Nitric Oxide Suppresses Tumor Cell Migration through N-Myc Downstream-regulated Gene-1 (NDRG1) Expression

ROLE OF CHELATABLE IRON

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Background: Expression of N-Myc downstream-regulated gene 1 inversely correlates with patient outcome. Results: Nitric oxide exposure leads to NDRG1 gene expression, which inhibits tumor cell migration. Conclusion: Nitric oxide-mediated sequestration of chelatable iron via dinitrosyliron complex formation is a major determinant of NDRG1 gene expression and phenotypic outcome. Significance: This mechanism of NDRG1 regulation is crucial for understanding the impact of ‘NO on metastasis.

N-Myc downstream-regulated gene 1 (NDRG1) is a ubiquitous cellular protein that is up-regulated under a multitude of stress and growth-regulatory conditions. Although the exact cellular functions of this protein have not been elucidated, mutations in this gene or aberrant expression of this protein have been linked to both tumor suppressive and oncogenic phenotypes. Previous reports have demonstrated that NDRG1 is strongly up-regulated by chemical iron chelators and hypoxia, yet its regulation by the free radical nitric oxide (‘NO) has never been demonstrated. Herein, we examine the chemical biology that confers NDRG1 responsiveness at the mRNA and protein levels to ‘NO. We demonstrate that the interaction of ‘NO with the chelatable iron pool (CIP) and the appearance of dinitrosyliron complexes (DNIC) are key determinants. Using HCC 1806 triple negative breast cancer cells, we find that NDRG1 is up-regulated by physiological ‘NO concentrations in a dose- and time-dependant manner. Tumor cell migration was suppressed by NDRG1 expression and we excluded the involvement of HIF-1α, sGC, N-Myc, and c-Myc as upstream regulatory targets of ‘NO. Augmenting the chelatable iron pool abolished ‘NO-mediated NDRG1 expression and the associated phenotypic effects. These data, in summary, reveal a link between ‘NO, chelatable iron, and regulation of NDRG1 expression and signaling in tumor cells.

N-Myc downstream-regulated gene 1 (NDRG1) is a predominantly cytosolic (1), ubiquitously expressed protein (2). As its name implies, it is repressed by the proto-oncogenes N-Myc (neuroblastoma-derived myelocytomatosis) and Myc (3). The NDRG family of proteins is a member of the α/β hydrolase superfamily (4, 5). These proteins contain mutations in all three catalytic residues of the active site, however, and possess no hydrolytic activity (4). Although the mechanism of action of NDRG1 has not been elucidated, its expression is associated with diverse physiologic processes ranging from developmental biology and endocrine signaling to immune responses and neuronal functioning (6). Pathologically, NDRG1 dysregulation has been linked to a host of disease states including neurological disorders. Most notably, however, is the strong association between NDRG1 and the metastatic progression of various cancers (2, 7).

Originally discovered by a differential display study on homocysteine-treated human umbilical vein endothelial cells (8), NDRG1 mRNA and protein were subsequently found to be markedly decreased in breast, prostate, esophageal, glioma, and colon cancers when compared with normal tissue (9–15). NDRG1 mRNA also was found to be more abundantly expressed in primary colon cancer tumors than their metastases (12). This protein was shown to be up-regulated by p53 (16–18) and PTEN (the phosphatase and tensin homolog deleted on chromosome 10) (19); thus it was classified as a metastatic suppressor. Examination of other cancer types, however, found that NDRG1 is more abundantly expressed in cervical cancer, renal cancer, and hepatocellular carcinoma (20–24). These findings highlight the context and tissue-specific functions of NDRG1. A recent study performing immunohistochemical analysis of tissue from prostate cancer patients found that when NDRG1 expression was examined in conjunction with KAI1 expression, the concomitant down-regulation of these genes was an independent prognostic marker of metastatic prostate cancer (25). Thus whereas NDRG1 expression
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may not be an effective prognostic indicator itself, it appears to be among a set of genes that have a characteristic expression indicative of metastasis in at least breast, prostate, and colon cancer.

Despite a knowledge gap in the mechanistic functioning of this protein and its associated phenotypes, numerous upstream regulatory effectors have been identified. These include generalized cell stress, small molecules (such as cAMP and Fe\(^{2+}\)), and numerous proteins including HIF-1\(\alpha\), p53, PTEN, and the MYC family. A considerable body of work has been done that describes the effect of hypoxia and HIF-1\(\alpha\) expression on NDRG1 up-regulation. There are also numerous reports implicating a role for metals in NDRG1 expression. Inducers of “chemical hypoxia” such as nickel (Ni\(^{2+}\)), cobalt (Co\(^{2+}\)), as well as chemical iron chelators, are known to strongly induce the expression of NDRG1 (NDRG1 regulation is well reviewed in Refs. 2, 6, and 7)). The link between metals, hypoxia, and NDRG1 up-regulation may be via HIF-1\(\alpha\) accumulation. One mechanism by which Co\(^{2+}\) and Ni\(^{2+}\) mimic hypoxia is by substituting for the Fe\(^{2+}\) atom in mononuclear non-heme iron oxygenases that contain the 2-His-1-carboxylate facial triad structural motif. For example, inhibition of HIF prolyl hydroxylase by this mechanism results in increased HIF-1\(\alpha\) levels (26, 27). Although compounds that elicit a hypoxic response have been shown to up-regulate NDRG1, both HIF-1\(\alpha\)-dependent and independent mechanisms of regulation have been described. NDRG1 expression was not observed in HIF-1\(\alpha\)-deficient mouse embryo fibroblasts (22), for example, although its expression was not hindered in HIF-1\(\alpha\)-deficient kidney cells (27).

Nitric oxide (NO, nitrogen monoxide) is a ubiquitous free radical signaling molecule that regulates many cellular processes including angiogenesis, smooth muscle tone, immune response, apoptosis, and synaptic communication (28). In addition to the many normal physiologic functions of NO, it has been implicated in the etiology and progression of many diseases including cancer (29). Although NO is produced in and around certain tumors, its unique physical and chemical properties dictate that under biological conditions it only reacts with a minority of chemical species; i.e. other radicals and transition metals (30). Of these biological targets, one of the potentially most significant and least studied is the chelatable iron pool (CIP). This small, but chemically significant, fraction of total cellular iron (0.2–3.0%, low \(\mu M\) range) (31, 32) is methodologically defined because it is accessible to chemical iron chelators (33). More importantly, it has recently been demonstrated that when cells are exposed to NO, the CIP is quantitatively converted into paramagnetic dinitrosyliron complexes with thiol-containing ligands (DNIC) (34, 35).

Nitric oxide is distinct from most signaling molecules in that it is not limited to classical receptor-ligand interactions, and it can directly target a wide variety of molecules within a cell (including heme and non-heme iron). Moreover, phenotypic consequences attributed to NO are often the indirect result of higher nitrogen oxides formed from secondary reactions with other radical species. However, like the effects of iron chelators and divalent metals (Co\(^{2+}\) and Ni\(^{2+}\)), NO has been shown to disrupt iron homeostasis and inhibit mononuclear non-heme iron oxygenases such as prolyl hydroxylase (36). Although signaling responses of NO can proceed through a multitude of possible mechanisms, it can be seen that there are distinctly recognizable biochemical similarities between the effects of NO and those of chelators and metals (Co\(^{2+}\) and Ni\(^{2+}\)) (37–40). For these reasons we asked whether NO might prove to be equally as efficacious as these other compounds at up-regulating NDRG1.

This is the first report of NDRG1 expression in response to NO. We note that triple negative breast cancer cells exposed to NO demonstrate NDRG1 mRNA and protein up-regulation consistent with the sequestration of chelatable iron in the form of DNIC. DNIC have been detected in numerous human tissue types and tumors (41, 42) yet explanations regarding their biological and potential therapeutic significance are limited. This may be significant because both NO production and NDRG1 protein levels are known to be increased in breast cancers. Although the specific upstream target for NO that can be attributed to NDRG1 expression has not been elucidated, the reaction of NO with chelatable iron is a significant contributor to this response. Furthermore, we demonstrate that the inhibitory effect of NO on triple negative breast cancer migration is a consequence of diminishing the chelatable iron pool and up-regulation of NDRG1. The amount of iron in the CIP is inversely related to the amount of iron in DNIC, such that the increase in NO-bound iron correlates with a proportional decrease in tumor cell migration and invasion.

EXPERIMENTAL PROCEDURES

Chemicals—Diethylenetriamine nonoate (DETA/NO) was a generous gift of Dr. Joseph Hrabie (National Institutes of Health, NCI). Ferric ammonium citrate, diethylenetriamine-pentaacetic acid, phosphate-buffered saline (PBS), desferoxamine mesylate, and \(N,N',N''\)-bis(2-hydroxybenzyl)ethylenediamine-
\(N,N''\)-diacetic acid (HBED) were purchased from Sigma. All cell culture supplies were purchased from Invitrogen.

Cell Culture—HCC 1806 triple negative breast cancer cells were obtained from the American Type Culture Collection (ATCC). The cells were grown to 80% confluence in 6-cm tissue culture plates in RPMI 1640 growth medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Prior to treatments, growth media was replaced with serum-free growth medium for 16 h. All experiments were conducted under these culture conditions.

Iron Supplementation—Cells were treated with ferric ammonium citrate (150 \(\mu g/\text{ml}\)) for the indicated time points. Plates were washed with PBS to remove excess extracellular iron.

Western Blot Analysis—Briefly, cells were lysed using CellLytic™ M Cell lysis reagent (Sigma) with 1% protease inhibitor mixture (Calbiochem) and 1 mM PMSF (Sigma). Protein samples were separated on denaturing polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes using the iBlot transfer system (Invitrogen). The membrane was blocked, and incubated overnight with primary antibodies for NDRG1 (Santa Cruz Biotechnology) and HIF-1\(\alpha\) (Transduction Laboratories). The blots were finally analyzed in a Fluor Chem HD2 imager (Alpha Innotech) using SuperSignal West Femto Max-
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**RESULTS**

**Nitric Oxide Induces NDRG1 mRNA Transcription and Protein Translation**—It is well established that NDRG1 is up-regulated in response to chemical iron chelators (37). We, and others, have recently demonstrated that ‘NO can react with the CIP to form cellular DNIC (34, 35). Because ‘NO targets the same pool of iron as chelators (the CIP), we hypothesized that ‘NO might have a comparable effect on NDRG1 up-regulation via its ability to diminish cellular iron availability.

We first performed a time course analysis of NDRG1 protein (Fig. 1A) and mRNA (Fig. 1B) regulation in response to ‘NO exposure. We treated HCC 1806 triple negative breast cancer cells with the ‘NO-donor DETA/NO (500 μM) and harvested the cells at various time points (0–24 h) for analysis. Changes in NDRG1 mRNA levels were evident by 4 h and maximal at 24 h. Protein accumulation was detectable by ~4–6 h and also maximal at 24 h.

To determine whether up-regulation of NDRG1 by ‘NO is dose-dependent, we examined changes in mRNA and protein levels over a range of DETA/NO concentrations (at 8 h). It can be seen in Fig. 1, C and D, that up-regulation of NDRG1 by ‘NO occurs in a concentration-dependent manner. Increases in NDRG1 mRNA and protein levels are evident with as little as 125 μM DETA/NO ([‘NO] = 5–50 nm) (Fig. 1C). Maximal induction is achieved with 500 μM DETA/NO ([‘NO] = 225 nm) (Fig. 1D). A further increase in the ‘NO concentration had no effect on the magnitude of NDRG1 up-regulation.

‘NO-donor compounds, like DETA/NO, enable continuous controlled treatment of cells with steady-state ‘NO concentrations. The concentration of any ‘NO-donor compound, however, is not indicative of the actual steady-state ‘NO concentration that the cells are exposed to (43). For this reason we used electrochemical detection to measure, in real-time, steady-state ‘NO concentrations in the media of DETA/NO-treated cells. The steady-state ‘NO concentrations from 500 μM DETA/NO were well within the physiologic range (~80–750 nm over the 24-h period, Fig. 2).

**Stability of NDRG1 mRNA and Protein after ‘NO Exposure**—Nitric oxide is often constitutively or intermittently increased in solid tumors (29, 45). We hypothesized that NDRG1 protein...
stability in response to its up-regulation by NO could be an important downstream phenotypic determinant of its metastatic suppressive function. Therefore, we set out to determine whether the elevated NDRG1 protein and mRNA levels persisted after NO removal. In Fig. 1 it can be seen that 4–24 h of NO exposure leads to an increase in NDRG1 mRNA and protein. It is not clear, however, if these increases were in response to continuous NO exposure or whether NDRG1 transcription might continue after shorter initial treatments. We evaluated this by treating cells with NO (DETA/NO 500 μM) for various lengths of time (1–8 h), removing the NO source, and measuring changes in NDRG1 protein levels at 8 h for all samples. This data indicated that a minimum of 4 h of NO exposure was required for NDRG1 up-regulation, but protein levels continued to rise with increasing duration of exposure (Fig. 3A).

Next we set out to determine the stability of NDRG1 mRNA and protein after its up-regulation by NO. Cells were exposed to NO for 8 h (time 0, Fig. 3, B and C), the NO source was then removed, and samples were collected at various time points to determine changes in NDRG1 mRNA and protein levels. Although it can be seen that NDRG1 protein levels are continually elevated for >48 h post NO removal, mRNA levels return to baseline values by 16 h.

Chelatable Iron Is Involved in the Regulation of NDRG1 by NO—NDRG1 is strongly up-regulated by treatment with iron chelators (39). Upon cellular NO exposure, the CIP is quantitatively converted into paramagnetic DNIC. The amount of DNIC formed is related to the dose and duration of NO exposure (35). To date, almost all known NO signaling pathways are through a process that involve heme binding or S-nitrosothiol formation. Although we cannot directly refute these mechanisms, our data suggest that the effect of NO on NDRG1 expression involves an alternate mechanism. Therefore, we asked if the ability of NO to alter cellular iron availability could be sufficient to regulate NDRG1 mRNA and protein.

This hypothesis was tested by augmenting the CIP in the presence of NO. We treated cells with iron (ferric ammonium citrate (FAC)) for various lengths of time and measured changes in the g = 4.3 signal by EPR (Fig. 4, A and B). This data...
confirmed that the CIP could be increased by exogenous iron administration and that measured increases in the CIP were proportional to the length of time the cells were treated. We then measured DNIC levels in these iron-loaded cells after ‘NO exposure (500 μM DETA/NO) and determined that increases in DNIC were proportional to increases in the CIP (Fig. 4, A and B). Finally, we compared the amount of DNIC formed in iron-loaded cells after treatment with two different concentrations of ‘NO (500 versus 1,000 μM DETA/NO, Fig. 4C). At the higher dose of ‘NO, the amount of DNIC approached the level of the augmented CIP. This indicates that the concentration of ‘NO may be a limiting factor for DINC formation at high CIP levels (i.e. at low ‘NO concentrations less of it will react with the CIP versus competing reactions with other cellular targets).

Although the biological activity of DNIC remains largely unknown, one clear consequence of their formation is a diminished CIP. It seems probable that sequestration of chelatable iron by ‘NO may profoundly affect enzymatic signaling pathways requiring iron cofactors. To verify the role of chelatable iron in NDRG1 regulation, we measured changes in NDRG1 protein levels following treatment of HCC 1806 cells with two different metal chelators and with ‘NO (Fig. 5A). The magnitude of NDRG1 gene expression induced by ‘NO or iron chelators was equivalent.

If ‘NO regulates NDRG1 via iron sequestration in the form of DNIC, it follows that greater amounts of ‘NO should be necessary to elicit the same response when the CIP is increased. As a proof of principle, we treated HCC 1806 cells with iron (FAC) for varying lengths of time to make stepwise increases in the CIP (as in Fig. 4). These cells were then exposed to ‘NO for 8 h and changes in NDRG1 mRNA (Fig. 5C) and protein levels (Fig. 5B) were measured. For a given amount of ‘NO, the degree of NDRG1 gene expression in response to ‘NO was less when the CIP was augmented. In a similar set of experiments, we further verified these findings by treating HCC 1806 cells with or without iron for 16 h to maximize the CIP. Iron loading was followed by measuring changes in NDRG1 mRNA levels subsequent to treatment with increasing concentrations of ‘NO (DETA/NO 250–1,000 μM) for 8 h (Fig. 5D). This figure clearly demonstrates that when the concentration of chelatable iron in a cell is artificially increased, greater amounts of ‘NO are necessary to obtain equivalent levels of NDRG1 mRNA up-regulation compared with basal iron levels.

**HIF-1α is Not Required for Up-regulation of NDRG1 by ‘NO**—Previous reports have demonstrated both HIF-1α-dependent and -independent mechanisms of NDRG1 up-regulation (6, 7, 46). Although HIF-1α is strongly up-regulated under conditions of hypoxia, there are also numerous reports documenting the rapid and robust accumulation of HIF-1α in response to ‘NO and iron chelators under normoxic conditions (47). Although ours is the first report of NDRG1 being up-regulated by ‘NO, it is also known to be a downstream target of HIF-1α.
under certain conditions. Therefore, it was important to specifically evaluate the contribution of HIF-1α signaling to NDRG1 up-regulation by NO. To this end we generated two stably transfected HCC 1806 cell lines. In one cell line we knocked-down NDRG1 mRNA (HCC 1806 N) and in the other we knocked-down HIF-1α mRNA (HCC 1806 H). Then we treated the cells with NO and measured the magnitude of NDRG1 and HIF-1α up-regulation at the mRNA and protein levels (Fig. 6, A and B). In the NDRG1 knockdown cells, the HIF-1α protein was still strongly up-regulated in response to NO, whereas the NDRG1 protein, as expected, was undetectable (Fig. 6A). Conversely, in the HIF-1α knockdown cells, NDRG1 mRNA and protein were significantly up-regulated, therefore, still responsive to NO. Under basal conditions HIF-1α is constitutively expressed and it is not transcriptionally regulated by NO. Not surprisingly, we did not see any increases in HIF-1α mRNA in response to NO exposure in either cell type (Fig. 6B). As predicted, however, NDRG1 mRNA was not increased in the HCC 1806 N cell line but was strongly increased in the HCC 1806 H cells. These data strongly suggest that NDRG1 gene expression as a result of NO exposure is not HIF-1α-mediated in this cell type.

NDRG1 Suppresses Tumor Cell Migration—In cancer biology NDRG1 is generally regarded as a metastasis suppressor protein. Because cell migration is a critical element in the metastatic process, we set out to determine whether changes in NDRG1 expression in response to NO would modulate this process. To evaluate this we used the HCC 1806 cell line because it is highly migratory in response to a serum stimulus. This enabled us to contrast differences in the migratory potential of HCC 1806 cells with that of the NDRG1 and HIF-1α knockdown cell lines we generated (HCC 1806 N and H). In response to serum, the NDRG1 knockdown cells migrated almost twice as much as either the wild-type or HIF-1α knockdown cells, suggesting that basal NDRG1 levels are an important inhibitor of cell migration (Fig. 7A). The HIF-1α knockdown cells migrated slightly more than, but similar to, the wild-type cells. This is expected because basal HIF-1α protein

FIGURE 4. EPR measurements of changes in the CIP and DNIC. A, HCC 1806 cells were treated with iron (FAC 150 μg/ml of media) for varying lengths of time (0–16 h) and either harvested for CIP measurements by EPR (left panel) or washed and exposed to NO (500 μM DETA/NO) for 8 h followed by DNIC analysis by EPR (right panel). B, quantification of the CIP and DNIC from A. C, HCC 1806 cells were exposed to iron (FAC) for 16 h and the CIP was measured (gray bar). The cells were then treated with two different concentrations of NO (DETA/NO 500 and 1,000 μM) for 8 h and the amounts of DNIC were measured (black bars). *, p < 0.5; **, p < 0.01 with respect to either DNIC or CIP untreated controls.
levels are almost undetectable under normoxic conditions; therefore, knocking down HIF-1α mRNA should not further decrease protein expression.

Having determined the migratory profile of these cell lines, we set out to examine the influence of NO on their behavior. Fig. 7B indicates that NO had a strong inhibitory effect on HCC
1806 cells. The migratory ability of the NDRG1 knockdown cells was also suppressed by NO, but to a lesser extent (Fig. 7C).

Interestingly, NO had almost no suppressive effect on cell migration in the HIF-1α knockdown cells (Fig. 7D). Fig. 7E illustrates the inhibitory effect of NO on each cell type when compared with their untreated control. Fig. 7F demonstrates the inhibitory effect of NO on each cell type when compared with the wild-type (HCC 1806).

These data indicate that basal levels of NDRG1 alone are sufficient to restrain cell migration, and HIF-1α alone has a suppressive effect on migration. However, marked inhibition of migration is only seen when NDRG1 is strongly up-regulated in conjunction with HIF-1α. Cumulatively then, NO-mediated suppression of migrations requires HIF-1α. When HIF-1α is present, the majority of these suppressive effects are due to NDRG1 and basal NDRG1 expression can suppress migration to an extent.

In addition to migration, invasion is a critical component of the metastatic process. In support of our hypothesis, therefore, we examined the ability of NO to modulate cell invasion. Fig. 7G illustrates that NO similarly suppresses tumor cell invasion. Cell viability was assessed for all conditions in Fig. 7 via standard growth plates (E plates). Knockdown cell lines and NO treatments had no effect on viability to 80 h (supplemental Fig. S3).

Chelatable Iron Regulates NO-mediated Migration—As we observed, increasing the chelatable iron pool dampened the effect of NO on NDRG1 induction. Therefore, we asked if...
excess iron would similarly effect the responsiveness of migration to NO. We treated HCC 1806 cells with iron (FAC) and then measured the suppressive effect of NO on migration (Fig. 7H). As shown, iron supplementation did not increase the migration of cells in the absence of NO. In support of our hypothesis, however, iron supplementation did reduce the overall suppressive effect of NO on migration when compared with non-supplemented control cells. This effect could not be attributed to changes in cell viability, which remained at 100% for the duration of the experiment (Fig. 7I).

**Nitric Oxide Regulates NDRG1 Expression in Other Cell Types**—In addition to breast cancer, NO is known to be up-regulated in a variety of diverse tumor types. We looked at five other tumor cell lines to determine the universality of the nitric oxides ability to regulate NDRG1 expression. We treated breast (MDA-MB-231 and MCF-7), colon (HT-29), ovarian (SKOV-3), and brain (SH-SY5Y) cell lines with NO and examined changes in NDRG1 mRNA and protein (Fig. 8, A and B). In all cell types except the SH-SY5Y cells, NDRG1 mRNA and protein are both significantly up-regulated. It is of interest that the N-Myc is well known to be up-regulated in neuroblastomas (48); therefore, it is likely that NDRG1 expression is suppressed to such a degree that NO has no effect on its regulation.

**The Role of MYC Genes in NDRG1 Regulation**—It is known that NDRG1 is down-regulated by both N-Myc and c-Myc (3). For this reason it was important to determine whether NDRG1 up-regulation by NO was in response to changes in N-Myc or c-Myc expression. First, we analyzed changes in N-Myc and c-Myc protein and mRNA in response to NO in HCC 1806 cells (Fig. 9, A and B). Although it is known that N-Myc is absent in HCC 1806 cells (49), we verified this by Western blot and qRT-PCR (Fig. 9A and data not shown). We were, however, able to detect a concentration-dependent increase in c-Myc mRNA and protein levels upon NO exposure. We also measured changes in c-myc mRNA in 5 additional cell lines (supplemental Fig. S4). Because NDRG1 is purported to be down-regulated by c-Myc, we suspect that NDRG1 up-regulation by NO is via alternate, dominating mechanisms.

**Excluding Other NO-mediated Mechanisms for NDRG1 Up-regulation**—The major biological mechanisms by which NO signals is through activation of soluble guanylyl cyclase (sGC) to form cGMP. We therefore wanted to rule out sGC-mediated signaling as a mechanism on NO-induced NDRG1 up-regulation. We treated HCC 1806 cells with the cGC inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) and measured the ability of NO to up-regulate NDRG1. We also treated these cells with YC-1 (3-(5-`hydroxymethyl-2-furyl)-1-benzylindazole), a NO-independent activator of sGC. Inhibition of sGC did not diminish the magnitude of NDRG1 up-regulation by NO and activation of sGC in the absence of NO did not up-regulate NDRG1 (supplemental Fig. S5).

**DISCUSSION**

**Mechanism for NDRG1 Regulation via NO Is Novel**—Many studies of NDRG1 gene expression have focused on hypoxia (HIF-1α), hypoxia mimetics (Co²⁺ and Ni²⁺), and metal chelators as mechanisms of regulation (2, 6, 7). The commonality between these positive regulators of NDRG1 suggests that a metal-coordinated or O₂-binding protein may be involved. Chemical metal chelators primarily target the chelatable iron pool within a cell and inhibit the catalytic function of non-heme, non-iron-sulfur iron-requiring proteins. Divalent metals such as Co²⁺ and Ni²⁺ are known to substitute for iron in mononuclear non-heme, iron oxygenases and will similarly inhibit enzyme function. Nitric oxide has many biological targets, one of which is the chelatable iron pool (28, 34, 35). The reaction of NO with the CIP results in iron sequestration in the form of DNIC. A logical result of DNIC formation, therefore, would be the observation of distinct phenotypic outcomes similar to treatment with metal chelators that are separate from other well defined NO signaling pathways. For these reasons, we tested the ability of NO to up-regulate NDRG1 and suppress metastasis in a CIP-dependant manner.

It is well known that NO signaling is disseminated through several dominant pathways. These include NO-heme interac-
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![Graph showing concentrations of N-Myc, c-Myc, and Actin](image)

**FIGURE 9.** The role of MYC genes in NDRG1 regulation. A, immunoblot for total N-Myc and c-Myc proteins in whole cell extracts after 8 h of treatment with increasing NO concentrations (125–1,000 μM DETA/NO) in HCC 1806 cells. B, analysis of c-myc mRNA by qRT-PCR after treating HCC 1806 cells with NO (62.5–1,000 μM DETA/NO) for 8 h. Representative immunoblots of n = 4 and qRT-PCR of n = 8. **, p < 0.01 with respect to untreated controls.

Although DNIC may not directly regulate NDRG1, their formation results in chelatable iron sequestration by NO, and this sequestration correlates strongly with NDRG1 up-regulation. This suggests that an upstream requirement for iron may exist for regulators whose target genes are suppressors of NDRG1 itself or genes that encode regulators for NDRG1. We cannot completely exclude other NO-mediated pathways as contributors to NDRG1 regulation. It is clear from our data, however, that lack of iron availability subsequent to NO exposure is a major upstream driving force.

Diffusion of NO and its reaction with chelatable iron is fast. One consequence of this is the rapid accumulation of the constitutively expressed HIF-1α protein. Similarly, we have shown that up-regulation of NDRG1 by NO requires the interaction of NO with chelatable iron. Unlike HIF-1α, however, there is a 4–8-h delay before the NDRG1 gene products accumulate. These kinetics are consistent with previous studies measuring NDRG1 up-regulation by iron chelators (2). Our findings suggest that the iron-dependent target for NO resulting in NDRG1 gene expression is well upstream of NDRG1 itself.

When HCC cells are treated with NO, DNIC form at concentrations that are equivalent to the concentration of the CIP (Fig. 4B). This is in agreement with work done by other groups, which demonstrated that the iron necessary for DNIC assembly is derived from the CIP (34). Under NO treatment conditions that resulted in DNIC formation, we saw NDRG1 strongly up-regulated at the mRNA and protein levels to the same extent as achieved with chemical iron chelators. We were able to make incremental increases in the CIP by incubating cells with FAC. The magnitude of the increase in the CIP was proportional to the length of time the cells were incubated with iron. We noted that as the CIP was increased NO had a comparatively lesser effect on NDRG1 mRNA and protein induction and at the same time the ability of DNIC to reach equivalent concentrations as the CIP was diminished. Therefore, if the CIP concentration is high, a greater proportion of iron remains in the CIP as opposed to forming DNIC in the presence of NO. In effect, this maintains the bioavailability of “free iron” leaving it accessible to support NDRG1 suppressive pathways.

In a similar set of experiments, we compared the amount of NO necessary to achieve maximal NDRG1 mRNA expression in cells with a high versus normal CIP (Fig. 5D). We noted that a greater amount of NO was required to achieve the same magnitude of NDRG1 up-regulation when the CIP was elevated compared with cells with basal iron levels. Maximal NDRG1 up-regulation by NO was not observed in iron-supplemented cells until the DNIC concentration increased to approximate the concentration of the CIP. These results further emphasize that the magnitude of NDRG1 expression in response to NO is not simply a function of the NO concentration but of the DNIC to CIP ratio.

Finally, we ruled out many known NDRG1 regulatory pathways. Although N-Myc is absent in HCC 1806 cells, c-Myc is up-regulated by NO. Previous reports have demonstrated, however, that c-Myc suppresses NDRG1 (3), and is thus not likely to be the means through which our observed changes are occurring. We also were able to rule out activation of sGC as being involved in NDRG1 gene expression, which is perhaps the best studied mechanism for NO signaling. RNA knockdown of HIF-1α revealed that in this cell type HIF-1α is not required for NDRG1 expression. This is a crucial observation because under normoxic conditions (21% O2) NO is known to strongly induce HIF-1α accumulation at concentrations consistent with NDRG1 up-regulation.

There are some reports of p53 being an upstream regulator of NDRG1 (7, 18) and it is also well known that p53 is post-translationally regulated by NO (47). This pathway could be excluded in our model because HCC 1806 are p53 null (53).

The mode of NDRG1 regulation that we propose depends on iron availability, and thus may have occurred through mechanisms involving the iron-regulatory proteins. Iron-regulatory proteins (IRP1 and −2) are important regulators of intracellular iron. In response to changes in cellular iron status, these proteins bind to conserved iron-responsive elements (IREs) in the 3′- and 5′-untranslated regions (UTRs) of specific mRNAs...
NO, NDRG1, and Cancer—Triple negative breast cancers, along with many other aggressive cancers, are highly resistant to conventional treatments such as cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) (60). Thus there is a pressing need to understand the underpinnings of the metastatic progression in these cancers to develop new therapies. Although this reality is certainly appreciated, obtaining molecular indices of metastatic progression will never be as simple as finding a single oncogene and will always remain highly tissue specific. These studies examine the regulation of a metastasis repressor gene by ‘NO. This is of considerable interest because both ‘NO and NDRG1 have both been shown to be positively and negatively correlated with metastatic progression (29, 44). Examined together, however, potential explanations for their seemingly contradictory behavior emerge.

Although NDRG1 expression has been found to be increased in certain cancers, it appears to function as originally defined in breast cancers, as a metastasis suppressor. In this setting it seems likely that loss of NDRG1 expression is a cause not a result of metastasis. Thus finding novel means to up-regulate its expression in these tumors is of considerable interest. When the highly metastatic breast cancer cell line HCC 1806 was exposed to ‘NO, there was marked suppression in cell migration. Conversely, in the absence of ‘NO, knocking down NDRG1 mRNA resulted in a doubling in the rate of cell migration. This indicates that basal levels of NDRG1 limit the migratory potential of cells and up-regulation of this protein by ‘NO is suppressive. The rate of cell migration in NDRG1 knockdown cells exposed to ‘NO was equivalent to the rate of wild-type cell migration in the absence of ‘NO. This indicates an NDRG1-independent means of migration suppression by ‘NO. Interestingly, the migration of HIF-1α knockdown cells was not suppressed by ‘NO even when NDRG1 was up-regulated. Taken together, this implies that there are HIF-1α-mediated, NDRG1-independent, mechanisms of migration suppression. Although HIF-1α accumulation is not directly involved in NDRG1 protein expression in these experiments, we observed an enhanced suppressive effect on migration when both proteins were present.

Under conditions of iron supplementation, where DNIC formation was less than the CIP, the suppressive effect of ‘NO on migration was completely abolished. This is consistent with our hypothesis that the chelation of iron by ‘NO leads to the up-regulation of NDRG1, and implies that the multitude of other iron-independent, ‘NO-induced pathways are not involved in suppression of migration. Furthermore, we were able to rule out cytotoxic effects of ‘NO as an explanation for its ability to diminish cell migration indicating that this is a true metastatic suppressive effect. Our findings are consistent with NDRG1 as a metastasis suppressor protein, but also hint at conditions where it might not be effective. When the HIF-1α knockdown cells were treated with ‘NO, there was a clear increase in NDRG1 mRNA and protein. Despite this increase, ‘NO had no effect on cell migration. It may be that in cases where NDRG1 expression is lost, metastasis becomes highly likely, but its expression might not be capable of suppressing migration in certain genetic backgrounds. Thus its high expression is more likely to be a failed compensatory mechanism in cases where it is associated with metastasis.

The redox status and microenvironmental conditions of any ‘NO-producing tissue can greatly impact both the concentration of ‘NO and the duration of its production. Our results demonstrate that ≥4 h of ‘NO exposure is required for NDRG1 up-regulation, suggesting that persistent rather than transient iron sequestration is essential. Although NDRG1 mRNA levels degrade rapidly after ‘NO removal, the amount of protein remains elevated for ≥48 h. Together, these results indicate that short exposure to ‘NO can have long lasting phenotypic effects. This may have important implications toward establishing ‘NO-attributable effects on tumor behavior even under conditions where there is discontinuous ‘NO production.

Conclusions—These results demonstrate that ‘NO, via its interaction and sequestration of chelatable iron, results in the up-regulation of NDRG1 in HCC 1806 (and other) cells. This represents a novel, previously unknown, mechanism of NDRG1 up-regulation and the first demonstration for a functional role of DNIC formation. Nitric oxide is up-regulated in a variety of tumor types and it is also generated by several classes of experimental chemotherapeutics. NDRG1 will likely be an effective therapeutic target in certain cancers. In fact, a recent study found that up-regulation of NDRG1 by thiosemicarbazones completely inhibited pancreatic tumor xenograft growth (46). In addition to the relevance of this data to cancer progression, these results may be extended to explain normal physiologic conditions where ‘NO is synthesized and NDRG1 is expressed. Continued research will be needed to confirm the existence of the actual chelatable iron-dependent target and to better understand the relevance of ‘NO signaling to mammalian pathophysiologic and physiological states.

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