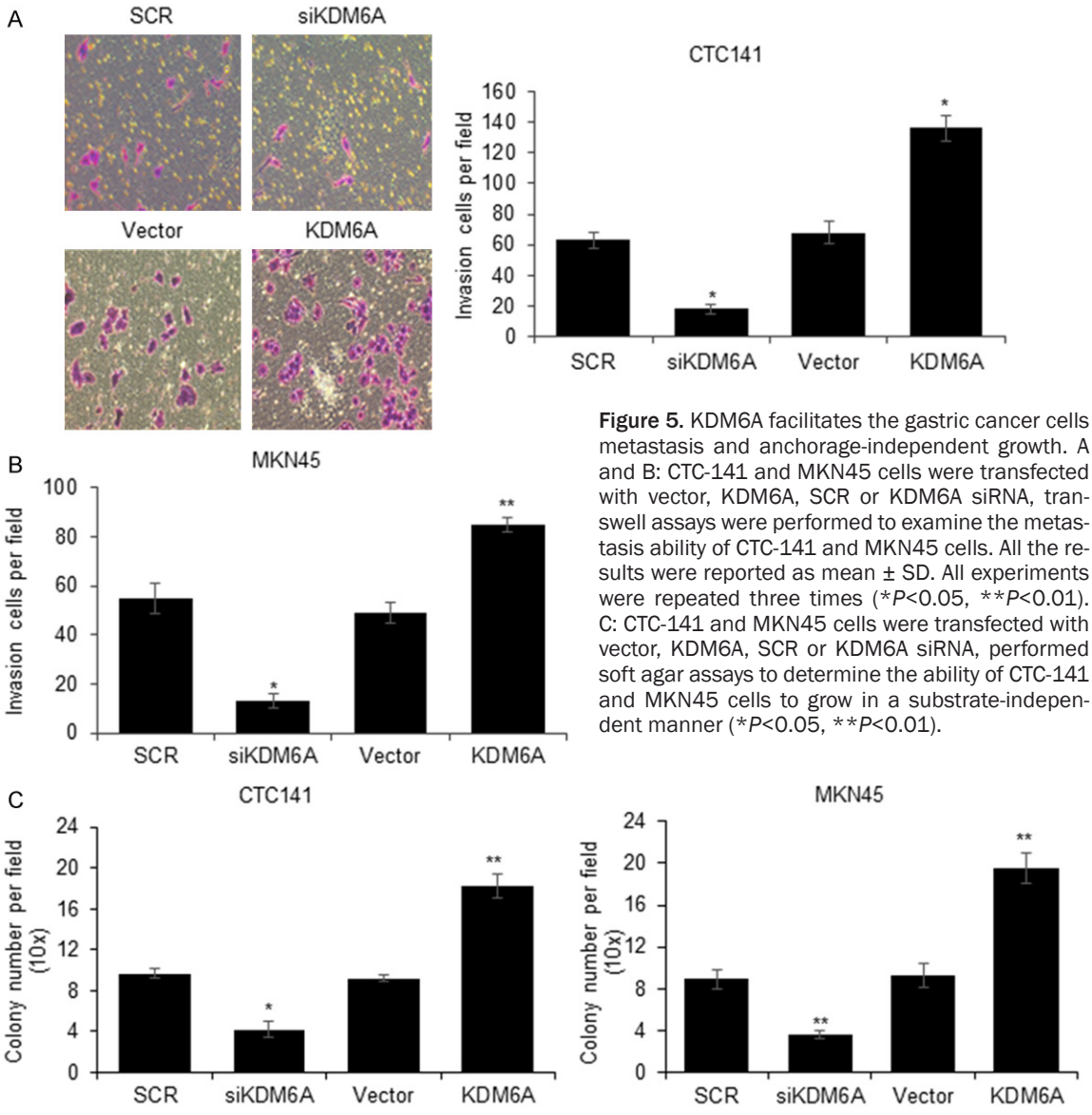

Figure 5. KDM6A facilitates the gastric cancer cells metastasis and anchorage-independent growth. A and B: CTC-141 and MKN45 cells were transfected with vector, KDM6A, SCR or KDM6A siRNA, transwell assays were performed to examine the metastasis ability of CTC-141 and MKN45 cells. All the results were reported as mean ± SD. All experiments were repeated three times (*P<0.05, **P<0.01). C: CTC-141 and MKN45 cells were transfected with vector, KDM6A, SCR or KDM6A siRNA, performed soft agar assays to determine the ability of CTC-141 and MKN45 cells to grow in a substrate-independent manner (*P<0.05, **P<0.01).

promoter region interacted with KDM6A, but *CCND1*, *CCNE1* and *p15*^{ink4b} promoter region did not interacted with KDM6A (**Figure 4C**). Moreover, luciferase reporter assays also confirmed that KDM6A suppressed the activity of *p16*^{ink4a}-luciferase in the CTC-141 and MKN45 cells (**Figure 4D**). Together, our work revealed that KDM6A transcriptional inhibited *p16*^{ink4a}.

KDM6A facilitates gastric cancer cells metastasis and anchorage-independent growth

Cancer cell metastasis is an important event which results in poor prognosis. To investigate whether KDM6A promotesgastric cancer cell metastasis, we evaluated cancer cell metastasis through transwell assay. As shown in **Figure 5A** and **5B**, the invasive cell numbers were obviously reduced following the inhibition of KDM6A in CTC-141 and MKN45 cells, respectively. While in the ectopic KDM6A expression group, the numbers of invasive cells were dramatically increased. Normal cells proliferation-required the solid substratum, but the cancer cells could grow without solid substratum and thereby facilitated cells metastasis. To further investigate whether KDM6A regulated gastric cancer cell anchorage-independent growth, we performed soft agar colony formation assay. We found that inhibition of KDM6A in CTC-141 and MKN45 cells contributed to fewer colonies, and ectopic expression of KDM6A contributed to more colonies compared with control group (**Figure 5C**). The above works demon-

strated that KDM6A improved the ability of CTC-141 and MKN45 cells anchorage-independent growth.

Discussion

Recent studies have revealed that KDM6A plays key functions in several human tumors, but the detail mechanism of KDM6A remains unknown [18, 19]. Moreover, KDM6A plays a crucial role in muscle regeneration and somatic cell reprogramming through its demethylase activity [20, 21]. However, various lineage suggested that KDM6A protein was required, KDM6A regulated early embryonic development and embryonic stem cell differentiation in a demethylase activity of KDM6A independent manner [22-24]. KDM6A is also a subunit of MLL3/4 complex which play key role during cell differentiation [25, 26]. It is still unknown the function of KDM6A on gene expression and tumor development.

In our study, we found that KDM6A was obviously up-regulated in gastric cancer cell lines and GC patients' tissues and high expression of KDM6A was found to be associated with stages of tumor development, tumor size and metastasis. In addition, high expression of KDM6A also predicated a poor prognosis.

Colony formation assay and CCK-8 analysis, suggested KDM6A promoted cell proliferation, moreover, cell cycle progression analysis revealed that KDM6A facilitates cell G_1/S transition. Moreover, we found $p16^{ink4a}$, a CKI that inhibited CDK4-cyclin D complex activity, was negative-regulated by KDM6A. In addition, cell apoptosis analysis demonstrated that KDM6A suppressed cell apoptosis, but the details mechanism were still unknown. We still need to find the downstream target of KDM6A which regulates cell apoptosis. Our previous work suggested KDM6A expression associated with gastric cancer cells metastasis. Transwell assay suggested that KDM6A promoted metastasis ability of gastric cancer cell. In addition, KDM6A also facilitated gastric cancer cell anchorage-independent growth.

KDM6A as a member of KDM family, depending on their enzyme activity, KDMs may act as either oncogenes or tumor suppressors [27]. Although there is a report indicated that deregulation of KDM6A facilitated the EMT in hepatocellular carcinoma (HCC) cells, thereby promotes tumor cell migration, our work suggested that high expression of KDM6A promotes gastric cancer cells metastasis. The different functions of KDM6A may depend on different tumor types or other unknown mechanism. Strikingly, KDM5A has been found to regulate drug resistance [12, 28]. So KDM6A maybe also participates in the regulation of drug resistance, thereby reduces the treatment effect and improves the survival ability of tumor cells.

In brief, the current study suggested that KDM6A promoted gastric cancer cells proliferation and metastasis, meanwhile, KDM6A also suppressed cell apoptosis. As a conclusion, KDM6A may serve as a novel biomarker for gastric cancer and provided a new therapy target.

Acknowledgements

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Disclosure of conflict of interest

None.

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