

# NDRG1 Inhibition of Tumor Progression in Caco2 Cells

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## Research

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# Abstract

**Background:** Colorectal cancer (CRC) is a leading cause of cancer death worldwide. Invasiveness and migration are the main cause of death, and so there is a need to find a sensitive, reliable molecular marker that can predict the migration of colorectal cancer at an early stage. NDRG1 (N-myc Downstream Regulated Gene 1) has been reported to be a multifunctional gene that has a strong relationship with tumor invasion and migration, but theories about the current role of NDRG1 in colorectal cancer remains to be conclusively determined.

**Methods and Results:** Through lentivirus infection and CRISPR/Cas9 methods, respectively, we established that NDRG1 stably overexpressed and knocked out Caco2 cell lines. CCK8(Cell Counting kit 8) data showed that NDRG1 inhibited Caco2 proliferation. Flow cytometry further confirmed that the cell cycle can be arrested at the G1/S phase when NDRG1 overexpresses, while the number of G2 phase cells significantly increased after NDRG1 was knocked out. This means that NDRG1 inhibited the proliferation of Caco2 cells by arresting the cell cycle in the G1/S phase. Our data also demonstrated that NDRG1 promotes early cell apoptosis. The strength of invasion and migration was decreased when NDRG1 overexpressed.

**Conclusions:** Our results underline that NDRG1 inhibits tumor progression in Caco2 cells. These findings may provide a new potential therapeutic strategy for the treatment of CRC.

## Introduction

Among new cancer cases in the world in 2018, colorectal cancer accounted for 10.2% of new cancer cases and 9.2% of cancer deaths. In general, colorectal cancer has become the third most common cancer [1]. Invasiveness and migration are the two malignant features of colorectal cancer that are the main causes of death. Research has shown that the invasion and migration of colorectal cancer is associated with genes as well as miRNA [2]. However, early, sensitive biological indicators that can predict the migration of colorectal cancer at an early stage have not yet been discovered.

NDRG1 is a multifunctional gene. Recent studies have shown that NDRG1 is related to tumor invasion and migration [3], apoptosis [4], tumor cell proliferation [5], drug response, and the drug resistance of tumor cells [6]. Its expression in tumors is tissue-specific. It acts as a tumor suppressing gene in prostate cancer [7] and ovarian cancer [8], but in lung cancer and esophagus squamous cell carcinoma NDRG1 promotes tumor development [9]. The current role of NDRG1 in colorectal cancer is also not conclusively known. Many researchers have found that NDRG1 inhibits tumor invasion and migration in colorectal cancer [10–12], while Wang [13] and Shah [14] found that NDRG1 promoted the development of colorectal cancer. Koshiji [15] found that the expression of NDRG1 differed with race and pathological stage in colorectal cancer patients.

To further investigate the role of NDRG1 in the development of colorectal cancer, we conducted the experiments described below aimed at establishing an early diagnosis and prognosis estimation for

colorectal cancer migration, thus providing new targets for molecular therapy for colorectal cancer.

## Material And Methods

### Cell culture

A Caco2 colorectal cancer cell line was purchased from the Kunming Cell Bank of the Kunming Institute of Zoology, Chinese Academy of Sciences. The Caco2 cell line was derived from a 72-year-old male Caucasian colorectal adenocarcinoma. The 293T cells were provided by the Kunming Institute of Zoology, Chinese Academy of Sciences. The medium required for Caco2 and 293T cells was a DMEM medium ( Dulbecco's Minimum Essential Medium ,Gbico, USA) containing 10% FBS(Fetal Bovine Serum ,Gbico, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. All cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Establishment of cell lines

The lentiviral plasmid used for NDRG1 overexpression was GV358-NDRG1, which was purchased from Genechem (Shanghai,China). The plasmid vector used for NDRG1 knockout was pL-CRISPR.EFS.GFP, which was provided by the Kunming Institute of Zoology, Chinese Academy of Sciences. We designed three sgRNAs which were both located in exon 3 of the NDRG1 gene: sgRNA1, sgRNA2,sgRNA3(Table 1), inserting these three sgRNAs into pL -CRISPR.EFS.GFP plasmid, respectively. All constructed plasmids were identified by sequencing. The GV358 and pL-CRISPR.EFS.GFP empty plasmids were used as a control. The plasmid and its corresponding packaging plasmid were co-transfected into 293T cells for virus packaging, and then the cells were infected with virus. Finally, they were sorted by flow cytometry. The monoclonal cells of NDRG1 knockout were isolated by flow cytometry sorting. Then the monoclonal cells were expanded and cultured to extract the DNA; the third exon of NDRG1 was amplified by PCR(Polymerase Chain Reaction). The PCR product was sequenced and identified, the successfully identified cells were subjected to a T-A clone, and T-A clone product was also sent for sequencing. (All the primers used are shown in Table 1.)

Table 1  
Names and Primers for the establishment of cell lines.

	<b>Forward Primer</b>	<b>Reverse Primer</b>
sgRNA1	5'ATCCTCACCTACCATGACAT3'	5' ATGTCATGGTAGGTGAGGAT3'
sgRNA2	5' ACGCTGTGTGGGACTCCCAA3'	5' TTGGGAGTCCCACACAGCGT3'
sgRNA3	5' GTTCATGCCGATGTCATGGT3'	5' ACCATGACATCGGCATGAAC3'
NDRG1	5'TTTGGTGCATTTAACAGCGCAGTCT3'	5'CAGGAAGTCCCAGGCAAAAAGAAAC3'

### RNA extraction and QPCR

The total cellular RNA was extracted (Qiagen miRNeasy Micro Kit, Germany, Co.,Ltd) and inverted into cDNA (Revertaid First Strand Cdna Synthesis Kit, Thermofisher,Co.,Ltd) according to the kit instructions. The ABI qPCR instrument was used to perform an amplification of the reactants. The reaction procedure was: 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s for 40 cycles, and 60 °C for 1 min. The results were normalized with that of GAPDH, and the relative gene expression levels were calculated by the  $\Delta\Delta C_t$  method. Three replicate wells were repeated for each sample. (All the primers used are shown in Table 2.)

Table 2  
Names and primers for QPCR.

	<b>Forward Primer</b>	<b>Reverse Primer</b>
GAPDH:	5'GCACCGTCAAGGCTGAGAAC3'	5'TGGTGAAGACGCCAGTGGAA3'
NDRG1	5'ATGTCTCGGGAGATGCAGGATGTAG3'	5'CTAGCAGGAGACCTCCATGGACTTG3'

### Western Blotting

High-efficiency RIPA lysate extracted the total cellular protein and quantified protein using the BCA method. 80  $\mu$ g protein was separated by electrophoresis on a 10%SDS-polyacrylamide gel. The proteins were electrotransferred from the gel to the PVDF membrane. The membrane was blocked with 5% non-fat milk solution for 2 h. The antibody rabbit, anti-human NDRG1 monoclonal antibody (1:500, cat.no. 9485S;CST Co., Ltd),  $\beta$ -tublin (1:5000, cat.no. 6046; Abcam Co., Ltd) was incubated for 1 h, and then incubated at 4 °C overnight. The next day, the corresponding secondary antibody (NDRG1 1:500,  $\beta$ -tublin 1:10000, cat.no. AS014; Abclonal Co., Ltd) was incubated for 1 h at room temperature. The membrane was washed and underwent detection by the enhanced ECL detection system.

### Cell counting kit-8 (CCK-8) assay

GV358-control, GV358-NDRG1, pL-control, and pL-NDRG1-knockout cells were seeded in 96-well plates at  $2 \times 10^3$  cells per well for the CCK-8 cell proliferation assay (CCK8 kit, Beyotime, Co., Ltd), then cultured for 1, 2, 3, and 4 days, respectively. According to the manufacturer's instructions, the cells were incubated with the CCK-8 reagent at 37 °C for 1 hour, with the absorbance of each sample scanned on a microplate reader equipped to read absorbance values at 450 nm.

### Assessment of cell cycle by flow cytometric analysis

For cell cycle synchronization,  $5 \times 10^5$  cells were plated in 6-well plates. After the cells were completely adherent, the cells were cultured in the serum-free medium for 24 h, and then cultured in a serum medium for 24 h. The cells were collected and underwent fixing with pre-cooled 75% ethanol at 4 °C for 14 ~ 24 h. After fixation, PI and RNaseA were added to the stain cells for 30 min in the dark, then detected by flow cytometry.

## Assessment of apoptosis by flow cytometric analysis

The cells were trypsinized without EDTA then washed twice with Cell Staining Buffer and resuspended with Annexin V Binding Buffer to adjust the cell concentration to  $(0.25-1.0) \times 10^7$  /mL. 100  $\mu$ L of the cell suspension was taken into a new 1.5 mL centrifuge tube and 5  $\mu$ L of APC Annexin V and 10  $\mu$ L of Propidium Iodide Solution were added, then incubated at room temperature for 15 min in the dark and tested by flow cytometry.

## 24-transwell for invasion and migration

The invasion experiment was done with the Chamber Matrigel Invasion 24-well DO (cat. no. 354480; Biocoat Co., Ltd) and the migration experiment was done with a Transwell Chamber (cat. no. 3422; Corning Co., Ltd). All experimental steps were carried out according to the instructions. The cells inoculated were  $1 \times 10^5$ , and the lower chamber was added with a DMEM medium containing 20% FBS. After 48 hours, the chambers were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet, and a number that passed through the microscope was observed. Then, the cells stained by crystal violet were eluted with 33% acetic acid, and the absorbance at 570 nm of the eluate was measured by a microplate reader.

## Statistical analysis

The data were statistically analyzed using the GraphPad Prism 7.0. The Student's t test was used for statistical analysis. Data are presented as a means  $\pm$  standard deviation.  $P < 0.05$  was considered a statistically significant difference.

## Results

### Flow cytometry sorting of cells

After the virus infected the cells, we sorted the positive cells (GFP positive) by sorting flow cytometry. The results showed that the positive rates of GV358-control cells, GV358-NDRG1 cells, pL-control cells, and pL-NDRG1-knockout cells were 58.3%, 54.1%, 0.506%, and 0.461%, respectively (Fig. 1A-D). For knockout cells, we used sorting flow cytometry for monoclonal cells with an efficiency of 4.73% (Fig. 2).

### Identification of gene knockout cell sequences

After the monoclonal cells were expanded and cultured, the DNA of the cells was extracted, and the third exon of NDRG1 was amplified. The product was sent for sequencing. The sequencing result showed that a DNA strand nick was generated on the third exon of NDRG1, then an A base was inserted. An analysis of the NDRG1 sequence in which the A base was inserted was performed using SnapGene software, and it was found that a TGA stop codon was formed after the inserted A base, thereby terminating the

translation of NDRG1. The amplified exon of NDRG1 was identified by T-A clone and sent to sequencing again. The sequencing results were consistent with the previous results (Fig. 3).

#### Verification of NDRG1 stable overexpression and knockout of the Caco2 cell line

After sorting GFP-positive cells by flow cytometry and sequencing identification, we detected the changes of NDRG1 mRNA and protein expression levels by QPCR and Western Blotting. The relative expression level of NDRG1 mRNA of GV358-NDRG1 cells increased about three-fold compared with the control group ( $P=0.0006$ ). In the pL-NDRG1-knockout cells, NDRG1 mRNA levels were clearly diminished relative to the controls ( $P=0.0001$ ) (Fig. 4A). Western Blotting results revealed that the protein expression level in the overexpression cells rose approximately doubled ( $P=0.0001$ ). After NDRG1 gene knockout, the level reduced significantly ( $P=0.0001$ ) (Fig. 4B, Fig. 4C, Fig. 4D). All the cells were expanded (Fig. 5).

#### NDRG1 arrested the cell cycle in the G1/S phase, which inhibited the proliferation of Caco2 cells

In different tumor cells, NDRG1 promoted or inhibited the proliferation of tumor cells. To confirm the role of NDRG1 in Caco2 cells, we detected cell proliferation by CCK8 and flow cytometry for the cell cycle. As shown in the figure (Fig. 6), the relative proliferative activity of GV358-NDRG1 cells was decreased at 48 h, 72 h and 96 h compared with the GV358-Control cells ( $P<0.001$ ) (Fig. 6A, Fig. 6B), while pL-NDRG1-knockout cells were higher than pL-control cells at 24 h, 48 h, 72 h, and 96 h ( $P<0.01$ ) (Fig. 6C, Fig. 6D), which indicates that overexpression of NDRG1 could inhibit the proliferation of Caco2 cells. Flow cytometry may illuminate the mechanism of these. The proportion of G1 phase cells was significantly increased ( $P<0.0001$ ) (Fig. 6E, Fig. 6F, Fig. 6G) after overexpression of NDRG1, while G2 phase cells were increased after NDRG1 knockout ( $P<0.0001$ ) (Fig. 6H, Fig. 6I, Fig. 6J). The above results showed that NDRG1 inhibits the proliferation of Caco2 cells by arresting the cell cycle in the G1/S phase.

#### NDRG1 inhibited metastasis and invasion of Caco2 cells

NDRG1 was also involved in tumor invasion and metastasis. Metastasis ability is also called exercise ability. Invasion and metastasis were two complementary processes. Metastasis ability can be regarded as the basis of tumor cell invasion. Thus, we also detected changes in the invasion and metastasis ability of cells through the 24-Transwell chamber. The number of cells passing through the chamber decreased after overexpression of NDRG1 ( $P=0.0001$ ) but increased after NDRG1 knockout ( $P=0.0001$ ). These results indicate that NDRG1 inhibited metastasis and invasion of Caco2 cells (Fig. 7).

#### NDRG1 promoted early apoptosis of Caco2 cells.

In order to further investigate the effect of NDRG1 on Caco2 cells, we used flow cytometry to detect cell apoptosis. The early apoptosis of the cells was increased after the overexpression of NDRG1 ( $P=0.0002$ ) (Fig. 8A, Fig. 8B, Fig. 8C), and the rate of pL-NDRG1-knockout cells was decreased compared with pL-control cells ( $P=0.0013$ ) (Fig. 8D, Fig. 8E, Fig. 8F), indicating that NDRG1 promoted early apoptosis of Caco2 cells.

## Discussion

According to the *2018 Global Cancer Statistics Report*, the third most common cancer is colorectal cancer [1], thus presenting a serious threat to people's life and health. Recent studies have shown that an excessive intake of red meat and processed meat increases the risk of colorectal cancer [16-18]. A large alcohol intake is also a risk factor [17, 18], and a high-fat diet may also be related to colorectal cancer [19].

Invasiveness and metastasis are synergistic results of a variety of factors that promote the transfer mechanism of tumor cells in response to changes in the living environment. Thus, the search for colorectal cancer migration suppressor genes is particularly important in current research. Further research on migration-associated genes in colorectal cancer cells will guide the early diagnosis of colorectal cancer, predicting early migration of cancer, which is of significance for reducing the mortality of patients with colorectal cancer.

NDRG1 is a multifunctional protein involved in cell growth [21], apoptosis [4], cell cycle regulation [22], tumor cell proliferation [5, 23] and tumor invasion and metastasis [3]. It can also be induced by a variety of drugs such as induced differentiation agents [24, 25]. NDRG1 not only regulates homeostatic and genomic stability [26], but is also involved in the regulation of the epidermal growth factor [27]. In addition, NDRG1 is related to the regulation of multiple signaling pathways, including the NF- $\kappa$ B pathway [12, 25], the PI3K/AKT/mTOR pathway [28, 29], the RAS/RAF/MEK/ERK pathway [28, 29], the TGF- $\beta$  Pathway [30], and the Wnt/ $\beta$ -catenin pathways [3, 9] - playing an important role in the development of tumors.

Recent studies have shown that NDRG1 may promote or inhibit the development of tumors, but its function in tumors is highly controversial. Some scholars believe that NDRG1 promotes the progression of liver cancer [31-33], lung cancer [34-36], bladder cancer [37], and gastric cancer [38, 39]. However, other researchers have found that NDRG1 inhibits the development of prostate cancer [7, 40], nasopharyngeal carcinoma [41], oropharyngeal squamous cell carcinoma [42], and ovarian cancer [8]. The role of NDRG1 in colorectal cancer is also not conclusively understood. Nathalie [43] found that NDRG1 mRNA levels were significantly reduced in colorectal cancer tissues. Wang [10] showed that silencing NDRG1 expression in colorectal cancer cells increased cell growth, invasion, and migration. Mi [11] also found that NDRG1 promoted epithelial-mesenchymal transition, invasion, and migration of colorectal cancer cells. These studies indicate that NDRG1 is a positive regulator in colorectal cancer. Interestingly, Koshiji [15] found that the expression of NDRG1 was different in colorectal cancer patients of different races and at different pathological stages, which resulted in different clinical outcomes. Some research also supports the premise that NDRG1 is a transfer-promoting gene [13, 44]. Why does NDRG1 have different effects in different tumor cells? In addition to the above factors, it may be related to specific cell types - pleiotropic functions of genes. We established that NDRG1 stably overexpressed and knocked out the Caco2 cell line, then measured changes in cell proliferation ability, cell cycle, apoptosis, invasion, and migration ability. The results show that NDRG1 can arrest the cell cycle in the G1/S phase, slowing the

proliferation rate of the cells. In addition, the overexpression of NDRG1 promoted the early apoptosis of Caco2 cells, and NDRG1 knockout inhibited the early apoptosis of Caco2 cells. In this experiment, we used a 24-Transwell chamber to detect the invasion and migration of cells at 48 h. The results show that NDRG1 overexpression inhibited cell invasion and migration, while cell invasion and migration were promoted after knockout. In conclusion, NDRG1 is a tumor suppressor in Caco2 cells.

The specific role of NDRG1 in colorectal cancer is currently the subject of controversy. To further clarify the mechanism by which NDRG1 inhibits tumorigenesis, we are proceeding to observe the signal pathway and the abilities of NDRG1 in nude mice. This preliminary basic research is useful in finding new targets for the molecular therapy of colorectal cancer, providing a greater theoretical basis for future clinical applications.

## **Conclusion**

In conclusion, we conducted analyses on the effect of NDRG1 on the Caco2 cell line. Through the analyses on the NDRG1 on the biological behaviors of Caco2 cell line, we found that NDRG1 would reduce the proliferation, apoptosis, migration and invasion abilities of Caco2 cell line. NDRG1 may have potential application value as a molecular biological index to predict the early invasion and metastasis of colorectal cancer.

## **Abbreviations**

CRC: Colorectal cancer

NDRG1: N-myc Downstream Regulated Gene 1

CCK8: Cell Counting kit 8

DMEM medium: Dulbecco's Minimum Essential Medium

FBS: Fetal Bovine Serum

## **Declarations**

### **Ethics approval and consent to participate**

The Caucasian colon cancer cell line Caco2 was purchased from Kunming cell bank of typical culture preservation Committee of Kunming Institute of zoology, Chinese Academy of Sciences. Caco2 cell line was derived from 72-year-old male Caucasian colorectal adenocarcinoma.

### **Consent for publication**

All participants agreed the consent of publication

## Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors have declared that no competing interests exist.

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## Authors' contribution

HYX and JYZ performed the experiments, analyzed the data and wrote the manuscript. ZY and HHR contributed analysis tools and manuscript preparation. YP and ZXF helped performed the experiments. ZXF helped perform the analysis with constructive discussions. WKX conceived and designed the experiments. WF conceived and designed the experiments as well as acquired research grant. All authors read and approved the final manuscript.

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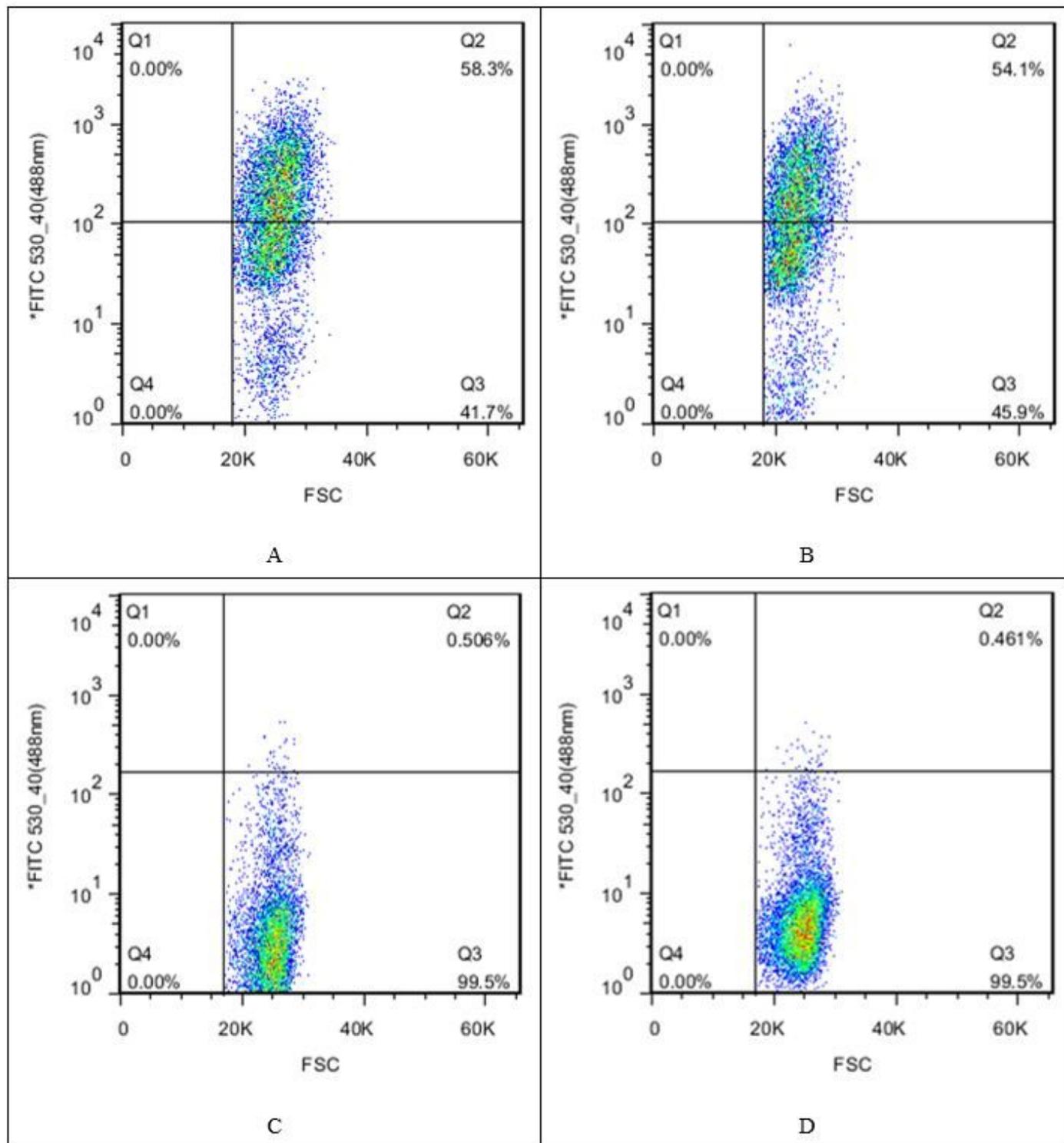
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# Figures



**Figure 1**

Sorting efficiency of cells. (A-B) Sorting efficiency of GV358-Control cells (58.3%), GV358-NDRG1 cells (54.1%) respectively. (C-D) Sorting efficiency of pL-control cells (0.506%), pL-NDRG1 -knockout cells (0.461%), respectively.

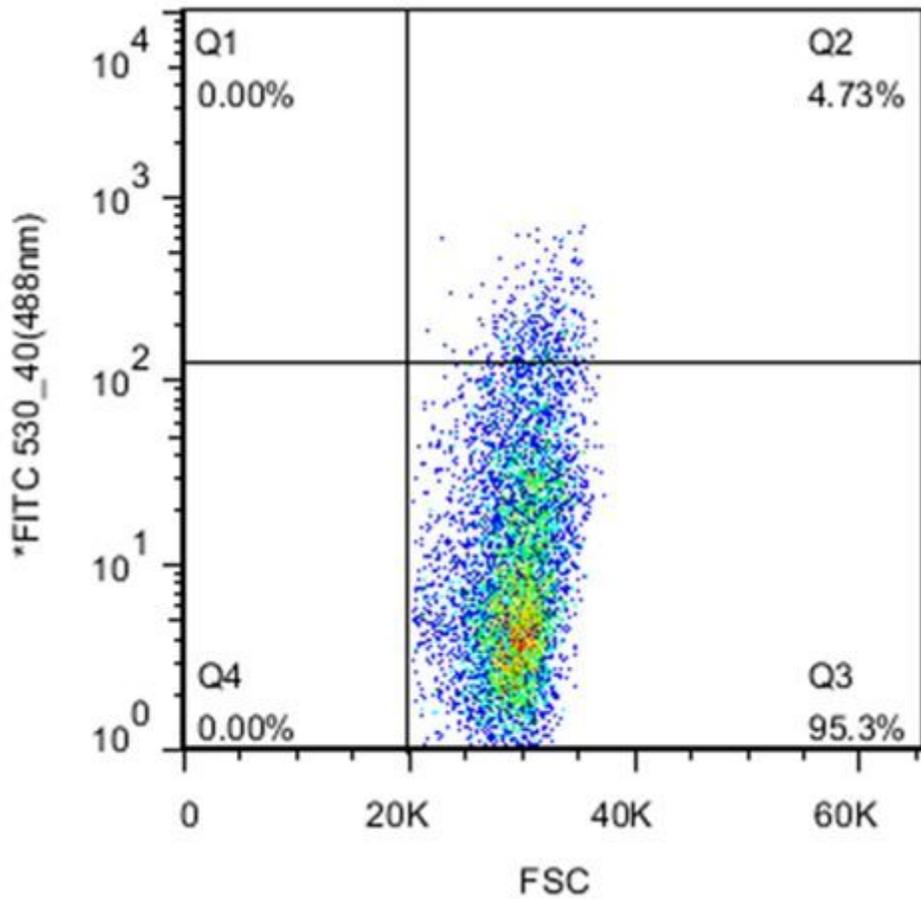
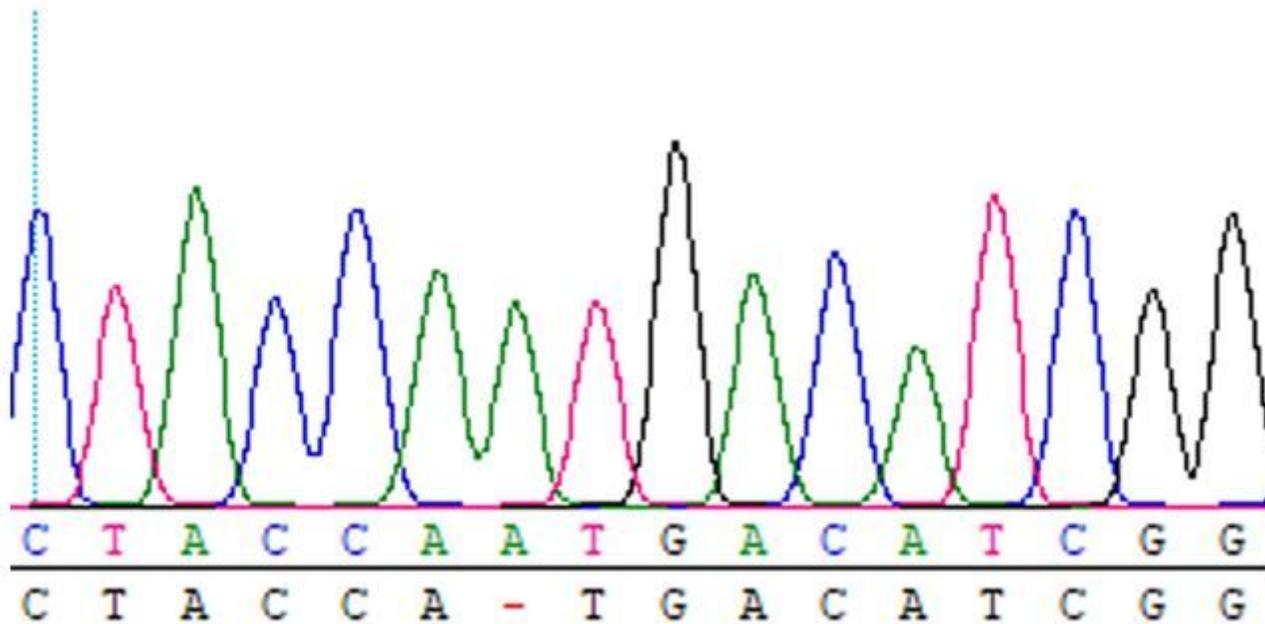


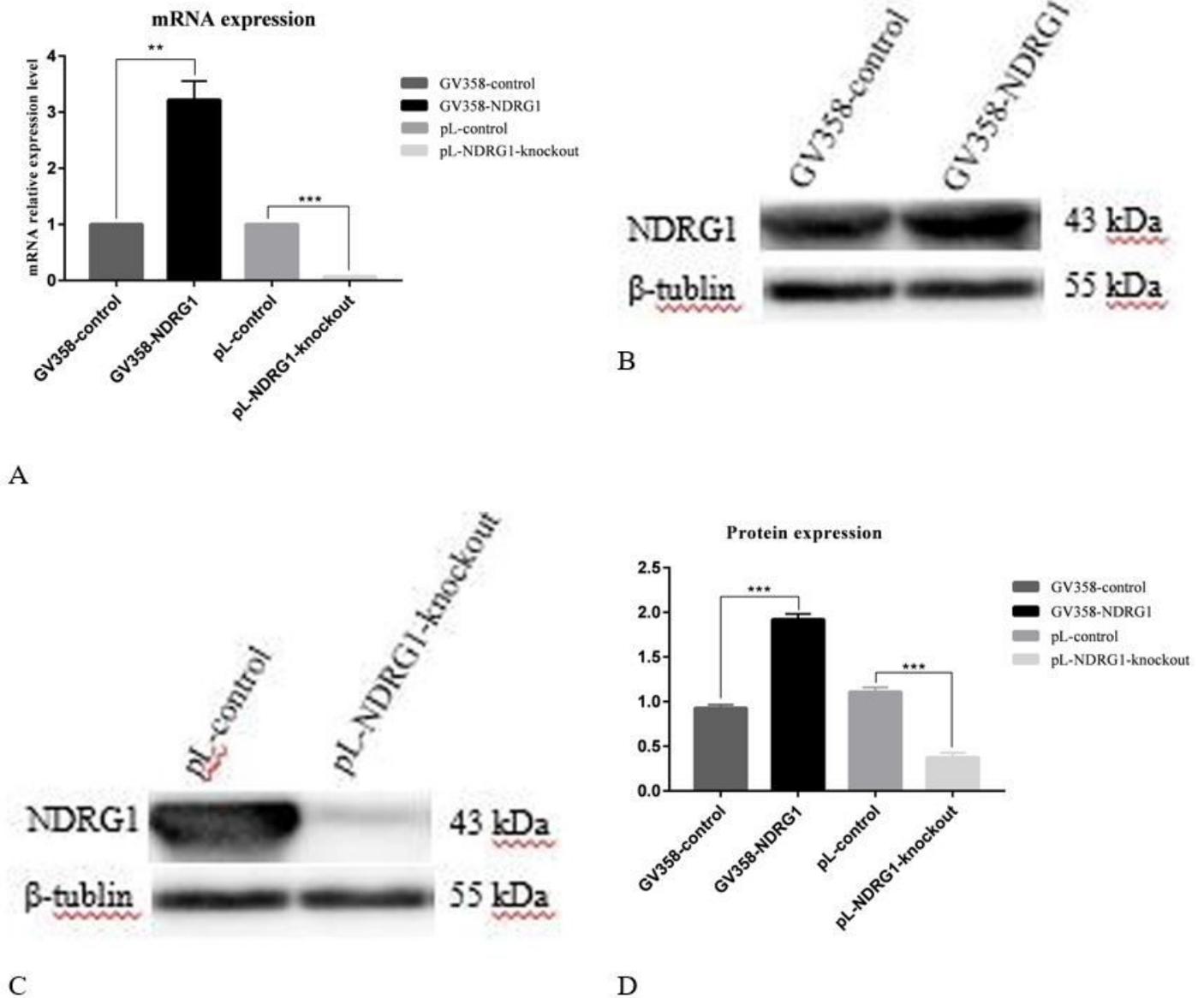
Figure 2

Sorting efficiency of monoclonal cells. Sorting efficiency of pL-NDRG1 -knockout cells for monoclonal cells (4.73%).



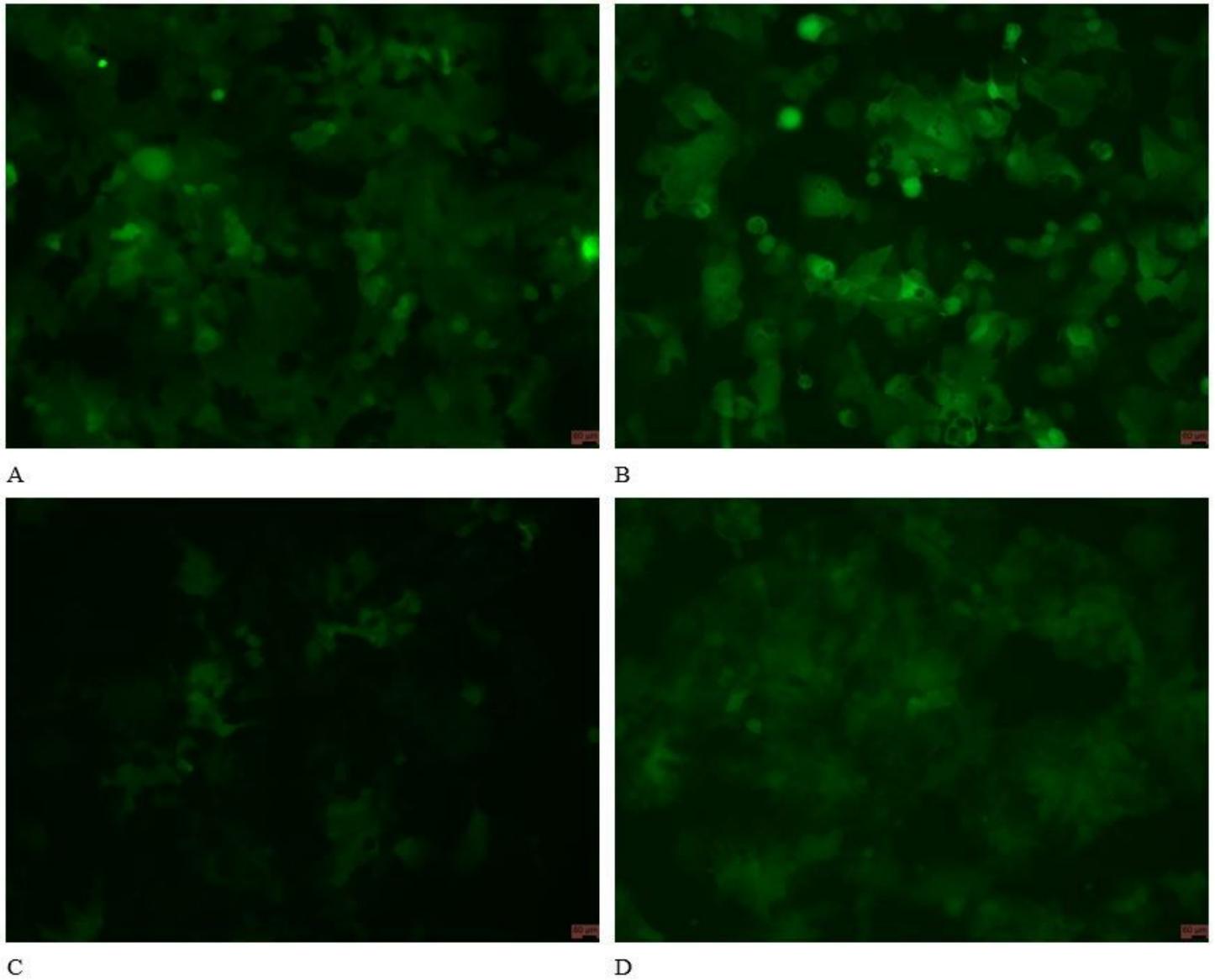
**Figure 3**

Sequencing results of NDRG1 exon 3 of NDRG1 knockout cells. A DNA strand nick was generated on the third exon of NDRG1, then an A base was inserted.



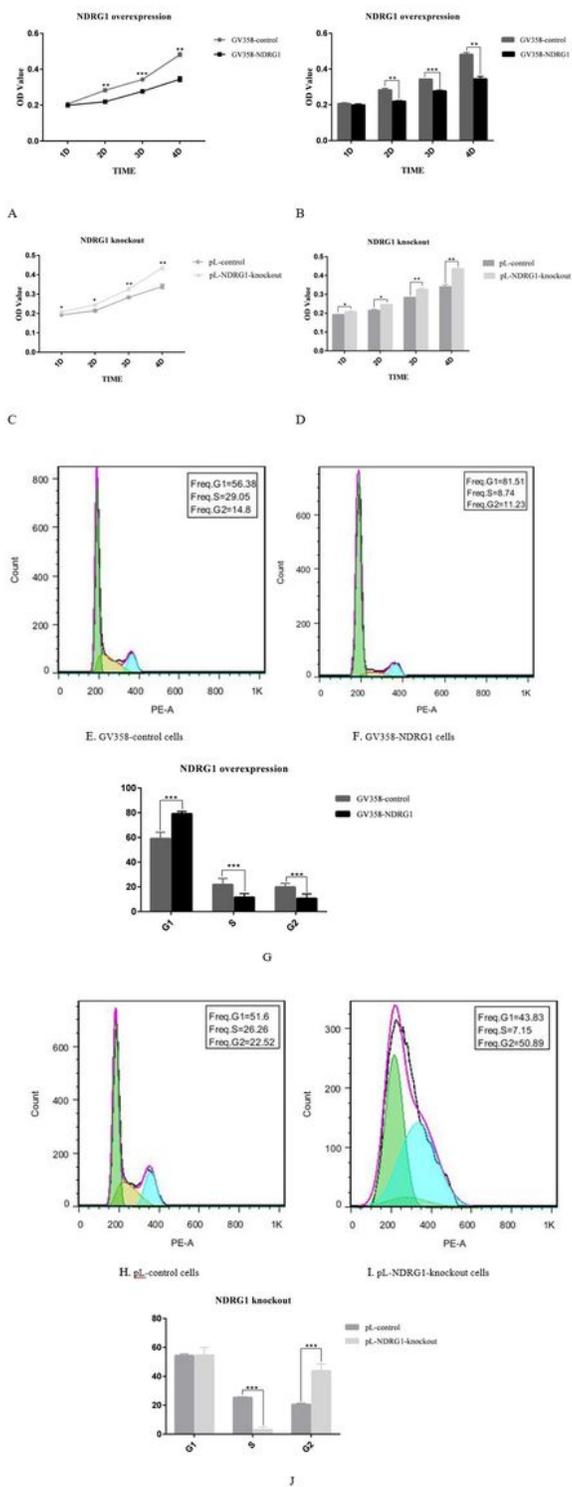
**Figure 4**

The results of QPCR and Western Blotting after NDRG1 overexpression and knockout. (A) The mRNA levels of NDRG1 were detected by the real-time polymerase chain reaction procedure. The relative levels of NDRG1 mRNA were calculated by the values of  $\Delta\Delta C_t$  by normalizing with that of GAPDH. \* $P < 0.05$  versus the control. Experiments were repeated three times. (B-D) Representative western blot bands and statistical results of NDRG1 protein expression normalized by  $\beta$ -tubulin. \* $P < 0.05$  versus control cells. Experiments were repeated three times.



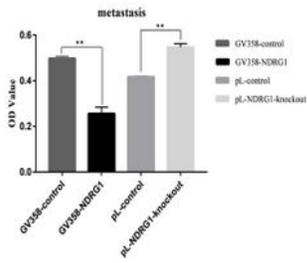
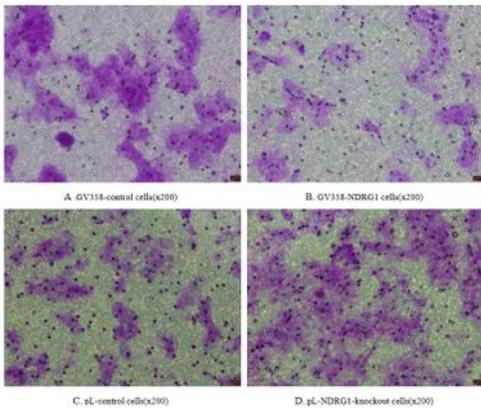
**Figure 5**

Cells under a fluorescence microscope. (A) GV358-Control cells (x200). (B) GV358-NDRG1 cells (x200). (C) pL-control cells (x200). (D) pL-NDRG1-knockout cells (x200). Scale bar: 60  $\mu$ M.

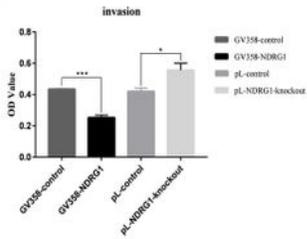
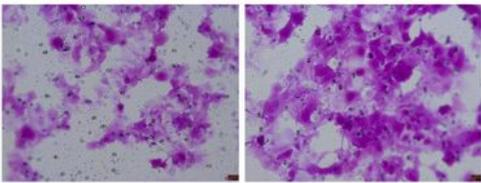
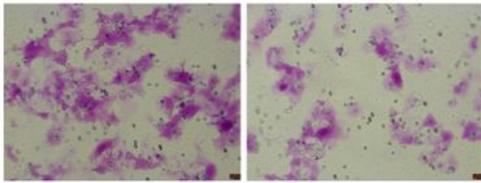


**Figure 6**

NDRG1 inhibits proliferation of Caco2 cells. (A-D) Cell viability was measured using the CCK-8 assay and the statistical results. \*P<0.05 versus control. (E-J) Cell cycle was measured using flow cytometry and the statistical results. \*P<0.05 versus control. Experiments were repeated three times.



E

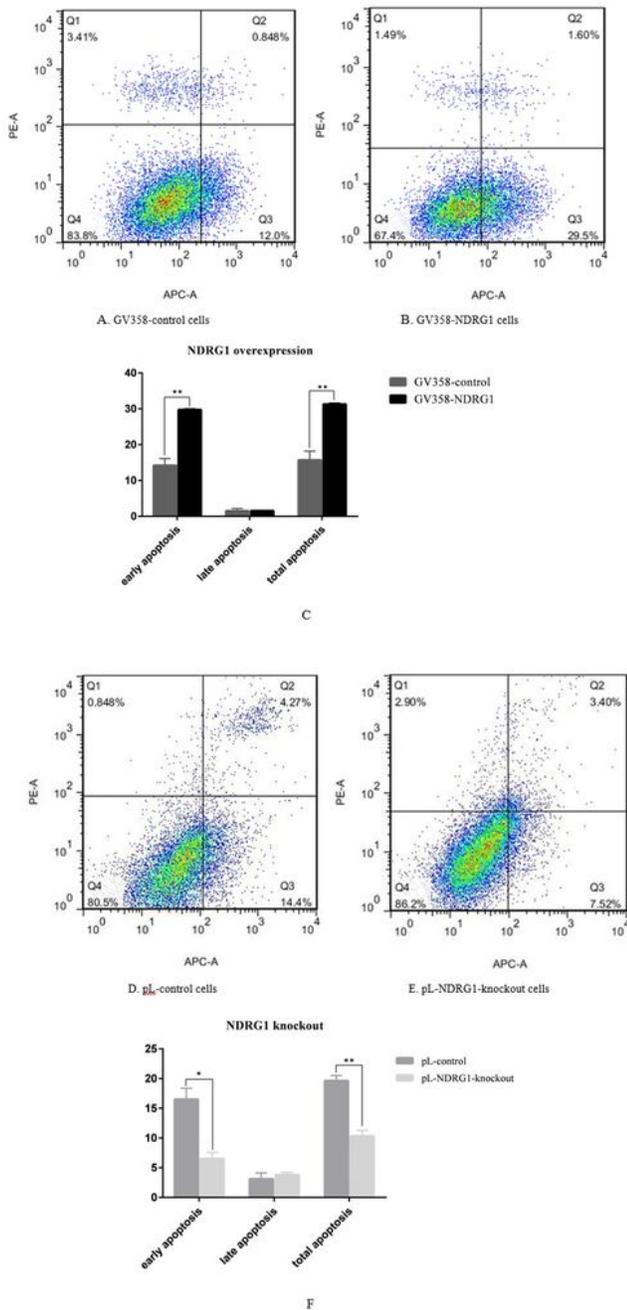


J

**Figure 7**

NDRG1 inhibited metastasis and invasion of Caco2 cells. 24-transwell was used to detect metastasis and invasion abilities. The cells stained by crystal violet were eluted with 33% acetic acid, and the absorbance at 570 nm of the eluate was measured. (A-E) number of cells passing through the chamber at 48h of metastasis experiment and the statistical results. \*P<0.05 versus control. (F-J) number of cells passing

through the chamber at 48h of the invasion experiment and the statistical results. \*P<0.05 versus control. Experiments were repeated three times.



**Figure 8**

NDRG1 promoted early apoptosis of Caco2 cells. (A-F) Annexin V-APC/propidium iodide (PI) staining by flow cytometry was performed to detect the apoptosis of cells. Early apoptosis (Annexin V-APC<sup>+</sup>/PI<sup>-</sup>), late

apoptosis (Annexin V-APC+/PI+), and necrosis (Annexin V-APC-/PI+), with the statistical results. \*P<0.05 versus control. Experiments were repeated three times.