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# The physical and functional interaction of NDRG2 with MSP58 in cells

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**Abstract**

*NDRG2*, a member of N-Myc downstream regulated gene family, exerts the important functions in cell differentiation and tumor suppression. Although the ectopic expressed Ndrg2 inhibits the proliferation of tumor cells, its intracellular signal transduction pathway is hardly known. Here, we identified MSP58, a 58-kDa microspherule protein, as an interacting partner of human Ndrg2 by using yeast two-hybrid screening. The interaction was confirmed by glutathione *S*-transferase pull-down assay *in vitro* and by co-immune-precipitation assay *in vivo*. The forkhead associated domain of MSP58 is essential for its interaction with Ndrg2. Ndrg2 could co-localize with MSP58 in nuclear of HeLa cell during cell stress. Furthermore, the modulation of Ndrg2 level influences the cell cycle process together with MSP58. In conclusion, the findings offered a novel insight into the physiological roles of Ndrg2.


*Keywords:* Ndrg2; Yeast two-hybrid; MSP58; Cell cycle regulation

*N-Myc* down regulated gene (*NDRG*) 2, which was first identified from a normal human brain cDNA library in 1999 (GenBank Accession No. AF159092), belongs to a new family of differentiation-related genes, the *NDRG* family [1,2]. This family includes four members, *NDRG*1, *NDRG*2, *NDRG*3, and *NDRG*4. The currently available bioinformatic analysis does not indicate any known motif or domain in *NDRG2* and other members of *NDRG* family [3].

The previous studies suggest that *NDRG* gene family may play a role in cell function regulation. Indeed, expression of *NDRG*1 is induced by hypoxia and has been implicated in Schwann cell signaling for axonal survival [4,5]. In addition, Ndrg1 is up regulated by differentiation-related retinoid and Vitamin D3 in human leukemia cells [6]. Ndrg2 is highly related to Ndrg1 both in structure and function. It was reported that Ndrg2 level was regulated by the agents including mineralocorticoid, androgen, and glucocorticoid [7–9]. *NDRG*2 gene expression was also induced during cell differentiation as well as embryo development [10–12]. Ndrg2 mRNA and protein levels were down regulated in tumors [1,13]. Stable ectopical expression of human Ndrg2 in glioblastoma cell lines decreases cell growth rates [1]. Recently, Ndrg2 is proved to be a novel target for Akt and may represent a site for PKC-mediated inhibition of insulin signal transduction [14]. These findings suggest that Ndrg2 seems to be broadly involved in stress responses, cell proliferation, and differentiation.

To identify Ndrg2, interacted proteins may provide important information for understanding its precise molecular and cellular functions. Therefore, we applied yeast two-hybrid system to screen an adult brain cDNA library using Ndrg2 as the bait. A cell cycle-dependent transcription factor MSP58 (58-kDa microspherule protein)/MCRS1 (microspherule protein 1) was picked up as the binding partner of Ndrg2.

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MSP58/MCRS1 has been reported to interact with several proteins, such as nucleolar protein p120, Mi-2β; transcription factors Daxx, STRA13, and also RNA-binding protein FMR [15–18]. These findings suggest that MSP58 may have functions of transcriptional regulation in the nucleus and nucleolus. In addition, TOJ3, a protein with high structural similarity to MSP58, exhibits transforming activity, whereas phosphatase and tensin homologue (PTEN) suppresses its transforming activity [19,20].

In the present study, we identified MSP58 as an Ndrg2-interacting protein by yeast two-hybrid screening. The physical association of Ndrg2 with MSP58 was shown both *in vitro* and *in vivo*. The co-localization of two proteins was confirmed and the functional significance of the interaction is discussed here.

## Materials and methods

*Plasmid construction*. The full-length of coding regions of *NDRG*1, *NDRG*2, *NDRG*3, *and NDRG*4 as well as five truncated *NDRG*2 cDNA fragments was inserted in-frame into the multiple cloning sites of pGBKT7 (Clontech, Palo Alto, CA) to generate different bait plasmids. The prey plasmids were constructed by subcloning the full-length MCRS2 or MSP58 cDNA into pACT2 (Clontech) and named as pACT2-MCRS2 and pACT2-MSP58, respectively. MSP58 cDNA was further truncated into five fragments (shown in Fig. 1A) and inserted into pACT2 vector.

Prokaryotic vectors expressing GST-fused MSP58, *NDRG*2 or their shortened form proteins were constructed by inserting corresponding fragments into pGEX4T-2 vector, respectively. The pCMV-Myc-*NDRG2*, pCIneo-FLAG- MSP58, and pCMV-Myc-MSP58$^{115–462}$, expressing the Myc-tagged Ndg2, Myc-tagged truncated MSP58 (amino acids 115–462), and FLAG-tagged MSP58 protein, respectively, were generated by subcloning corresponding cDNA into pCMV-Myc or pCIneo-FLAG vector separately. PDsRed2-N1-NDRG2, expressing a RFP (red fluorescent protein) fused Ndrg2 protein, and pEGFP-C3-MSP58, expressing a GFP (green fluorescent protein) fused MSP58 protein, were constructed by inserting *NDRG*2 or MSP58 full-length cDNA into pDsRed2-N1 (Clontech) or pEGFP-C3 vector (Clontech). All the newly constructed plasmids were confirmed by sequencing.

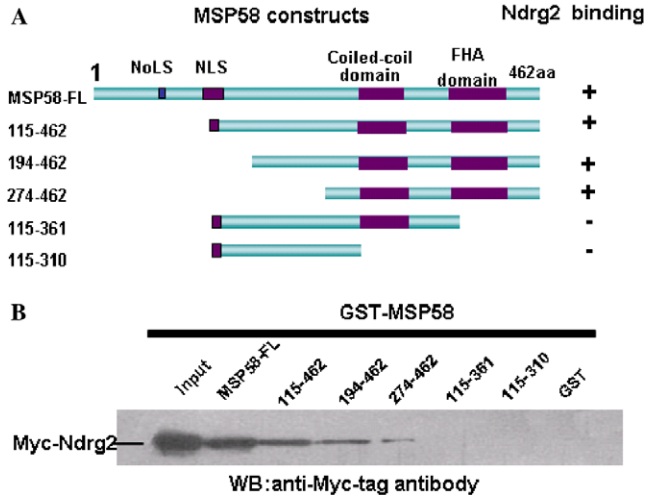


Fig. 1. Ndrg2 interacts with the COOH-terminal of MSP58. (A) Schematic of the full-length MSP58 and its truncated fragments. The cDNAs encoding them were fused to Gal4-AD in pACT2 vector,and co-transformed into yeast *AH109* with full-length Ndrg2-Gal4-BD vector. The grown clones on SD/-Trp-Leu-His-Ade/X-α-Gal dish were transferred onto nitrocellulose membrane and tested for β-galactosidase activity. The "+" stands for positive interaction and "−" for negative clones. (B) The Ndrg2 protein bound to GST fusions. The numbers on each lane stand for the first and last amino acids of truncated MSP58 fused with GST.

*Yeast two-hybrid assay*. Human brain cDNA library was purchased from Clontech. The yeast two-hybrid was performed according to the manual provided by Clontech. The plasmid DNA was recovered from the positive clones and sequenced. To test the protein–protein interaction in yeast, the bait and prey vectors were co-transformed into yeast AH109, reporter gene test was performed following the manual.

*Cell culture and gene transfection*. HHCC (human hepatocellular carcinoma), HeLa, and COS-7 cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies) in a humidified 5% $CO_2$ atmosphere at 37 °C. In all of transfection experiments, the cells were seeded in a 60- or 100-mm plate at proper density and transfected the next day with the various plasmid DNA using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. The transfected cells were collected for further experiments at indicated time.

*GST pull-down assay*. Various GST fusion proteins were expressed in *Escherichia coli*. The fusion proteins were purified with glutathione–agarose beads (BD Biosciences Pharmingen, San Diego, CA). Ten micrograms of GST fusion proteins (on agarose) was incubated with cell lysate for 2 h at room temperature in 500 μL of pull-down buffer (10 mM Tris, pH 7.4, 150 mM NaCl, and 0.5% NP-40) containing Protease Inhibitor Cocktail (Roche, IL). The agarose beads were collected and washed four times with the pull-down buffer. After adding 40 μL of SDS sample buffer and 3 min heating at 95 °C, the supernatant was used for 12% SDS–PAGE, followed by Western blotting with anti-Myc antibody (9E10, Clontech, 1:1000).

*Co-immune-precipitation and Western blot*. The cell lysates were prepared with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% NP-40, 0.1 mM PMSF, and Protease Inhibitor Cocktail). The cell lysates were incubated with an anti-Myc (Clontech), anti-Flag antibody (Sigma–Aldrich, St. Louis, MO) or anti-Ndrg2 antibody (Santa Crutz Biotechnology, CA). The immune-complexes were precipitated with protein A- or protein G–Sepharose 4B (Amersham Biosciences, Piscataway, NJ). After washing with the lysis buffer, the co-precipitated proteins were separated by SDS–PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). The blots were probed with various antibodies. To visualize antibody-bound protein, the proper secondary antibodies conjugated to horseradish peroxidase (1:4000 dilution; Santa Cruz Biotechnology) and ECL detection solutions (Pierce, Rockford, IL) were applied.

*Fluorescence Microscopy analysis*. For visualizing the localization of GFP- or RFP-fused MSP58 or Ndrg2 in cells, pDsRed2-N1-NDRG2 and pEGFP-C3-MSP58 were transfected into HeLa cells. Thirty hours after transfection, cell was washed twice with PBS (20 mM potassium phosphate, pH 7.4, and 150 mM NaCl) and fixed with 4% paraformaldehyde, then observed by Fluorescence microscope (Olympus, Japan).

*Cell cycle assay*. The cells cultured in 60-mm dishes were resuspended in 285 μL cold PBS mixed with 15 μL fetal bovine serum and fixed with 0.7 mL ice-cold ethanol. After the ethanol was washed out, the fixed cells were treated with 50 μg/mL RNAse A at 37 °C, incubated with 50 μg/mL propidium iodide (PI) at 4 °C for 30 min, and then analyzed with a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA). Cell cycle distribution was determined using ModFIT software (Verity Software House, Inc., Topsham, ME).

## Results and discussion

### *MSP58 is one of the binding partners of Ndrg2 in yeast*

To identify Ndrg2-interacting proteins, the yeast two-hybrid assay was used to screen a human brain cDNA

library using Ndrg2 as bait. One of the positive candidates encodes a truncated form (lacking 115 amino acids from its N-terminus) of MSP58 protein. This sequence is the common part of MSP58, MCRS1, and MCRS2 protein family.

Then the interactions between Ndrg2 and full-length MSP58, MCRS1, and MCRS2 were further confirmed in yeast. The results showed that all of full-length of MSP58, MCRS1, and MCRS2 proteins could interact with Ndrg2 in yeast significantly, while the control plasmid, pACT2 or PGBKT7, had no interaction with either MSP58 or Ndrg2. Although the four members of Ndrg family are highly homologous, only Ndrg2 was found to interact with MSP58 in yeast (data not shown).

Among the members of *NDRG* family, *NDRG*1 is the most studied and has been reported to involve in multiple cellular responses [21–24]. Recently, accumulating studies indicate that Ndrg2 also participates in cell proliferation and differentiation regulation [1,10–13]. However, the precise molecular mechanism of Ndrg2 is unknown. We identified MSP58 as a novel Ndrg2-interacting protein. To our knowledge, this study is the first report on Ndrg2-interacting protein. It would be helpful for understanding the physiological role of Ndrg2.

### *Mapping of Ndrg2 and MSP58 interacting domain in yeast*

To map the region(s) of Ndrg2 and MSP58 involving their interaction, various truncated mutants of Ndrg2 and MSP58 were subjected to the analysis in yeast two-hybrid assay. Except for the full-length of Ndrg2, any truncation of Ndrg2 could completely abolish its interaction with MSP58 (data not shown). These results implicated the integrity of Ndrg2 protein is necessary for stable interaction with MSP58. The N-terminal-deleted MSP58 proteins ($MSP58^{115–462}$, $MSP58^{194–462}$, and $MSP58^{274–462}$) are keeping the interaction ability with Ndrg2. In contrast, the C-terminal truncated MSP58 proteins ($MSP58^{115–361}$ and $MSP58^{115–310}$) failed to interact with Ndrg2 (Fig. 1A). The results implicated the C-terminal region of MSP58 is necessary for its interaction with Ndrg2.

### *Ndrg2 interacts with MSP58 directly in vitro*

Next, we tested whether the direct association exists between Ndrg2 and MSP58 by using GST pull-down assay. GST-MSP58 fusion protein or GST fusions with the deletion mutants of MSP58 were incubated with the COS7 cell lysate containing Myc-tagged Ndrg2 proteins produced by pCMV-Myc-NDRG2 transfection. The Ndrg2 binding to the various GST fusions was detected by anti-Myc antibody blotting. As shown in Supplement 1and Fig. 1B, Ndrg2 protein could be specifically pulled down by GST-MSP58 fusion protein but not by GST alone.

In agreement with the results of yeast two-hybrid assays, the N-terminal truncated MSP58 proteins could bind Ndrg2, whereas the C-terminal truncated one failed to do so. Therefore, the C-terminal region of MSP58 is sufficient and indispensable for its interaction with Ndrg2.

The C-terminal of MSP58 contains a conserved FHA domain and a coiled coil domain. The FHA domain is a phosphopeptide-binding domain first identified in a group of forkhead transcription factors. In human, many proteins containing an FHA domain are found in the nucleus and involved in DNA repair and cell cycle arrest [25]. It has been proved that FHA domains are competent to bind to phosphopeptides or to peptides in a phosphorylation-dependent manner [26]. Sequence analysis has shown that C-terminus of Ndrg2 is enriched in arginine and serine residues, and thus contains a number of potential phosphorylation sites. Moreover, recent studies proved that Ndrg2 was a novel physiological substrate for both AKT and SGK1 [14]. Therefore, it will clearly be of interest to further examine the influence of Ndrg2 phosphorylation on its binding to MSP58 in future.

### *Co-localization of Ndrg2 and MSP58 in cells*

The data above strongly suggested that MSP58 is a binding partner of Ndrg2. However, Ndrg2 and MSP58 have been reported to be in the different subcellular compartments. Transiently expressed GFP-Ndrg2 in cells showed cytoplasmic localization, whereas MSP58 was supposed to be mainly a nuclear or nucleolar protein [15–18]. To determine whether Ndrg2 is co-localizing with MSP58 in cells, GFP-MSP58 and RFP-Ndrg2 expression vectors were co-transfected into HeLa cells. The expressed proteins were visualized under fluorescence microscope (Fig. 2). The most of GFP-MSP58 proteins were seen in nucleus, meanwhile the small fraction of GFP-MSP58 displayed cytoplasm localization. However, RFP-Ndrg2 localized predominantly to the cytoplasm. Hence, only small fraction of MSP58 and Ndrg2 are co-localized in cytoplasm and nucleus, respectively. It is known that $NiCl_2$ can induce the translocation of Ndrg2 from cytoplasm to nuclear (Wang et al., submitted paper). Therefore, we stimulated

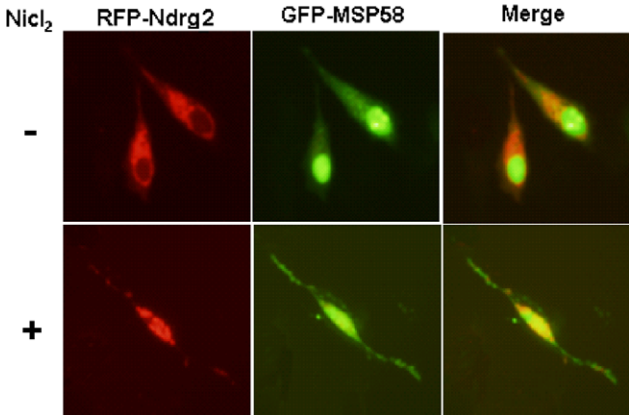


Fig. 2. Co-localization of Ndrg2 and MSP58 in HeLa cells. The co-localization of GFP-MSP58 and RFP-Ndrg2 in HeLa cells upon being treated with $NiCl_2$ for 12 h observed under fluorescence microscope.

the cells expressing GFP-MSP58 and RFP-Ndrg2 with 1 mM $NiCl_2$, a mimic agent of hypoxia, for 12 h. There was much more significant co-localization of Ndrg2 and MSP58 in the nucleus of $NiCl_2$ treated cells.

The case of such cytoplasm protein as Ndrg2, which shuttles from cytoplasm to nuclear, is not rare in cells. For instance, MDMX, a homolog of MDM2, does not have conserved NLS sequence but can translocate to nuclear upon DNA damage [27]. More important, Ndrg1 has been characterized as a shuttle protein [28,29]. The co-localization of Ndrg2 and MSP58 does not mean that they were transported together. The over-expressed MSP58 does not induce the translocation of Ndrg2. Hence, nuclear translocation of Ndrg2 might result from an alternate mechanism, perhaps by interaction with other nuclear proteins.

*Interaction of MSP58 with Ndrg2 in vivo*

To establish whether MSP58 physically associates with Ndrg2 in mammalian cells, the expression constructs encoding FLAG-tagged MSP58 and Myc-tagged Ndrg2 were co-transfected into HeLa cells. The cell lysates were subjected to immune-precipitation assays with anti-FLAG antibody followed by Western blot analysis with anti-Myc antibody. The direct association of MSP58 with Ndrg2 cannot be detected in transfected cells (data not shown). However, consistent to the co-localization data, we do detect the co-precipitation of Ndrg2 and MSP58 if the transfected cells were treated with 1 mM $NiCl_2$ for 12 h (Fig. 3). In addition, if the expression vector encoding Myc-tagged MSP58 fragment ($MSP58^{115–462}$), which lacks the nuclear localization signal in the N-terminal part of MSP58, was transfected into cells, Ndrg2 could be detected in the immune-complex of MSP58 and *vice versa* (shown in Supplement 2). The result suggests that Ndrg2 translocation from the cytoplasm to the nuclear under cell stress, such as hypoxia, is necessary for its physical association with MSP58.

*Regulation of cell cycle by interaction of Ndrg2 and MSP58*

It is known that P78, a splicing alternative isoform of MSP58, plays a role in G2/M cell cycle checkpoint control [30]. Ndrg2 was also proved to markedly reduce the cell proliferation. For better understanding the significance of Ndrg2 and MSP58 interaction, we tested whether the Ndrg2 protein level influences the effect of MSP58 on cell cycles. The cells containing different level of Ndrg2 were established by stably transfecting Ndrg2 cDNA or Ndrg2 SiRNA in HeLa cells (data not shown). When MSP58 cDNA expression vector was introduced into the cells, the percentage of cells in the S phase of cell cycle increased as expected and the extent of S phase cell increasing had no difference in all of cells no matter Ndrg2 level was manipulated or not (Fig. 4). However, if the MSP58 ectopic expressed cells were treated with 1 mM $NiCl_2$ for 12 h

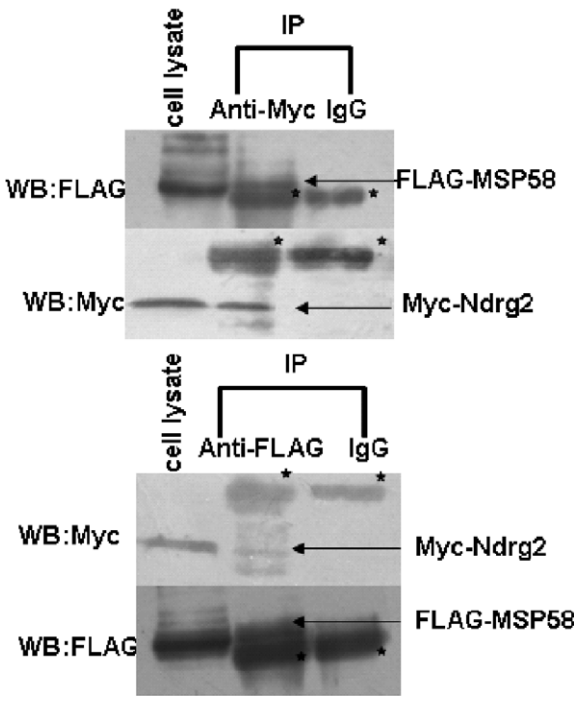


Fig. 3. Ndrg2 interacts with MSP58 *in vivo*. HeLa cells co-transfected with 8 μg pCMV-Myc-*NDRG2* and pCIneo-FLAG- MSP58, then treated with 1 mM of $NiCl_2$ for 12 h. The antibodies for immunoprecipitation and Western blot are as indicated. The location of various proteins is indicated with arrowhead. WB, Western blot; IP, immunoprecipitation; ∗, Ig heavy chain.

before cell cycle analysis, the clear difference of cell number in S phase as well as G2 phase could be observed among the cells with different level of Ndrg2. $NiCl_2$ itself almost has no effects on the cell cycle, but it abolished the S phase cell augmentation in HeLa cells caused by MSP58 overexpression. However, in cells with the lower level of Ndrg2, $NiCl_2$ was unable to affect the cells in S phase caused by MSP58 overexpression. It is reasonable to see this phenomenon because the most of Ndrg2 and MSP58 interaction occurs in nuclear and under cell stress condition. In the case of cells with the higher level of Ndrg2, the cell cycle change was similar to that in original HeLa cells except for the percentage of cells in the G2/M phase was slightly higher than other groups (Table 1).

The above data imply that the protein–protein interaction between Ndrg2 and MSP58 may play an important role in controlling cell cycle, especially in the situation of cell stress response. This result is similar to what was observed following RNA interference of P78, an isoform of MSP58 [30]. In this sense, function of Ndrg2 might be similar to PTEN tumor suppressor [20], which acts on the upstream of MSP58 in the signaling pathway to inhibit its oncogenicity. However, the mechanism of the cell cycle changes caused by Ndrg2 and MSP58 interaction cannot be elucidated with the current data yet and further study will facilitate the functional recognition of both molecules.

In summary, we identified MSP58 as a novel Ndrg2-interacting protein and found that Ndrg2 could move from

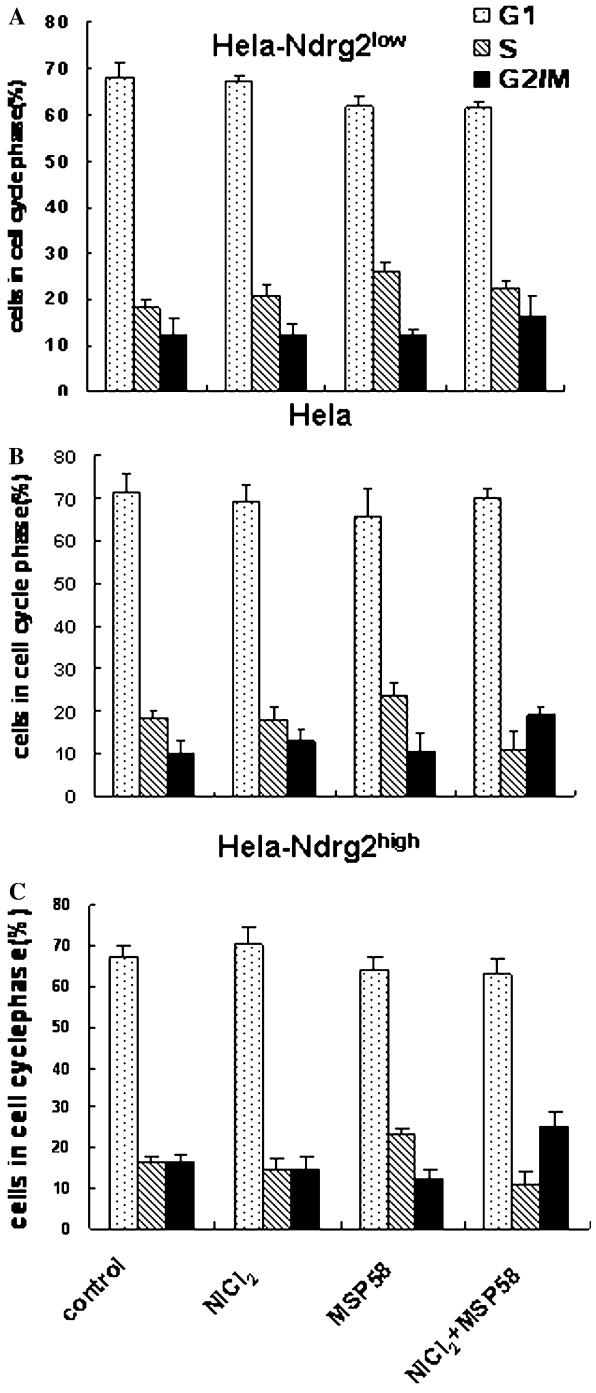


Fig. 4. The regulation of cell cycle progression by the interaction of Ndrg2 and MSP58. The Ndrg2 down or up regulated transfectant was called HeLa-Ndrg2$^{low}$ HeLa-Ndrg2$^{high}$. The cell distributions in different cell cycle phase were analyzed in FLAG-MSP58 expression vector transfected HeLa-Ndrg2$^{low}$, HeLa, and HeLa-Ndrg2$^{high}$ cells (A–C), respectively. Some transfectants were treated with $NiCl_2$ for 12 h before cell cycle analysis. The values shown in the graph are the means ± SD of four independent experiments performed in triplicate.

cytosol to nucleus to form the complex with MSP58 upon $NiCl_2$ treatment. It was showed that the overexpression of MSP58 increases S phase cell number. Furthermore, Ndrg2 was proved to inhibit MSP58-induced cell proliferation. These studies give us insight into function of Ndrg2.The role of Msp58 in cell proliferation and its interaction with Ndrg2 might be important clue to elucidate the role of Ndrg2 in tumor suppression.

Table 1
The comparison of cell cycle distribution in the cells expressing MSP58 after being treated by $NiCl_2$

| Cell line | Cell cycle | | |
|---|---|---|---|
| | $G_0/G_1$ phase | S phase | G2 phase |
| HeLa-Ndrg2$^{low}$ | 61.48 ± 1.19 | 22.27 ± 1.99 | 16.25 ± 4.32 |
| HeLa | 70.20 ± 2.19 | 10.80 ± 4.16 | 19.01 ± 1.87 |
| HeLa-Ndrg2$^{high}$ | 63.14 ± 3.75 | 11.33 ± 3.1 | 25.53 ± 3.3 |

The numbers are the means ± SD as the percentage of total cell number.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.141.

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