

## RESEARCH ARTICLE

# Overexpression of NDRG2 Can Inhibit Neuroblastoma Cell Proliferation through Negative Regulation by CYR61

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## Abstract

Several recent studies have showed that the n-myc downstream regulated gene 2 (NDRG2) is a new tumor suppressor gene, and that it plays an important role in tumor suppression in several cancers or cancer cell lines. However, few studies focused on its function in neuroblastoma cells. In the present investigation, we demonstrated that NDRG2 overexpression inhibited their proliferation. Using a cDNA microarray, we found that overexpression of NDRG2 inhibited the expression of cysteine-rich protein 61 (CYR61), a proliferation related gene. From our research, CYR61 may partially hinder NDRG2-mediated inhibition of cell proliferation. Overexpression of NDRG2 resulted in accumulation of cells in the G1 phase, which was accompanied by upregulation of p21 and p27 and downregulation of CDK4 and cyclin D1. Taken together, these data indicate that NDRG2 inhibits the proliferation of neuroblastoma cells partially through suppression of CYR61. Our findings offer novel insights into the physiological roles of NDRG2 in neuroblastoma cell proliferation, and NDRG2 may prove to be effective candidate for the treatment of children with neuroblastoma.

**Keywords:** NDRG2 - CYR61 - neuroblastoma - proliferation

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## Introduction

Neuroblastoma (NB) is one of the most common solid tumors in childhood, and is remarkable due to its broad spectrum of clinical behavior. It accounts for approximately 15% of pediatric cancer deaths (Matthay et al., 1995). Over the past several years, therapeutic options for children with NB have increased substantially, including improved radical surgery, neoadjuvant chemotherapy, and bone marrow transplantation. Despite these improvements, many NB patients remain incurable. To explain the wide variability in clinical outcome observed between patients, and to provide important, clinically relevant insights into disease management, we must elucidate the biological mechanisms underlying NB development and progression. The human NDRG2 (n-myc downstream regulated gene 2) gene is located at chromosome 14q11.2 and encoding a 41 kDa protein. It was first cloned in our laboratory from a cDNA library derived from normal human brain tissue (Deng et al., 2003). The human NDRG family includes four highly homologous members, NDRG1, NDRG2, NDRG3, and NDRG4. These members have different tissue expression patterns, indicating that they may play distinct roles, but some exhibit similarities in either regulation of expression or biological functions (Yao et al., 2008). In previous studies, NDRG2 was identified

as a new tumor suppressor gene, and its expression was decreased or undetectable in various tumors and tumor cell lines when compared to the normal tissue or cell lines (Lorentzen et al., 2007; Ma et al., 2008; Zhao et al., 2008; Kim et al., 2009; Lorentzen et al., 2011). These findings suggested that NDRG2 participates in modulation of the malignant progression of tumors. Indeed, accumulation evidence suggests that NDRG2 plays a role as a tumor suppressor, but its exact functional role in cells remains to be clarified, especially in the unreported tissues or cell lines like NB.

In this study, we established stable NB cell lines with NDRG2 and evaluated their biological functions. Our results demonstrate that NDRG2 overexpression can inhibit proliferation of NB cells, and this may be partially through suppression of CYR61.

## Materials and Methods

### *NB cell lines and cell culture conditions*

Two NB cell lines, SK-N-SH and SH-SY5Y, were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. Both cell lines were subcultured at 3-day intervals.

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### Lentivirus packaging and infection

The lentivirus system included three plasmids (pLenti.6, psPAX2, and pMD2.G) and was obtained from Addgene (Cambridge, MA, USA). The vectors of pLenti.6-NDRG2 and the control pLenti.6-Cherry were kindly provided by Dr. Jian Zhang (Dept. of Biochemistry and Molecular Biology, The Fourth Military Medical University, Xi'an, China). At 60% confluence, HEK-293T cells were transfected with pLenti.6 (2 µg), psPAX2 (1.5 µg) and pMD2.G (0.5 µg) using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. After 12 hours of transfection, the media was changed to remove the transfection reagent. The cells were incubated at 37 °C in 5% CO<sub>2</sub> for 48 hours and the media was harvested from the cells and filtered through a 0.45 µm filter. The lentiviral particle solution was added to SK-N-SH and SH-SY5Y cells. Then the stable colonies were selected with blasticidin (10 µg/ml; Sigma, St Louis, MO, USA).

### Western blot analysis

Cells were harvested from 60-mm culture dishes. Total proteins were extracted in lysis buffer [0.05 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40 (NP-40), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 1 mg/ml leupeptin]. Protein concentrations were measured using the Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Protein samples were separated by SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. Western blot analysis was carried out using the following primary antibodies: anti-NDRG2 (Sigma, USA), anti-CYR61, anti-p21, anti-p27, anti-CDK4, anti-cyclinD1 (Cell Signaling, USA), and anti-β-actin antibodies (Santa Cruz Biotechnology, CA, USA). Finally, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Promega) for at least 1 h at room temperature and detected using the ECL method (Amersham Biosciences).

### Isolation of total RNA and real-time PCR analysis

Total RNA was isolated from parental cells or stable clones using Trizol reagent (Invitrogen, CA, U.S.A.) according to the manufacturer's protocol. Total RNA (2 µg) was reverse transcribed with reverse transcriptase (Fermentas). The resulting first strand cDNA was used as the template for real-time PCR analysis. Real-time PCR was performed on an ABI 7500 system (Applied Biosystems). β-actin cDNA was used as an internal control to normalize variance in amounts of input cDNA. Real time quantitative PCR primers were designed using Primer Express v3.0 Software, and the sequences were: NDRG2 cDNA: forward primer: 5'-GAGATATGCTCTTAACCAACCCG-3', reverse primer: 5'-GCTGCCCAATCCATCCAA-3'; β-actin cDNA: forward primer: 5'-TGGCACCCAGCACAATGAA-3', reverse primer: 5'-CTAAGTCATAGTCCGCCTA GAAGCA-3'. The PCR reaction consisted of 12.5 µl of

SYBR Green PCR Master Mix, 300 nM each for forward and reverse primers, and 1.5 µg template cDNA in a total volume of 25 µl. Thermal cycling conditions were: 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s and 60 °C for 30 s. Thermal dissociation plots were examined for biphasic melting curves, which would indicate nonspecific products could be contributing to the amplification signal.

### cDNA microarray analysis

Total RNA was extracted from SK-N-SH-cherry and SK-N-SH-NDRG2 using TRIZOL reagent (Invitrogen), and two micrograms of total RNA were reverse-transcribed to generate cDNA probes. cDNA microarray analysis was performed to analyze whole genome information by Agilent whole human genome oligo microarray (4x44K) according to the manufacturer's instructions, and mRNA levels were quantitated by Agilent Array Analyzer software. Relative mRNA expression of cell differentiation-associated genes was normalized to signals from four different housekeeping genes on the same membrane and expressed in arbitrary units. For the cDNA array expression analysis, a twofold change in gene expression between control and treated cultures was considered significant.

### Methyl thiazolyl tetrazolium assay (MTT)

Each of the SK-N-SH, SK-N-SH-Cherry, SK-N-SH-NDRG2, and SH-SY5Y, SH-SY5Y-Cherry, SH-SY5Y-NDRG2 cells were seeded separately at a density of 1x10<sup>4</sup> cells/well in 96-well plates containing 200 µl DMEM (with 10% FBS) and cultured for 5 days. Five wells from each group were randomly selected for the MTT (Sigma) assay each day. After a 4h incubation with MTT at 37 °C, 150 µl of DMSO (Sigma) was added to each well for 10 min to stop the reaction. The percentage of viable cells was determined by measuring the absorbance at 490 nm on a multiscanner reader (TECAN-spectra mini Grodig).

### EDU assay

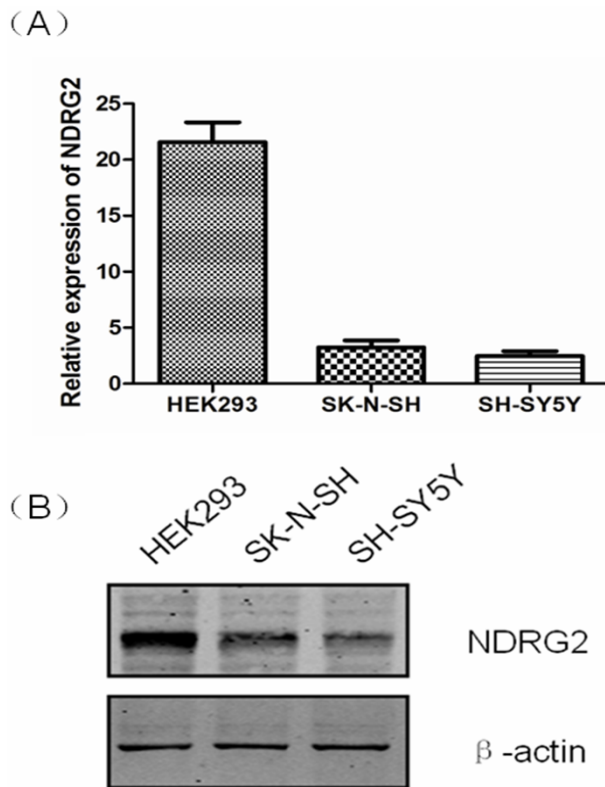
EdU staining was performed as previously described (Salic et al., 2008). Cells were grown in 24-well plates containing DMEM with 10% FBS. Following a 6 h incubation with EdU (Rui Bo Co., China), cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 for 10 min, and stained with 100 µl of 1xApollo® for 30 min at room temperature. Positively stained cells were counted in five randomly selected visual fields.

### Plate colony formation assay

For colony formation assays, 1,000 cells were seeded into 60 mm dishes with 5 ml of DMEM supplemented with 10% FBS. After 15 days, the resulting colonies were washed with PBS, fixed with methanol for 10 min, and stained with Giemsa (Sigma, USA) for 20 min. Only clearly colonies visible (diameter > 50 µm) were counted. Assays were conducted in duplicate in three independent experiments.

### Plasmid constructs and cell transfection

The human CYR61 was amplified from SK-N-SH cDNA.



**Figure 1. NDRG2 Expression Decreases in Two NB Cell Lines.** NDRG2 was assessed in SK-N-SH and SH-SY5Y cells by real-time PCR (A) and Western blot analysis (B). HEK293 cells were used as control cells.  $\beta$ -actin was used as an internal control for both in real-time PCR and Western blot analyses. All results are representative of three independent experiments

The primers sequences were: CYR61 forward primer: 5'-ATTGGATCCATGAGCTCCCGCATCGCC-3', reverse primer: 5'-CGGGAATTC TTAGTCCCTAAATTTGTG-3'. The resulting amplicon was cloned into the pcDNA3.1 (+) vector (Invitrogen) to generate the pcDNA3.1-CYR61 vector.

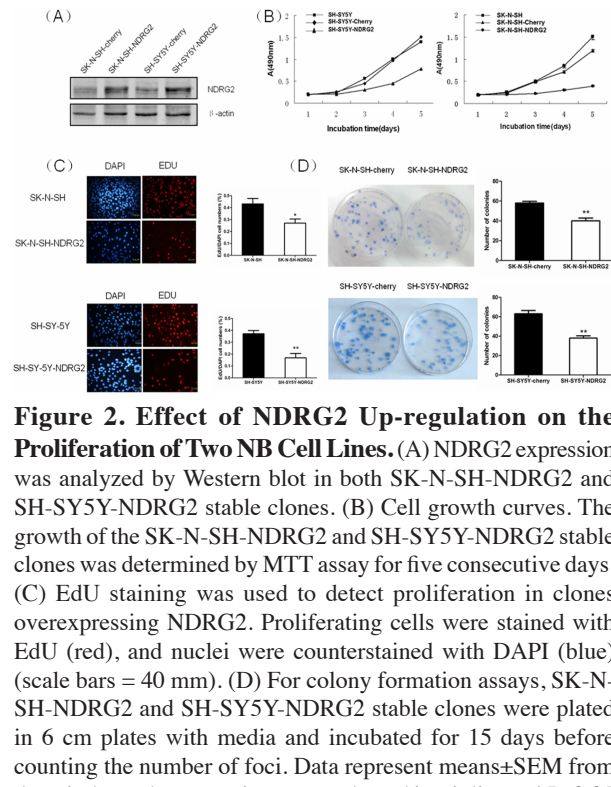
Cells were seeded in 6-well plates at a density of  $5 \times 10^5$  cells/well and transfected the next day using Lipofectamine 2000 (Invitrogen) at a confluency of about 80%. After transfected for 48 h, the expression level was measured by using Western blot.

#### Flow cytometric analysis of the cell cycle

Cells were seeded in 25 ml flasks with DMEM containing 10% FBS until they reached 80-85% confluency. Approximately  $1 \times 10^6$  cells were harvested, washed twice with ice-cold PBS, and fixed in 70% ethanol at 4 °C overnight. The cells were then stained with 20  $\mu$ g/ml propidium iodide (PI) in a final volume of 300  $\mu$ l for 20 min. DNA content and cell cycle stage were analyzed by flow cytometry (FACSCalibur).

#### Statistical analysis

Data were generally expressed as mean  $\pm$  standard error values. Groups of data were compared by analysis of variance (ANOVA) and post hoc analysis using Student-Keuls method. A value of  $p < 0.05$  was considered statistically significant.



**Figure 2. Effect of NDRG2 Up-regulation on the Proliferation of Two NB Cell Lines.** (A) NDRG2 expression was analyzed by Western blot in both SK-N-SH-NDRG2 and SH-SY5Y-NDRG2 stable clones. (B) Cell growth curves. The growth of the SK-N-SH-NDRG2 and SH-SY5Y-NDRG2 stable clones was determined by MTT assay for five consecutive days. (C) EdU staining was used to detect proliferation in clones overexpressing NDRG2. Proliferating cells were stained with EdU (red), and nuclei were counterstained with DAPI (blue) (scale bars = 40  $\mu$ m). (D) For colony formation assays, SK-N-SH-NDRG2 and SH-SY5Y-NDRG2 stable clones were plated in 6 cm plates with media and incubated for 15 days before counting the number of foci. Data represent means  $\pm$  SEM from three independent experiments conducted in triplicate. \* $P < 0.05$  and \*\* $P < 0.01$  compared with control cells. EdU, 5-ethynyl-20-deoxyuridine

## Results

### NDRG2 expression was decreased in NB cell lines

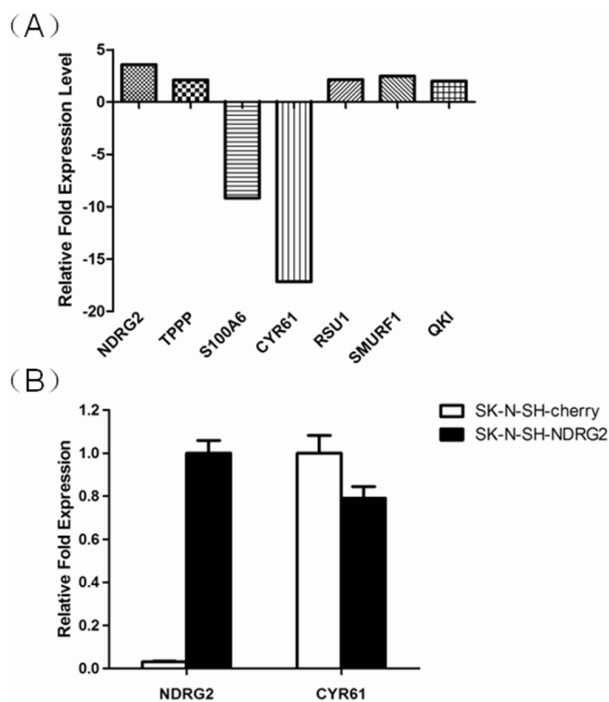
Previous studies showed that NDRG2 expression was decreased in several cancer cells, but no analysis in NB cells was performed. In this study, we evaluate the expression of NDRG2, including both RNA and protein level by quantitative real-time PCR and Western blot, respectively. Consistent with previous studies, we found that NDRG2 expression was significantly decreased in SK-N-SH and SH-SY5Y cells (Figure 1A, 1B).

### NDRG2 overexpression inhibited cell proliferation

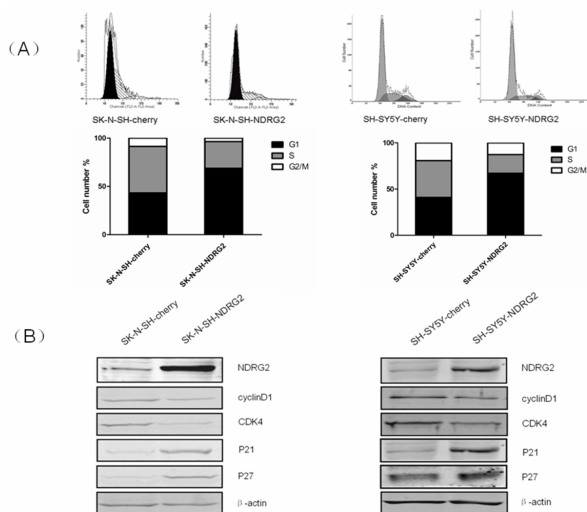
In our previous study, NDRG2 was identified as a tumor suppressor gene for inducing cell proliferation or inhibiting cell proliferation. To determine the exact role of NDRG2 in NB, we used lentivirus constructs to overexpress NDRG2 in stable NB clones (Figure 2A) and analyzed cell growth of two stable cell lines. Cell growth curves showed that the growth of both SK-N-SH-NDRG2 cells and SH-SY5Y-NDRG2 cells was inhibited when compared to control cells (Figure 2B). EdU assay also showed that NDRG2 could suppress the proliferation of SK-N-SH-NDRG2 cells and SH-SY5Y-NDRG2 cells (Figure 2C). The effects of NDRG2 overexpression on colony formation were also analyzed. Fewer clones were observed in SK-N-SH-NDRG2 cells and SH-SY5Y-NDRG2 cells when compared to control cells (Figure 2D).

*cDNA microarray showed the proliferation-related gene CYR61 was downregulated in SK-N-SH-NDRG2 stable clones*

Both our current results and previous studies support

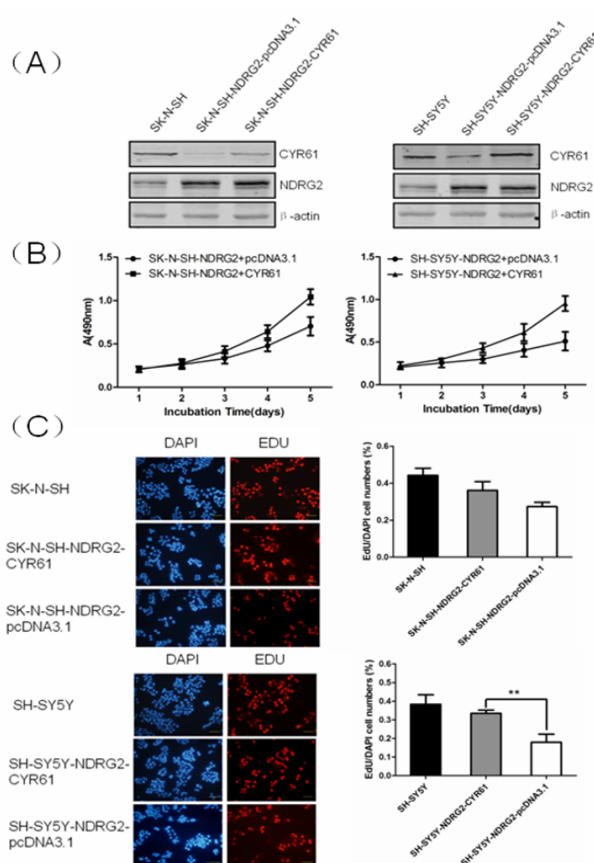


**Figure 3. Comparison of CYR61 Expression between cDNA Microarray and qRT-PCR.** (A) Expression levels of a subset of genes analyzed by cDNA microarray in SK-N-SH-cherry and SK-N-SH-NDRG2 stable clones. (B) CYR61 expression levels were validated by qRT-PCR. These results are representative of three independent experiments



**Figure 5. Overexpression of NDRG2 Induces Cell Cycle Arrest at G1 Phase in SK-N-SH-NDRG2 and SH-SY5Y-NDRG2 Stable Cell Clones.** (A) Cell cycle was determined by flow cytometric analysis of two NDRG2 stable cell clones and control cells. (B) Effect of NDRG2 on cell cycle effector levels by Western blot. Cyclin D1, cyclin-dependent kinase 4 (CDK4), p21 and p27 proteins were evaluated. β-actin was used as an internal control. One representative experiment of three performed is shown

a role for NDRG2 in cell proliferation. To further explore the molecular mechanisms of growth inhibition caused by NDRG2 overexpression, we performed a cDNA microarray to assess the mRNA expression levels of cell proliferation-related genes. Different gene expression profiles of the SK-N-SH-cherry and SK-N-SH-NDRG2 cells were observed (Figure 3A). NDRG2 overexpression



**Figure 4. Effect of NDRG2 Up-regulation on the Proliferation of Two NB Cell Lines by Introducing CYR61 and the Control.** (A) CYR61 expression was analyzed by Western blot in both parental cells and SK-N-SH-NDRG2 and SH-SY5Y-NDRG2 stable clones. (B) Cell growth curves. The growth of SK-N-SH-NDRG2-CYR61 and SH-SY5Y-NDRG2-CYR61 cells was determined by MTT assay for five consecutive days. (C) EdU staining was used to detect proliferation in NDRG2 overexpression clones by introducing CYR61. Proliferating cells were stained with EdU (red), and nuclei were counterstained with DAPI (blue) (scale bars =40 μm). \*\**P*<0.01 compared to control cells

induced up-regulation of tumor suppression gene such as protocadherin 17, and some differentiation related genes such as Rsu1 and Smurf1. Conversely, the proliferative factor CYR61 was significant down-regulated. To validate expression data obtained from the microarray analysis, the expression of CYR61 were tested by quantitative real-time PCR. As shown in Figure 3B, quantitative real-time PCR confirmed the regulation pattern observed on the arrays.

#### *NDRG2 inhibited cell proliferation partially induced by CYR61 suppression*

To explore whether CYR61 participates in NDRG2-mediated inhibition of cell proliferation, we introduced CYR61 into two NB cell lines by pcDNA3.1-CYR61 vector; expression was confirmed by Western blot (Figure 4A). As shown in Figure 4B, CYR61 partially suppressed the proliferation effect of NDRG2 in SK-N-SH-NDRG2 and SH-SY5Y-NDRG2 stable clones analyzed by MTT assay. EdU assay results also indicated that the proliferation ability of cells over-expressing NDRG2 was decreased by CYR61 (Figure 4C). These results showed

that CYR61 could impair the function of NDRG2 by inhibiting cell proliferation in NB cell lines.

#### *Overexpression of NDRG2 induced cell cycle arrest in G1 phase*

NDRG2 is reported to cause cell cycle arrest in G1 phase in several cell lines, such as colon cancer cells, liver cell lines, and gastric cancer cell lines. To explore the potential contribution of NDRG2 to cell cycle progression in NB cells, flow cytometry was used to evaluate the distribution of cells in cell cycle. As shown in Figure 5, SK-N-SH-NDRG2 cells and SH-SY5Y-NDRG2 cells accumulated in the G1 phase. The expression of specific cell cycle regulators such as CDK4 and cyclin D1 were decreased, while that of p21 was significantly increased in both SK-N-SH-NDRG2 and SH-SY5Y-NDRG2 cells when compared to control cells. Cumulatively, these results indicate that NDRG2 is essential for cell repression of cell proliferation and plays a critical role in cell cycle procession.

## Discussion

NDRG2 is identified as a new tumor suppressor, and its roles in cell differentiation and cell proliferation in various types of cancer have been reported. In several studies, low-expression of NDRG2 was found in multiple types of cancers or cells (Lusis et al., 2005; Lorentzen et al., 2007; Lee et al., 2008; Zhao et al., 2008; Kim et al., 2009; Shi et al., 2009). For example, NDRG2 was highly expressed in normal colonic mucosa and colonic adenomatous tissues, but was expressed at lower levels in all invasive cancer tissues (Kim et al., 2009). Decreased NDRG2 expression was also found in thyroid carcinomas (Zhao et al., 2008), esophageal squamous cell carcinoma (Shi et al., 2010), and primary AML cells (Tschan et al., 2010). Conversely, overexpression of NDRG2 has been measured in decreased cancer cell growth (Deng et al., 2003; Choi et al., 2007; Kim et al., 2009; Yang et al., 2010). NDRG2 can inhibit MSP58 (a 58-kD nuclear microspherule protein)-induced cell proliferation in glioma cells or colon cancer cells (Xu et al., 2012). Consistent with all the previous results, we observed that NDRG2 expression was decreased in two NB cell lines, SK-N-SH and SH-SY5Y (Figure 1A). To reveal the molecular mechanism of NDRG2 in NB cell proliferation, we established SK-N-SH and SH-SY5Y stable cell lines that constitutively expressed NDRG2 (Figure 1B). NDRG2 overexpression by lentivirus in NB cells resulted in suppression of proliferation (Figure 2A, 2B, 2C). These results suggest that NDRG2 inhibits the tumorigenesis of NB cells by inhibiting cell proliferation.

CYR61, a member of the CCN family of tissue growth factors (CYR61/CTGF/NOV), is highly expressed in various cancer tissues (Li et al., 2012; Sabile et al., 2012). CYR61 regulates multiple cellular activities such as cell proliferation, adhesion, migration, survival and apoptosis (Chien et al., 2004; Yu et al., 2008; Jandova et al., 2012; Lee et al., 2012). However, the role of CYR61 in cancer development is complex. Several studies have shown that CYR61 can promote tumorigenesis, progression and invasion in breast cancer (Tsai et al., 2002), gliomas

(Xie et al., 2004), and hepatocellular carcinoma (Li et al., 2012). It was also found that CYR61 played important roles in inducing apoptosis, inhibiting tumor growth in non-small-cell lung cancer (Tong et al., 2001). To date, there have been no studies on the functions of CYR61 in NB cells. In the present study, we used a cDNA array to identify decreased expression of CYR61 in cell lines stably transfected with NDRG2, and validated this result by real-time PCR (Figure 3A, B). In NB cells over-expressing NDRG2, the proliferation inhibiting effect of NDRG2 could partially reversed by introducing CYR61 (Figure 4A, B). These results suggested that CYR61 promoted NB cell proliferation, and functioned a negative regulation role by inhibiting NDRG2-mediated cell growth.

Studies have shown that NDRG2 can inhibit cell proliferation, invasion, and metastasis through regulating of several target genes, such as AP-1, TCF/ $\beta$ -catenin, NF- $\kappa$ B, TGF- $\beta$ 1, JAK2/STAT (Kim et al., 2009; Kim et al., 2009; Kim et al., 2009; Kang et al., 2011; Yang et al., 2011; Kim et al., 2012). NDRG2 overexpression directly down-regulated AP-1 activity and induced a decrease in cyclin D1 expression in SW620 cells (Kim et al., 2009); overexpression could also down-regulated TCF/ $\beta$ -catenin signaling in human colon carcinoma (Kim et al., 2009). Through suppression of NF- $\kappa$ B activity, NDRG2 could also inhibit melanoma cells invasion (Kim et al., 2009). In contrast, other reports showed CYR61 could affect the growth of non-small-cell lung cancer cells via the  $\beta$ -catenin-c-myc-p53 pathway (Tong et al., 2004). Cyr61 could enhance glioma cells tumorigenicity through activation of integrin-linked kinase (ILK) to stimulate TCF/ $\beta$ -catenin signaling pathways (Xie et al., 2004). Thus, based on these results, we hypothesize that NDRG2 may inhibit cancer cell proliferation partially by negative regulation of CYR61 expression through TCF/ $\beta$ -catenin signaling pathway. Further studies are required determine the exact molecular mechanism.

Studies have shown that NDRG2 plays an important role in cell cycle control. Overexpression of NDRG2 leads to G1/S cell cycle arrest in several cell lines, such as human colon cancer cells (SW620), rat liver-derived cell lines (BRL), mouse myoblasts (C2C12) (Fioletta et al., 2009; Kim et al., 2009; Yang et al., 2010). Our data showed that the introduction of NDRG2 inhibited the proliferation of two NB cell lines and resulted in the accumulation of cells in G1 phase (Figure 5), consistent with data from others studies. To further define the potential molecular mechanism of cell cycle arrest, we analyzed several cell cycle related genes. Among these molecules, CDK4 and cyclin D1 protein levels were reduced, while levels of p21 and p27 were elevated when NDRG2 expression was up-regulated (Figure 5). These results suggest that up-regulation of NDRG2 could result in anti-proliferative effect in NB cells through cell cycle inhibition.

In our present study, we provided solid evidence that NDRG2 inhibited the proliferative ability of NB cells. Moreover, we demonstrated for the first time that the inhibitory effect of NDRG2 on the proliferation of SK-N-SH and SH-SY5Y cells was partly due to the inhibition of CYR61 suppression. NDRG2 overexpression could lead to cell cycle arrest in G1 phase. These findings advance our

understanding of the role of NDRG2 in tumor suppression and suggest that it may serve as a potential therapeutic target for NB treatment.

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