N-Myc downstream-regulated gene 2 suppresses proliferation and induces oncosis of OS-RC-2 human renal cancer cells

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Abstract. V-myc avian myelocytomatosis viral oncogene homolog (Myc) regulates cell proliferation, differentiation and apoptosis in several types of cancer. N-myc downstream-regulated gene 2 (NDRG2) is known to exhibit reduced expression in renal cell carcinoma (RCC) tissues compared with adjacent non-neoplastic tissues and is an independent poor prognostic factor predicting survival in RCC. In the present study, green fluorescent protein (GFP)-NDRG2 and control GFP recombinant adenovirus plasmids were constructed and used to infect human renal cancer (OS-RC-2) cells. NDRG2 expression was measured using western blot analysis and the subcellular localization of NDRG2 was detected using confocal microscopy. The rate of proliferation of the cells was measured using colony formation and MTT assays, and the cell cycle was analyzed using flow cytometry. The results showed that the OS-RC-2 cells expressed little NDRG2 prior to infection with GFP-NDRG2 recombinant adenovirus; however, following infection, NDRG2 was found to be overexpressed, primarily in the mitochondria. The proliferation rate of the OS-RC-2 cells was reduced by NDRG2. Approximately 84.8% of the NDRG2-expressing cells were in S phase compared with 58.7% in the control virus-infected cells ($P<0.05$). In addition, the upregulation of NDRG2 induced a higher proportion of OS-RC-2 cells to undergo oncosis instead of apoptosis. In conclusion, the results from this study suggest that NDRG2 expressed in mitochondria may arrest renal cancer cells in S phase, decrease cell proliferation and induce oncosis. This indicates that NDRG2 is not only a biomarker, but may also be a therapeutic target for the treatment of RCC.

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Introduction

As one of earliest discovered oncogenes, v-myc avian myelocytomatosis viral oncogene homolog (Myc) regulates cell proliferation, differentiation and apoptosis (1). The N-Myc downstream-regulated gene (NDRG) family is novel class of Myc-repressed genes. At present four members (NDRG1-4) have been identified, which are expressed in the brain, heart, skeletal muscle and kidneys (2,3). Since it is cloned as a downregulated gene in glioblastoma, NDRG2 has different expression patterns in tumor and normal tissue (4-6). As a novel p53-inducible gene, NDRG2 may be involved in the p53-mediated apoptosis pathway in response to DNA damage; however, NDRG2 may also have a role in the inhibition of proliferation that is independent of p53 (7).

Renal cell carcinoma (RCC), a major neoplasm arising from the kidney, accounts for 3% of all malignant tumors in adults (8). The incidence rates are higher in Western countries than those in Asian countries. NDRG2 expression in two cancer cell lines, A-498 and 786-O, has been found to be markedly decreased compared with that in two human kidney proximal tubular cell lines, HK-2 and HKC. The expression of NDRG2 is also downregulated in RCC tissues compared with that in adjacent normal tissue, indicating that NDRG2 may have an important role in carcinogenesis (9). Furthermore, a high proportion of RCC cases exhibit reduced NDRG2 expression. According to a recent multivariate analysis, NDRG2 is an independent poor prognostic factor predicting survival in RCC (10).

In the present study, NDRG2 was overexpressed in human renal cancer OS-RC-2 cells in order to determine whether NDRG2 is a potential therapeutic target in RCC.

Materials and methods

Cell culture. The human renal cancer cell line, OS-RC-2 (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China), was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/l streptomycin in a humidified atmosphere containing 5% CO_2, which was maintained at 37˚C. The present study was approved by the Ethics Committee of Guangdong Medical College (Guangdong, China).

Constructs and infection. The recombinant adenoviruses pAV.EX1d-NDRG2/IRES/eGFP and pAV.EX1d-eGFP were constructed as previously described (11). A multiplicity of infection of 40 was determined experimentally for the OS-RC-2 cells. Cells were seeded in six-well plates and incubated to reach ~80% confluence. The growth medium was removed and adenoviruses expressing NDRG2 (OS-RC-2-NDRG2 group) or the control gene green fluorescent protein (GFP) (OS-RC-2-GFP group) were added to OS-RC-2 cells with serum-free medium, and the cells were then incubated for 16 h. The medium was subsequently replaced with growth medium and the cells were incubated for different periods of time. Uninfected cells served as another control (OS-RC-2 group).

Western blot analysis. Protein from lysed cells was measured using the bicinchoninic acid protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). A total of 100 μg lysate was loaded per lane onto SDS polyacrylamide gels for separation by electrophoresis and transfer onto Hybond™ nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). Following transfer, the membranes were incubated with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h at 37˚C. Anti-NDRG2 (Abnova, Taiwan, China) was then added and the membranes were incubated overnight at 4˚C. The membranes were subsequently washed three times with phosphate-buffered saline (PBS) prior to incubation with the secondary antibody for 1 h at room temperature. The blots were then developed using chemiluminescence substrate solution (Pierce Biotechnology, Inc.) and exposed to X-ray film for visualization.

Cell growth assays. The cell growth was monitored using the MTT assay. Briefly, cells were seeded into 96-well plates at an initial density of 2,000 cells/well in triplicate. At each time-point, the cells were washed and incubated with tetrazolium salt (100 mg/ml; Sigma, St. Louis, MO, USA) at 37˚C for 4 h. The supernatant was removed, and 150 μl dimethyl sulfoxide was added to each well. The absorbance (optical density) of the reaction solution at 570 nm was recorded.

Colony formation assay. OS-RC-2 cells, which were stably infected with either the control virus (pAV.EX1d-eGFP) or the virus carrying NDRG2 (pAV.EX1d-NDRG2/IRES/eGFP), were seeded into 100-mm dishes at a density of 500 cells/dish. The cells were grown for two weeks in culturing medium. The colonies were then fixed and stained with Coomassie Brilliant Blue.

Cell cycle analysis. Cells were seeded overnight on 60-mm-diameter plates in a complete medium, placed in a serum-free medium for 48 h to synchronize the cells and then maintained again in the complete medium. After 24 h, the cells were recovered and washed with ice-cold PBS, prior to being suspended in ~0.5 ml 70% alcohol and maintained at 4˚C for 30 min. The suspension was filtered through a 50-mm nylon mesh, and the DNA content of the stained nuclei was analyzed using a flow cytometer (Epics XL; Beckman Coulter, Miami, FL, USA). The cell cycle was analyzed using Multicycle-DNA cell cycle analysis software (FACScan™; Becton-Dickinson, San Jose, CA, USA). The proliferation index (PI) was calculated using the following formula: $PI = (S + G_2)/(S + G_2 + G_1)$.

NDRG2 subcellular localization analysis. The cells were grown on glass coverslips. At different time-points subsequent to transfection, the cells were incubated for 30 min at 37˚C with either 5 nm Rhodamine 123 (a cell-permeable mitochondria-selective dye; Molecular Probes®, Invitrogen Life Technologies, Carlsbad, CA, USA), Bodipy (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene), a cell-permeable Golgi apparatus-selective dye, or Lucifer Yellow (a cell-permeable lysosome-selective dye) obtained from Sigma. The cells were then rinsed with PBS three times and fixed in freshly prepared cold 4% paraformaldehyde in PBS for 30 min at 4˚C. The glass coverslips were then placed on the glass slides, and mounting medium was used to hold the specimens in place. Slides were observed under a confocal laser fluorescence microscope.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analyses were performed using the SPSS software package (SPSS, Inc., Chicago, IL, USA). A one-way analysis of variance followed by the t-test for independent groups was used. The χ^2-test or Fisher exact test was used to assess dichotomous variables. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Upregulation of NDRG2 in OS-RC-2 cells. To confirm that the cells had been successfully transfected, the cell morphology was observed using light and fluorescence microscopes. Almost all the cells were found to express GFP in the GFP virus-infected group, compared with only 60% of cells in the GFP-NDRG2 virus-infected group (Fig. 1).

The protein expression levels of NDRG2 were then analyzed using western blotting. In the normal cultured OS-RC-2 cells, little NDRG2 expression was detected. However, following infection with the recombinant GFP-NDRG2 adenovirus, the NDRG2 levels were significantly elevated, while the NDRG2 expression remained low in the cells infected with the control GFP vector (Fig. 2).

Localization of NDRG2 in OS-RC-2 cells. To evaluate the subcellular localization of NDRG2, organelle-specific dye was used. Following GFP-NDRG2 infection, NDRG2 was found to be mainly expressed in the mitochondria, but not in the Golgi apparatus or lysosome. The expression of NDRG2 in the mitochondria increased between 6 and 72 h after transfection (Fig. 3).

NDRG2 inhibits OS-RC-2 cell proliferation. The results from the colony formation assay demonstrated that NDRG2 inhibited the colony formation of OS-RC-2 cells at two weeks (OS-RC-2-NDRG2 cloning efficiency, 24.8%, vs. OS-RC-2-GFP cloning efficiency, 51.6% and OS-RC-2 cloning efficiency, 76.4%; Fig. 4A, Table I)

Cell proliferation was then observed over time. The results demonstrated that uninfected OS-RC-2 cells continued proliferating up until day 7. However, the growth curve for the cells infected with GFP-NDRG2 was significantly lower than that

Table I. Cloning efficiency of the three groups of cells.

Cells	Number of cloning cells	Cloning efficiency (%)
OS-RC-2	382	76.40
OS-RC-2-GFP	258	51.60
OS-RC-2-NDRG2	124	24.80

Cloning efficiency was calculated by determining the number of cloning cells per 500 cells. GFP, green fluorescent protein; NDRG2, N-Myc downstream-regulated gene 2.

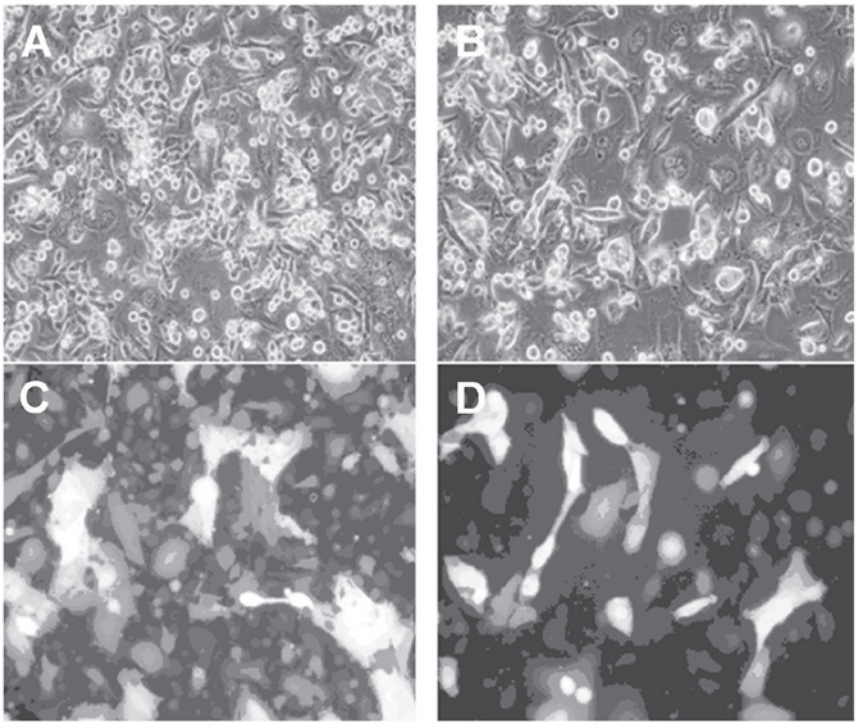

Figure 1. Validation of transfection using (A and B) light (x200) and (C and D) fluorescence microscopy (x400). (A and C) GFP gene transfection into OS-RC-2 cells. (B and D) GFP-NDRG2 gene transfection into OS-RC-2 cells. GFP, green fluorescent protein; NDRG2, N-Myc downstream-regulated gene 2.

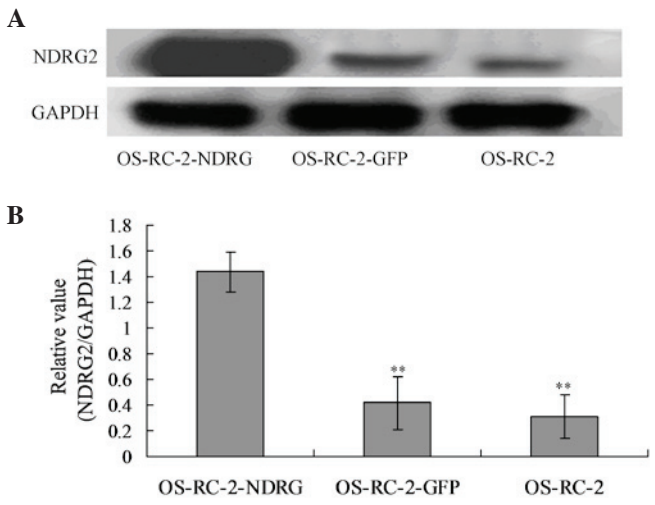

Figure 2. Overexpression of NDRG2 following infection with NDRG2 recombinant adenovirus. (A) Western blot analysis of NDRG2 protein expression in OS-RC-2 cells. (B) Relative expression levels of NDRG2 from the western blot bands. **P<0.01 compared with the OS-RC-2-NDRG2 group. GFP, green fluorescent protein; NDRG2, N-Myc downstream-regulated gene 2.

for the control cells infected with GFP (P<0.05; Fig. 4B). This suggested that NDRG2 had the potential to inhibit the proliferation of OS-RC-2 cells.

NDRG2 induces cell cycle arrest in OS-RC-2 cells. To further investigate the mechanism by which NDRG2 inhibits OS-RC-2 cell growth, the effect of NDRG2 expression on the cell cycle was analyzed using fluorescence-activated cell sorting analysis. The results demonstrated that 84.3% of the GFP-NDRG2-transfected cells were in S phase compared with 58.7% of untransfected cells. Furthermore, 11.8% of the GFP-NDRG2-transfected cells were in G_1 phase compared with 22.4% of untransfected cells (P<0.05; Fig. 5).

NDRG2 induces OS-RC-2 cell oncosis instead of apoptosis. Forty-eight hours after infection, a small number of cells appeared to have shrunk and showed marked nuclear chromatin condensation, which are markers of apoptosis. By contrast, a greater number of cells showed swelling and nuclear chromatin clumping, which are markers of oncosis,

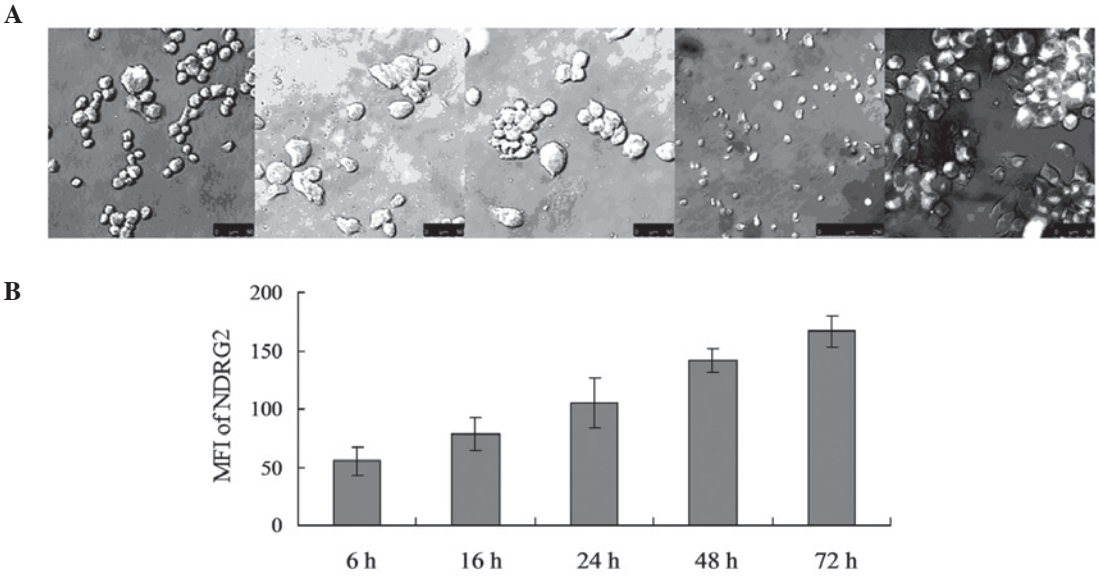

Figure 3. Subcellular localization of NDRG2 following infection. (A) Subcellular localization of NDRG2 protein in the mitochondria 6, 16, 24, 48 and 72 h subsequent to transfection. (B) Statistical analysis. NDRG2, N-Myc downstream-regulated gene 2; MFI, mean fluorescence intensity.

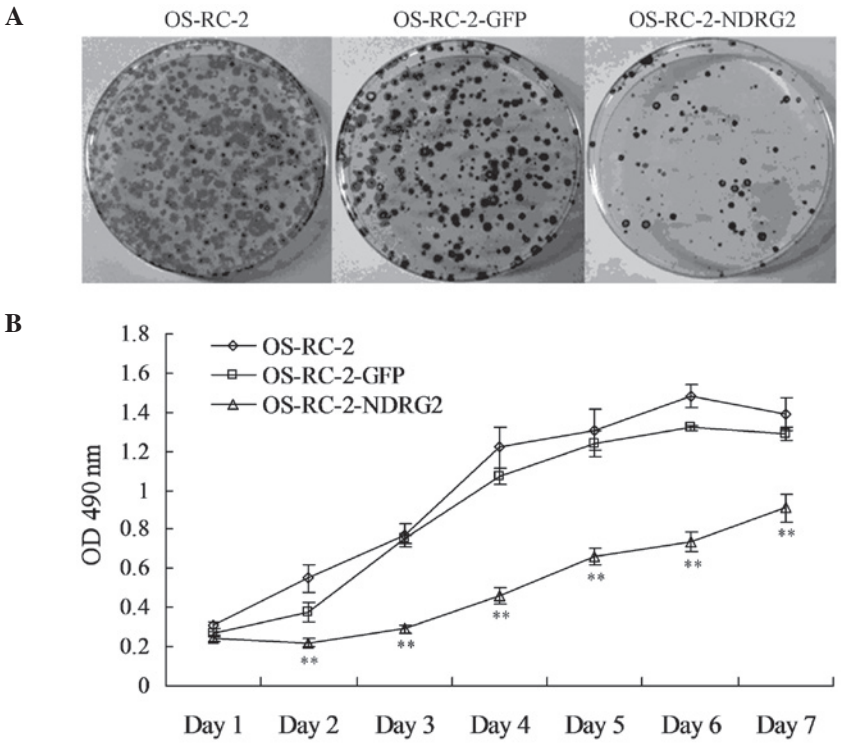

Figure 4. Overexpression of NDRG2 inhibits growth of OS-RC-2 cells. (A) Colony formation assay for the OS-RC-2 cell proliferation in the three groups. (B) Colony formation on days 1-7. $^{**}P<0.01$ compared with the OS-RC-2-GFP and OS-RC-2 groups. NDRG2, N-Myc downstream-regulated gene 2; OD, optical density; GFP, green fluorescent protein.

in the GFP-NDRG2 infection group compared with the GFP infection (Fig. 6).

Discussion

Several studies have previously shown that the downregulation of NDRG2 in RCC is correlated with aggressive clinicopathological features in patients with RCC (8-10). The mechanism underlying this effect may involve p53-dependent apoptosis and the p53-independent pathway. In the present study it was demonstrated that NDRG2 is not only a biomarker of RCC, but also a therapeutic target for the treatment of RCC.

It was demonstrated in this study that the upregulation of NDRG2 by infection with NDRG2 recombinant adenovirus inhibits OS-RC-2 cell proliferation. Additionally, it was found that NDRG2 tumor suppressor activity is mediated by the inhibition of cell cycle progression with an increased accumulation of cancer cells in S phase. It has been previously reported that

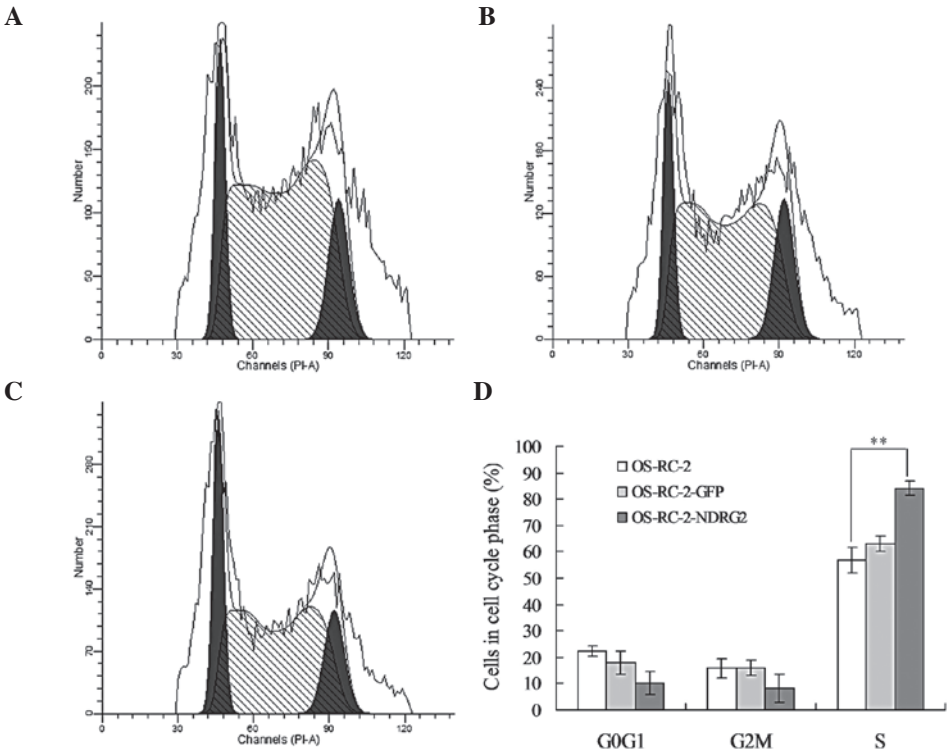

Figure 5. NDRG2 induces cell cycle arrest in OS-RC-2 cells. Cell cycle detection of cells in the (A) OS-RC-2-GFP-NDRG2, (B) OS-RC-2-GFP and (C) OS-RC-2 groups. (D) Analysis of the G_0G_1, G_2M and S phase cells in the three groups. $^{**}P<0.01$, OS-RC-2 group versus the OS-RC-2-GFP-NDRG2 group. NDRG2, N-Myc downstream-regulated gene 2; GFP, green fluorescent protein; PI, propidium iodide.

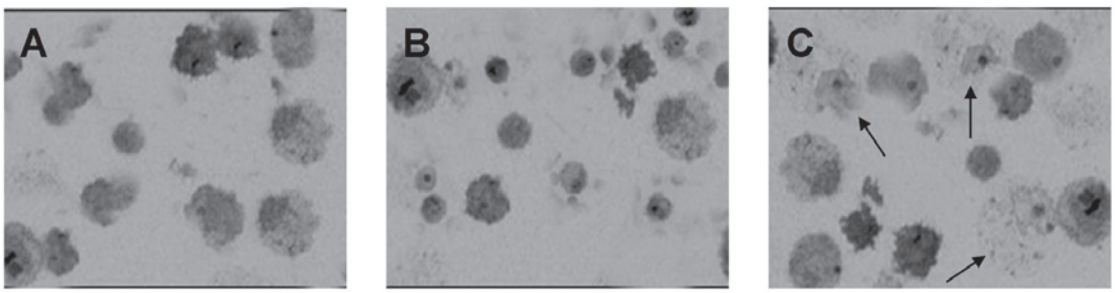

Figure 6. NDRG2 transfection triggers oncosis in the OS-RC-2 group. Oncosis was not observed in the (A) OS-RC-2 or the (B) OS-RC-2-GFP groups. (C) Oncosis was observed in the OS-RC-2-GFP-NDRG2 group. The black arrows indicate cells undergoing oncosis. NDRG2, N-Myc downstream-regulated gene 2; GFP, green fluorescent protein.

NDRG2 suppresses activator protein-1 activity and regulates cyclin D1 expression via the phosphorylation pathway in human colon carcinoma cells (12). The results from the present study are in accordance with those of Ma *et al* (13), which also showed that NDRG2 inhibits cancer cell proliferation. However, Ma *et al* found an increase in the accumulation of cancer cells in the G_1 phase and a reduction in the number of cells in the S phase of the cell cycle (13). The different cell lines and adenovirus vectors used may account for the different findings.

Another difference between the findings of the present study and previous studies is that, in the present study, NDRG2 induced oncosis instead of apoptosis (9,10). Oncosis is a type of accidental cell death that is characterized by cellular and organelle swelling, blebbing and an increase in membrane permeability. These alterations in membrane permeability are a result of the failure of the ion pumps in the plasma membrane (14). A rapid decrease in intracellular adenosine triphosphate (ATP) is an important biochemical event leading to oncosis, as opposed to apoptosis (15). Interference with ATP synthesis rapidly leads to deactivation of the Na^+/K^+-ATPase in the cell membrane, resulting in an increase in the intracellular concentrations of Na^+ and Cl^- accompanied by water influx, cellular swelling and a rapid increase in the intracellular Ca^{2+} concentration (16). A number of chemotherapeutic agents, including arsenic trioxide, cisplatin and etoposide, have also been found to induce both oncosis and apoptosis (17-19). The interrelation between apoptosis and oncosis is increasingly being recognized, including in models characterized by the initial activation of apoptosis followed by oncosis and, conversely, oncosis followed by apoptosis (20).

The differences between the findings of the present study and previous reports may also be due to the localization of

NDRG2. In cells in a normal state, NDRG2 protein is primarily located in the cytoplasm, although it is also associated with the cell membrane and adherens junctions (21). Following damage to the DNA, NDRG2 can be translocated from the cytoplasm to the nucleus. Wang *et al* (22) demonstrated that the NDRG2 fragment comprising amino acid residues 101 to 178 is responsible for the nuclear translocation. It has been proposed that nuclear NDRG2 expression may affect cancer biology. However, in the present study, NDRG2 was primarily expressed in the mitochondria and its expression increased in a time-dependent manner. Mitochondrial function is impaired in cancer cells. The metabolism of proliferative tumor cells utilizes mitochondria as functional biosynthetic organelles. In proliferating cells, the use of mitochondrial enzymes in the synthesis of anabolic precursors takes priority over the oxidative phosphorylation-dependent production of ATP. It has been reported that NDRG2 overexpression in malignant breast cancer cells specifically inhibits Akt phosphorylation and induces the phosphorylation of p38 mitogen-activated protein kinase and stress-activated protein kinase/ c-Jun NH_2-terminal kinase, which drives anabolic metabolism and tumorigenesis by reprogramming the mitochondria (23,24). It has also been shown that a modest increase in uncoupling protein 2 expression results in a reduction in intracellular ATP levels and a marked, rapid fall in mitochondrial membrane potential, which causes morphological oncosis (25). However, whether NDRG2 regulates renal cancer cell proliferation and oncosis through mitochondrial reprogramming requires further investigation.

In conclusion, it was found in the present study that NDRG2 is expressed in the mitochondria of OS-RC-2 cells, and that NDRG2 expression arrests cells in the S phase, decreases renal cancer cell proliferation and induces oncosis. These results further expand the present understanding of NDRG2 and its role in tumor cells, and may have important implications for targeting NDRG2 for cancer therapy.

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