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MiR-22-3p regulates the proliferation, migration and invasion of colorectal cancer cells by directly targeting KDM3A through the Hippo pathway

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Summary. Colorectal cancer (CRC) has one of the highest incidences and mortality rates of all malignancies worldwide. microRNAs (miRNAs) have been reported to be involved in many biological processes of diseases. MiR-22-3p is considered to be involved in cancer progression, but its role in CRC remains unclear. In this study, we detected that in CRC, the level of miR-22-3p is downregulated. MiR-22-3p has antitumor effects in CRC. miR-22-3p can reduce the proliferative, invasive and migrative capacity of CRC cells. Luciferase reporter analyses confirmed that KDM3A was a target of miR-22-3p, which can directly target the 3'UTR of KDM3A and decrease the expression of KDM3A in CRC cells. Our study also confirmed that KDM3A plays a role as an oncogene in CRC. KDM3A overexpression attenuated the tumor suppressor effects of miR-22-3p in CRC cells, demonstrating that miR-22-3p exerts antitumor effects by targeting KDM3A. Overexpression of miR-22-3p in CRC reduced YAP1 expression, whereas overexpression of KDM3A restored the expression of YAP1. In summary, miR-22-3p might inhibit the progression of CRC by targeting KDM3A to regulate the HIPPO signaling pathway, which may provide an opportunity for the treatment of CRC.

Key words: miR-22-3p, KDM3A, Colorectal cancer (CRC), Hippo pathway, Epithelial-mesenchymal transition (EMT)

Introduction

Colorectal cancer (CRC) is the leading cause of cancer-related death in China and among the leading causes of cancer-related death worldwide (Siegel et al., 2021). Even through improvements in oncological and surgical management of patients with CRC in the last 10 years, no effective cure exists when surgery does not obtain a satisfactory therapeutic effect (Salem et al., 2020). Thus, a better understanding of the pathological mechanism of this cancer must be gained. Previous studies have demonstrated that aberrant gene transcription and aberrant posttranscriptional changes are involved in the pathophysiological process of CRC. However, the exact mechanism of CRC metastasis, recurrence, and drug resistance is not fully understood. To improve outcomes for patients with CRC, further exploration of the exact mechanism of CRC development is necessary.

The deregulation of miRNAs has been confirmed in many types of human malignancies and is associated with tumor cell growth, migration and invasion. MiRNAs refer to small noncoding RNAs that take part in the posttranscriptional regulation of genes. Additionally, miRNAs are precisely denoted as the -3p and -5p mature miRNAs. miR-22 and its mature strand miR-22-3p have been increasingly indicated to be involved in a variety of biological processes and diseases. For example, recent studies have shown that miR-22 can inhibit the malignant behavior of tumor cells (Hu et al., 2019; Sun et al., 2019; Cong et al., 2020). Zhang et al. reported that miR-22-3p alleviates cerebral ischemic injury by modulating the KDM6B/BMP2/BMF

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axis (Zhang et al., 2021). However, the role and exact mechanisms of miR-22-3p in CRC remain unclear.

Lysine demethylase 3A (KDM3A), formerly known as JMJD1A, is highly expressed in CRC and promotes proliferation and invasion (Liu et al., 2019). Mechanistically, KDM3A is fundamental for the enhancer activation of Hippo pathway-related genes in CRC (Wang et al., 2019). The Hippo pathway has been proven to take part in tumorigenesis and is also regarded to play an important role in CRC progression (Pan et al., 2018; Zheng et al., 2019). Recently, a myocardial infarction-induced myocardial injury study showed that miR-22-3p can regulate the expression of KDM3A (Zhang et al., 2020). Considering that KDM3A has been shown to have pro-carcinogenic effects in colorectal cancer, we speculate that miR-22-3p may inhibit colorectal cancer progression by regulating the KDM3A/YAP1 signaling axis.

Currently, KDM3A and the Hippo pathway are valuable targets for therapeutic intervention in CRC. In this research, the results revealed that miR-22-3p was downregulated in CRC. Functional biology experiments revealed that upregulation of miR-22-3p markedly decreased CRC cell proliferation, migration and invasion. We further confirmed that KDM3A is a direct target of miR-22-3p and is an oncogene in CRC progression by regulating the Hippo pathway. Collectively, the results from these studies have the potential to provide insights into the carcinogenesis mechanisms in CRC.

Materials and methods

Cell culture

Human CRC cell lines (CaCO2, DLD-1, SW480 and SW620) and a normal colon mucosal epithelial cell line (NCM460) were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium (Gibco Company, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, and 50 mg/ml streptomycin. The cell incubator conditions were 37°C with a humidified atmosphere containing 5% CO_2.

Transfection assay

Cells were transfected using Lipofectamine™ 3000 (Thermo Fisher Scientific, Inc.) and 10 or 25 nM miR-22-3p mimic (5'-AAGCUGCCAGUUGAAGAACUGU -3'), miR-22-3p inhibitor (5'-ACAGUUCUUCAACUG GCAGCUU-3') or scrambled miRNA control (all purchased from Nanjing Jinbai Co., Ltd., China) were added. Lentiviruses encoding KDM3A (KDM3A-LV), control lentivirus and enhanced infection reagents and polybrene were constructed by JI KAI Gene Technology Co., Ltd. (Shanghai, China). Cells were treated with the lentivirus according to the product manual and selected with puromycin.

Bioinformatics analysis

Published transcriptional profiling data of CRC patients were downloaded from the GEO database (GSE32323) for expression analysis and correlation analyses (Edgar et al., 2002; Khamas et al., 2012; Barrett et al., 2013). The association between rectal adenocarcinoma miR-22 expression levels and overall survival was obtained using the kmplot database (Nagy, et al., 2021). The comparison of KDM3A protein expression between CRC tissues and normal tissues was examined in UALCAN (Chandrashekar et al., 2017). The correlations between KDM3A expression levels and survival rates have been featured in UALCAN. In the survival analysis, samples were categorized into two groups: high expression (with TPM values above the upper quartile) and low/medium expression (with TPM values below the upper quartile). miRBase (Kozomara et al., 2019), TargetScan 7.2 (Agarwal et al., 2015) and miRDB (Chen and Chen, 2020) were used to predict the potential target genes for miR-22-3p.

Real-time quantitative PCR

Total RNA was extracted with TRIzol reagent (TransGen Biotech, Beijing), phase-separated with chloroform, and precipitated using isopropanol. Then, the RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The reverse transcription of total RNA and microRNA was separately performed using PrimeScript RT Master Mix (Takara; RR036A) and the miRcute Plus miRNA First-Strand cDNA Synthesis Kit (KR211, Tiangen) according to the manufacturer's instructions. Similarly, quantitative PCR was performed with the reagents (RR420A, Takara) to detect KDM3A mRNA levels. To detect miR-22-3p levels, an miRcute Plus miRNA qPCR Detection Kit (SYBR Green) (FP411, Tiangen) was used to complete the experiment, and small nuclear U6 was used as an internal standard. In this study, the sequences of primers for RT-qPCR were as follows: KDM3A forward: 5'-3 AAGGTGTGTGTGGAATTTGATG and reverse: 5'-3' AAAATGCTCTCCTTAGAAGGCT. GAPDH forward: 5'-3'CCATGTTCGTCATGGTGTG and reverse: 5'-3'GGTGCTAAGCAGTTGGTGGTG. miR-22-3p: 5'-3'AAGCTGCCAGTTGAAGAACTGT. U6 forward: 5'-3'CTCGCTTCGGCAGCACA and reverse: 5'-3'AACGCTTCACGAATTTGCGT.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8; Yeasen) was used to complete the cell proliferation assay. Cells were seeded in 96-well plates at a density of 10^3 cells per well, incubated at 37°C for 24h, and transfected with miR-22-3p mimic, miR-22-3p inhibitor, or negative controls. After cultivation for 24h, 48h, and 72h, 10 µl CCK-8 reagent was added to each well and incubated for 2h. The

optical density (OD) values were read at 450 nm.

Dual-luciferase reporter assay

Luciferase reporters containing the wild-type (WT) KDM3A 3′UTR were constructed. Relying on the same method, luciferase reporters containing a mutated KDM3A 3′UTR sequence of the miR-22-3p binding site were also constructed (Public Protein/Plasmid Library, Nanjing, China). Following the protocol, SW480 and DLD-1 cells were transfected with miR-22-3p mimics and luciferase reporters by Lipofectamine™ 3000 (Invitrogen). As a control group, the same cells were transfected with a negative control mimic and luciferase reporters.

After 24 hours of transfection, firefly reagent and Renilla reagent were added in turn, and the luciferase activities were detected.

Cell migration and invasion assays

Transwell chambers were used to determine the invasion and migration of CRC cells. SW480 and DLD-1 cells were transfected with nucleic acids or lentiviral virus. After transfection, $10-3\times10^5$ SW480 and DLD-1 cells were seeded into the upper Transwell chamber (8 μm pore size, Corning, USA) inserts covered with or without Matrigel (BD Biosciences, USA). Then, 800 μl medium with 20% FBS was added to the lower chamber. The Transwell chambers were incubated for 48 hours with 5% CO_2 at 37°C. Transwell chambers were then placed in 4% paraformaldehyde. Then, the chambers were stained with 0.5% crystal violet for 30 min. The transmembrane cells were quantified using a microscope at 200× magnification. We randomly selected five visual fields, recorded the number of cells in each field, and calculated the mean value.

Western Blot

Total protein was extracted from CRC cells using radioimmunoprecipitation assay (RIPA) buffer (Solarbio Life Science, Beijing, China), which was mixed with protease inhibitor on precooled plates. Then, the protein concentration was measured via a bicinchoninic acid (BCA) protein assay kit (Solarbio Life Science). Next, the CRC proteins were transferred onto nitrocellulose membranes (Millipore), and 10% SDS-PAGE gels were used for separation. The membranes were coated with specific primary antibodies overnight on a rotary shaker in a 4°C environment. The next day, the membranes were coated with HRP-linked secondary antibodies on a rotary shaker at room temperature for 1h. Finally, Western blot data were quantified by a ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA, USA). Throughout this study, primary antibodies targeting the proteins are listed as follows: PCNA (ab29, 1:1000, Abcam), GAPDH (1:1000, TransGen Biotech, Beijing), β-actin (1:1000, TransGen Biotech, Beijing), KDM3A

(12835-1-ap, 1:10000, Proteintech), and YAP1 (1:1000, Wanlei bio, Shenyang).

Statistical analysis

SPSS 22 software was used for statistical analysis. Data are presented as the means ± SDs of the mean. The data for two group comparisons were first subjected to normality tests. If the datasets fit a normal distribution, an unpaired, two-tailed t test was used, and if not, nonparametric Mann–Whitney and Wilcoxon signed-rank tests were used. Differences among more than two groups were evaluated by one-way ANOVA. All statistical tests were 2-sided with a P value less than 0.05 considered statistically significant.

Results

MiR-22-3p was downregulated in CRC

To assess miR-22-3p expression in CRC tissues, we downloaded the GEO database (GDS4382), which contains the expression profile of 17 tumor tissues and corresponding adjacent normal tissues. By analyzing the GEO database, we observed that miR-22 mRNA expression in CRC tissues was lower than that in normal CRC tissues (Fig. 1A). Furthermore, using quantitative real-time PCR, we analyzed the expression levels of miR-22-3p in CRC cell lines and NCM460 cells. A consistent downregulation of miR-22-3p was observed across CRC cell lines compared with NCM460 (Fig. 1B). To confirm the correlation between the survival rate and expression level of miR-22-3p, Kaplan–Meier analysis of patients with rectal adenocarcinoma using TCGA datasets from Kmplot was performed. The results demonstrated that CRC patients with a high miR-22-3p level had a longer overall survival rate than those with low miR-22-3p expression (Fig. 1C). However, these differences did not reach statistical significance. The above results show that miR-22-3p expression is widely attenuated in CRC and may be inversely associated with prognosis.

MiR-22-3p functions as a tumor suppressor in CRC cells

To test the impact of miR-22-3p on CRC tumor progression, SW480 and DLD-1 cells were transiently transfected with miR-22-3p mimics or negative control mimics. On the other hand, the same cells were transiently transfected with miR-22-3p inhibitor or negative control inhibitor. After 48 hours, RT-qPCR analysis revealed that miR-22-3p was markedly upregulated in the cells treated with miR-22-3p mimics (Fig. 2A). A CCK-8 assay demonstrated that upregulation of miR-22-3p resulted in greater suppression of cell proliferation than the control (mimics NC). (Fig. 2B,C). To assess the prometastatic role of miR-22-3p in CRC, cell migration and invasion were evaluated through a Transwell chamber. The results

showed that the upregulation of miR-22-3p significantly decreased the cellular migration and invasion of SW480 and DLD-1 cells (Fig. 2D-G). Additionally, we used Western blotting to detect pivotal markers of proliferation and EMT, such as PCNA and E-cadherin. In SW480 and DLD-1 cells treated with miR-22-3p mimics, the protein levels of E-cadherin increased, and the protein levels of PCNA decreased (Fig. 2H,I). RT-qPCR analysis revealed that miR-22-3p was markedly downregulated in the same cells treated with miR-22-3p inhibitor (Fig. 3A). The CCK-8 assay demonstrated that downregulation of miR-22-3p enhanced cell proliferation (Fig. 3B,C). Transwell cell migration and Matrigel-coated invasion assays showed that downregulation of miR-22-3p markedly enhanced the migration and invasion abilities (Fig. 3D,G). In contrast, in cells treated with the miR-22-3p mimic, the protein levels of E-cadherin and PCNA were reversed when treated with the miR-22-3p inhibitor (Fig. 3H,I). This suggests that miR-22-3p suppresses tumorigenesis, in part, through its suppressive effects on cellular proliferation, cell migration and invasion.

KDM3A is a direct target of miR-22-3p in CRC cells

To clarify the direct mechanism of miR-22-3p, we forecasted the potential targets of miR-22-3p using miRBase, TargetScan 7.2 and miRDB. Then, bioinformatics analysis demonstrated that KDM3A is the target of miR-22-3p (Fig. 4A). To confirm whether miR-22-3p directly targets KDM3A, we constructed two

luciferase reporters: one had a wild-type (WT) KDM3A 3′UTR, and the other had a mutated KDM3A 3′UTR sequence. Co-transfection experiments confirmed that miR-22-3p mimics markedly inhibited the luciferase activities of the wild-type KDM3A 3′UTR reporter (Fig. 4B), but not those of the mutant 3′UTR (Fig. 4C). Furthermore, correlation analysis showed that there was a negative correlation between miR-22-3p expression and KDM3A mRNA expression (Fig. 4D). To further investigate the function of miR-22-3p in regulating endogenous KDM3A expression, we detected KDM3A mRNA and protein expression in SW480 and DLD-1 cells. Relative to the negative controls, cells treated with miR-22-3p mimics markedly inhibited the expression of KDM3A mRNA (Fig. 4E) and protein (Fig. 4F,G). The results confirmed our predictions that KDM3A was a target of miR-22-3p, which was negatively regulated by miR-22-3p in CRC cells.
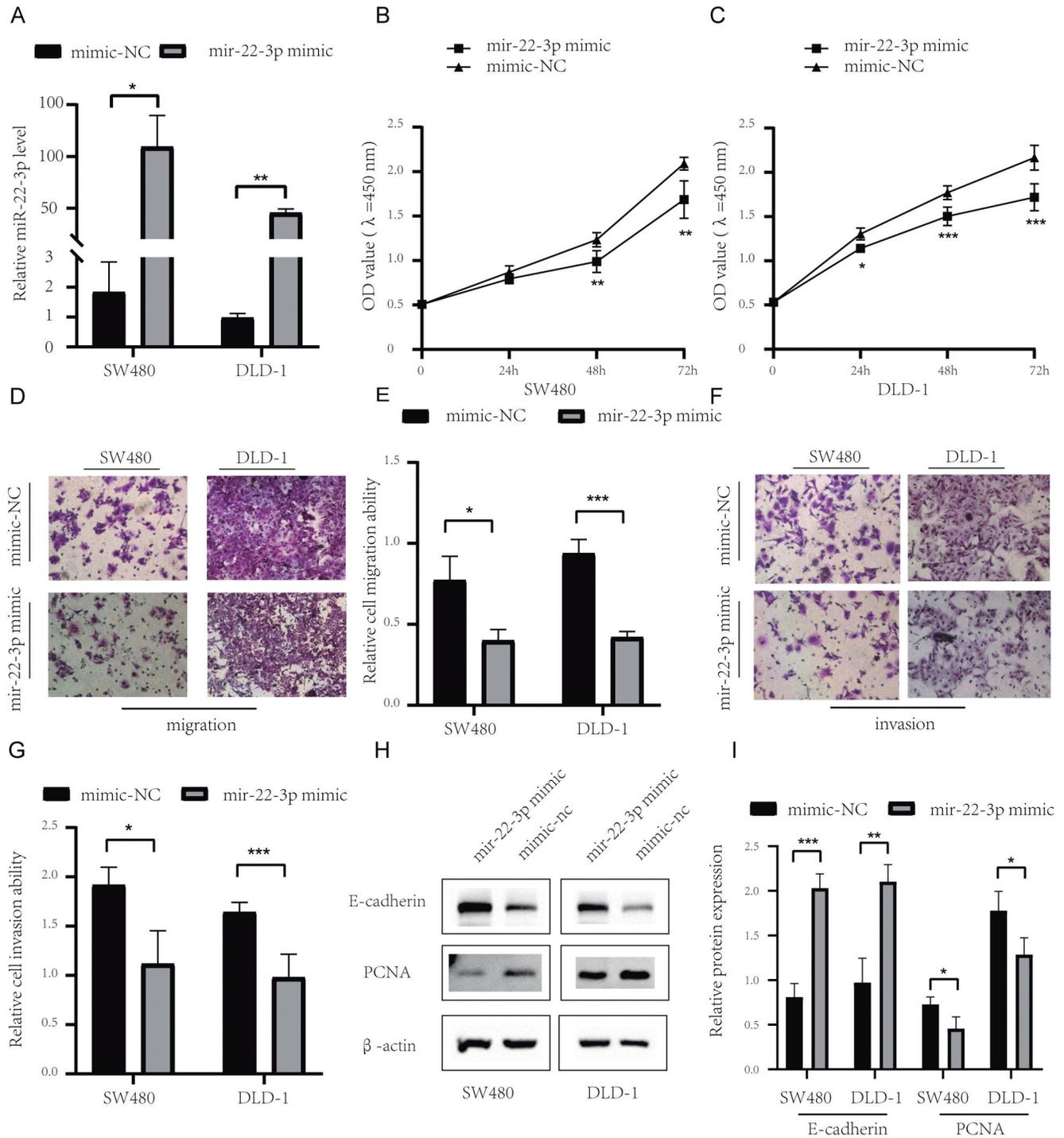
KDM3A serves as an oncogene in CRC cells

To analyze the clinical value of KDM3A in CRC patients, UALCAN was used based on the TCGA database. The results showed that KDM3A expression was highly upregulated in CRC tissues compared with normal tissues (Fig. 5A). Furthermore, the KM curve showed that the group of patients with high expression of KDM3A had higher death rates than the group of patients with low expression of KDM3A (p<0.05) (Fig. 5B). RT–qPCR analysis revealed that compared with those in NCM460 cells, the mRNA levels of KDM3A
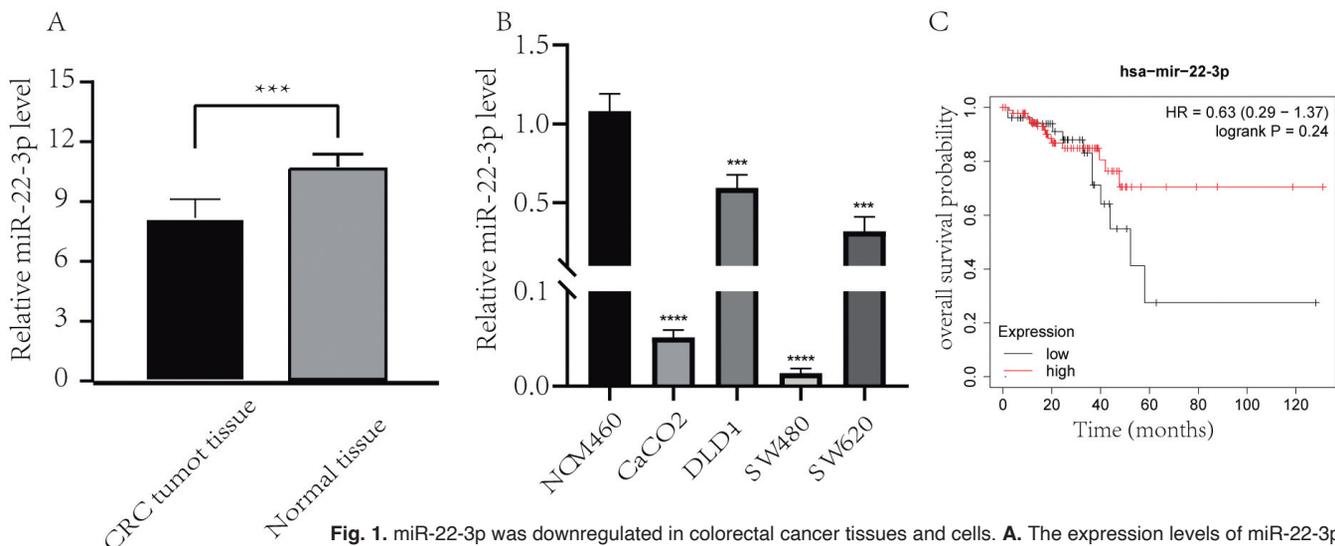

Fig. 1. miR-22-3p was downregulated in colorectal cancer tissues and cells. **A.** The expression levels of miR-22-3p in CRC (n=17) and tumor-adjacent normal tissues (n=17) derived from the GEO database (GSE32323). **B.** An RT-qPCR assay was used to detect miR-22-3p expression levels in NCM460, CaCO2, DLD-1, SW480 and sw620 cells. **C.** Association between miR-22-3p expression and OS time was analyzed using the online Kaplan-Meier survival analysis (KMplot, http://kmplot.com/analysis/). *, p<0.05; **, p<0.01;***, p<0.001; ****p<0.0001. Each experiment with three replicates was repeated three times. A t test or nonparametric test was used for the comparisons of two groups. Differences among more than two groups were evaluated by one-way ANOVA.

Fig. 2. miR-22-3p mimic inhibits tumorigenesis in colorectal cancer. **A.** The expression of miR-22-3p was analyzed in SW480 cells and DLD-1 cells via RT-qPCR after transfection with miR-22-3p mimic or miR-22-3p mimic NC. **B, C.** Analysis of the miR-22-3p mimic on SW480 cells and DLD-1-cell viabilities was confirmed by CCK-8 assay. **D-G.** The miR-22-3p mimic inhibited CRC cell migration and invasion abilities, as demonstrated via Transwell migration assays or Transwell invasion assays. **H, I.** Western blot assays showed the protein levels of E-cadherin and PCNA in SW480 cells and DLD-1 cells transfected with miR-22-3p mimic or miR-22-3p mimic NC. *, p<0.05; **, p<0.01, ***, p<0.001. Each experiment with three replicates was repeated three times. A t test or nonparametric test was used for the comparisons of two groups. Differences among more than two groups were evaluated by one-way ANOVA.

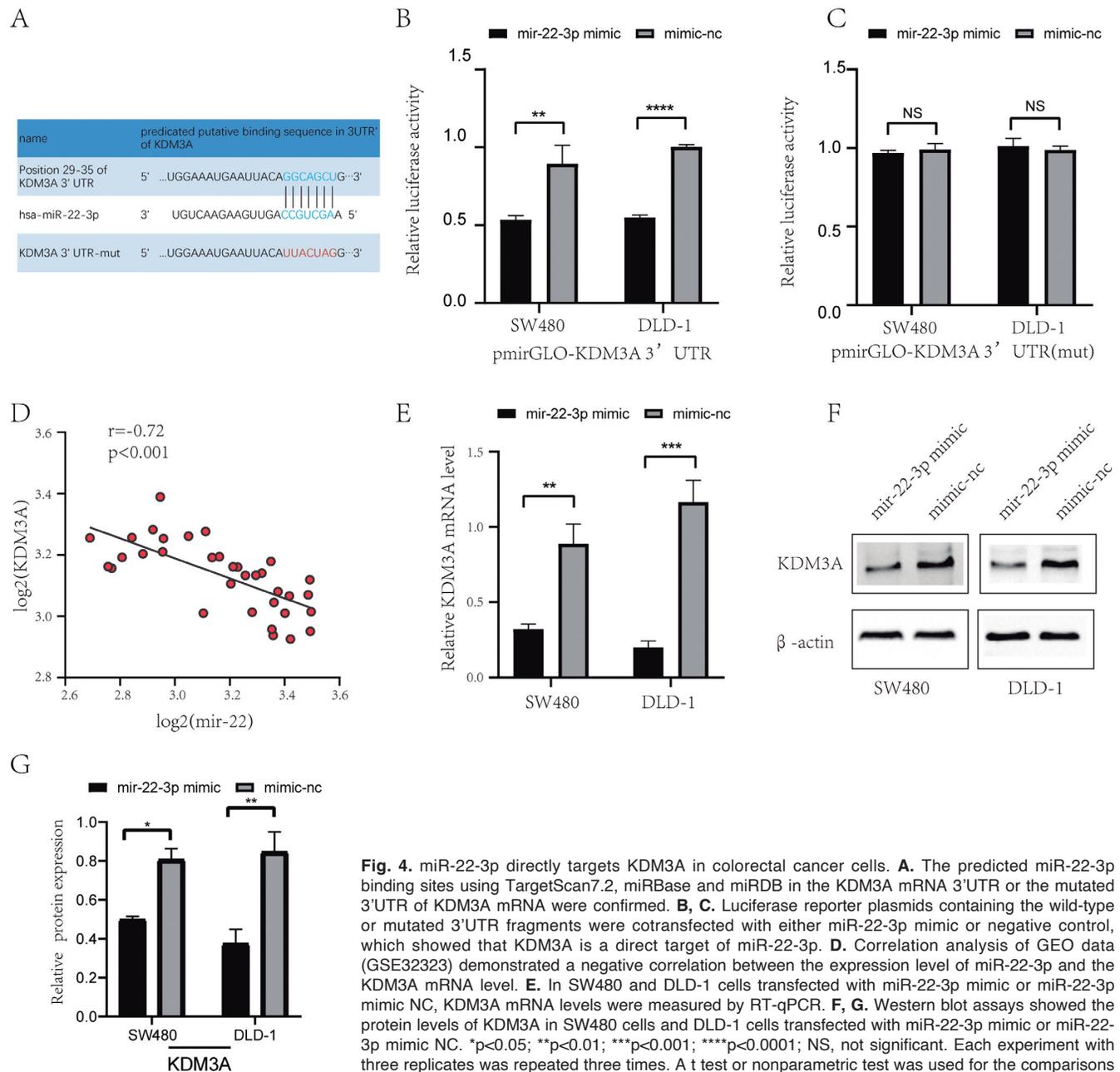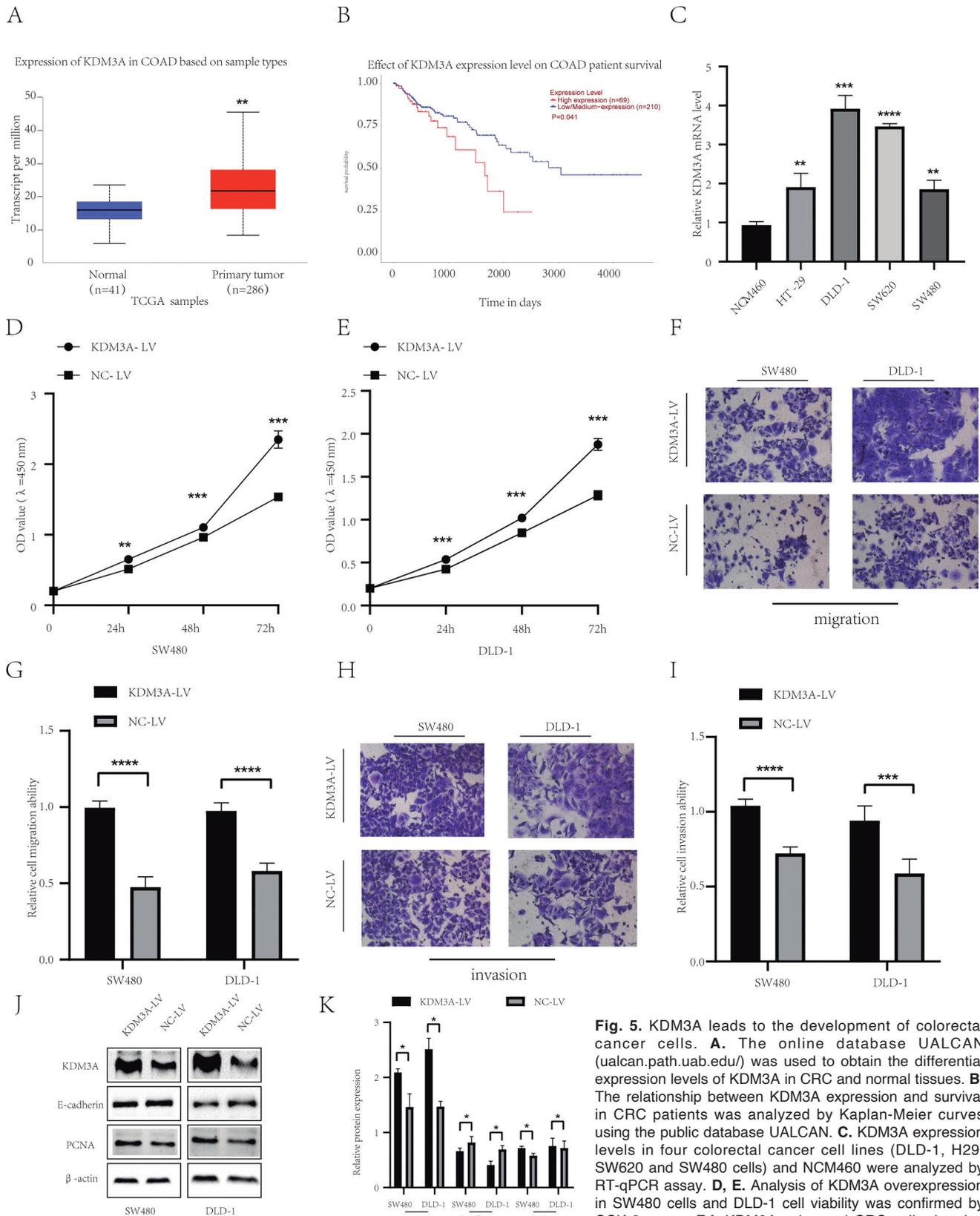*MiR-22-3p/KDM3A modulates the progression of CRC cells*
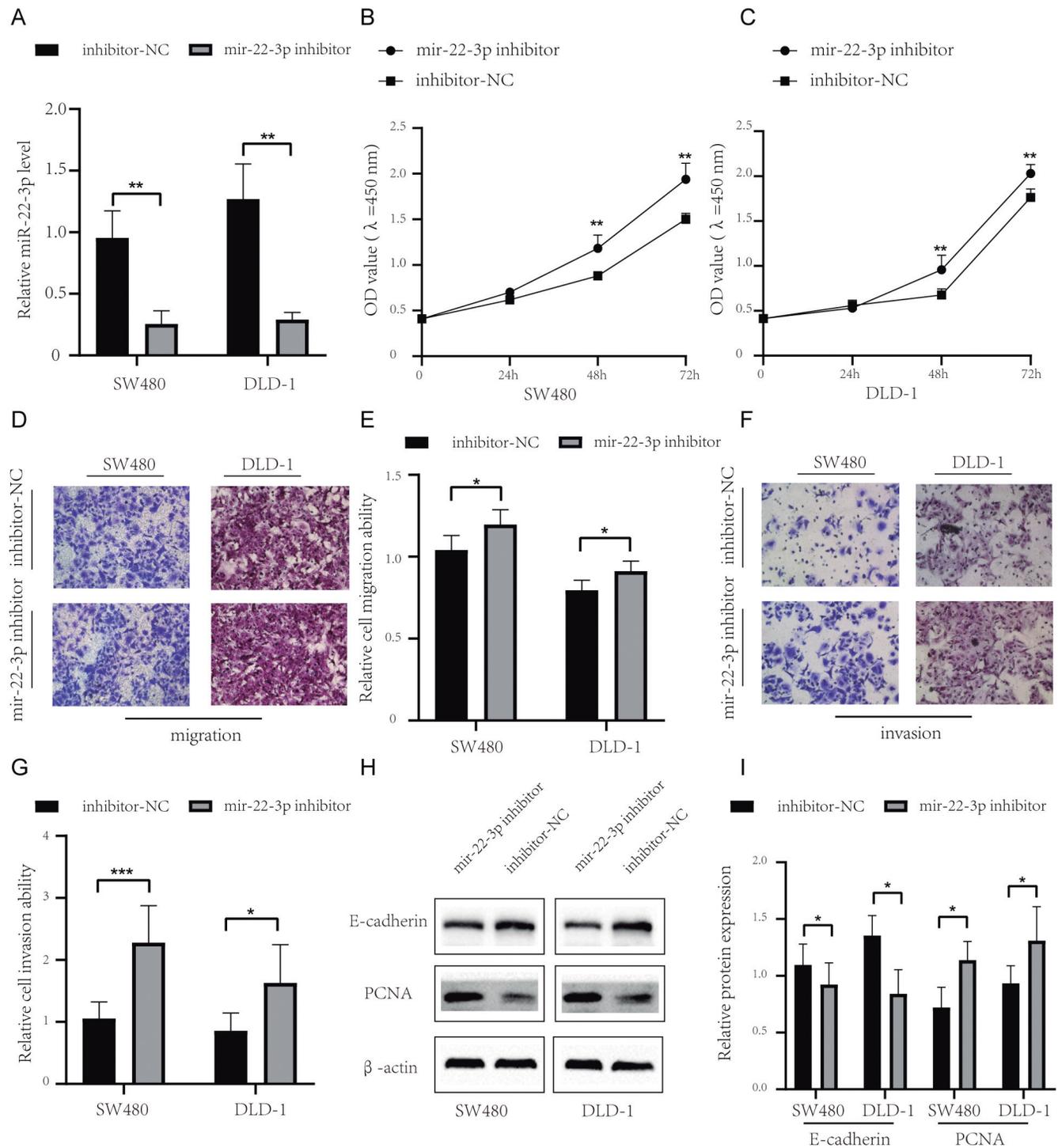
Fig. 3. miR-22-3p inhibitor promotes tumor progression in colorectal cancer. **A.** The expression of miR-22-3p was analyzed in SW480 cells and DLD-1 cells via RT-qPCR after transfection with miR-22-3p inhibitor or miR-22-3p inhibitor NC. **B, C.** Analysis of the miR-22-3p inhibitor on SW480 cell and DLD-1-cell viabilities was confirmed by CCK-8 assay. **D-G.** The miR-22-3p inhibitor-enhanced CRC cell migration and invasion abilities were demonstrated via Transwell migration assays or Transwell invasion assays. **H, I.** Western blot assays showed the protein levels of E-cadherin and PCNA in SW480 cells and DLD-1 cells transfected with miR-22-3p inhibitor or miR-22-3p inhibitor NC. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. Each experiment with three replicates was repeated three times. A t test or nonparametric test was used for the comparisons of two groups. Differences among more than two groups were evaluated by one-way ANOVA.

were markedly upregulated in the 4 CRC cell lines (H29, DLD-1, SW620 and SW480) (Fig. 5C). To confirm whether KDM3A promotes CRC progression, we established SW480 and DLD-1 cells that stably overexpressed KDM3A or negative control by lentiviral transfection. The CCK-8 results showed that the overexpression of KDM3A significantly enhanced cell proliferation in SW480 and DLD-1 cells (Fig. 5D,E). We next used a Transwell chamber to assess the effects of

KDM3A on migration and invasiveness. The results showed that overexpression of KDM3A significantly enhanced SW480 and DLD-1 cell migration and invasion (Fig. 5F-I). Western blotting demonstrated a significant decrease in E-cadherin protein expression as well as an increase in KDM3A and PCNA protein expression in KDM3A-overexpressing cells (Fig. 5J,K). All these results indicated that KDM3A plays a role as an oncogene in CRC.

Fig. 4. miR-22-3p directly targets KDM3A in colorectal cancer cells. **A.** The predicted miR-22-3p binding sites using TargetScan7.2, miRBase and miRDB in the KDM3A mRNA 3'UTR or the mutated 3'UTR of KDM3A mRNA were confirmed. **B, C.** Luciferase reporter plasmids containing the wild-type or mutated 3'UTR fragments were cotransfected with either miR-22-3p mimic or negative control, which showed that KDM3A is a direct target of miR-22-3p. **D.** Correlation analysis of GEO data (GSE32323) demonstrated a negative correlation between the expression level of miR-22-3p and the KDM3A mRNA level. **E.** In SW480 and DLD-1 cells transfected with miR-22-3p mimic or miR-22-3p mimic NC, KDM3A mRNA levels were measured by RT-qPCR. **F, G.** Western blot assays showed the protein levels of KDM3A in SW480 cells and DLD-1 cells transfected with miR-22-3p mimic or miR-22-3p mimic NC. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; NS, not significant. Each experiment with three replicates was repeated three times. A t test or nonparametric test was used for the comparisons of two groups. Differences among more than two groups were evaluated by one-way ANOVA.

Fig. 5. KDM3A leads to the development of colorectal cancer cells. **A.** The online database UALCAN (ualcan.path.uab.edu/) was used to obtain the differential expression levels of KDM3A in CRC and normal tissues. **B.** The relationship between KDM3A expression and survival in CRC patients was analyzed by Kaplan-Meier curves using the public database UALCAN. **C.** KDM3A expression levels in four colorectal cancer cell lines (DLD-1, H29, SW620 and SW480 cells) and NCM460 were analyzed by RT-qPCR assay. **D, E.** Analysis of KDM3A overexpression in SW480 cells and DLD-1 cell viability was confirmed by CCK-8 assay. **F-I.** KDM3A-enhanced CRC cell migration and invasion abilities were demonstrated via Transwell migration assays or Transwell invasion assays. **J, K.** Western blotting demonstrated a decrease in E-cadherin protein expression and an increase in PCNA protein expression in KDM3A-overexpressing cells relative to the control group in SW480 cells and DLD-1 cells after transfection with KDM3A-LV or NC-LV. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; NS, not significant. Each experiment with three replicates was repeated three times. A t test or nonparametric test was used for the comparisons of two groups. Differences among more than two groups were evaluated by one-way ANOVA. Abbreviations: KDM3A-LV, lentivirus encoding KDM3A; NC-LV, lentivirus encoding the negative control.
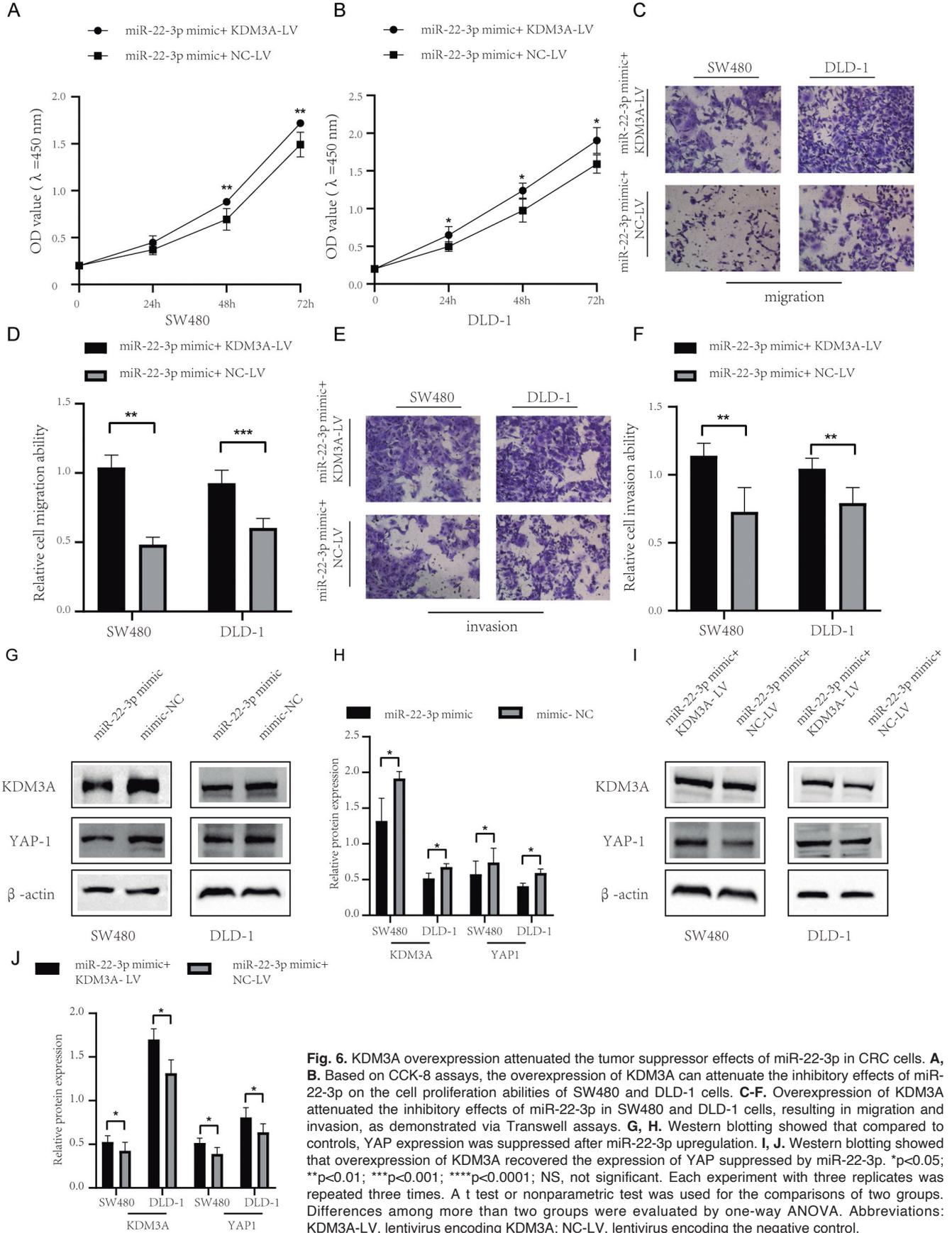
Fig. 6. KDM3A overexpression attenuated the tumor suppressor effects of miR-22-3p in CRC cells. **A, B.** Based on CCK-8 assays, the overexpression of KDM3A can attenuate the inhibitory effects of miR-22-3p on the cell proliferation abilities of SW480 and DLD-1 cells. **C-F.** Overexpression of KDM3A attenuated the inhibitory effects of miR-22-3p in SW480 and DLD-1 cells, resulting in migration and invasion, as demonstrated via Transwell assays. **G, H.** Western blotting showed that compared to controls, YAP expression was suppressed after miR-22-3p upregulation. **I, J.** Western blotting showed that overexpression of KDM3A recovered the expression of YAP suppressed by miR-22-3p. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; NS, not significant. Each experiment with three replicates was repeated three times. A t test or nonparametric test was used for the comparisons of two groups. Differences among more than two groups were evaluated by one-way ANOVA. Abbreviations: KDM3A-LV, lentivirus encoding KDM3A; NC-LV, lentivirus encoding the negative control.

KDM3A overexpression attenuated the tumor suppressor effects of miR-22-3p in CRC cells

To investigate whether miR-22-3p's antitumor activity is through the silencing of KDM3A in CRC, CRC cells stably overexpressing KDM3A or negative control were transfected with the mir-22-3p mimics. Based on CCK-8 assays, the overexpression of KDM3A can attenuate the inhibitory effects of miR-22-3p on the cell proliferation abilities of SW480 and DLD-1 cells (Fig. 6A,B). Furthermore, Transwell results assessing migration and invasion showed that overexpression of KDM3A attenuated the inhibitory effects of miR-22-3p in SW480 and DLD-1 cells (Fig. 6C-F). Taken together, these results indicated that KDM3A mediates the cell proliferation, migration and invasion effect of miR-22-3p in CRC.

MiR-22-3p inhibits the Hippo pathway by downregulating KDM3A in CRC cells

To confirm the role of the Hippo pathway in miR-22-3p antitumor activity, the expression of total YAP1 was validated by Western blotting in different intervention groups. The results show that compared to controls, YAP1 expression was suppressed after miR-22-3p upregulation (Fig. 6G,H). However, on the basis of the above intervention, overexpression of KDM3A recovered the expression of YAP1 (Fig. 6I,J). Altogether, the results demonstrate that miR-22-3p inhibits the Hippo pathway by downregulating KDM3A in CRC cells.

Discussion

CRC is one the most frequently diagnosed gastrointestinal neoplasms worldwide. It remains one of the main causes of cancer-related death (Siegel et al., 2021). Although the treatment of CRC has made progress in multiple dimensions, the prognosis of a considerable number of patients is still poor. More therapeutic targets and treatment options are clearly needed; therefore, further studies will be needed to clarify this mechanism of CRC progression. It has been shown that miRNAs play an important role in the pathophysiological process of diseases (Mikami et al., 2021; Walgrave et al., 2021). Furthermore, the biogenesis of cancer-related miRNAs, including those with a role as oncogenes or those with tumor suppressor functions, often takes part in many biological processes, such as tumor initiation and tumor metastasis of cancers, including CRC (Peng et al., 2020a,b; Cao et al., 2021; Liu et al., 2022a,b). It is interesting to note that the same miRNA may indeed have distinct roles in different tumor microenvironments. For example, researchers recently found that miR-22-3p can suppress the growth of triple-negative breast cancer (Gorur et al., 2021). Remarkably, miR-22-3p was thought to promote tumor progression in a glioblastoma study (Han et al., 2020). However, there are few studies (Sha et al., 2019; Wang et al., 2021) regarding the role of miR-22-3p in CRC, and its role remains unclear.

In this study, we confirmed that the miR-22-3p levels were significantly downregulated in CRC. This result is consistent with the findings of other studies showing that miR-22-3p is usually weakly expressed in most cancers (Saccomani et al., 2020; Wang et al., 2021). We also found that low expression of miR-22-3p was associated with poor prognosis in CRC patients. upregulation of miR-22-3p expression in CRC cells inhibits the proliferation, migration and invasive ability of CRC cells. Conversely, downregulation of miR-22-3p expression in CRC cells promoted the ability of CRC cells to proliferate, migrate and invade. This is consistent with previous studies in which miR-22-3p was shown to has an antitumoral effect (Guan et al., 2021; Tian et al., 2021).

MiRNAs usually function by binding to the target mRNA to induce degradation of the mRNA. This bioinformatic analysis and dual luciferase assays demonstrated that KDM3A is a direct target of miR-22-3p. Meanwhile, after upregulation of miR-22-3p in CRC cells, KDM3A was found to be significantly decreased in both mRNA and protein levels, which further supported the above findings. In previous studies, KDM3A has been shown to function as an oncogene in a number of cancers, such as renal cell carcinoma, pancreatic tumor (Dandawate et al., 2019; Zhang et al., 2022). In this study, by bioinformatic analysis, we found that high KDM3A expression was associated with poor prognosis in CRC patients. Meanwhile, overexpression of KDM3A in CRC cells promotes the proliferation, migration and invasion ability of CRC cells. It is suggested that KDM3A functions as an oncogene in CRC. In rescue experiments, we confirmed that overexpression of KDM3A partially antagonized the inhibitory effect of miR-22-3p on CRC cells. These results suggest that miR-22-3p reduces the malignant behavior of CRC cells by targeting KDM3A.

Hippo signaling pathway is one of the tumor-associated signaling pathways, first identified in Drosophila. The Hippo signaling pathway is involved in important biological functions in animals such as cell proliferation, apoptosis and control of organ size (Cho et al., 2020; Peng et al., 2020a,b; Ding et al., 2022). YAP1 is a key molecule in the Hippo pathway. Unphosphorylated YAP1 can be translocated to the nucleus to regulate transcription factors and induce the expression of relevant target genes involved in cancer progression (Tsinias et al., 2020). Studies in CRC show that YAP1 promotes the proliferation and liver metastasis of CRC cells (Jiang et al., 2021; Liu et al. 2022b). Studies on the mechanism of action of YAP1 in CRC show that YAP1 can promote the development and progression of CRC by regulating transcription factor PRDM14 and inhibiting autophagy (Jin et al., 2021; Kim et al., 2022). Furthermore, recent study has shown that KDM3A is associated with YAP1 expression (Wang et

al., 2019). Therefore, we hypothesize that miR-22-3p inhibits CRC progression via the Hippo signaling pathway. In the present experiments, we detected a decrease in YAP1 protein by overexpression of miR-22-3p in CRC cells. In contrast, YAP1 expression was restored after overexpression of KDM3A in the rescue experiment. These results suggest that miR-22-3p inhibits CRC progression by targeting KDM3A to regulate the expression of YAP1.

Our study is the first to report the role of miR-22-3p/KDM3A/hippo pathway signaling axis in CRC. This finding will help us to provide a new theoretical basis in the treatment of CRC. However, there are still shortcomings in this study, including the lack of validation in animal studies and validation on a large clinical sample.

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