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The metastasis suppressor, N-myc downstream regulated gene 1 (NDRG1), upregulates p21 via p53-independent mechanisms

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The metastasis suppressor, *N-myc downstream regulated gene-1* (*NDRG1*), has been shown to markedly reduce metastasis of numerous tumors. The current study was focused on further elucidating the molecular mechanisms behind the antitumor function of NDRG1. We have identified for the first time that NDRG1 upregulates the potent cyclin-dependent kinase inhibitor, p21. This effect was observed in three different cancer cell types, including PC3MM and DU145 prostate cancer cells and H1299 lung carcinoma cells, and occurred independently of p53. In addition, reducing NDRG1 expression using short hairpin RNA in PC3MM and DU145 cells resulted in significantly reduced p21 protein levels. Hence, p21 is closely correlated with NDRG1 expression in these latter cell types. Examining the mechanisms behind the effect of NDRG1 on p21 expression, we found that NDRG1 upregulated p21 via transcriptional and posttranscriptional mechanisms in prostate cancer cells, although its effect on H1299 cells was posttranscriptional only. Further studies identified two additional NDRG1 protein targets. The dominant-negative p63 isoform, ΔNp63, which has been found to inhibit p21 transcription, was downregulated by NDRG1. On the other hand, a truncated 50 kDa MDM2 isoform ($p50^{MDM2}$), which may protect p21 from proteasomal degradation, was upregulated by NDRG1. The downregulation of ΔNp63 and upregulation of $p50^{MDM2}$ are potential mechanisms by which NDRG1 increases p21 expression in these cells. Additional functional studies identified that NDRG1 inhibits cancer cell migration, suggesting that p21 is a molecular player in its antimetastatic activity.

Introduction

N-myc downstream regulated gene-1 (NDRG1) functions as a metastasis suppressor in numerous cancers including breast, prostate, colon, lung and pancreatic cancer (1–6). Interestingly, increased NDRG1 expression is correlated with increased survival of prostate, breast, pancreatic and colon cancer patients (1,2,5,7).

The function of NDRG1 and the mechanisms behind its antitumor effects remains elusive and is dependent on the cell type (8,9). Due to its antimetastatic activity, NDRG1 upregulation in cancer cells may be a promising strategy to prevent tumor progression (1,5). In fact, a new series of anticancer drugs were demonstrated to significantly upregulate NDRG1 in numerous cancer cells (9,10). Furthermore, these agents selectively induced growth arrest *in vivo* in a variety of tumor xenografts (11,12). As the ability of such agents to upregulate NDRG1 may be involved in their mechanism of action, it was important to elucidate the antitumor effects of NDRG1.

Abbreviations: mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; MMP9, matrix metallopeptidase 9; NDRG1, N-myc downstream regulated gene-1; NLS, nuclear localization signal; PARP, poly (ADP-ribose) polymerase; RT–PCR, reverse transcription–polymerase chain reaction; shRNA, short hairpin RNA; TET, tetracycline.

Considering the vital role of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ (referred to here as 'p21') in G_1/S arrest (13,14), we investigated if NDRG1 may act through this mechanism. Indeed, p21 plays a crucial role in inhibiting cell cycle progression and apoptosis and is a target of p53 (13–15). Moreover, p21 has also been negatively correlated with cell migration (16,17) and metastatic progression of cancers (18,19). Since NDRG1 may affect proliferation and apoptosis in addition to metastasis (8,20), it was crucial to examine a possible link between NDRG1 and p21.

In these studies, we demonstrate that NDRG1 was able to upregulate p21 in a p53-independent manner. Examining the molecular mechanisms involved in the NDRG1-mediated upregulation of p21, we identified that the p63 isoform ΔNp63 and also a 50 kDa MDM2 isoform ($p50^{MDM2}$) are targeted by NDRG1.

Materials and methods

Cell culture

Human prostate cancer cells (PC3MM) were transfected with tetracycline (TET)-inducible (TET-ON) human *NDRG1* (pcDNA5/TO/Flag-Drg-1) and were obtained from Dr K.Watabe (Southern Illinois University School of Medicine, USA) (3). Human lung cancer cells, H1299, transfected with the human *NDRG1* vector pBI-*NDRG1*-EGFP in a TET-OFF system (6) were obtained from GenHunter (Nashville, TN). Both the TET-ON and -OFF systems were able to efficiently induce NDRG1 expression (Figure 1A and C). With the TET-ON system (PC3MM), the relevant control was these cells incubated in the absence of TET, which did not induce NDRG1 (e.g. Figure 1A). For the TET-OFF system (H1299), we used empty vector control H1299 cells (+/− TET) for all experiments but did not include these data as it was same as the +TET control illustrated in Figure 1C. Human prostate cancer cells (DU145) were purchased from the American Tissue Culture Collection, Manassas, VA. All cells were incubated in 5% CO_2 at 37°C and were cultured for <3 months after resuscitation.

Expression plasmids and transfection

DU145 cells were transiently transfected with either pCMV-tag2-FLAG-*NDRG1* (GenHunter) or pCMV-tag2-FLAG (Stratagene, Santa Clara, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

Western blot and RNA analysis

Protein was extracted from either whole cell lysates (22) or nuclear and cytoplasmic fractions (NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit; Pierce, Rockford, IL). Western analysis was performed via established protocols (22). Primary antibodies used were against NDRG1 (Abcam, Cambridge, UK), p15, p16, p18, p21, p27, p57, cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, MA), MDM2 (SMP14, C-18, N-20 and 2G2), p63 (C-12 and 4A4) and histone deacetylase 1 (HDAC1—H-51) (Santa Cruz Biotechnology, MD). The secondary antibodies used were horseradish peroxidase-conjugated anti-goat, anti-rabbit and anti-mouse (Sigma–Aldrich Co., St. Louis, MO). β-Actin (Sigma–Aldrich) was used as a loading control.

Isolation of messenger RNA (mRNA) was performed using TRIzol® (Invitrogen) by standard procedures (23). Reverse transcription–polymerase chain reaction (RT–PCR) was carried out via established methodology (23) using the primers in supplementary Table I, available at *Carcinogenesis* Online. RT–PCR was shown to be semiquantitative by an optimization protocol, demonstrating that it was in the log phase of amplification. β-actin was used as a loading control.

Densitometry was performed using Quantity One software (Bio-Rad, Hercules, CA) and normalized using the relative β-actin loading control.

Immunofluorescence

Immunofluorescence was performed as described previously (24). Images were analyzed by counting cells with nuclear p21 expression, with 300 cells being examined under each condition in three experiments.

Luciferase reporter assays

Control cells or cells hyperexpressing NDRG1 were transfected with a pWWP-Luc construct (from Dr B.Vogelstein, John Hopkins University), encoding the human wild-type $p21^{WAF1}$ promoter, using Lipofectamine 2000 (Invitrogen).

After 24 h incubation, cells were harvested and prepared using the Luciferase Assay System (Promega, New South Wales, Australia). Luciferase activity was measured with the FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany).

DNA synthesis assay

NDRG1 expression was initiated 48 h prior to the assay. Incorporation of [^{3}H]thymidine into DNA was examined using standard methods (25).

Cellular proliferation assay

Proliferation was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay (26).

Cell cycle analysis

PC3MM and H1299 cells (~60% confluence) were induced to express NDRG1 for 48 h followed by serum starvation for 24 h. Cells were then incubated in fresh medium (10% fetal calf serum) with (for PC3MM) or without TET (for H1299) for 24 h. Cell cycle analysis was then performed (27).

Colony formation assay

Colony formation was examined using the CytoSelect™ 96-Well Cell Transformation Assay (Cell Biolabs, San Diego, CA) following manufacturer's protocol.

Cell migration assays

Wound healing assays to assess cell migration were performed by standard methods (28). Further quantitative cell migration assays were performed by standard methods (29) using the Cytoselect™ 24-well plate Cell Migration Assay (Cell Biolabs).

Statistics

Data are presented as mean ± standard deviation (number of experiments) and were compared using the Student's *t*-test. Results were considered significant when $P < 0.05$.

Results

NDRG1 upregulates p21 protein expression in multiple tumor cell types independently of p53

Considering the marked antitumor activity of NDRG1, we hypothesized that it affects the expression and localization of p21. To examine this, three different cancer cell types transfected with *NDRG1* were implemented. These included H1299 lung cancer cells and the prostate cancer cell types, PC3MM and DU145. The H1299, PC3MM and DU145 cells have been used by others to demonstrate the antitumor effects of NDRG1 and thus were suitable models (3,6,30).

Expression of p21 protein was tested in the nuclear (N) and cytoplasmic (C) fractions by western blot analysis (Figure 1A–C). With all three cell types, NDRG1 upregulation resulted in a significant increase in p21. The upregulation of NDRG1 in PC3MM prostate cancer cells led to a significant ($P < 0.001$) 5-fold increase in p21 protein levels in both the nucleus and cytoplasm relative to control (Figure 1A). The identity of the fractions as nuclear or cytoplasmic was confirmed by probing for HDAC1(Figure 1A) (21). Another prostate cancer cell line, DU145, which was transiently transfected with *NDRG1* showed a significant ($P < 0.05$) 2-fold increase in nuclear p21 levels at 72 h posttransfection when compared with vector controls, although there was no increase of cytosolic p21 (Figure 1B). The induction of NDRG1 in H1299 cell significantly ($P < 0.01$) increased p21 protein expression 4- to 5-folds in the nuclear and cytoplasmic fractions relative to the control (Figure 1C).

It is important to note that with each of the three cell lines examined, multiple bands of NDRG1 were detected at ~43 kDa (Figure 1) and could correspond to different phosphorylation states of NDRG1, as reported by others (31,32). In addition, a higher band at 46 kDa was also detected in DU145 cells transfected with NDRG1 (Figure 1B). All three cell types have been characterized as either p53-null (H1299 and PC3MM) or have mutated p53 (DU145) that does not transactivate target genes (6,33). Therefore, the ability of NDRG1 to upregulate p21 in these cells was independent of p53.

Since there are a variety of cyclin-dependent kinase inhibitors that are potent effectors of inducing cell cycle arrest (34), we also assessed the effect of NDRG1 on p15, p16, p18, p27 and p57 (35). However, we observed no significant effect ($P > 0.05$) on the expression of these proteins when NDRG1 was overexpressed in the PC3MM and H1299 cells (supplementary Figure 1A and B is available at *Carcinogenesis* Online).

Decreasing NDRG1 in PC3MM and DU145 cells using shRNA reduced p21 expression. Considering our results showing that NDRG1 overexpression significantly increased p21 protein levels, we further examined whether reducing NDRG1 would have the opposite effect in PC3MM and DU145 cells. Both cell types were transiently transfected with short hairpin RNA (shRNA) specific for *NDRG1*. These latter prostate cancer cells were used in this study due to their higher endogenous NDRG1 levels when compared with H1299 cells, which have very low NDRG1 expression [Figure 1C; (6)].

Our results demonstrate that shRNA specific for *NDRG1* (shNDRG1 1/2 and shNDRG1 3/4) significantly ($P < 0.01$) reduced NDRG1 protein expression in both PC3MM and DU145 cells when compared with control shRNA (shControl; Figure 1D). Moreover, p21 protein levels were also significantly ($P < 0.01$) reduced in both cell types in response to NDRG1 shRNA (Figure 1D). This further demonstrates that the expression of p21 is correlated with NDRG1 levels and confirms the results described above.

NDRG1 upregulates nuclear p21 expression in PC3MM, DU145 and H1299 cells. Considering that nuclear localization of p21 is crucial for its function as a cyclin-dependent kinase inhibitor (13), we examined the nuclear levels of p21 in response to NDRG1 in PC3MM, DU145 and H1299 cells using immunofluorescence microscopy (Figure 2). This was achieved by fixing cells with ice-cold methanol, which is optimal for detecting nuclear proteins (36). In all three cell types, there was a marked increase in nuclear p21 (Figure 2). Induction of NDRG1 in PC3MM cells significantly ($P < 0.01$) increased nuclear p21 levels from 41 ± 7% to 63 ± 9% (Figure 2A). Similarly, only 13 ± 4% of control DU145 cells expressed nuclear p21 and this was significantly ($P < 0.01$) increased to 26 ± 4% in the presence of NDRG1 (Figure 2B). Furthermore, NDRG1 overexpression in H1299 cells significantly ($P < 0.001$) increased nuclear p21 levels from 25 ± 19% to 71 ± 10% (Figure 2C). Collectively, these results confirmed our western results showing NDRG1 increased nuclear p21 (Figure 1). To further characterize p21 localization, 4% paraformaldehyde fixation was used. This demonstrated nuclear and cytosolic p21 was comparable (data not shown) with that observed by western blotting (Figure 1).

p21 mRNA levels increase upon NDRG1 expression in PC3MM and DU145 cells. To further examine the mechanisms involved in p21 upregulation by NDRG1, we investigated whether p21 was transcriptionally or posttranscriptionally regulated by NDRG1. Initially, the effect of NDRG1 expression on *p21* mRNA in each of these three cell types was examined. PC3MM cells with TET-inducible NDRG1 expression were examined for *p21* and *NDRG1* mRNA expression at different time points (0, 2, 5, 8 and 24 h) following supplementation with TET (Figure 3A). These experiments showed that *NDRG1* mRNA was significantly ($P < 0.001$) increased 6-fold at 2 h following incubation with TET and this was followed by a significant ($P < 0.001$) 5-fold elevation in *p21* mRNA after 8 h (Figure 3A).

To determine whether NDRG1 increased *p21* mRNA in DU145 cells, this cell type was transiently transfected with *NDRG1* and the *p21* mRNA levels were then examined using RT–PCR (Figure 3B). Our results showed that 30 h posttransfection, *p21* mRNA levels were significantly ($P < 0.01$) increased 4-fold in cells transfected with *NDRG1* compared with those transfected with an empty vector (Figure 3B).

H1299 cells were stably transfected with a TET-regulated *NDRG1* expression vector where the removal of TET induces NDRG1 expression (Figure 3C). These cells were incubated in the absence of TET for 2–36 h and the mRNA then extracted at each time point. However, we detected no significant ($P > 0.05$) increase in *p21* mRNA following up to a significant ($P < 0.001$) 5-fold induction of NDRG1 (Figure 3C), suggesting a posttranscriptional event was responsible for the increased p21 expression (Figures 1C and 2C).

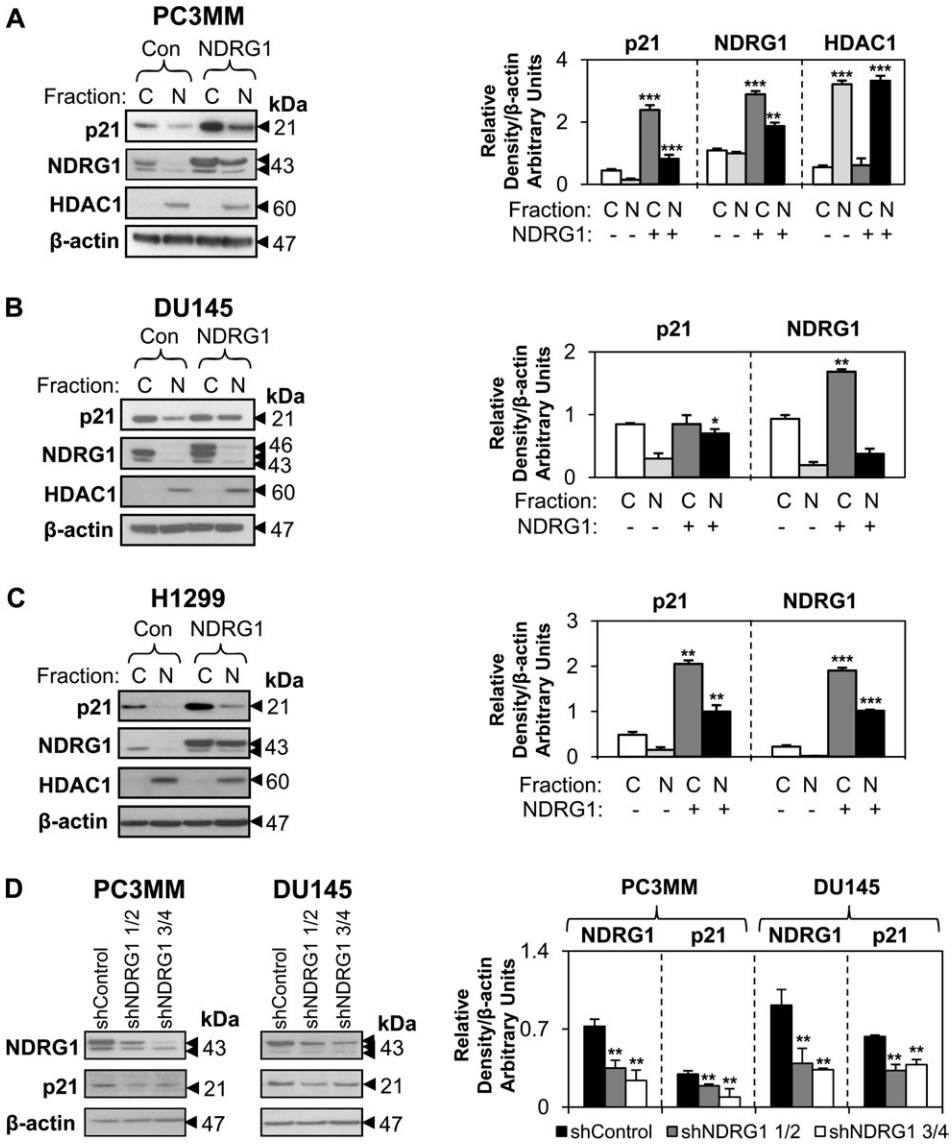

Fig. 1. Western analysis demonstrates that NDRG1 expression increases p21 protein levels in the cytoplasmic and nuclear fractions of (**A**) PC3MM cells, (**B**) DU145 cells and (**C**) H1299 cells. PC3MM and H1299 cells were examined 24 h following NDRG1 induction with TET. DU145 cells were transiently transfected with NDRG1 and p21 examined 72 h following transfection. Effective separation of lysates into cytoplasmic (C) and nuclear (N) fractions was determined by the relative levels of HDAC1 which demonstrates predominately nuclear expression (21) (**D**) Decreasing NDRG1 in PC3MM and DU145 cells using shRNA leads to reduced p21 protein levels. PC3MM and DU145 cells were treated with control scrambled shRNA and shRNA-targeting NDRG1 (100 nM) for 72 h. Densitometric analysis is expressed relative to the loading control, β-actin. The western blot shown is typical of three to five experiments and the densitometry values represent the means ± standard deviation (three to five experiments). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

NDRG1 activates the p21 promoter in PC3MM and DU145 cells. To further investigate the effect of NDRG1 on *p21* mRNA expression, we examined a luciferase-linked *p21* promoter construct transiently transfected into H1299, PC3MM and DU145 cells (Figure 3D). Upregulation of NDRG1 in PC3MM and DU145 cells led to a significant ($P < 0.01$) 3-fold increase in *p21* promoter activation (Figure 3D). This indicated that NDRG1 activated the *p21* promoter, leading to increased *p21* mRNA. On the other hand, H1299 cells showed no significant ($P > 0.05$) difference in luciferase activity when NDRG1 was overexpressed (Figure 3D), confirming the RT–PCR observations that the effect of NDRG1 on increasing p21 was a posttranscriptional event in these cells (Figure 3C).

Mechanisms behind the NDRG1 effect of upregulating p21: ΔNp63 and p50^{MDM2}

To explore the mechanism behind the effect of NDRG1 on upregulating p21, we examined proteins that have p53-independent effects on p21, including p63 and MDM2 (19,37), in PC3MM and H1299 cells. These two cell types were chosen as they were stably transfected with *NDRG1* under the control of TET-regulated promoters and because both demonstrated robust changes in p21 after NDRG1 induction (Figure 1A and C). Moreover, the PC3MM and H1299 cell types were used previously to show the antitumor function of NDRG1 (3,6).

NDRG1 decreases ΔNp63 expression in PC3MM cells. p63 is a transcription factor that has a function similar to p53 and can compensate for this tumor suppressor in its absence (37). There are two major isoforms of p63, namely TAp63 and ΔNp63, whose functions differ significantly (37). TAp63 contains the transactivation domain and is responsible for classical p63 effects, including upregulation of p53-target genes (37). On the other hand, ΔNp63 is truncated at the N-terminal and lacks the transactivation domain (37). Further, both isoforms can also be truncated at the C-terminal to generate α, β and γ variants (37). Interestingly, the ΔNp63 isoforms function in

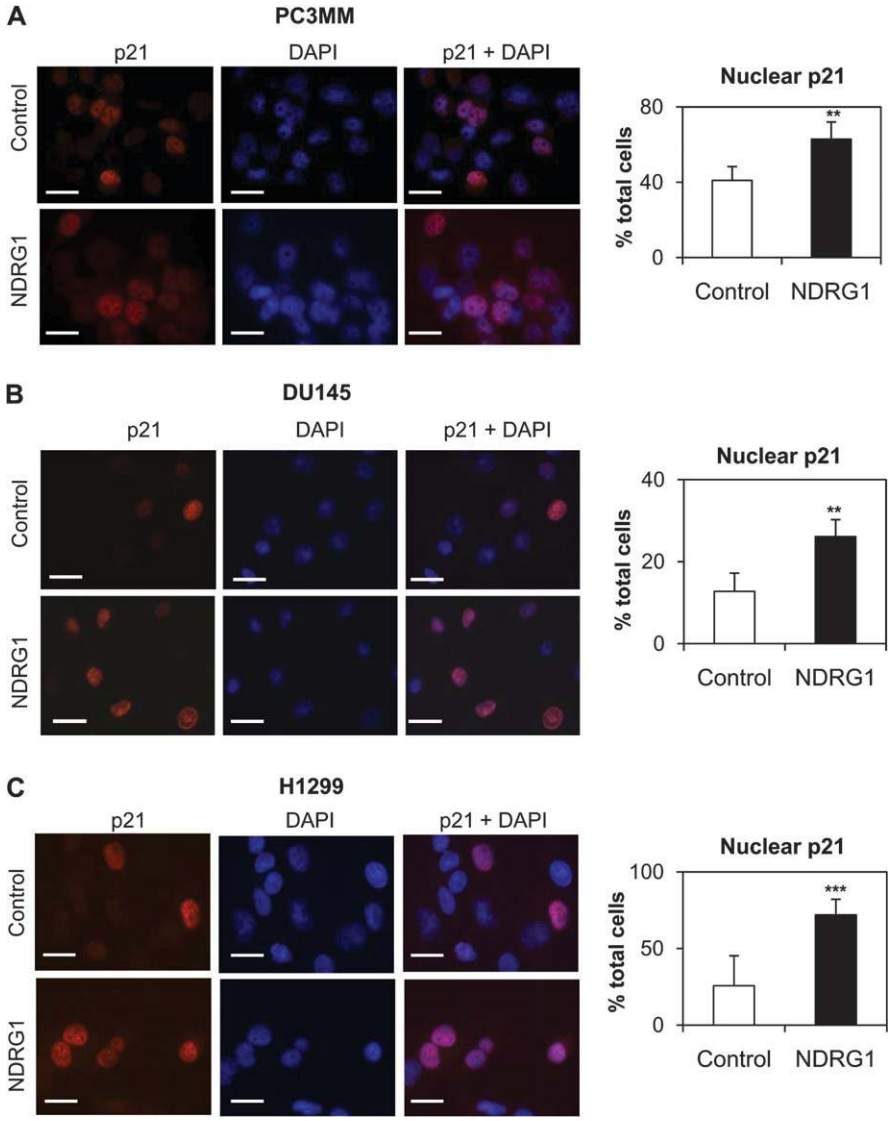

Fig. 2. Immunofluorescence demonstrates that NDRG1 expression significantly increases nuclear p21 protein levels in (**A**) PC3MM cells, (**B**) DU145 cells and (**C**) H1299 cells. Photographs are from cells incubated with primary p21 antibody and 4′,6-diamidino-2-phenylindole (DAPI) with an electronic merge of both photos to demonstrate the nuclear localization of p21. NDRG1 was induced as described in Figure 1 legend. The immunofluorescence photographs shown are typical of three to five experiments. The quantitation of p21 expression was performed by assessing 300 cells in each condition in three different experiments for each cell type. The results of this analysis are represented as % total cells with nuclear staining and are mean ± standard deviation. The scale bar in each image represents 20 μm. $^{**}P < 0.01$ and $^{***}P < 0.001$.

a dominant-negative manner to inhibit the function of TAp63 by binding to the promoter of TAp63-target genes (e.g. *p21*) and preventing transcription (37,38).

To examine whether NDRG1 was able to modulate p63 activity, we assessed the mRNA and protein levels of both TAp63 and ΔNp63 in PC3MM and H1299 cells. We found that NDRG1 overexpression in PC3MM cells did not significantly affect *TAp63* mRNA levels (Figure 4A). On the other hand, *ΔNp63* mRNA was significantly ($P < 0.001$) reduced by NDRG1 overexpression (Figure 4A). At the protein level, we detected two bands for TAp63, which represent α (80 kDa) and γ (56 kDa) isoforms, which do not alter in expression in response to NDRG1 (Figure 4B). However, nuclear ΔNp63 protein levels were significantly ($P < 0.001$) reduced by 5-fold after NDRG1 overexpression (Figure 4B), which is in agreement with the decrease in its mRNA levels (Figure 4A). Moreover, the presence of ΔNp63 in the nucleus (Figure 4B) is known to have an inhibitory function on *p21* transcription (37,38). Hence, the ability of NDRG1 to reduce nuclear ΔNp63 (Figure 4A and B) may lead to decreased inhibition of *p21* transcription (Figure 3D) and result in increased *p21* mRNA and protein levels, as observed in PC3MM cells (Figures 1–3A). When NDRG1 was overexpressed in H1299 cells, we also observed no significant effect on the TAp63α and γ protein isoforms, whereas ΔNp63 protein expression could not be detected despite extensive attempts (supplementary Figure 1C is available at *Carcinogenesis* Online). This suggested that in H1299 cells, a different mechanism was involved in the upregulation of p21 by NDRG1. Indeed, as indicated by RT–PCR and promoter analysis assays (Figure 3C and D), the upregulation of p21 in H1299 cells was due to a posttranscriptional process and this was investigated below.

NDRG1 upregulates a p50 ^{MDM2} isoform in PC3MM and H1299 cells. To further examine the mechanism of p21 upregulation in H1299 cells, we assessed other molecules that have a posttranscriptional effect on p21 expression. One such molecule, which modulates p21 in a p53-independent manner, is full-length MDM2 (p90^{MDM2}), which degrades p21 (19). This effect is independent of ubiquitination, with the nuclear localization signal (NLS) of MDM2 being necessary for p21 degradation (19).

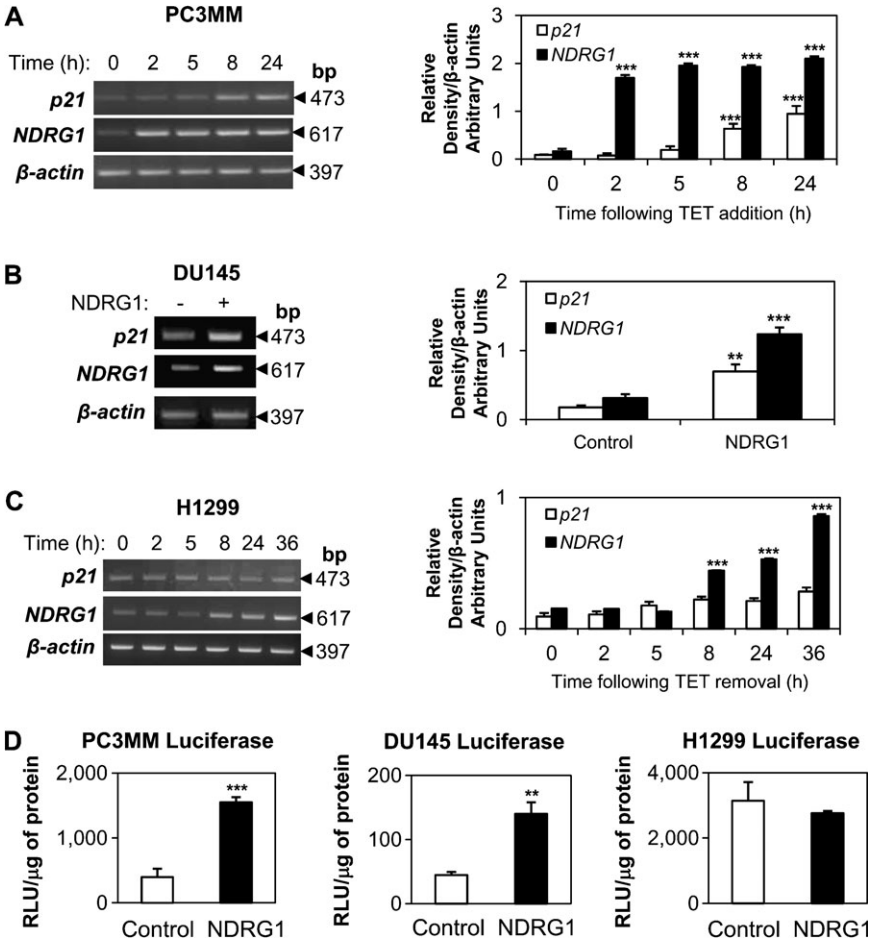

Fig. 3. RT–PCR demonstrates that NDRG1 expression upregulates *p21* mRNA levels in (**A**) PC3MM and (**B**) DU145 cells but not (**C**) H1299 cells. (**D**) p21 promoter activity is increased in both PC3MM and DU145 cells after NDRG1 expression following incubations of up to 24 and 30 h, respectively. On the other hand, no effect on *p21* mRNA levels is observed in H1299 cells following up to 36 h of NDRG1 overexpression. (D) Control cells or cells hyperexpressing NDRG1 were transfected with a pWWP-Luc construct encoding the human wild-type $p21^{WAF1}$ promoter for 24 h and luciferase activity was measured as a direct indicator of promoter activity. The gel photographs in (A), (B) and (C) are representative of three experiments performed, whereas the densitometric analysis is mean ± standard deviation (three experiments). Luciferase activity assays in (D) are mean ± standard deviation (three to four experiments). $^{**}P < 0.01$ and $^{***}P < 0.001$.

We examined the effect of NDRG1 on *MDM2* mRNA expression in PC3MM and H1299 cells using RT–PCR primers initially designed to amplify a region corresponding to the N-terminal of this molecule (supplementary Table I is available at *Carcinogenesis* Online) and found it was not significantly affected by NDRG1 overexpression (supplementary Figure 1D and E is available at *Carcinogenesis* Online). However, in both cell types, in response to NDRG1 overexpression, there was a significant ($P < 0.001$) 20-fold increase in a truncated (50 kDa) isoform of MDM2 ($p50^{MDM2}$; Figure 4C and D) in the cytoplasm as detected by the SMP14 MDM2 antibody (SMP14 Ab). Similar MDM2 isoforms have previously been reported (39,40). A slight, but not significant, increase in nuclear $p50^{MDM2}$ expression was also observed in cells overexpressing NDRG1 relative to the control (Figure 4C and D). In contrast, full-length $p90^{MDM2}$ was not detected in either cell type. As there was no change in *MDM2* mRNA expression, this suggested a posttranslational modification of $p90^{MDM2}$ led to the increase in $p50^{MDM2}$. We detected two additional MDM2 isoforms of 60–70 kDa in H1299 cells only, whose expression was not significantly altered by NDRG1 (Figure 4D).

To further characterize the $p50^{MDM2}$ isoform, we examined PC3MM and H1299 cell lysates with three other MDM2 antibodies specific for the N-terminal (Ab N-20), C-terminal (Ab C-18) or the NLS (Ab 2G2). The $p50^{MDM2}$ isoform was not detected with these antibodies in control or NDRG1-expressing cells (supplementary Figure 1F and G is available at *Carcinogenesis* Online), suggesting this 50 kDa isoform was missing regions of the C- and N-terminals and that it lacks the NLS. This NLS is crucial for $p90^{MDM2}$ to induce p21 degradation (19,41), and hence, this $p50^{MDM2}$ isoform, which is primarily expressed in the cytoplasm, cannot carry out this latter function. In contrast, elevated $p50^{MDM2}$ expression can hinder $p90^{MDM2}$ function and reduce its ability to degrade p21 (19,39,42).

Downstream functional effects of NDRG1-mediated p21 upregulation

To examine whether the increase in p21 in response to NDRG1 has functional effects on the cells *in vitro*, we examined the downstream effects of NDRG1 in PC3MM and H1299 cells, which have been shown to be highly metastatic (3,6).

Effect of NDRG1 on DNA synthesis and proliferation. With regard to the marked effects of NDRG1 on increasing p21 and the role of the latter as a cyclin-dependent kinase inhibitor, we examined whether this would result in reduced DNA synthesis and proliferation of PC3MM and H1299 cells. We therefore performed [^{3}H]thymidine incorporation and MTT proliferation assays with both cell types. We found NDRG1 overexpression did not significantly modulate DNA synthesis (Figure 5A and B) or proliferation (supplementary Figure 2A and B is available at *Carcinogenesis* Online). Furthermore, we observed no significant effects of NDRG1 on colony formation (supplementary Figure 2C and D is available at *Carcinogenesis* Online) or the distribution of cells in different cell cycle phases in control

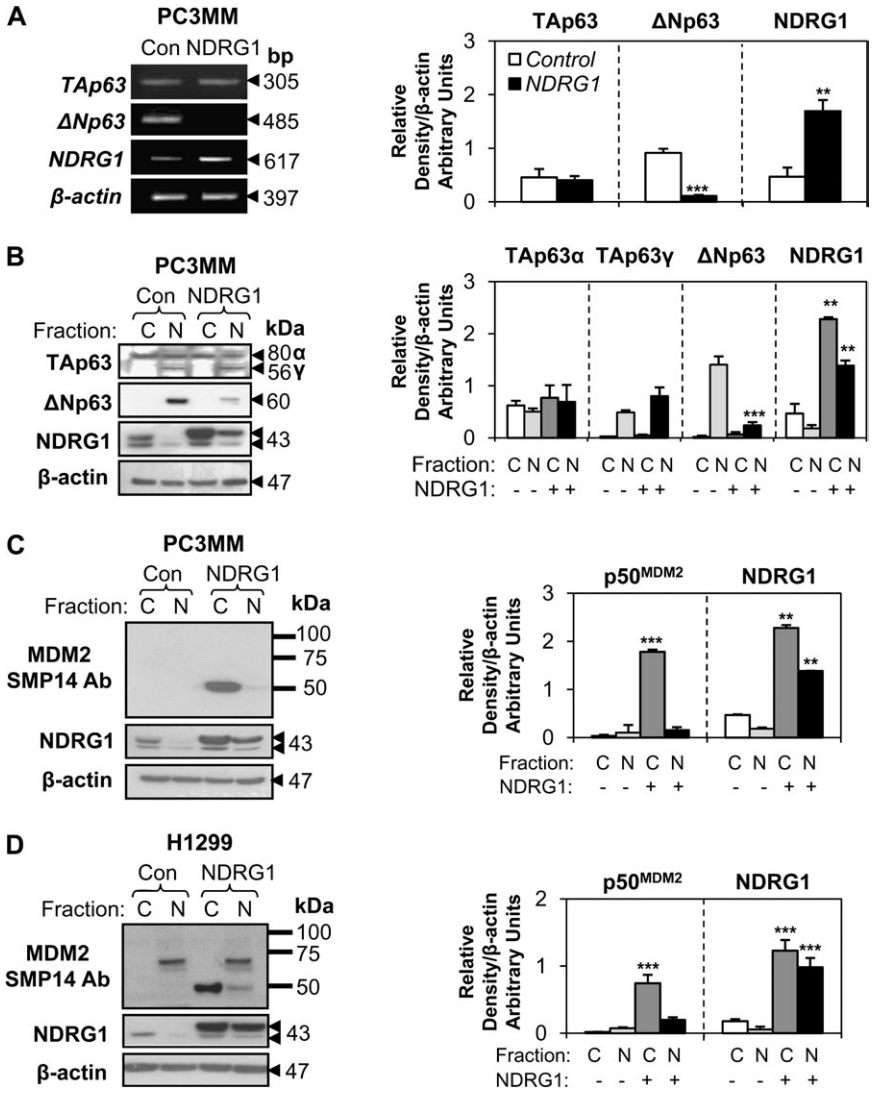

Fig. 4. NDRG1 expression downregulates *ΔNp63* mRNA (**A**) and protein (**B**) levels in PC3MM cells while increasing $p50^{MDM2}$ protein levels in PC3MM cells (**C**) and H1299 cells (**D**). The gel photographs are representative from three experiments and the densitometric analysis is mean ± standard deviation (three experiments). The TAp63 was detected as two bands corresponding to α and γ isoforms (37). NDRG1 expression was induced for 24 h prior to examination of its target proteins. $^{**}P < 0.01$ and $^{***}P < 0.001$.

versus NDRG1-expressing cells (supplementary Figure 3 is available at *Carcinogenesis* Online).

Moreover, NDRG1 did not appear to induce marked apoptosis, there being no significant difference in the pre G_1/G_0 phase of the cell cycle in both PC3MM and H1299 cells (supplementary Figure 3 is available at *Carcinogenesis* Online). However, we did observe that in the PC3MM and DU145 cells, the protein levels of cleaved PARP were increased in response to NDRG1, suggesting that this metastasis suppressor is able to initiate early apoptotic events (supplementary Figure 4A is available at *Carcinogenesis* Online). In fact, earlier studies have demonstrated that p21 is necessary for PARP cleavage to occur in response to apoptosis-inducing agents (43). Hence, NDRG1 may potentially play an important role in apoptosis through its effects on p21 and PARP. Interestingly, we detected no cleaved PARP in the H1299 cells, suggesting that there is no effect on apoptosis in this cell type.

Considering our negative results, regarding the role of NDRG1 in proliferation and the fact that this molecule is classed as a metastasis suppressor (1,2,4,5,44), we next focused on its ability to inhibit cell migration, an important component of metastasis (45).

Effect of NDRG1 on the metastatic potential of PC3MM and H1299 cells. To assess metastatic potential, we examined the effect of NDRG1 expression on this parameter using wound healing and migration assays. Using PC3MM and H1299 cells, NDRG1 overexpression significantly ($P < 0.05$) inhibited wound healing after 60 and 40 h, respectively (Figure 5C and D). We also performed transwell migration assays with both cell types and found that overexpression of NDRG1 significantly inhibited migration (Figure 5E and F).

We also found that matrix metallopeptidase 9 (*MMP9*), a protein playing an important role in cell migration and metastasis (46), was markedly reduced in both PC3MM and DU145 cells at the mRNA level following NDRG1 overexpression (supplementary Figure 4B is available at *Carcinogenesis* Online). Indeed, this may contribute to the reduced cell migration observed in the PC3MM cells. On the other hand, NDRG1 did not affect *MMP9* in H1299 cells, suggesting a different mechanism was responsible for the reduced migration of these cells. We also examined an angiogenesis marker, interleukin 8 (47), and found that it was not modulated by NDRG1 in any of the three cells types examined (supplementary Figure 4B is available at *Carcinogenesis* Online).

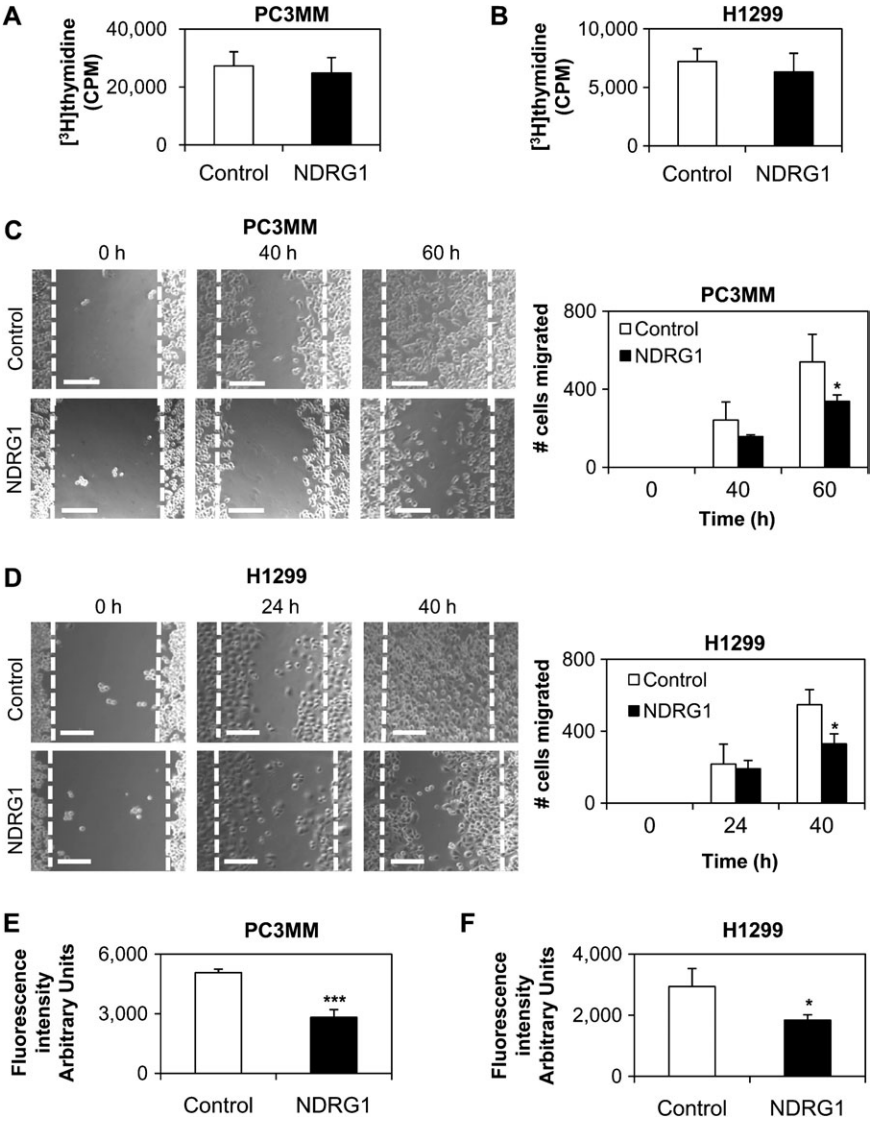

Fig. 5. Functional analysis of NDRG1 upregulation. NDRG1 does not modulate DNA synthesis in either PC3MM (**A**) or H1299 (**B**) cells. NDRG1 inhibits wound healing (**C** and **D**) as well as cell migration (**E** and **F**) of both PC3MM and H1299 cells. The scale bar in each image in (C) and (D) represents 100 μm. The data presented are representative of three separate experiments and are shown as mean ± standard deviation. NDRG1 expression was induced for at least 24 h prior to examination of its functional effects. $^{*}P < 0.05$ and $^{***}P < 0.001$.

Discussion

We have identified that NDRG1 significantly increased the expression of the crucial cyclin-dependent kinase inhibitor, p21, in each of the three cell types tested. In addition, decreasing endogenous NDRG1 expression in PC3MM and DU145 cells resulted in markedly reduced p21 protein levels. Hence, the effect of NDRG1 on p21 expression may be a key underlying mechanism behind its antitumor functions. Furthermore, the upregulation of p21 at the transcriptional and post-transcriptional levels in PC3MM cells, compared with only post-transcriptional regulation in H1299 cells, suggests that NDRG1 modulates p21 expression by different mechanisms depending upon the cell type. This is of interest considering the cell-dependent effects of many metastasis suppressors, including NDRG1 (8,9,20,48).

Despite different mechanisms involved in p21 regulation by NDRG1, the effect was independent of p53 as the cell types used were either *p53* null (PC3MM and H1299) or have mutant *p53* (DU145) (6,33). At present, there is no evidence to suggest NDRG1 itself directly acts as a transcription factor (49). However, a number of studies have found that NDRG1 has numerous phosphorylation sites, which may affect its function (31,32). Furthermore, NDRG1 has previously been demonstrated to directly bind to other protein complexes (30,50) and these may contribute to its antitumor activity. This led us to the hypothesis that NDRG1 may modulate other proteins/pathways to mediate p21 upregulation. Considering this, our studies focused on proteins involved in regulating p21 levels in a p53-independent manner, namely p63 and MDM2.

The function of p63 is similar to p53, as it can transcriptionally activate p21 (37). This is mainly through the TAp63 isoform (30,43), whereas ΔNp63 inhibits this function (37,38). The downregulation of ΔNp63 in PC3MM cells suggests the effect of NDRG1 on upregulating p21 may be mediated by the decrease in inhibition of TAp63-mediated *p21* transactivation (Figure 6). In regards to the post-transcriptional effects of NDRG1 on p21, we detected a significant increase in $p50^{MDM2}$ in our *NDRG1*-transfected PC3MM and H1299 cells. Our studies demonstrated the $p50^{MDM2}$ isoform lacks the NLS and is primarily localized in the cytoplasm. In fact, this truncated isoform interacts with full-length MDM2, sequestering its normal function to degrade p21 and allowing increased p21 expression (42). The overall effect of an increase in $p50^{MDM2}$ could be increased

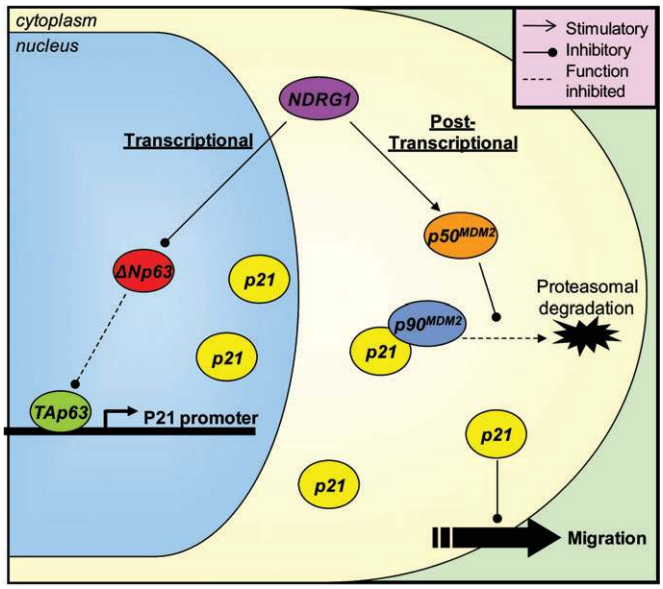

Fig. 6. Schematic diagram of transcriptional and posttranscriptional effects of NDRG1 on regulating p21 expression. Transcriptional regulation: under control conditions in the absence of NDRG1 expression, ΔNp63 acts to prevent *p21* promoter transactivation by TAp63. However, NDRG1 expression leads to repression of nuclear ΔNp63 levels, which allows transactivation of the *p21* gene by TAp63 leading to increased p21 levels. Posttranscriptional regulation: in control cells where NDRG1 levels are low, p21 may be degraded via the proteasome by full-length $p90^{MDM2}$ or other MDM2 isoforms with an intact NLS. Upregulation of NDRG1 expression results in increased levels of $p50^{MDM2}$. The $p50^{MDM2}$ isoform may interact with full-length MDM2 protein and prevents its normal function. This may lead to reduced proteasomal degradation of p21 and results in increased protein levels of this molecule. Once upregulated, p21 can function to inhibit migration and reduce the metastatic potential of cancer cells.

p21 (Figure 6) and this could explain the posttranscriptional effect of NDRG1 on upregulating p21 in H1299 cells. Additional studies examining the interaction between NDRG1 and both MDM2 and p63 isoforms and how they contribute to p21 upregulation will enhance our understanding of the mechanisms behind the function of this metastasis suppressor.

Our results show that NDRG1 can upregulate p21 by a p53-independent process. This is crucial to consider since 50% of all cancers lack wild-type p53 and an alternate therapeutic target is important to identify (51). Hence, the development of treatments aimed at upregulating NDRG1 may be beneficial for the treatment of cancers that lack p53 and show resistance to chemotherapeutics (52). We identified a novel anticancer agent that significantly upregulates NDRG1 and markedly inhibits cancers that do not possess wild-type p53 (9,10,12). Therefore, our studies have important consequences in understanding the response of p53 mutant/null cancer cells to such treatments via NDRG1.

Considering the role of p21 as an inhibitor of tumor progression (13–15), the ability of NDRG1 to upregulate this protein may be key to its antitumor functions (3,6). *NDRG1* has been classified as a metastasis suppressor and is involved in inhibiting metastatic progression of cancers rather than proliferation (44). This is observed with other metastasis suppressors, which inhibit cancer dissemination while having no effect on primary tumor growth (44,53). Furthermore, NDRG1 acts in a pleiotropic fashion, similarly to other metastasis suppressors (44), where factors such as the cell type and extracellular environment can modulate its function (8,20). This is highlighted by studies reporting that NDRG1 inhibits growth of lung and pancreatic cancers (5,6) while having no effect on primary tumor growth in prostate and breast cancer, but leading to inhibition of metastasis (1,2).

We demonstrated that NDRG1 did not modulate proliferation or colony formation of PC3MM and H1299 cells. However, when overexpressed in these cells, NDRG1 impaired cell migration, which is a key property of metastasis (Figure 5C–F). NDRG1 also reduced mRNA levels of *MMP9* in PC3MM and DU145 prostate cancer cells and this may in part contribute to its antimetastatic activity. This was in agreement with previous studies where NDRG1 reduced PC3MM cell metastasis *in vivo* (3). Furthermore, NDRG1 overexpression in PC3MM and DU145 cells also resulted in increased cleavage of PARP, suggesting that NDRG1 may initiate early apoptosis in these latter cells. Considering the role of p21 in preventing cell migration, metastasis and inducing apoptosis (16–18,54), we hypothesize that its upregulation plays a role in the antimetastatic function of NDRG1.

Together, these results further demonstrate the different molecular effects of NDRG1 on the prostate cancer cells (PC3MM and DU145 cells) when compared with the lung cancer (H1299) cells. Moreover, they show additional NDRG1 targets namely, MMP9 and PARP that may contribute to the antimetastatic activity of this protein and may be connected to the increase in p21 expression.

It is important to note that low levels of p21 have been found to promote the assembly of active cyclin/CDK complexes, whereas higher p21 expression is able to inhibit these latter complexes and induce cell cycle arrest (55,56). Our studies demonstrate that in p53-independent systems, the upregulation of p21 does not modulate cell proliferation but may contribute to the inhibition of cell migration. Others have previously demonstrated that the cyclin/CDK complex can interact with filamin A to promote migration of breast cancer cells (57). Hence, the increased p21 levels observed in our studies may inhibit migration, at least in part, through its ability to bind to the cyclin/CDK complex.

In conclusion, we demonstrate that NDRG1 overexpression increases p21 via transcriptional and/or posttranscriptional mechanisms depending upon the cell type. The mechanisms responsible involve p63 and MDM2 isoforms, both of which are targeted by NDRG1. Moreover, NDRG1 significantly suppressed cellular migration and this could be mediated through the known role of p21 in inhibiting this process.

Supplementary material

Supplementary Table I and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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