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Identification of NDRG1-regulated genes associated with invasive potential in cervical and ovarian cancer cells

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ABSTRACT

N-myc downstream regulated gene 1 (NDRG1) is an important gene regulating tumor invasion. In this study, shRNA technology was used to suppress NDRG1 expression in CaSki (a cervical cancer cell line) and HO-8910PM (an ovarian cancer cell line). In vitro assays showed that NDRG1 knockdown enhanced tumor cell adhesion, migration and invasion activities without affecting cell proliferation. cDNA microarray analysis revealed 96 deregulated genes with more than 2-fold changes in both cell lines after NDRG1 knockdown. Ten common upregulated genes (LPXN, DDR2, COL6A1, IL6, IL8, FYN, PTP4A3, PAPPA, ETV5 and CYGB) and one common downregulated gene (CLCA2) were considered to enhance tumor cell invasive activity. BisoGenet network analysis indicated that NDRG1 regulated these invasion effector genes/proteins in an indirect manner. Moreover, NDRG1 knockdown also reduced pro-invasion genes expression such as MMP7, TMPRSS4 and CTSK. These results suggest that regulation of invasion and metastasis by NDRG1 is a highly complicated process.

1. Introduction

N-myc downstream regulated gene 1 (NDRG1), also known as Drg1, Cap43, RTP, Rit42 and PROXY-1, is a member of the NDRG gene family. The other members of this family, NDRG2, NDRG3 and NDRG4, are homologous to NDRG1, sharing 57–65% identity [1]. NDRG1 is a 43 kD protein, composed of 394 amino acids, is highly conserved among multicellular organisms, and is a predominantly cytosolic protein expressed ubiquitously in normal and neoplastic tissues [2].

NDRG1 plays an important role in carcinogenesis and tumor progression, especially in invasion and metastasis. It has been reported to be down-regulated in highly metastatic colon cancer [3] and gastric cancer [4]. Up-regulated NDRG1 may play a role in the suppression of malignant cell invasion and metastasis [5]. However, there are also reports showing that NDRG1 may be a putative tumor metastasis promoter gene. Increased expression of NDRG1 is associated with tumor invasion, metastasis and poor survival in thyroid cancer [6], cervical adenocarcinoma [7], and colon cancer [8]. These studies suggest that NDRG1 could regulate tumor invasion and metastasis both positively and negatively.

In the present study, we suppressed the expression of NDRG1 in the human cervical cancer cell line, CaSki [9], and the human ovarian cancer cell line, HO-8910PM [10], by using short hairpin RNA (shRNA) interference technique and studied the possible roles of NDRG1 in invasion. Furthermore, cDNA microarray approach was employed to identify possible downstream targets of NDRG1 shedding new light on the role of NDRG1 in the regulation of tumor invasion and metastasis.

2. Material and methods

2.1. Cell culture

The human cervical cancer cell line, CaSki, and the human ovarian cancer cell line, HO-8910PM, were purchased from the Shanghai Institutes for biological sciences. These cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicilium/streptomycin in a 37 °C incubator supplied with 5% CO_2.

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2.2. Construction of shRNA plasmids

Four oligonucleotides targeting NDRG1 were designed and individually cloned into pGPU6/GFP/Neo-shRNA expression vector (Genepharma) according to manufacturer's instruction. CaSki and HO-8910PM cells were stably transfected with recombinant NDRG1-targeted shRNA plasmids and control shRNA vector in the presence of Lipofectamine 2000 (Invitrogen). Clones with the highest suppression of NDRG1 expression, carrying the oligonucleotide 5′-GCTGATCCAGTTTCCGGAA- 3′, were selected for further experiments.

2.3. Western blot analysis

The cells were lysed in RIPA lysis buffer containing protease inhibitors (Beyotime). Proteins were separated in 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to PDMF membranes (Millipore). The blots were then probed with goat anti-NDRG1 polyclonal antibody (1:2000, Abcam) and mouse anti-GAPDH monoclonal antibody (1:5000, Abcam). Protein signal was detected by ECL system (Pierce).

2.4. Cell proliferation assay

Three thousand cells were seeded on a 96-well plate and incubated in normal condition. Cells were assessed using an AlamarBlue proliferation assay [11] at 24-h interval for 4 days. Briefly, AlamarBlue (Biosource International) was directly added to each well at a ratio of 100 μl indicator to 1 ml media at each detection point. The plates were further incubated for about 4 h. The 540 and 600 nm absorbance values (OD) of each well were examined with a microplate reader. Cell proliferation was evaluated with reduction rate of AlamarBlue according to the manufacturer's protocol. Each experiment consisted of 6 replications.

2.5. Cell adhesion assay

The wells of the 96-well culture plate were coated with Matrigel (BD Bioscience) at a concentration of 5 μg/well. Cells in medium containing 0.1% FBS were added to the wells (2×10^4/well) and incubated at 37 °C for 2 h. After the medium and detached cells were removed, normal media with 10% FBS were then added. After incubation at 37 °C for another 12 h, cell density was assessed with the AlamarBlue assay. Each experiment consisted of 6 replications.

2.6. Cell motility and invasion assays

For the motility assay, 10^5 cells were suspended in 500 μl of serum-free DMEM and seeded into the cell culture inserts with microporous membrane (8 μm) (BD Bioscience) without any extracellular matrix (ECM) coating. 600 μl DMEM medium, containing 20% FBS was added to the bottom chamber. After 24 h of incubation at 37 °C, cells on the upper surface of the chamber were scrapped out by a cotton swab. Cells migrated through the chamber were stained by hematoxylin and eosin (H&E). Each experiment consisted of three replications. At least three independent experiments were performed. For in vitro invasion assay, the working method was similar to that described above, except that the inserts of the chambers were coated with Matrigel.

2.7. cDNA microarray analysis

The cells were lysed in Trizol reagent (Invitrogen). Total RNA was further purified using the RNeasy Mini kit (Qiagen). The isolated RNA was amplified and labeled with Cy3 using a NimbleGen One-Color DNA Labeling Kit (Roche NimbleGen). The labeled cDNA samples were hybridized to NimbleGen human gene expression 12 × 135k microarrays (Roche NimbleGen) that represent 44,049 transcripts. Hybridized arrays were scanned using an Axon GenePix 4000B microarray scanner (Molecular Devices). The data was extracted from scanned images using NimbleScan and the Robust Multichip Average (RMA) algorithm was used to generate gene expression values. Differentially expressed genes were identified through Fold-change and *T*-test screening. The gene expression profiling was performed, which identified a subset of the total number of genes that are differentially expressed in both cell lines. GO analysis and pathway analysis were performed on this subset of genes using Agilent GeneSpring (version11.0). Visualized molecular interaction network was built and analyzed using BisoGenet [12].

2.8. Quantitative real-time PCR

Total RNA was isolated from cells as described above. 5 μg of total RNA was reverse-transcribed into cDNA using a reverse transcription system (Promega). cDNA was detected using a SYBR Green realtime PCR master mix kit (TOYOBO). Gene expression levels were normalized with GAPDH, and data were analysed with StepOne software v2.1 (Applied BioSystems). To assess NDRG1 mRNA expression and to confirm differential expression observed with cDNA microarray, NDRG1 and 11 common invasion-related genes with more than 2-fold changes were quantified by RT-PCR. The primers used are listed in Tabe S1.

2.9. Statistical analysis

Statistical analysis was carried out using SPSS 16.0 for Windows. Two-tailed Student's *t*-test was used for analyzing continuous variables. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Suppression of NDRG1 expression by shRNA

To investigate the role of NDRG1 in human cervical and ovarian cancer invasion, we established stable NDRG1 knockdown in CaSki-shNDRG1 and HO-8910PM-shNDRG1 cells by using the same shRNA vectors. Microscopically, NDRG1 knockdown did not show any detectable morphological alterations of these cells (Fig. 1A). The NDRG1 expression was suppressed by ~80% in the stable transfected cells, as determined by both protein levels (Fig. 1B) and the mRNA levels (Fig. 1C).

3.2. Effects of NDRG1 knockdown on cell proliferation

To investigate the effects of NDRG1 suppression on cell growth, AlamarBlue assay was employed to analyze the growth rate of NDRG1 knockdown cells and control cells for 4 days. Data showed that NDRG1 knockdown did not affect proliferation of CaSki-shNDRG1 or HO-8910PM-shNDRG1 cells (Fig. 1D). Furthermore, NDRG1 knockdown did not significantly alter apoptosis and cell cycle distribution of these cells either, as determined by flow cytometry (data not shown).

3.3. Effects of NDRG1 knockdown on cell adhesion, motility and invasion

The adhesion and motility of tumor cells in the ECM are considered to be important indicators of invasive property of metastatic tumor cells. We observed that NDRG1 knockdown resulted in increased adherence of CaSki and HO-8910PM cells to the

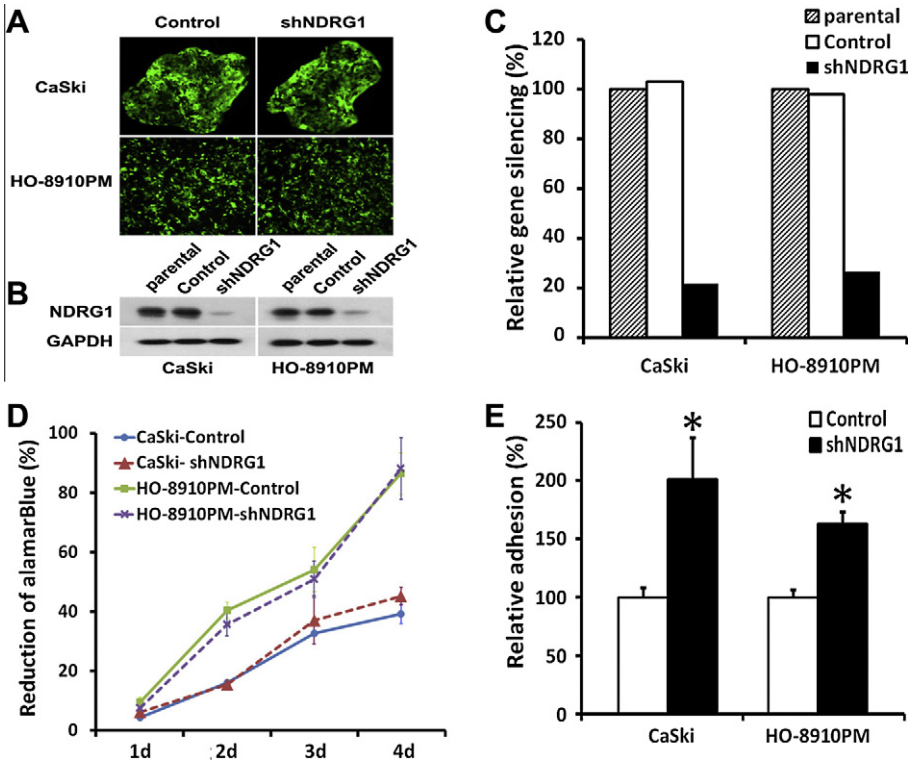

Fig. 1. Reducing NDRG1 gene expression by shRNA. (A) Fluorescence microscopy examination of control and shNDRG1 cells. (B) Western blot analyses and (C) Real-time PCR quantification of NDRG1 among parental, control and shNDRG1 cells. (D) Cell proliferation assay for 4 day. (E) Cell adhesion assay. $^{*}p < 0.01$.

Matrigel-coated substrate (Fig. 1E). Motility assay showed that NDRG1 knockdown increased the number of migrated cells by 2-fold after 24 h (Fig. 2A and B). NDRG1 knockdown also led to 3.9- and 2.6-fold increase in invasive ability of CaSki and HO-8910PM cells, respectively (Fig. 2C and D). These results indicate that down-regulation of NDRG1 promotes tumor cell migration and invasion of CaSki and HO-8910PM cells.

3.4. Identification of downstream targets of NDRG1

To further evaluate the functional outcomes caused by NDRG1 knockdown, we performed cDNA microarray analysis using RNA isolated from control or the NDRG1 knockdown cells. Among the 44,049 transcripts (135,000 probe sets) represented in the NimbleGen human gene expression microarray, we found 45 up-regulated and 51 down-regulated genes whose changes reached more than 2-folds in both CaSki-shNDRG1 and HO-8910PM-shNDRG1 cells as compared to control cells (Table S2). To explore the potential biological functions of the common deregulated genes in both NDRG1 knockdown cell lines, annotations were derived from GO biological processes and BisoGenet network. A total of 96 differential expression genes were involved in different biological processes, including cell adhesion, cell-cell signaling and tissue development. Functional categories containing more than three genes are listed in Table 1. In addition, 11 common invasion-related genes with more than 2-fold alterations in both NDRG1 knockdown cell lines were listed in Table 2 and their relations to NDRG1 were shown in visualized BisoGenet network (Fig. 3). Changes in the expressions of these invasion-related genes were also similarly observed using qRT-PCR analysis, and most of the results were consistent with the findings from cDNA microarray analysis (Table 2).

4. Discussion

NDRG1 plays a significant role in invasion and metastasis of cancer. However, there is controversy regarding the inhibitory versus stimulatory effects of NDRG1 on tumor metastasis. Although the majority of studies have identified NDRG1 as a gene that is associated with metastasis suppression [3–5], there are some reports claiming the opposite [6–8]. To assess role of NDRG1 in tumor metastasis, we employed shRNA technology to suppress the expression of NDRG1 gene in cervical cancer cell line and ovarian cancer cell line. Although it was reported that NDRG1 affected tumor cell proliferation [24], we did not observe any significant difference in growth rate, apoptosis or cell cycle distribution after NDRG1 knockdown in the present study. However, we found that suppression of NDRG1 expression resulted in increased in vitro invasion activities including adhesion and migration abilities.

Identification of common downstream targets of NDRG1 in different cell lines would provide better understanding on its regulation mechanism in tumor cells. With this in mind, cDNA microarray analysis was performed to identify differential genes in CaSki-shNDRG1 and HO-8910PM-shNDRG1 cells compared with control cells. Remarkably, NDRG1 knockdown lead to common deregulation of 96 genes with more than 2-fold alterations in both NDRG1 knockdown cell lines (Table S2), suggesting that NDRG1 can affect large numbers of downstream targets and may play an important regulatory role in cancer cells. After review of all these genes, 10 common upregulated genes (LPXN, DDR2, COL6A1, IL6, IL8, FYN, PTP4A3, PAPPA, ETV5 and CYGB) and one common down-regulated gene (CLCA2) were considered as effector genes to promote invasion and metastasis [13–23]. Their functions and roles in tumor invasion and metastasis were summarized in Table 2.

The molecular interactions of NDRG1 and these effector genes were summarized using a BisoGenet network (Fig. 3), which was

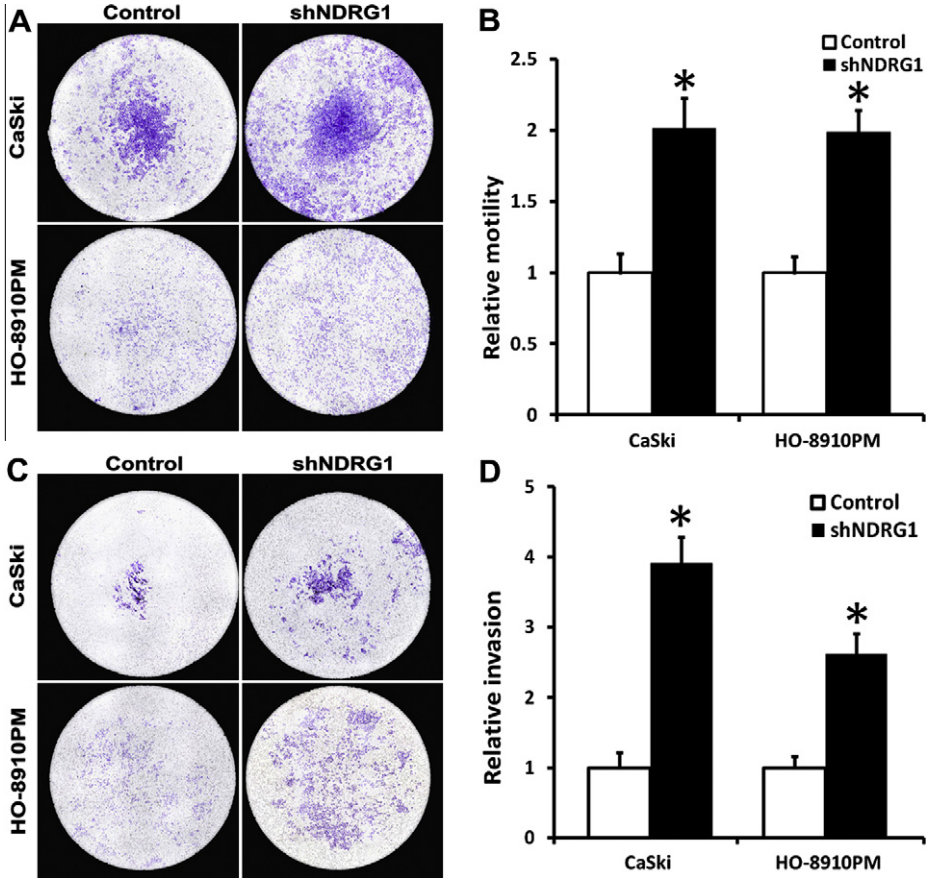

Fig. 2. NDRG1 knockdown enhances the invasive behavior of CaSki-shNDRG1 and HO-8910PM-shNDRG1 cells. (A) and (B) Cell motility assay and (C and D) cell invasion assay. $^{*}p < 0.01$.

Table 1
Functional categories of common deregulated genes in both NDRG1 knockdown cell lines.

Accession	GO Term	Enrichment	*p*-value	Count	Gene
GO:0007155	Cell adhesion	2.286	0.016	9	NRXN3, MAG, CLCA2, CD22, IL8, LPXN, COL6A1, DDR2, KIAA0894
GO:0007267	Cell-cell signaling	2.168	0.042	7	NRXN3, DLG7, TNFSF10, LALBA, GRB10, FST, IL6
GO:0009888	Tissue development	2.119	0.046	7	ALOX15, FOXQ1, CA2, MSX1, CACNA1H, TNNI3, FST,
GO:0007167	Enzyme linked receptor protein signaling pathway	2.844	0.019	6	MSX1, FST, DDR2, GRB10, MAPK8IP2, KIAA0894
GO:0046903	Secretion	2.760	0.035	5	NRXN3, CA2, SCNN1A, FST, IL6
GO:0048583	Regulation of response to stimulus	2.607	0.043	5	IL8, MAPK8IP2, GRB10, IL6, FYN
GO:0006936	Muscle contraction	4.290	0.014	4	TNNI2, CNN1, CACNA1H, TNNI3
GO:0048729	Tissue morphogenesis	3.911	0.019	4	FOXQ1, CA2, TNNI3, FST
GO:0008202	Steroid metabolic process	3.773	0.022	4	OSBPL6, LIPE, AKR1B10, DHRS2
GO:0070887	Cellular response to chemical stimulus	3.492	0.028	4	IL8, IL6, GRB10, KIAA0894
GO:0001944	Vasculature development	3.027	0.043	4	DLX3, IL8, TNNI3, IL6
GO:0032963	Collagen metabolic process	15.219	0.001	3	MMP7, IL6, ADAMTS14
GO:0002274	Myeloid leukocyte activation	11.492	0.002	3	NDRG1, IL8, DHRS2
GO:0044236	Multicellular organismal metabolic process	11.492	0.002	3	MMP7, IL6, ADAMTS14
GO:0015758	Glucose transport	10.625	0.003	3	SLC2A5, GRB10, KIAA0894
GO:0006821	Chloride transport	10.428	0.003	3	CLCN4, CLCA2, P2RY6
GO:0008643	Carbohydrate transport	7.039	0.009	3	GRB10, SLC2A5, KIAA0894
GO:0007586	Digestion	6.704	0.010	3	AKR1B10, AMY2B, AMY1A
GO:0006470	Protein amino acid dephosphorylation	4.299	0.033	3	PTP4A3, DUSP2, PTPRZ1
GO:0006820	Anion transport	4.266	0.033	3	CLCN4, CLCA2, P2RY6

based upon a remote database that integrates data from multiple public sources including NCBI, UniProt, KEGG, GO, DIP, BIND and HPRD and allowing the search of data about molecular interactions and functional relations of a group of genes/proteins [12]. Intriguingly, we found that none of the 11 dysregulated genes were related directly to NDRG1 in terms of this network. Thus, NDRG1 appears to regulate them indirectly through more than one mediator gene or protein. Moreover, there are no significant expression changes in most of the mediator genes (Table S2), which imply that NDRG1 may regulate its mediator genes by modulating their functions and/or activities, instead of their gene expression. It was reported that NDRG1 can directly interact with ER chaperons and transcriptional regulation factors to alter a large number of downstream gene expression [25]. This kind of indirect regulation has an amplification effect, but it also implies that the regulation may be complicated and unstable. For instance, we also found that NDRG1

Table 2
List of common invasion effector genes in both NDRG1 knockdown cell lines.

Gene name	CaSki-shNDRG1 vs. CaSki-control		HO-8910PM-shNDRG1 vs. HO-8910PM-control		Description; relationship with cancer	Ref.
	Fold change (Microarray)	Fold change (qRT-PCR)	Fold Change (Microarray)	Fold change (qRT-PCR)		
ETV5	13.4	19.7	3.1	4.3	Enhances cell migratory activity in endometrial cancer	[13]
LPXN	4.8	9.7	2.0	2.5	Enhances cell migratory activity in prostate carcinoma	[14]
IL6	4.3	6.8	3.9	5.2	Induces malignant features in progenitor cells from human ductal breast carcinoma	[15]
CYGB	3.6	4.4	4.5	6.2	Hypoxic up-regulation of Cygb in tumor tissues allow cancer cells to survive	[16]
PAPPA	3.5	4.1	3.5	5.9	PAPPA proteolytic activity markedly increased cell invasion	[17]
CLCA2	-3.0	-2.3	-2.4	-3.1	Tumor suppressor in human breast cancer	[18]
FYN	2.5	2.7	2.8	5.3	A critical mediator of the Ras-stimulated invasive cell phenotype	[19]
PTP4A3	2.3	3.9	5.0	8.1	Enhances cell motility activity, promotes cancer metastasis	[20]
DDR2	2.1	3.1	3.5	4.0	Enhances nasopharyngeal carcinoma invasion	[21]
IL8	2.1	2.2	2.0	2.3	Increased expression in invasive oral cancer	[22]
COL6A1	2.1	2.9	2.9	3.5	Enhances the metastatic ability of the lung cancer cell	[23]

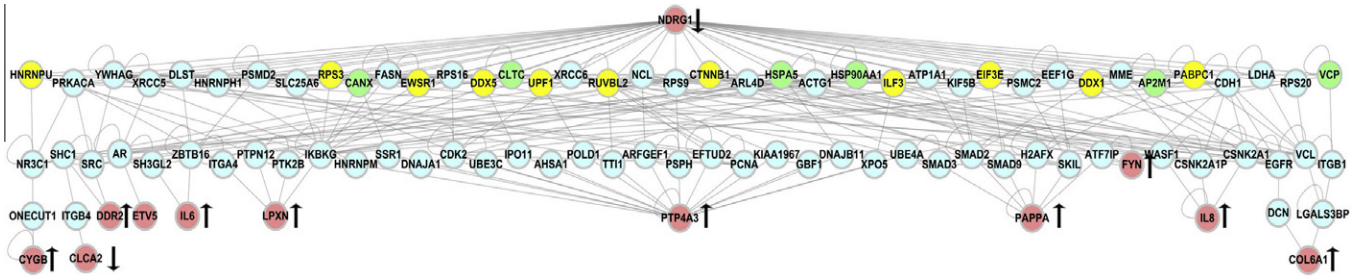

Fig. 3. The molecular interactions of NDRG1 and 11 invasion effector genes/proteins. The nodes represent genes/proteins, and the edges indicate their functional relations. Pink nodes indicate NDRG1 or effector genes/proteins. Powderblue, yellow and palegreen nodes indicate mediator genes/proteins. Yellow nodes indicate transcriptional regulation factors. Palegreen nodes indicate ER chaperons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

knockdown reduced expressions of some pro-invasion genes, such as MMP7, TMPRSS4, and CTSK (Table S2). These genes have been shown to induce tumor invasion and metastasis [26–28]. Moreover, the expression of E-cadherin, a closely related-target of NDRG1 [29], did no show a significant change in NDRG1 knockdown cells. These contradictory gene expression patterns suggested that regulation of invasion and metastasis by NDRG1 appeared to be a highly complicated process.

Our results suggest that NDRG1 knockdown could significantly increase invasive ability of ovarian cancer cell line HO-8910PM and cervical cancer cell line CaSki. To date, there is no report about correlation between NDRG1 and ovarian cancer invasion. In consistent to our observations, Wang et al. demonstrated that up-regulated NDRG1 significantly reduced invasion and migration of cervical cancer cells, but caused no cell apoptosis [30]. However, it has also been reported that NDRG1 expression was increased with progression from normal cervix to invasive cervical cancer and was considered as a candidate gene associated with invasion of cervical cancer [7,31]. Indeed, part of evidence from clinical data showed expressions of NDRG1 in clinical specimens have positive correlation with degree of metastasis and patients' survival, suggesting that NDRG1 could promote metastatic processes [6–8,32–35]. However, NDRG1 can be markedly upregulated by pleiotropic factors including hypoxia, hormones, oncogenes, tumor-suppressor genes, and metal ions [36]. Because the process of tumor expansion and invasion may aggravate tumor cell hypoxia, NDRG1 upregulation in invasive and metastatic cells could be due to the hypoxic state that cancer cells endure [37]. By contrast, hypoxia condition is almost non-existent in in vitro cell study. By reviewing the existing literature on the relation of NDRG1 to tumor invasion and metastasis, we found that NDRG1 has been most clearly linked to metastasis suppressor function in almost all in vitro cell study, including cell lines from pancreatic cancer [5], cervical cancer [30], prostate cancer [38], lung cancer [38], gastric cancer [39] and colon cancer [40]. Therefore, based on above mentioned analysis, hypoxia effects on clinical tumor specimens need to be considered when evaluating the role of NDRG1 in tumor invasion and metastasis, as they have an important impact on NDRG1 expression.

Acknowledgments

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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.2011.03.140.

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