ORIGINAL RESEARCH

ATG3, a Target of miR-431-5p, Promotes Proliferation and Invasion of Colon Cancer via Promoting Autophagy

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Background: Studies have indicated that ATG3 could mediate the effects of other tumor-related regulators in carcinogenesis. However, the expression, role, and mechanism of ATG3 itself in cancers are rarely revealed. Thus, we explored the expression, function, and mechanism of ATG3 in colon cancer.

Materials and methods: The expression of ATG3 was detected in colon cancer tissues and cell lines, as well as in adjacent tumor tissues and normal colon epithelial cells. The effects of ATG3 alteration on proliferation and invasion were further analyzed. The expression and role of miR-431-5p, a potential negative regulator of ATG3, were also studied. Eventually, the role of autophagy in ATG3 related effects in colon cancer was checked.

Results: ATG3 is upregulated in colon cancer tissues and cells demonstrated by qPCR and IHC. ATG3 knockdown significantly suppressed proliferation and invasion of colon cancer cells indicated by plate clone formation and Transwell invasion assays. The expression of miR-431-5p is downregulated and negatively correlates with ATG3 in colon cancer. Furthermore, luciferase report system, plate clone formation and Transwell invasion assays demonstrated that miR-431-5p could prohibit cell proliferation and invasion via directly targeting ATG3 in colon cancer. Eventually, Western blot, plate clone formation and Transwell invasion assays proved that autophagy block could antagonize the promotive functions of ATG3 on proliferation and invasion in cancer suggesting autophagy activation accounts for the promotive role of ATG3 on proliferation and invasion in colon cancer.

Conclusion: Collectively, ATG3 upregulation, caused by downregulated miR-435-5p, promotes proliferation and invasion via an autophagy-dependent manner in colon cancer suggesting that miR-431-5p/ATG3/autophagy may be a potential therapeutic target in colon cancer.

Keywords: colon cancer, proliferation, invasion, ATG3, miR-431-5p, autophagy

Introduction

Colon cancer is one of the most prevalent and fatal malignant diseases both in developing and developed countries. Surgical resection and chemoradiotherapy, the main treatments for colon cancer, is effective for colon cancer at an early stage (stage 0 and I); however, the prognostic outcome of colon cancer patients at advanced stages (II-IV) is unsatisfactory.[1–3] Therefore, more efforts should be taken to identify critical regulators in carcinogenesis and to reveal the corresponding mechanism in colon cancer, which can potentially provide new diagnostic markers and therapeutic targets and eventually improve the treatment efficacy of colon cancer.

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Studies have revealed that autophagy, a conserved process of self-degradation, plays vital roles in mediating the functions of tumor relative factors almost at every stage of cancer, including carcinogenesis, recurrence, metastasis, as well as therapeutic sensitivity.[4–7] Serving as the constitutive components of autophagy process, autophagy-associated proteins are critical regulators of autophagy. On the one hand, autophagy-associated proteins are in charge of autophagy modification upon receiving the regulatory signals from upstream regulators of autophagy, such as PI3K/AKT,[8] AMPK,[9] and MAPK.[10] On the other hand, the aberrant expressions of autophagy-related proteins largely account for the abnormal status of autophagy.[11–13] Notably, although the roles of autophagy in cancers are widely explored, few studies have focused on the role and mechanism of autophagy-associated proteins themselves in the malignant progression of tumors. Thus, studying the expression of autophagy-related proteins and exploring the underlying mechanisms may help to elucidate the molecular mechanism of cancer and present potential targets for clinical therapy.

ATG3, serving as an E2-like enzyme, is an important autophagy regulator involving in LC3-B generation via promoting covalent binding of phosphatidylethanolamine to the C-terminal glycine of LC3-A, a key step for the membrane association of ATG8-like proteins.[14] According to the available studies, ATG3 could mediate the functions of other tumor-related factors either in an autophagy-dependent or in the autophagy-independent manners, suggesting ATG3 may exert critical roles in carcinogenesis.[15–18] Up to now, the expression and functions of ATG3 in colon cancer have not been revealed. Thus, in this study, we analyzed the expression of ATG3 in colon cancer and further explored functions and the underlying mechanism of aberrant expression of ATG3 in colon cancer. Our results demonstrate that ATG3 is upregulated in colon cancer tissues and cell lines, and ATG3 can promote proliferation and invasion of colon cancer cells by activating autophagy. Loss of miR-431-5p accounts for the overexpression of ATG3 and miR-431-5p can antagonize the oncogenic functions of ATG3 in colon cancer.

Materials and Methods
Tissue Samples

Twenty-four cases of colon cancer tissues and paired adjacent tumor tissues were collected from the resection tissues of newly diagnosed colon cancer patients with surgery in the department of general surgery of Xiangya Hospital of Central South University. The collected tissues were immediately divided into two parts, one was frozen in liquid nitrogen for RNA extraction, and one was fixed with formalin for paraffin sectioning. The study was approved by the Ethical Committee of Xiangya Hospital of Central South University and informed consents were signed by all patients before tissue collection.

Immunohistochemistry

Immunohistochemistry (IHC) was performed according to our previous study.[19] Simply, paraffin-embedded sections were successively immersed in xylene and graded ethanol for dewaxing and re-hydration. After antigen retrieval and endogenous peroxidase activity blocking, the slides were subsequently incubated with rabbit anti-ATG3 polyclonal antibody (dilution: 1:150, catalog number: D221649, BBI, Shanghai, China) overnight at 4°C. Following three times washing, the slides were successively subjected to the rest processes including incubation with biotin-linked secondary antibody, PBS washing, incubation with streptavidin-HRP, DAB staining, and hematoxylin counterstaining. Eventually, the pictures were captured with the microscopic imaging system microscope (Olympus, Tokyo, Japan) and analyzed by two independent pathologists. Semiquantitative analysis of IHC results was carried out based on staining intensity and area according to our previous description.[19,20]

Cell Culture

Colon cancer cell lines, SW480, SW620, HCT116, and HCT15, being purchased from ATCC, were maintained with RPMI-1640 (BI, Jerusalem, Israel) plus 10% fetal bovine serum (BI, Jerusalem, Israel). NCM460, an immortalized colon epithelial cell line, was purchased from ATCC and cultured with M3 Base medium (INCELL, TX, USA) plus 10% FBS (BI, Jerusalem, Israel). Human embryonic kidney 293T cells were brought from Cell Bank of Typical Culture Preservation Committee of Chinese Academy of Sciences and cultured with DMEM plus 10% FBS (BI, Jerusalem, Israel). All cells were maintained in a humidified cell incubator at 37°C with 5% CO_2. Chloroquine (CQ, Sigma, MI, USA) at 50μM was added into the culture medium for inhibition of autophagy flux.

siRNA and Plasmid Transfection

siATG3, miR-431-4p mimics, and pcDNA3.1-ATG3 plasmids were transfected into SW480 and HCT116 cells using Highgene transfection reagent (Abclonal, Wuhan, China) according to the manufacturer's instruction. Specific siRNAs targeting ATG3 at 3′-untranslated region and miR-431-5p

mimics were synthesized by RiboBio Inc (Guangzhou, China). The sequences of siATG3 were 5′-CAAGCTG TCATTCCAACAATA-3′. The sequences of miR-431-5p mimic, mimic control, and siNC (negative control) were not offered by RiboBio Inc for commercial confidentiality.

RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)

Total RNA extraction and mRNA reverse transcription were performed according to the previous study.[21] AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) was applied to analyze the expression of ATG3 and miR-431-5p in colon cancer tissues and cells. The PCR reaction was run on the CFX Connect Real-Time System (Bio-Rad, CA, USA) and the results were analyzed with CFX Manager 2.0 (Bio-Rad, CA, USA). The sequences of primers are listed as follow. ATG3 mRNA forward primer:5′-ACATGGCAATGGGCTA CAGG-3′(Tm, 60.40; GC%,55.00); ATG3 mRNA reverse primer: 5′-CTGTTTGCACCGCTTATAGCA-3′ (Tm, 59.26; GC%, 47.62), product length, 108bp. ATG3 hnRNA forward primer: 5′-GCTCGACTTTTCAGGGACCA-3′ (Tm, 59.97; GC%,55.00), ATG3 hnRNA reverse primer:5′-TATGACG GGAGAAGGCAGGA-3′ (Tm, 60.03; GC%,55.00), product length, 202bp. GAPDH forward primer: 5′- CAGCAAGAGC ACAAGAGGAA-3′(Tm, 58.11, GC%, 50.00), GAPDH reverse primer: 5′- ATGGTACATGACAAGGTGCGG -3′ (Tm, 60.68, GC%, 52.38), product length, 156bp. The primers of miR-431-5p and U6 were purchased from the RiboBio Inc for commercial confidentiality.

Western Blot

Western blot was performed as previously described.[3] Briefly, total proteins were extracted with RIPA buffer high-speed centrifugation at 4°C. After denature by 5×SDS loading buffer, proteins were loaded and separated by 10% SDS PAGE (30 μg/ lane). Subsequently, the proteins were transferred onto poly-vinylidene difluoride membranes (PVDF). Then, the membranes were blocked with 5% non-fat milk and incubated with the primary antibodies including ATG3 (dilution: 1:500, catalog number: D221649, BBI, Shanghai, China), β-Tubulin (dilution: 1:2000, catalog number: 100109-MM05T, Sino Biological, Beijing, China), LC3A/B (dilution: 1:1000, catalog number: A11280, Abclonal, Wuhan, China) at 4°C overnight. Next, after washing by TBST, subsequently incubated with secondary antibodies, and washing by TBST again, the protein bands were visualized using chemiluminescent HRP substrate

(EpiZyme, Shanghai, China) via a FluorChem FC3 system (Proteinsimple, CA, USA).

Dual Luciferase Reporter Assay

Both psiCHECK2-wild-ATG3-3′UTR and psiCHECK2-mutant-ATG3-3′UTR luciferase report plasmids of ATG3 were constructed, respectively, according to manufacturer's instruction (Promega Corporation, WI, USA). Then, luciferase reporter vectors were co-transfected with miR-451-5p mimics into 293T cells by using Lipofectamine™ 2000 reagent (Thermo, MA, USA). Forty-eight hours later, the luciferase activity was detected by using a Dual-Luciferase Reporter detection System (Promega Corporation, WI, USA) and the signaling was collected with an Epoch2 spectrophotometer (BioTek, VT, USA). The relative luciferase activity was presented by the ratio of firefly luciferase to Renilla luciferase activity.

Plate Clone Formation Assay

Plate clone formation assay was performed as previously described.[3] Briefly, colon cancer cells were seeded into six-well plates at a density of 800 cells/well. Eight days later, the cells were fixed with methanol and stained by 0.5% crystal violet. Clones containing more than fifty cells were counted under an inverse microscope (Nikon, Tokyo, Japan).

Transwell Invasion Assay

Transwell assay was applied as described in our previous paper with minor modifications.[21] Briefly, 8-μm pore size transwell chambers (Costar, ME, USA) pre-coated with Matrigel (Corning, NY, USA) were used to evaluate the ability of cell invasion. 300 μL pure RPMI-1640 containing 2.5×10^4 cells were added into the upper chamber. The lower well was filled with 750μL RPMI 1640 medium plus 5% FBS as a chemoattractant. After culture at 37°C for twenty-four hours, the cells were fixed with methanol and stained by 0.5% crystal violet. After swabbing the cells above on the upper side of the membrane, the invasive cells were photoed and counted under a microscopic scope.

Statistical Analysis

All experiments were independently repeated for at least 3 times. Statistical analyses and statistical charts were conducted and produced using SPSS18.0 software and GraphPad Prism version 8. For comparisons between two groups, a Student's t-test or chi-square test was used. Pearson correlation coefficients was adopted to analyze the expression correlation

between miR-451-5p and ATG3. *P* <0.05 were considered to be statistically significant.

Results

ATG3 Is Upregulated in Colon Cancer

Firstly, we analyzed the expression of ATG3 in colon cancer based on the online data from TCGA and GEO using UALCAN[22] and Oncomine database. As Figure 1A shows, ATG3 is significantly upregulated in colon cancer tissues, which is confirmed by two GEO data sets (Figure 1B and C). Compared with adjacent tumor tissues, upregulation of ATG3 is also validated in collected colon cancer tissues demonstrated by qPCR and IHC assays (Figure 1D and E). Thus, these results proved that ATG3 is upregulated and may serve as an oncogenic regulator in colon cancer.

ATG3 Knockdown Inhibits Growth Proliferation and Invasion of Colon Cancer Cells

Next, we checked the expression of ATG3 in colon cancer cell lines. As indicated by Figure 2A and B, compared with

the expression level in NCM460 cells, ATG3 was slightly upregulated in HCT15 and SW480 and significantly upregulated in SW620 and HCT116. Therefore, to explore the functions of ATG3 in colon cancer, specific siRNAs of ATG3 were introduced into SW620 and HCT116 cells to knock down ATG3 expression. Successful ATG3 knockdown was achieved, indicating by notably decreased protein level (Figure 2C). Subsequently, the proliferation and invasion of SW620 and HCT116 were analyzed by plate clone formation and Transwell invasion assays. Significant inhibitory effects of ATG3 knockdown on cell proliferation and invasion were observed demonstrated by fewer clones (Figure 2D), and less invasive cells (Figure 2E). Therefore, these results indicated ATG3 could promote proliferation and invasion in colon cancer.

Downregulated miR-431-5p Accounts for the High Expression of ATG3 in Colon Cancer Cells

Both transcriptional and post-transcriptional mechanisms may account for the expression regulation.[23,24] Hence, we detected the level of ATG3 hnRNA (heterogeneous
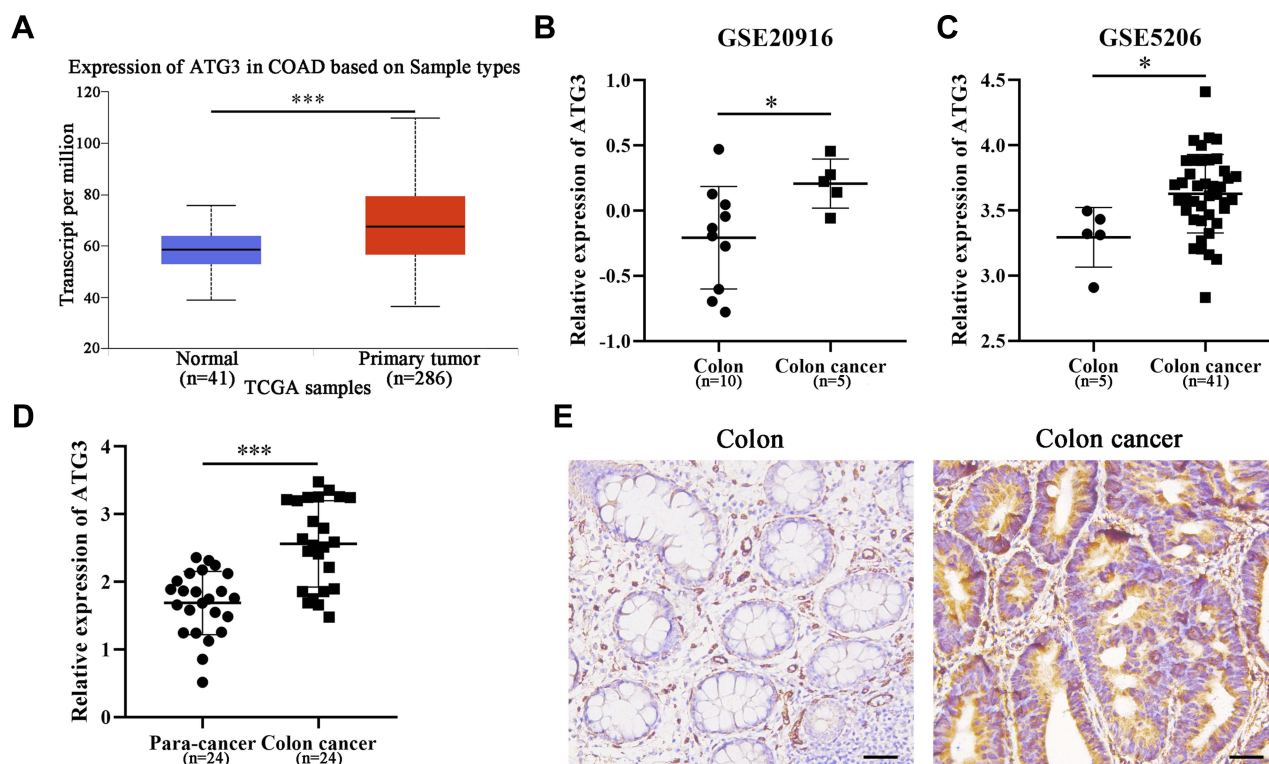
Figure 1 ATG3 is upregulated in colon cancer tissues and cells.
Notes: Upregulation of ATG3 in colon cancer is supported by online data from TCGA (**A**) and GEO database (**B** and **C**). Upregulation of ATG3 in colon cancer is confirmed by qPCR (**D**) and IHC (**E**) in our collected tissues. COAD: colon adenocarcinoma, "Colon" and "Para-cancer" means normal tissues in this part. *Stands for *P* < 0.05; ***Stands for *P* < 0.001.
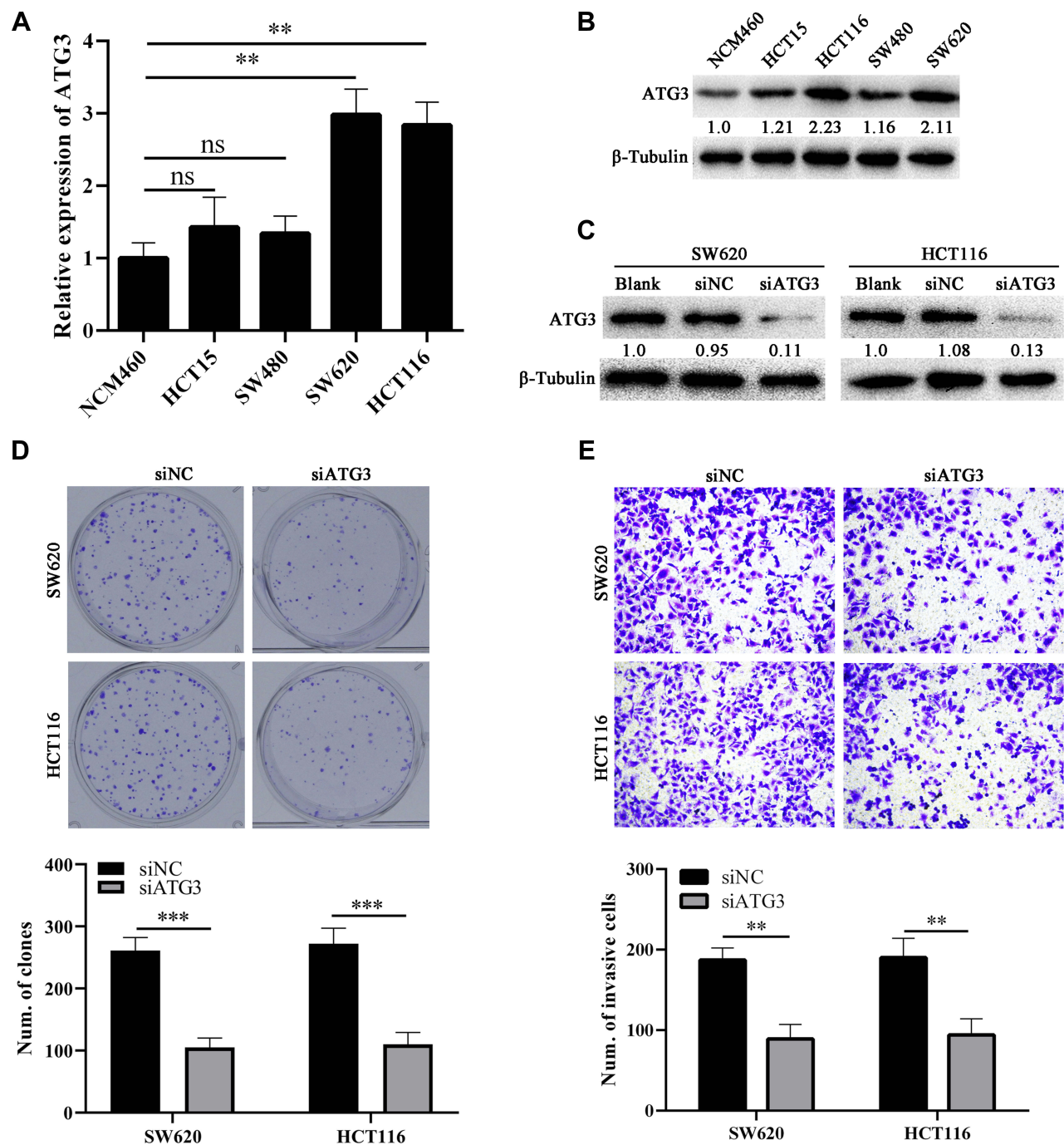
Figure 2 ATG3 knockdown inhibits proliferation and invasion of colon cancer cells.
Notes: qPCR (**A**) and Western blot (**B**) show that ATG3 is upregulated in colon cancer cells compared with colon epithelial cell NCM460. (**C**) Western blot indicates that ATG3 is successfully knocked down in SW620 and HCT116 cells. ATG3 knockdown significantly suppresses proliferation and invasion of colon cancer cells demonstrated by plate clone formation (**D**) and Transwell assays (**E**). siNC stands for negative control small RNA; Ns stands for no significant difference; **Stands for $P < 0.01$; ***Stands for $P < 0.001$.

nuclear RNA), the primary transcript products which can directly indicate transcript activity, between colon cancer cells and normal colon epithelium cells using qPCR. No significant difference was observed among SW620, HCT116, and NCM460 cells (Supplementary Figure 1A).

Furthermore, the TCGA data shows that methylation status of ATG3 promoter is comparable between colon and colon cancer tissues (Supplementary Figure 1B). Thus, these results imply that post-transcript mechanisms such as miRNAs are involved in the regulation of ATG3 in colon

cancer. Subsequently, potential miRNAs for targeting ATG3 were predicted via Starbase 2.0,[25] miR-431-5p was selected for experimental validation for more supported AGO CLIP-seq experiment evidence, negative correlation to ATG3 in colon cancer, and positive correlation to the overall survival of colon cancer (data from OncomiR[26]) (Supplementary Figure 2A–C). Indeed, downregulated miR-431-5p in colon cancer cells and tissues was indicated by qPCR (Figure 3A and B), and the expression of miR-431-5p was inversely correlated to ATG3 in colon cancer (Figure 3C). Accordingly, the level of ATG3 mRNA and protein could be dramatically decreased by miR-431-5p mimics in SW620 and HCT116 cells (Supplementary Figure 3A and B). Importantly, compared to the negative control, miR-431-5p mimics could significantly decrease luciferase activity of 293T cells transfected with wild ATG3 3′UTR report plasmids; whereas, the inhibitory effect did not appear in 293T cells transfected with mutant ATG3 3′UTR report plasmids (Figure 3D). Thus, our results confirmed that miR-431-5p could directly target and inhibit ATG3 in colon cancer.

Ectopic miR-431-5p Expression Suppresses Colon Cancer Progression by Targeting ATG3

Following, we explored the functions of miR-431-5p and the roles of ATG3 in miR-431-5p related functions in colon cancer. By ectopic expression of ATG3, we successfully rescued the expression of ATG3 in colon cancer cells transfected with miR-431-5p mimics (Figure 3E), which ensured the reliability of function experiments. As Figure 4A and B indicated, miR-431-5p mimics can significantly suppress the proliferation and invasion of colon cells, and these inhibitory effects on colon cancer cells were almost released upon ectopic expression of ATG3. Thus, miR-431-5p can inhibit the proliferation and migration of colon cancer cells by targeting ATG3.

ATG3 Promotes Proliferation and Invasion of Colon Cancer Cells in an Autophagy-Dependent Manner

Considering ATG3 is an autophagy-related protein, we further explored whether ATG3 exerts its functions by modulating autophagy in colon cancer. As Figure 5A demonstrated, ATG3 knockdown significantly inhibited the accumulation of LC3B, an autophagy marker for indicating autophagosome formation, and these inhibitory effects could

be observed under chloroquine (CQ) treatment, excluding the possibility of degradation obstacle accounting for LC3B accumulation, and, suggesting that ATG3 can suppress autophagy flux in colon cancer. Next, we investigated the role of autophagy in ATG3 related functions in colon cancer. Re-expression of ATG3 with or without CQ addition was successfully applied in colon cancer cells with ATG3 knockdown (Figure 5B). Subsequently, function experiments showed that CQ treatment could significantly antagonize the rescue effects of ATG3 restoration on proliferation and invasion in colon cancer (Figure 5C and D). Consequently, our results manifested that ATG3 can promote proliferation and invasion of colon cancer cells in an autophagy-dependent manner.

Discussion

Although the expression patterns of autophagy-related proteins have been exclusively revealed in multiple cancers, almost all studies focus on only three proteins: BECN1, p62, and LC3B.[27–29] As for ATG3, its expression in tumors has been rarely revealed. According to the expression profiles from the TCGA database, ATG3 upregulation is observed in most type of tumors (data not shown) suggesting ATG3 is an important regulator in carcinogenesis. A recent study shows that ATG3 is upregulated in gastric cancer tissues, but acts as a favorable prognostic factor supported by overall survival analysis.[30] Accordingly, we proved that ATG3 is also upregulated in colon cancer tissues and cell lines indicating ATG3 may serve as an oncogenic regulator in colon cancer.

No significant difference of ATG3 hnRNAs between colon epithelial cells and colon cancer cells was observed indicating post-transcriptional regulators involved in the regulation of ATG3. Micro RNAs (miRNAs), a type of non-coding RNA with 21–24 nucleotides in length, are the most common negative regulators of expression which can cause mRNA translation inhibition or degradation via directly binding its target mRNAs at 3′ untranslated region (3′UTR). Several miRNAs including miR-495,[31] −23a,[32] −155,[33] −206,[34] −1,[35] and −365[36] have been confirmed to target ATG3 in both malignant and non-malignant cells suggesting miRNAs may account for the dysregulation of ATG3 in colon cancer. Indeed, we validated that miR-431-5p is downregulated and inversely correlated to ATG3 in colon cancer. Moreover, miR-451-5p can directly bind and decrease ATG3 level in colon cancer. Therefore, we originally reveal that downregulation of miR-431-5p accounts for ATG3 upregulation in colon cancer.
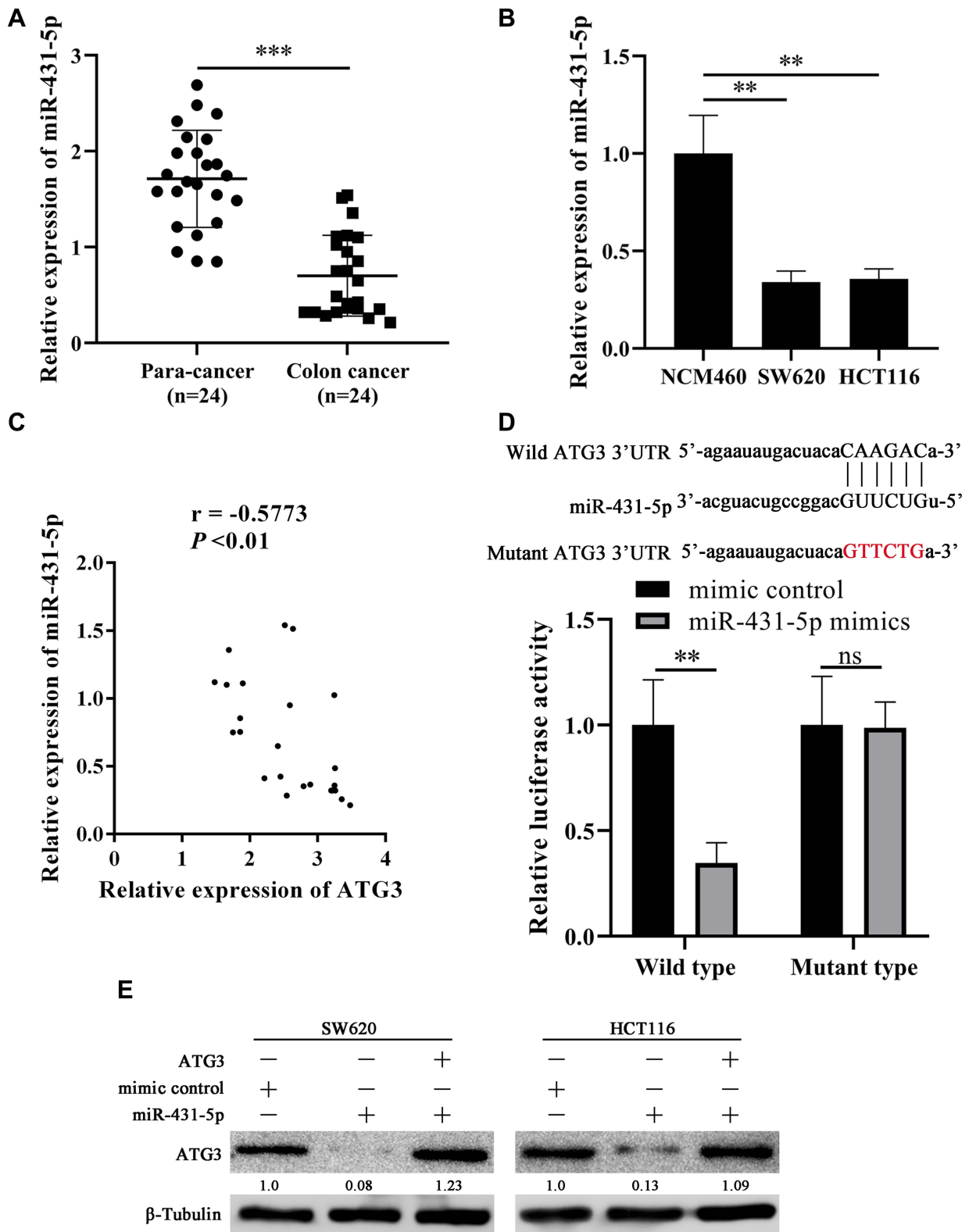
Figure 3 miR-431-5p is downregulated, inversely correlates with ATG3, and directly targets ATG3 in colon cancer.
Notes: (**A** and **B**) miR-431-5p is downregulated in colon cancer tissues and cells indicated by qPCR assay. (**C**) miR-431-5p expression is inversely associated with that of ATG3 in colon cancer indicating by Pearson correlations analysis ($r = -0.5773$, $P < 0.01$). (**D**) Sketch of wild and mutant luciferase reports plasmids and dual luciferase reporter system assay shows that miR-432-5p can directly target ATG3. (**E**) Western blot shows that ATG3 can be inhibited by miR-431-5p mimics, which can be reversed by ectopic expression of ATG3. **Stands for $P < 0.01$; ***Stands for $P < 0.001$.
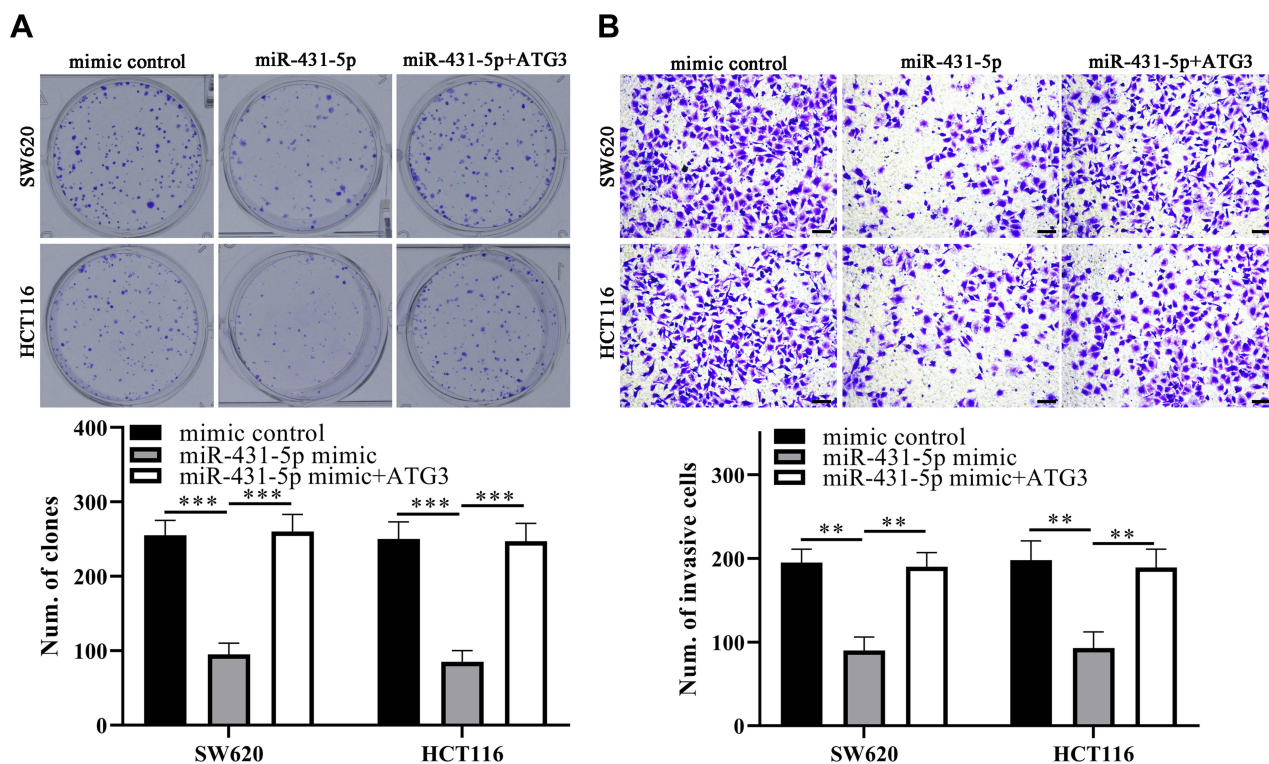
Figure 4 miR-431-5p could inhibit proliferation and invasion of colon cancer cells by targeting ATG3.
Notes: (**A**) As the results of plate clone formation assay indicated, miR-431-5p mimic significantly inhibits proliferation of SW620 and HCT116 which could be subsequently rescued by restoration of ATG3. (**B**) miR-431-5p mimic significantly inhibits invasion of SW620 and HCT116 which could be subsequently rescued by ectopic expression of ATG3, demonstrating by Transwell assay. **Stands for $P < 0.01$; ***Stands for $P < 0.001$.

The roles of ATG3 in tumorigenesis and progression are tissue-dependent demonstrated by the available studies. Generally, ATG3 serves as a positive and protective factor in tumors. Oncogenic lncRNAs (long noncoding RNAs), including lnc-HOTAIR,[17] -Meg3,[15] -PVT1[36] and -GAS5,[32] promote tumorigenesis and progression via stabilizing ATG3 mRNA by specifically sponging miRNAs targeting ATG3 like miR-365 and miR-23a, or directly interacting with ATG3 mRNA in distinct cancers. Meanwhile, sustained levels of ATG3 can impair the resistance of malignant cells to DNA-damaging drugs.[16] Consistent with most research findings, the oncogenic roles of ATG3 in colon cancer, such as promoting proliferation and invasion, are confirmed by our results. Moreover, miR-431-5p, serving as a tumor-suppressive regulator, can significantly inhibit colon cancer progression by targeting ATG3. However, additional efforts should be paid to reveal the underlying mechanism of miR-431-5p deregulation in colon cancer.
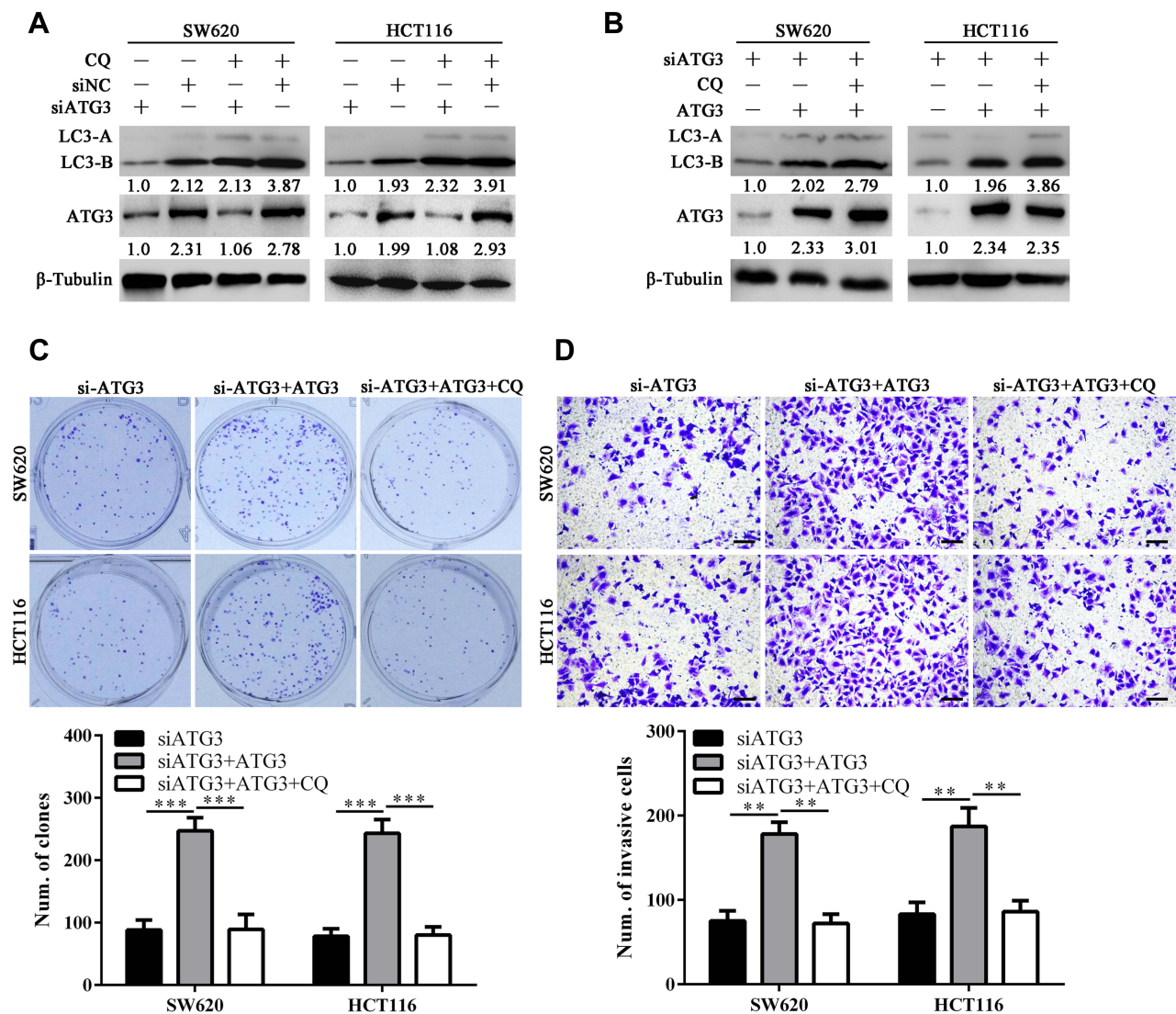
In addition to acting in an autophagy-dependent manner, autophagy-related proteins can also regulate tumorigenesis and progression in an autophagy-independent manner manifesting by numerous studies.[18,37–39] For example, BECN1 can promote degradation of MCL1, one of oncogenes, via physical interaction, being independent of autophagy.[40] Similarly, Y203 phosphorylation of ATG3 is not critical for autophagy induction but is important in sensitizing cancer cells to DNA-damaging agents.[16] Moreover, the pro-apoptotic roles of ATG3 for attached intestinal epithelial cells are independent of ATG3 related autophagy.[18] Thus, it is necessary to distinguish the function of autophagy in autophagy-related proteins. In our study, CQ treatment significantly antagonized the promotive effects of ATG3 on colon cancer cells, suggesting that ATG3 promotes colon cancer progression in an autophagy-dependent manner.

Conclusions

Collectively, we demonstrate that upregulation of ATG3, a result of the loss of miR-431-5p, can promote cell proliferation and invasion in colon cancer. miR-431-5p restoration can significantly inhibit proliferation and invasion via targeting ATG3 in colon cancer. Moreover, we further reveal that ATG3 promotes colon cancer progression in an

Figure 5 ATG3 promotes proliferation and invasion of colon cancer cells in an autophagy-dependent manner.
Notes: (**A**) ATG3 knockdown inhibits autophagy indicated by less LC3B accumulation with or without CQ (50μM) addition. (**B**) Ectopic expression of ATG3 restores autophagy flux of colon cancer cells demonstrated by LC3B accumulation. (**C** and **D**) CQ (50μM) treatment antagonizes the promotive effects of ATG3 on proliferation and invasion of colon cancer cells indicated by plate clone formation and Transwell assays. **Stands for $P < 0.01$; ***Stands for $P < 0.001$.

autophagy-dependent manner suggesting that miR-451-5p/ATG3/autophagy may be an effective therapeutic target in colon cancer.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

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