The metastasis suppressor, Ndrg-1: a new ally in the fight against cancer

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Tumor metastasis is an important clinical problem, contributing to the majority of cancer-related deaths. The recent discovery of metastasis suppressor genes, such as N-myc downstream-regulated gene-1 (Ndrg-1), has introduced a novel approach to treating cancer and preventing metastasis. Ndrg-1 has been identified as a protein involved in the differentiation of epithelial cells. In addition, Ndrg-1 expression can be regulated by androgens and is involved in the pathology of the disease, Hereditary Motor and Sensory Neuropathy-Lom (HMSNL). However, one of the most well documented links between Ndrg-1 and pathophysiology is its association with inhibition of tumor metastasis. The expression of Ndrg-1 was found to be significantly downregulated in a variety of different neoplasms including breast, colon and prostate cancer. Furthermore, Ndrg-1 expression was shown to be negatively correlated with tumor metastasis. Studies in vitro and in vivo have demonstrated a significant reduction in the metastatic ability of cells overexpressing Ndrg-1. The ability of these cells to invade was also compromised. The Gleason grade of prostate and breast cancers was found to correlate with Ndrg-1 expression, with more advanced and poorly differentiated tumors having lower Ndrg-1 levels. Recently, Ndrg-1 expression was demonstrated to be regulated by cellular iron levels and induced by iron chelators. These latter compounds were recently identified as potential anticancer agents as they selectively prevent cancer cell proliferation and lead to apoptosis. The discovery that iron chelators also increase Ndrg-1 expression further augments their antitumor activity and provides a novel strategy for the treatment of cancer and its metastasis.

General introduction

Metastasis involves the spread of cancer cells from a primary tumor site to distant organs [for review see (1)]. Due to the difficulty to treat and contain metastasis, it is a significant clinical problem and the major cause of cancer-related death (1). Over the past 7 years, there has been increasing interest in the growing family of tumor metastasis suppressor proteins (2–4). Currently, there are 13 well-characterized metastasis suppressors described in the literature. These include RKIP, Nm23, KISS1, KA11, BRMS1, TIMPs, E-cadherin, MKK4, TXNIP, CRSP3, SScCKs, RhoGD12 and N-myc downstream-regulated gene-1 (Ndrg-1) (4). The growing number of metastasis suppressor genes may potentially lead to the development of new therapeutic strategies. That is, pharmacological interventions that could upregulate their expression to inhibit tumor metastasis. This review will focus on Ndrg-1, which has been well characterized to have significant anti-metastatic effects in a number of different cancers.

Human Ndrg-1 is also known as Drg-1, Cap43, RTP and RIT42 and has been identified as a metastasis suppressor in prostate, colon and breast cancers (5–7). From here on, the human molecule will be referred to as Ndrg-1, as this terminology has been agreed to by a recent consortium (8). Ndrg-1 is part of the NDRG family in which there are four members, namely, Ndrg-1, -2, -3 and -4, which have 57–65% amino acid sequence homology to each other (Figure 1) (9,10). The NDRG family is divided into two subfamilies, one consisting of Ndrg-1 and -3, and the other including Ndrg-2 and -4 (9). The mouse analog, Ndr-1 (also known as TDD5), is highly homologous to Ndrg-1, suggesting that this gene is well conserved between species (11,12). Surprisingly, Ndrg-1 homologs have been found in sunflowers (13), although their role in plant tumorigenesis remains unknown.

The expression of Ndrg-1 appears to be regulated in a variety of ways by diverse agents in normal and neoplastic cells. For instance, Ndrg-1 was identified by Kokame et al. (14) as a homocysteine-responsive gene that was induced by sulfhydryl reagents such as cysteine and 2-mercaptoethanol in human umbilical vein endothelial cells. Later, Ndrg-1 was identified as being upregulated during colon epithelial cell differentiation and downregulated in colon neoplasms (15,16). Ndrg-1 expression was also found to be increased by androgens in prostate cancer cells (17).

Other stimuli related to metal ions or metal ion metabolism are also involved in Ndrg-1 regulation. For instance, nickel compounds were shown to transcriptionally induce Ndrg-1 in a number of human cell lines by potentially activating hypoxia inducible factor-1 (HIF-1) (18). Interestingly, increased intracellular calcium levels were found to significantly upregulate Ndrg-1 (19). More recently, iron chelators with high antitumor activity were observed to increase Ndrg-1 expression by HIF-1α-dependent and -independent mechanisms (see sections: “Ndrg-1 and stress response”, and “Link of Ndrg-1 to iron metabolism and tumor cell proliferation”) (20). Therefore, this molecule appears to be involved in a number of biological systems and may play different roles in various organs and tissues.

The current review will examine the state of knowledge regarding the structure and function of Ndrg-1 and the potential to modulate its expression using pharmacological interventions to inhibit tumor metastasis.

Abbreviations: HDL-C, high-density lipoprotein-cholesterol; HIF-1, hypoxia inducible factor-1; HMSNL, Hereditary Motor and Sensory Neuropathy-Lom; Ndrg-1, N-myc downstream-regulated gene-1.
The molecular structure of Ndrg-1

The Ndrg-1 gene is mapped to chromosome 8q24 (21), a region commonly amplified in some tumors (8). However, while there are no studies detailing amplification of the Ndrg-1 locus, it is of interest that c-myc is located in this region and is commonly amplified in cancer (22,23). This is significant, as c-myc has been shown to downregulate Ndrg-1 expression which could potentially lead to a more metastatic phenotype (8). The Ndrg-1 gene encodes a 3.0 kb mRNA that is translated into a protein with a molecular weight of 43,000 Da and an isoelectric point of 5.7 (14,16,18). The Ndrg-1 amino acid sequence encodes three tandem repeats of GTRSRSHTSE in its C-terminal region, which are not present in the other members of the NDRG family (10). This may indicate that Ndrg-1 has a unique function amongst this group of proteins.

Examining the sequence of Ndrg-1 protein, it is clear there is no apparent transmembrane domain, signal sequence or endoplasmic reticulum retention sequence (14). A phosphorylation site search using PROSITE software (http://ca.expasy.org/prosite/) revealed a phosphopantetheine attachment site as well as protein kinase C, casein kinase II and tyrosine kinase phosphorylation sites. In addition to this, Agarwala et al. (24) discovered there were more than seven phosphorylation sites in Ndrg-1, two of them being protein kinase A- and calmodulin kinase II-sensitive. Studies examining the function of Ndrg-1 in mast cells also showed that it existed as a multi-phosphorylated protein (25). Interestingly, the amount of phosphorylated Ndrg-1 was demonstrated to decrease as cell density increased, suggesting a possible role of Ndrg-1 in proliferation (24). Collectively, these data denote that Ndrg-1 may play a regulatory role in cells, whose levels or functions could be controlled at least in part by phosphorylation.

To identify potential molecules involved in Ndrg-1 regulation, the nucleotide sequence of the Ndrg-1 promoter was analyzed for motifs using Genomatix Suite 3.0 software (Genomatix, Munchen, Germany) (26) and was found to contain a number of motifs for cell cycle regulators and transcription factors such as E2F and MYC. In fact, there are a number of studies describing N-myc as a transcription factor that downregulates Ndrg-1 expression (see section: ‘Ndrg-1 and MYC’) (8,11). Further analysis revealed that the 5’-end of the Ndrg-1 cDNA contains multiple CpG sites, suggesting that Ndrg-1 expression may be controlled by DNA methylation (7). The expression of Ndrg-1 was also upregulated by the histone deacetylase inhibitors, trichostatin A and N-hydroxy-N0-phenol-octane-1,8-diotic acid diamide suberoylanilide hydroxamic acid (7). Hence, DNA methylation and histone acetylation, as well as a number of transcription factors are involved in regulating Ndrg-1 levels.

Studies examining the protein structure of Ndrg-1 have revealed interesting characteristics. The AceView database from NCBI indicates that Ndrg-1 and other NDRG family members have an α/β hydrolase-fold motif, which is commonly found in hydrolytic enzymes (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/) (9). However, further investigation revealed that all of the residues required for hydrolytic activity have been eliminated from this motif (27). Considering this, it can be suggested that the presence of the α/β hydrolase fold in these genes indicates that they evolved from a hydrolytic enzyme and have retained the structure, but not the functionality of this fold. On the other hand, there is the possibility of convergent evolution, where these genes have developed this molecular architecture for a different purpose.

Considering the data described above, it can be concluded that Ndrg-1 is a protein regulated by DNA methylation, histone deacetylation and certain transcription factors such
as E2F and MYC. Furthermore, Ndrg-1 is likely to play a regulatory role in cells, due to its ability to be phosphorylated, ultimately altering its function. Finally, the protein structure reveals that this molecule may have evolved from a hydrolytic enzyme.

**Cellular and tissue distribution of Ndrg-1**

The tissue expression of Ndrg-1 mRNA is ubiquitous and is especially high in the prostate, brain, kidney, placenta and intestine (7,9,10,14,16,18,28,29). Although it has been shown that Ndrg-1 mRNA is expressed in most human tissues, Ndrg-1 protein is mainly found in epithelial cells, suggesting that Ndrg-1 has a specific function (28). In contrast, Ndrg-2 was strongly expressed in the spinal cord, Ndrg-3 was highly expressed in the testis, while Ndrg-4 was expressed in the brain and heart only (9,10). Three Ndrg-4 isoforms have been identified and named Ndrg-4B, Ndrg-4B\*ar and Ndrg-4H (10). Both Ndrg-4B and Ndrg-4B\*ar are alternatively spliced isoforms (10). However, all three isoforms of Ndrg-4 are produced from one gene, possibly by tissue-specific alternative promoter use (10). In fact, Ndrg-4B mRNA was only detected in the brain, while Ndrg-4H mRNA was more abundant in the heart than brain (10). Hence, the distinct expression patterns of the members of the NDRG family suggests they each have different specific functions (9).

Ndrg-1 appears to be a predominantly cytoplasmic protein, but its cellular localization is dependent on the cell type assessed (28). For instance, intestinal and lactating breast epithelia have Ndrg-1 associated with the plasma membrane, while epithelial cells of the prostate have predominant nuclear Ndrg-1 localization (28). In contrast, in kidney proximal tubule cells, Ndrg-1 is associated with the mitochondrial inner membrane (28). Analysis of human Ndrg-1 localization using PSORT II software (http://psort.hgc.jp/) predicted Ndrg-1 to be predominantly expressed in the cytoplasm, nucleus and the mitochondrion at a probability of 47.8, 26.1 and 8.7%, respectively, Ndrg-1 was also predicted to be localized to the cytoskeleton, vacuoles, plasma membrane and the peroxisome, although this was found to be less likely.

While Ndrg-1 has been shown to localize in the nucleus of some cell types, there was no nuclear localization signal detected in its amino acid sequence (28). Therefore, it has been proposed that Ndrg-1 interacts with nuclear proteins, allowing it to enter the nucleus (28). Indeed, it has been shown that Ndrg-1 associates with heat-shock cognate protein 70 (Hsc70) in mast cells (30). Hsc70 is a nucleocytoplasmic transport protein (31) that has been shown to enter the nucleus accompanying Ndrg-1 (30). Upon exposure to low concentrations of actinomycin D, which acts as a DNA-damaging agent rather than a transcriptional inhibitor (32,33), Ndrg-1 was demonstrated to translocate from the cytoplasm to the nucleus (21). This suggested a role for Ndrg-1 in DNA repair and identified it as a stress response gene (21). Alternatively, this observation could suggest that Ndrg-1 may be acting as a transcription factor. In fact, similar translocation to the nucleus in response to DNA damage has been observed with other transcription factors such as p53 (34).

Electron microscopy studies showed that Ndrg-1 was associated with membranes being often observed adjacent to adherens junctions, as well as the intermediate and microfilament bundles which insert into these structures (28). This suggests that Ndrg-1 plays a role in cell adhesion. In fact, Ndrg-1 was demonstrated to upregulate the levels of the adhesion molecule E-cadherin when overexpressed in colon tumor cells, further supporting this hypothesis (7).

The studies above examining the expression profile of Ndrg-1 suggest that it may be involved in cell adhesion and/or DNA damage repair. Therefore, changes in Ndrg-1 expression could have profound consequences in cancer development and metastasis.

**Biological function and regulation of Ndrg-1**

Although the exact function of Ndrg-1 remains unknown, a variety of studies have reported the expression of this gene under many different conditions (5–8,18,25,29,30,35–42). As outlined in Figure 2, Ndrg-1 has a number of potential functions and downstream effects, ranging from myelin sheath maintenance, differentiation, metastasis suppression and enhanced exocytosis in mast cells (5–8,18,25,29,30,35–42). The range of functions attributed to Ndrg-1 are discussed briefly below, since knowledge of these may provide clues to its function as a metastasis suppressor in tumor cells.

**Ndrg-1 and its role in differentiation**

It was first suggested by van Belzen and coworkers (16) that Ndrg-1 was involved in differentiation and a number of subsequent studies have confirmed this. For instance, rat peripheral gliona cells (D6P2T) which were induced to differentiate by the cAMP phosphodiesterase inhibitor, isobutylmethyl xanthine (IBMX), stopped proliferating (8). These changes were accompanied by a reduction in N-myc expression and a significant increase in Ndrg-1 (8). In addition to this, Ndrg-1 was also shown to increase during keratinocyte differentiation (35). Studies examining Ndrg-1 overexpression in metastatic colon cancer cells have reported morphological changes resembling differentiation (7). Additionally, ligands of nuclear transcription factors involved in cell differentiation, such as PPARγ, have been shown to induce Ndrg-1 expression (7,29). Hence, there is substantial evidence to support the hypothesis that Ndrg-1 is involved in the induction of differentiation.

In agreement with these studies, Ndrg-1 has been identified as a gene upregulated under conditions where U937 myelomonocytic cells stop proliferating, suggesting this protein is involved in inducing terminal differentiation (29). These results along with data showing the involvement of Ndrg-1 in differentiation, potentially indicate that Ndrg-1 may play a role in regulating the cell cycle. A study assessing the expression of Ndrg-1 throughout the cell cycle has revealed it to be biphasic, with expression peaking in the G1 and G2/M phases and being lowest in S phase (21). Further evidence that Ndrg-1 is involved in cell cycle regulation was provided by studies showing that it was required to maintain spindle structure during cell division in human mammary epithelial cells (43). In fact, it was shown that Ndrg-1 is a microtubule-associated protein that localized to centrosomes and participated in the spindle checkpoint in a p53-dependent manner (43). Moreover, inhibition of Ndrg-1 expression resulted in significant changes in microtubule structure and the disappearance of α-tubulin protein (43). Therefore, it was suggested that the loss of Ndrg-1 may contribute to genomic instability in cancer cells (43).
**Ndrg-1 and nerve myelination**

In addition to its role in cell differentiation, Ndrg-1 has been reported to play an important role in the disease, Hereditary Motor and Sensory Neuropathy-Lom (HMSNL) (37). This condition is the most common form of demyelinating Charcot-Marie-Tooth disease in the Roma (Gypsy) population (36,37). It is an autosomal recessive disease caused by demyelination of peripheral nerves that progresses to severe disability in adulthood (37). Gene mapping analysis has shown that all individuals with this disease have a truncating mutation known as the Gypsy founder mutation, R148X, in Ndrg-1 (37). It has been shown that Ndrg-1 is abundantly expressed in peripheral nerves, especially Schwann cells, where it plays a significant role in Schwann cell differentiation and signaling which is necessary for axonal survival (37). Specifically, Ndrg-1 is mainly expressed in the cytoplasm of Schwann cells, suggesting that a deficiency in Ndrg-1 is the primary cause of Schwann cell dysfunction (39). In fact, Ndrg-1 knockout mice exhibit progressive demyelination in peripheral nerves, suggesting this protein is essential for myelin sheath maintenance (39). Other key observations in Ndrg-1 knockout mice include hind limb weakness and leg muscle atrophy, all suggesting neurological abnormalities (39). Indeed, severe degeneration of the sciatic nerves was observed at 3 months of age, along with thinly myelinated axons (39). The myelin sheaths of Ndrg-1 knockout mice began to degenerate at 5 weeks of age, but were normal before this time. (39). This suggested that the defect was not the ability to form myelin sheaths in Schwann cells, but due to a problem in their maintenance (39). The brain of Ndrg-1 knockout mice showed no abnormalities, suggesting that the absence of Ndrg-1 in the brain may be compensated for by other members of the NDRG family (39).

**Role of Ndrg-1 in lipid biosynthesis and metabolism**

The phosphopantetheine-binding domain in Ndrg-1 may indicate an additional role in the lipid biosynthetic pathways operating in myelin-producing Schwann cells (37). Indeed, yeast two-hybrid analyses have revealed that Ndrg-1 interacts with the high-density lipoproteins, apolipoprotein A-I and A-II (36). Considering this, it has been suggested that Ndrg-1 contributes to high-density lipoprotein-cholesterol (HDL-C) levels (36). Because the risk of developing atherosclerosis is inversely related to the serum concentration of HDL-C, Ndrg-1 may be involved in the pathogenesis of this disease (36). In fact, the Gypsy founder mutation in Ndrg-1, which was identified as the cause of HMSNL (37), was also correlated with low HDL-C levels (36). Males homozygous with this mutation had a high total cholesterol:HDL-C ratio, increasing their risk of cardiovascular disease (36). Taken together with its recent link to the myelin biosynthetic pathway in Schwann cells (37), these observations underline the possible roles of Ndrg-1 in lipid metabolism.

**Ndrg-1 and stress response**

A link of Ndrg-1 to stress-response has been identified in a study that demonstrates that the sulphydryl group-containing amino acid, homocysteine, upregulates the expression of this gene (14). Elevated levels of homocysteine have been correlated with atherosclerosis and thrombosis (14,44,45). Human umbilical vein endothelial cells incubated with homocysteine showed a significant increase in Ndrg-1 mRNA expression after only 4 h (14). It has been suggested that elevated homocysteine levels damage cells, leading to a stress response and subsequent changes in gene expression, such as Ndrg-1 upregulation (14). These results suggest that Ndrg-1 may play a cytoprotective role upon exposure to conditions that lead to stress.

The regulation of Ndrg-1 by HIF-1 (20,46,47) is a further indication of its function as a stress response gene. HIF-1 is a transcription factor that is activated under hypoxic conditions and acts to initiate a signaling pathway promoting cell survival (48). This protein is composed of two subunits, a constitutively expressed α subunit and an α subunit which is regulated by the hypoxic state and iron levels (48,49). Under
conditions of normal oxygen tension and iron levels, HIF-1α is regulated by prolyl hydroxylase which allows its binding to the von Hippel-Lindau (VHL) protein. This protein activates a ubiquitin E3 ligase resulting in the subsequent degradation of HIF-1α (50). However, under conditions of oxygen or iron-depletion or both, the prolyl hydroxylase fails to function, leading to the accumulation of HIF-1α in the cell (48–50). HIF-1α is then able to translocate to the nucleus where it binds to HIF-1β to form the HIF-1 complex (51). Once assembled, HIF-1 can regulate a number of genes by binding to their hypoxia response elements (HREs) located in the promoter (52).

A number of studies have shown that Ndrg-1 is regulated by HIF-1 (20,46,47). In fact, two HREs were identified upstream of the Ndrg-1 promoter, at −1376 and −7503 bp, suggesting that HIF-1 may act to induce Ndrg-1 expression (20). It was found that short-term hypoxia induced Ndrg-1 expression in HIF-1α−/− cells, while no Ndrg-1 induction was observed in HIF-1α+/− cells (46). Studies examining the HIF-1 mediated upregulation of Ndrg-1 in response to iron-depletion have also found that this transcription factor leads to a greater extent of Ndrg-1 expression (20).

Another possible role for Ndrg-1 as a stress-response gene has been identified in human trophoblasts (53). Trophoblasts form the surface of the human placenta villi and are essential for gas exchange, nutrition and removal of waste from the developing fetus (53). Trophoblast hypoxia in later stages of pregnancy is often caused by factors such as smoking and may lead to hypoxic injury of the placenta (53). Microarray studies examining the differences in gene expression between normal and hypoxic trophoblasts have identified that Ndrg-1 is upregulated in response to hypoxia (53). Increased Ndrg-1 levels in trophoblasts were found to correlate with increased expression of syncytin, human chorionic gonadotropin (hCG) and human placental lactogen (hPL), which are all markers of differentiated trophoblasts (53). Conversely, reducing Ndrg-1 expression in hypoxic trophoblasts using small interference RNA (siRNA) resulted in a decrease in syncytin, hCG and to a lesser extent, hPL (53). This was accompanied by diminished trophoblast viability due to increased apoptosis (53). This study also found that Ndrg-1 overexpression in trophoblasts led to a decline in p53 expression, suggesting a protective role for Ndrg-1 in these cells against p53-induced apoptosis (53). Furthermore, NDRG1 expression was regulated by the activity of Sir2-like protein 1 (SIRT1), which promotes cell survival. Together, these data indicated that NDRG1 interacts with SIRT1/p53 signaling to attenuate hypoxic injury in human trophoblasts (53). In contrast, another investigation showed that Ndrg-1 was involved in the p53-mediated apoptotic pathway, where it sensitized cells to apoptosis (54). However, it should be noted that this latter study examined cancer cells, as opposed to normal trophoblasts (53). This again suggests the cell-specific functions of Ndrg-1.

Ndrg-1 and exocytosis

In mast cells, Ndrg-1 was the most frequently upregulated gene during in vitro maturation (42). In fact, Ndrg-1 overexpression induced mast cells to degranulate more rapidly, leading to an enhanced exocytotic response to various stimuli (42). Intriguingly, Ndrg-1 was shown to associate with proteins such as prenylated rab acceptor protein 1 (Pra1) and reticulon 1C (RTN1C), both of which interact with the soluble N-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE) that is required for exocytosis (36). Hence, Ndrg-1 may help transform immature mast cells into a mature phenotype, which is more efficient in responding to secretagogues (42).

The many functions of Ndrg-1 discussed in the sections above suggests it plays roles in processes such as cell division, differentiation and metabolism. In fact, it could be suggested that Ndrg-1 acts in a pleiotropic manner. However, it is likely that Ndrg-1 has cell-specific functions, which could account for the diversity of roles it has been found to play in different biological systems. Clearly, altered expression of Ndrg-1 could lead to significant pathological consequences that may become profound in conditions such as cancer.

Ndrg-1 and cancer

As already discussed, the expression of Ndrg-1 in normal cells and tissues is widespread and prominent (28). However, in cancer cells, Ndrg-1 has often been found to be downregulated (Figure 3) (5–7,16,21,40). Immunohistochemical analyses of a range of prostate cancer specimens have shown that Ndrg-1 expression is lower in cancers than normal tissues (5). In addition to this, the level of Ndrg-1 expression has been found to be inversely related with the Gleason grade of the tumor, with higher grade (more advanced) and poorly differentiated tumors expressing less Ndrg-1 (5,21). Studies examining colon cancer have also reported higher Ndrg-1 mRNA levels in primary colon cancers than their metastases (7). In fact, in colon cancer, the Ndrg-1 mRNA levels appeared to decrease with progression from normal colonic epithelium to carcinoma (16). Similarly, the expression of Ndrg-1 mRNA was also shown to be downregulated in breast cancer (6).

Factors contributing to low Ndrg-1 expression in tumor cells include DNA methylation. The DNA methylation inhibitor, 5-azacytidine, significantly elevated Ndrg-1 expression in breast cancer cells in vitro, although this did not affect their rate of proliferation (6). Also, the expression of Ndrg-1 throughout the cell cycle in cancer cells was found to be consistent throughout all phases, as opposed to the biphasic expression in normal cells, as mentioned earlier (21). Together, these results contribute to the hypothesis that Ndrg-1 plays a significant role in cancer progression and metastasis.

Ndrg-1: a gene targeted by tumor suppressors

Further evidence suggesting involvement of Ndrg-1 in tumorigenesis comes from studies showing upregulation of Ndrg-1 by the transcription factor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (41). Overexpression of PTEN resulted in increased levels of Ndrg-1, while PTEN inhibition decreased Ndrg-1 expression in a dose- and time-dependent manner (41). Although no PTEN binding sequence was found in the promoter region of Ndrg-1, a protein involved in the PTEN signaling pathway, BPOZ (55), has a possible binding region in the Ndrg-1 promoter, as determined by the Genomatix suite 3.0 program (26). Significantly, BPOZ has been implicated as a candidate mediator of the PTEN growth-suppressive signaling pathway (55) and further studies assessing the relationship to Ndrg-1 are necessary.
Inactivation of PTEN was found to significantly correlate with invasion and metastasis of a wide variety of cancers both in vitro and in vivo (41). Additionally, PTEN was shown to markedly diminish lymph node metastasis in mice with prostate cancer (56). Therefore, the ability of PTEN to act as a metastasis suppressor could be mediated, at least in part, by its downstream target, Ndrg-1 (41). A clinical study examining PTEN and Ndrg-1 expression in prostate and breast cancer demonstrated an almost identical staining pattern for both proteins, with high expression in normal tissue and low expression in poorly differentiated tumor cells (41). Finally, the combination of high PTEN and Ndrg-1 expression in breast and prostate cancers was found to correlate with better survival rates, as opposed to the high expression of one, or neither, of these molecules (41).

The VHL protein is another tumor suppressor involved in regulating Ndrg-1 expression (38). However, unlike PTEN, VHL downregulates Ndrg-1 expression in renal cancer cells. VHL normally targets HIF-1 for degradation, thus leading to lower Ndrg-1 levels. (50). This tumor suppressor is often mutated in renal cell carcinoma, which consequently often overexpress HIF-1 and other hypoxia-inducible proteins (38). It has been reported that Ndrg-1 is more highly expressed in cancerous regions of renal tissue (38), which is probably due to the loss of VHL leading to decreased HIF-1 degradation. However, this study did not report the effects of high Ndrg-1 levels in the VHL-negative renal cancer cells and whether they are more aggressive when compared with their VHL-positive counterparts. These observations need to be confirmed and extended, as they would be useful in assessing the role of Ndrg-1 in tumorigenesis.

**Ndrg-1 and p53**

The tumor suppressor, p53, has been reported to regulate Ndrg-1 levels (21). Stein et al. isolated Ndrg-1 as one of the genes necessary for p53-dependent apoptosis and have shown that Ndrg-1 is upregulated following p53 induction in the colon cancer cell line DLD-1 (54). This response was reported to occur only 8 h after p53 was induced, suggesting that Ndrg-1 is an early target for p53 (54). Additionally, this study showed that selective knockdown of p53 using siRNA resulted in reduced Ndrg-1 expression in DLD-1 cells (54). These authors also showed using H1299 cells transfected with Ndrg-1 and inducible p53 that both proteins were required for p53-mediated apoptosis (54). In agreement, Kurdistani et al. (21) have shown that Ndrg-1 is a p53-responsive gene which may control cellular proliferation. Both studies suggest that Ndrg-1 expression is induced by DNA-damaging agents in a p53-dependent manner (21,54). Furthermore, a luciferase reporter assay showed that Ndrg-1 was directly regulated by p53 (54). Together, these data suggest that the frequent loss of p53 expression in cancers is responsible for reduced Ndrg-1 expression (21). Paradoxically, another study found that Ndrg-1 inhibits p53 expression in human placental trophoblasts, acting to alleviate apoptosis and promote cell survival in response to hypoxic injury (53). However, as mentioned above, the role of Ndrg-1 in normal trophoblasts may be quite different to its role in cancer cells and this requires further investigation.

Other studies have shown no correlation between Ndrg-1 expression and DNA damage, despite the upregulation of p53 (6,20). An increase in Ndrg-1 was observed in the p53-null lung cancer cell line H1299 in response to iron-depletion, indicating that Ndrg-1 induction under these conditions was not p53-dependent (20). Also, when p53 expression was increased using a tetracycline-responsive system in H1299 cells, Ndrg-1 expression was not affected (54). Immunohistochemical analyses of prostate and breast cancer tissue samples found no significant correlation of Ndrg-1 protein expression with p53 (41). Indeed, because the effect of p53 on Ndrg-1 expression was only observed in certain cell types, this may partly explain the contradictory reports describing a role of p53 in Ndrg-1 expression (54).

It is significant that p53 knockout mice do not show a complete loss of Ndrg-1 expression, suggesting that there are other mechanisms regulating its levels (21). Considering the controversial role of p53 in Ndrg-1 expression, both Le et al. (20) and Kurdistani et al. (21) used MCF-7 cells and similar concentrations of the DNA-damaging agents, actinomycin D (6.27 ng/ml and 10 ng/ml, respectively) and mitomycin C (10 μg/ml), to increase p53 expression. However, their results in terms of Ndrg-1 induction were significantly different, with one study observing no effect (20) and the other finding that Ndrg-1 expression was upregulated in response to these agents (21). At present, it appears that the regulation of Ndrg-1 expression by p53 may depend on the conditions and cell types assessed and remains an important subject for further investigation.

**Ndrg-1 and MYC**

As described earlier, the oncogene N-myc was demonstrated to downregulate Ndrg-1 expression (8). During mouse
embryogenesis, it has been shown that the expression of N-myc decreased as the levels of Ndr-1 (the mouse Ndrg-1 homolog) increased, indicating an inverse relationship between the two (11). In fact, Ndr1 was transcriptionally repressed by the N-myc:Max heterodimer, this effect being dependent on direct binding of N-myc:Max to the promoter region of Ndr-1 (11). Interestingly, c-myc, and c-myc:Max were also shown to repress the Ndr-1 promoter in much the same way as N-myc. This indicates that the suppression of Ndr-1 is a general property of the MYC family. Human Ndrg-1 has an N-myc binding motif close to the initiation site (11). However, the effect of N-myc:Max on Ndr-1 suppression may not depend on the direct binding of this complex to the Ndr-1 promoter, and may be due to the interaction of this complex with the basal transcriptional machinery (11).

Because human cancers such as neuroblastoma often demonstrate N-myc overexpression, this is likely to be a contributing factor to Ndrg-1 suppression in these neoplasms (8). In addition, another member of the MYC family, namely c-myc, is also overexpressed in many tumor types (11,57). In cells with naturally high levels of c- or N-myc, Ndrg-1 expression was found to be downregulated (8). Similarly, in cells with forced N- or c-myc expression, Ndrg-1 was suppressed (8). Therefore, the overexpression of c- and N-myc in either epithelial or fibroblastic cell types resulted in the repression of Ndrg-1 (8). On the other hand, suppression of N-myc levels by retinoids was found to induce Ndrg-1 expression in IMR-32 and NGP neuroblastoma cells (8). However, this latter study is a special case, as retinoids can induce differentiation which could lead to increased Ndr-1 through other pathways. As described previously, Ndrg-1 was found to be upregulated by nickel in some cell types (18). Interestingly, nickel also suppresses N-myc expression, indicating that this is a potential mechanism of nickel-mediated Ndrg-1 upregulation (8) in addition to that mediated by HIF-1 (see section: ‘Ndrg-1 and stress response’) (18).

### The role of Ndrg-1 in primary tumor growth

Inhibition of primary tumor growth by Ndrg-1 was demonstrated by Kurdistani and associates (21). This study was performed using breast, prostate and bladder cancer cell lines which were stably transfected with Ndrg-1 so that they overexpress this protein. The result was a reduction in the proliferation rates of these cells by up to 70%, as well as the formation of smaller colonies in soft agarose (21). In vivo studies using nude mice injected with EJ bladder cancer cells overexpressing Ndrg-1 also showed significantly smaller tumor formation compared to controls (21).

More recent studies examining the effect of Ndrg-1 in pancreatic cancer cells found that although overexpression of this protein did not effect cell growth in vitro, there was a significant reduction of primary tumor xenograft growth in vivo (58). This difference was explained by the potential ability of Ndrg-1 expression to modulate tumor stroma and angiogenesis in pancreatic cancer (58). Examination of samples from 65 pancreatic cancer patients for Ndrg-1 expression using immunohistochemistry demonstrated a significant association between high Ndrg-1 expression and lower tumor microvascular density, invasion depth and histopathological grading (58). Moreover, the survival of patients with high tumor Ndrg-1 expression was significantly greater (P < 0.0062) than those patients negative for the molecule (58).

Paradoxically, in contrast to the investigations above, in vivo studies assessing colon (7) and prostate (5) cancers showed no significant effect of Ndrg-1 on the primary tumor growth in mice. Furthermore, studies examining breast cancer found that there was no correlation with the size or histological grade of the tumor with Ndrg-1 expression (6). Again, this may indicate that Ndrg-1 has tissue-specific functions.

### Ndrg-1 as a metastasis suppressor

As Ndrg-1 has a significant role in differentiation (see section: ‘Ndrg-1 and its role in differentiation’), it is no surprise that it may also lead to metastasis suppression in many cancers. In fact, Ndrg-1 was shown to be an effective metastasis suppressor in prostate, colon and breast cancer (5–7,40). The overexpression of Ndrg-1 in colon and prostate cancer cells has been shown to reduce the invasive ability of these cells (5,7). This reduction in metastasis was significant, ranging from 70% in colon cancer cells (SW620) to 90% in rat prostate cancer cells (AT6.1) and 20–30% in the human prostate cancer cell line, ALVA (5,7).

Prostate cancer cells transfected with Ndrg-1 were reported to have a markedly lower invasive ability (58). In contrast, similar cells expressing low Ndrg-1 levels were more invasive, when tested by a Matrigel® invasion assay (58). Similarly in breast cancer, the invasiveness of MDA-MB-468 cells was significantly reduced when Ndrg-1 was overexpressed, which is consistent with the immunohistochemical analyses showing lower Ndrg-1 levels in tumor metastases (6). In human prostate cancer specimens, the expression of Ndrg-1 was much higher in organ-confined tumors, than in lymph node or bone metastases (5). Interestingly, upregulation of Ndrg-1 in metastatic H1299 lung cancer cells resulted in an inhibition of proliferation, while in non-metastatic DLD-1 colon cancer cells there was no effect (54). Collectively, these studies clearly indicate that Ndrg-1 is negatively correlated with tumor metastasis.

In vivo studies in animal models of metastasis have shown even more dramatic results in both colon and prostate cancer. For example, mice injected with SW620 colon cancer cells overexpressing Ndrg-1 resulted in only 23% of these developing liver metastases, compared to 75% in the control group (7). Similarly, mice injected with AT6.1 rat prostate cancer cells overexpressing Ndrg-1 showed significantly lower incidence of lung metastases than control mice (5).

To date, there has been little assessment of the molecular targets of Ndrg-1 that mediate its anti-metastatic activity. The adhesion molecule and metastasis suppressor, E-cadherin (59), was found to be upregulated by Ndrg-1 (7). Increased expression of E-cadherin has been shown to reduce the motility of metastatic breast cancer cells in vitro (59). However, it is unlikely that E-cadherin is the only molecular target of Ndrg-1 that leads to metastasis suppression. Further studies assessing the targets of Ndrg-1 using techniques such as gene array may be useful in understanding the molecular mechanisms of metastasis suppression.

Considering the role of Ndrg-1 in metastasis suppression, it was proposed that its expression could be used as a prognostic marker in cancer patients (5,6). This follows the observation that individuals with higher Ndrg-1 tumor levels have greater survival rates, as indicated in 5 year follow-up studies in breast and prostate cancer patients (5,6). This is also supported by a study examining Ndrg-1 expression in
In vivo studies examining colorectal cancers have found Ndrg-1 expression lower in cancers with high Ndrg-1 levels compared to those with low Ndrg-1 levels (58). Importantly, in a study examining Ndrg-1 expression in African-American prostate cancer patients, it was found that they have significantly reduced expression of this protein when compared with Caucasian prostate cancer patients (60). Indeed, African-American men have been shown to have a greater risk of contracting prostate cancer, presenting at a younger age and with more aggressive tumors than Caucasians (60). However, whether Ndrg-1 is the only factor involved in the more aggressive pathogenesis of prostate cancer in African-Americans remains to be determined.

**Ndrg-1 and angiogenesis**

The ability of a tumor to induce new blood vessel generation (angiogenesis) is one of the key factors which allows it to grow and invade (61). Intriguingly, a recent study has found that Ndrg-1 is involved in regulating both tumor growth and angiogenesis (58). In fact, pancreatic cancer cells expressing high levels of Ndrg-1 were shown to have reduced matrix metalloproteinase-9 activity, a critical component of the angiogenic switch during carcinogenesis (58). In addition to this, the levels of two angiogenic factors, vascular endothelial growth factor (VEGF-1) and IL-8 (interleukin-8) were reduced at the mRNA level in cells overexpressing Ndrg-1 (58). In vivo studies examining the angiogenic potential of pancreatic tumors with high or low Ndrg-1 expression also confirmed that cancers with high Ndrg-1 levels had significantly lower protein levels of both VEGF-1 and IL-8. Furthermore, the ability of these tumors to form microvessels was significantly compromised (58). The authors of this paper conclude that Ndrg-1 could play a role in the angiogenic ‘on- or off-switch’ of tumor stroma in pancreatic ductal adenocarcinoma. At present, this is the only information concerning Ndrg-1 and angiogenesis and these intriguing results remain to be confirmed.

**Chemotherapy and Ndrg-1**

In addition to the potential of Ndrg-1 as a prognostic indicator, the extent of its expression may also modulate the response of a tumor to treatment. An investigation examining the sensitivity of metastatic colon cancer to the topoisomerase inhibitor, CPT-11 (a camptothecin analog), have found that Ndrg-1 is involved in the resistance to this agent (62). Two human colon cancer cell lines, SW620 and Hct116 expressing high and low levels of Ndrg-1, respectively, were used to demonstrate that high Ndrg-1 expression leads to resistance to CPT-11 (62). Conversely, the suppression of this protein sensitizes these cells to CPT-11 both in vitro and in vivo (62).

Further clinical studies using CPT-11 have also demonstrated that high Ndrg-1 expression confers resistance to this agent (40). Potentially, this may be mediated through the ability of Ndrg-1 to act as a stress response gene and cytoprotectant (53). CPT-11 also upregulated Ndrg-1 expression further increasing the resistance of metastatic colon cancer cells to this drug (40). Additionally, in studies where CPT-11 upregulated Ndrg-1, subsequent treatment with the drug, flavopiridol, then led to a decrease in Ndrg-1 expression resulting in a better therapeutic outcome (63).

On the other hand, the upregulation of Ndrg-1 could be a beneficial therapeutic strategy as this gene is correlated with inhibition of metastasis and a better clinical outcome (5, 6, 40, 41). The upregulation of Ndrg-1 can be mediated through p53 and/or HIF-1 (20, 21) and theoretically agents which increase expression of these transcription factors could potentially elevate Ndrg-1 levels. Recently, the expression of Ndrg-1 was found to be upregulated by the depletion of intracellular iron using chelators which show anti-proliferative activity (see section: “Link of Ndrg-1 to iron metabolism and tumor cell proliferation”) (20). Investigations using other types of agents such as the peroxisome proliferator-activated receptor-gamma ligand, thiozolidinedione, have demonstrated that it upregulates Ndrg-1 in colon cancer cells suppressing growth and metastases (64). Hence, this new antitumor strategy of increasing Ndrg-1 expression may be a useful addition to the pharmacological armamentarium against cancer.

**The controversy of Ndrg-1 expression in cancer**

Although the overwhelming majority of studies have identified Ndrg-1 as a gene that is downregulated in cancer and associated with metastasis suppression, there are some reports claiming the opposite. Some studies have suggested that Ndrg-1 expression is upregulated in prostate cancer compared with corresponding normal tissue, due to its response to hormones such as androgens (46). These studies suggest that the loss of hormone-dependence is responsible for the previously observed decrease of Ndrg-1 expression in some cancers (5–7, 16, 21, 40, 41). In fact, Ndrg-1 expression was found to be increased by androgens in prostate cancer cells (i.e. the LNCaP cell line) which are androgen receptor positive (17). Furthermore, prostate cancer cells that are androgen receptor negative, namely PC-3 and DU-145 cells, had no Ndrg-1 induction when exposed to androgens (17). However, an association of Ndrg-1 with growth arrest in these cells is indicated by the fact that its expression is maximal at androgen concentrations which promote differentiation and reduce proliferation (10^-9 M), rather than at lower androgen levels that are optimal for proliferation (10^-10 M) (17). In addition, Ndrg-1 was suggested to be a marker of androgen-induced differentiation in the human prostate (17).

Interestingly, in contrast to the situation in human prostate cells where androgens increased Ndrg-1 expression (17), the mouse homolog of Ndrg-1 (known as TDD5 or Ndr-1), was reported as a gene differentially repressed by androgens in a T cell hybridoma (12). Also, in breast cancer cells, Ndrg-1 was shown to be downregulated by 17β-estradiol and this was dependent on estrogen receptor-α (ER-α) pathways (65). Cells expressing ER-α were shown to have lower levels of Ndrg-1 than ER-α-negative cells (65). Together, these studies suggest that androgens play an important role in mediating Ndrg-1 expression and this needs to be considered when determining the level of Ndrg-1 in tumor tissue.

Studies examining colorectal cancers have found Ndrg-1 expression to be higher in more advanced lesions, leading the authors to speculate that Ndrg-1 is a metastasis promoter gene
(66). However, this appears to be an isolated observation, particularly considering the accumulating evidence of Ndrg-1 downregulation in cancer and its involvement in metastasis suppression (5–8, 16, 21, 40, 41). As mentioned above, Ndrg-1 can be markedly upregulated by hypoxia, which may explain its higher levels in advanced colorectal cancers.

It has also been proposed that Ndrg-1 upregulation in tumor cells could be due to the hypoxic state that cancer cells endure, as indicated by their elevated HIF-1α protein levels (67). Theoretically, this is an attractive hypothesis, as Ndrg-1 expression can be upregulated in a HIF-1α-dependent manner (see section: “Ndrg-1 and stress response”) (20).

A study exploring the role of Ndrg-1 in mouse skin carcinogenesis has found that the expression of Ndrg-1 is also increased in mouse skin tumors (35). However, the increase was observed in early papillomas that were well differentiated, and was present in more advanced and less differentiated lesions (35). Also, the majority of the papillomas arising from treatment with the carcinogens, 12-O-tetradecanoylphorbol-13-acetate and dimethylbenzanthracene, were found to contain an activating mutation in the c-Ha-ras gene (35). Similarly, nearly all cell lines showing increased Ndrg-1 expression were also found to contain a mutation in c-Ha-ras. This not only indicates that mutations in Ha-ras may upregulate Ndrg-1 in some tumors, but also suggests certain treatments used to induce cancer could be responsible for increasing Ndrg-1 expression, possibly overcoming other processes in the tumor that result in reduced expression of this gene (35).

A recent study examining Ndrg-1 expression in a large number of clinical samples (223 prostate cancer specimens) has found that there is no conclusive Ndrg-1 upregulation or downregulation in these samples (60). Instead, there are three different expression patterns of this protein: (i) intense, predominantly membranous staining, similar to benign prostate epithelium; (ii) intense nucleo-cytoplasmic localization, and (iii) low or undetectable expression (60). These expression patterns were suggested to reflect different responses to hypoxia and androgens in prostate epithelia (60). Previous studies showing Ndrg-1 expression levels in clinical specimens have used smaller sample numbers, which could account for the inconsistencies in the literature. Furthermore, the effects of factors such as hormones and the chemicals used to induce carcinogenesis, as well as the extent of hypoxia, need to be considered when evaluating the role of Ndrg-1 in cancer, as they have an important impact on its expression. In addition, mutations in other Ndrg-1-regulatory proteins such as MYC and p53 also need to be considered.

From the investigations described above, it is clear that Ndrg-1 has an important role in the growth of primary and metastatic tumors. While there is some controversy regarding the relationship of Ndrg-1 expression to suppression of metastasis in certain cancers, the majority of studies have demonstrated that higher levels lead to a less aggressive phenotype. Moreover, Ndrg-1 expression in some cancers may be a good prognostic indicator.

**Link of Ndrg-1 to iron metabolism and tumor cell proliferation**

Recently, several investigations from different laboratories have shown that the expression of Ndrg-1 is linked to intracellular iron levels (20, 68). Iron plays a key role in cell proliferation and growth as it is required for DNA synthesis and cell cycle progression [for reviews see (69–71)]. In fact, the rate-limiting enzyme of DNA synthesis, ribonucleotide reductase, requires iron in its active site and is crucial for the conversion of ribonucleotides to deoxyribonucleotides (72). Iron chelators are agents which deprive cells of iron leading to cell cycle arrest and apoptosis [for reviews see (73, 74)].

Recently, novel chelators have been developed and found to be efficient and selective anticancer agents (73–76). For instance, in vivo studies using the iron chelator, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), showed a 47% reduction in the lung tumor mass in mice after only 5 days of treatment (77). In addition, another related chelator, Triapine®, has also been shown to be effective in tumor models in animals (78) and has entered phase I and phase II clinical trials (79).

Considering the important role of iron in proliferation, several genes involved in growth (e.g. the transferrin receptor 1) have been shown to be regulated by intracellular iron levels [for review see (74)]. Interestingly, Ndrg-1 has been shown to be upregulated by iron chelators in a variety of cancer cell lines (20). The upregulation of Ndrg-1 following iron-depletion could also be reversed by the incubation of cells with iron-donors which restored intracellular iron levels (20). The regulation of Ndrg-1 mRNA was not mediated by the iron-regulatory protein-iron responsive element system that mediates the regulation of the transferrin receptor 1 (69).

However, when Act D was used at high concentrations (4 µM) to inhibit transcription, it prevented the ability of chelators to upregulate Ndrg-1, suggesting transcriptional upregulation following iron-depletion (20). Further investigation demonstrated that the upregulation of Ndrg-1 in response to iron-depletion was shown to be mediated by mechanisms that were dependent and independent of HIF-1α (20). Of interest, previous studies have also suggested that calcium upregulates Ndrg-1 via a HIF-1α-independent pathway (19).

Mimosine is a toxic L-amino acid that is also an iron chelator that blocks cell cycle progression by binding iron (80). Two-dimensional gel electrophoresis analyses of the proteins affected by mimosine treatment in HeLa cells, revealed a significant upregulation of Ndrg-1 by a transcriptional mechanism (68). Studies examining the effect of mimosine on the expression and activity of the transcription factors HIF-1α, p53, c-Jun/AP-1 and N-Myc/c-Myc identified an increase in c-Jun/AP-1 only (68). Furthermore, an AP-1 inhibitor completely eliminated Ndrg-1 induction in response to mimosine (68). This may indicate that AP-1 mediates the mimosine-induced Ndrg-1 expression (68). It is intriguing that both hypoxia and the subsequent elevation in intracellular calcium lead to the activation of both the AP-1 transcription factor and Ndrg-1 (19, 20). Furthermore, an AP-1 response element was found on the Ndrg-1 promoter, suggesting that it may be regulated by this transcription factor (19). It can be speculated that the HIF-1α-independent pathway that upregulates Ndrg-1 expression after iron depletion (20) could be potentially mediated through AP1.

In summary, while the role of iron in the expression of Ndrg-1 has only recently been established, it could provide a potential mechanism for modulating the expression of this molecule using iron chelators. This may be a useful new therapeutic strategy to inhibit tumor growth and metastasis.
Conclusions

Ndrg-1 plays a significant role in preventing the metastasis and invasion of cancer cells and it has also been suggested to play a role in primary tumor growth. Potentially, these effects may be mediated through the ability of Ndrg-1 to induce differentiation. In addition, the adhesion molecule E-cadherin may be at least one molecular target of Ndrg-1 that could partly explain its biological activity. Tumor suppressor genes often mutated in cancer such as p53 and PTEN have been implicated in regulating Ndrg-1 expression and would explain its lower expression in neoplastic cells. Furthermore, oncogene products such as c-myc and N-myc also suppress Ndrg-1 expression, theoretically enhancing metastatic potential of cancer cells.

The majority of studies have implied that Ndrg-1 is negatively correlated with cancer progression, suggesting that a loss of Ndrg-1 expression results in a more aggressive, metastatic phenotype and identifying Ndrg-1 as a potential prognostic indicator. More recently, several research groups have shown that intracellular iron depletion using specific iron chelators upregulate Ndrg-1 expression through HIF-1α-dependent and -independent mechanisms. Hence, iron chelators provide for the first time, a therapeutic strategy for upregulating Ndrg-1 expression to potentially prevent metastasis and tumor growth.

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References


33. Le,N.T. and Richardson,D.R. (2003) Potent iron chelators increase the mRNA levels of the universal cyclophosphamide kinase inhibitor p21(CIP1/WAF1), but paradoxically inhibit its translation; a potential mechanism of cell cycle dysregulation. **Carcinogenesis**, 24, 1045–1058.


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