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Review

Molecular functions of the iron-regulated metastasis suppressor, NDRG1, and its potential as a molecular target for cancer therapy

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ABSTRACT

N-myc down-regulated gene 1 (NDRG1) is a known metastasis suppressor in multiple cancers, being also involved in embryogenesis and development, cell growth and differentiation, lipid biosynthesis and myelination, stress responses and immunity. In addition to its primary role as a metastasis suppressor, NDRG1 can also influence other stages of carcinogenesis, namely angiogenesis and primary tumour growth. NDRG1 is regulated by multiple effectors in normal and neoplastic cells, including N-myc, histone acetylation, hypoxia, cellular iron levels and intracellular calcium. Further, studies have found that NDRG1 is up-regulated in neoplastic cells after treatment with novel iron chelators, which are a promising therapy for effective cancer management. Although the pathways by which NDRG1 exerts its functions in cancers have been documented, the relationship between the molecular structure of this protein and its functions remains unclear. In fact, recent studies suggest that, in certain cancers, NDRG1 is post-translationally modified, possibly by the activity of endogenous trypsins, leading to a subsequent alteration in its metastasis suppressor activity. This review describes the role of this important metastasis suppressor and discusses interesting unresolved issues regarding this protein.

Contents

Abbreviations: Akt, protein kinase B; CPT-11, irinotecan; CMT4D, Charcot–Marie–Tooth disease, type 4D; DFO, deferoxamine (or desferrioxamine B); DpC, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone; DpT, di-2-pyridylketone thiosemicarbazone; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; ECM, extracellular matrix; ERG-1, v-ets avian erythroblastosis virus E26 oncogene homologue; EGR1, early growth factor-1; ETS, v-ets avian erythroblastosis virus E26 oncogene homologue 2; HDL-C, high-density lipoprotein cholesterol; HIF-1α, hypoxia inducible factor-1α; HMSNL, hereditary motor and sensory neuropathy-Lom; Hsc70, 70-kDa heat shock cognate protein; HTE, human tracheal epithelial cell; HUVEC, human umbilical vein endothelial cell; IL, interleukin; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; MMP, matrix metalloproteinase; NDRG1, N-myc down-stream regulated gene 1; NF-κB, nuclear factor-κB; NIH, 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone; PAR-1, protease activated receptor; PKA, protein kinase A; PKA, protein kinase C; PrEC, prostate epithelial cell; pMLC2, phosphorylated myosin light chain 2; PTEN, phosphatase and tensin homologue deleted on chromosome 10; pVHL, von Hippel–Lindau protein; ROCK1, Rho-associated, coiled-coil containing protein kinase 1 (ROCK1); ROS, reactive oxygen species; SGK, serum- and glucocorticoid-induced kinase; TAT, tumour-associated trypsinogen; TAT2, tumour-associated trypsinogen-2; TCF/LEF, T-cell factor/lymphoid enhancer-binding factor; Thtpa, thiamine triphosphatase; VEGF, vascular endothelial growth factor

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1. Introduction

Cancer is a major public health issue, with one in four deaths in the United States attributable to the disease [1]. Cancers encompass multiple entities of complex and multifactorial diseases [2], each having its own specific aetiology and genomic, histological and clinical characteristics [3,4]. Despite recent advances, effective clinical management remains elusive due to intra-tumoural heterogeneity and therapeutic resistance [5–11]. Therefore, novel therapies and further investigation into the pathophysiology of cancer are essential [12,13].

Metastasis is the process by which cancer cells disseminate from a primary tumour site to a distal secondary tissue microenvironment [14] and therefore represents an important therapeutic target [15]. Metastasis markedly complicates clinical management and is a major cause of death due to cancer [16]. Growing evidence has underscored the involvement of oncogenes, tumour suppressor genes and metastasis suppressor genes in the development and progression of cancer [17–20]. One metastasis suppressor, namely N-myc down-stream regulated gene 1 (NDRG1), is of particular interest and plays an anti-oncogenic role in cancers of the brain [21], breast [22,23], colon and rectum [24–28], oesophagus [29], pancreas [30] and prostate [31]. Paradoxically, NDRG1 is highly expressed in cancers of the kidney [32], liver [33–35], mouth [36], skin [37] and uterine cervix [38,39], where it has been suggested to play a role in promoting tumourigenesis.

Human NDRG1, the inaugural member of the NDRG family, was first identified as a predominantly cytoplasmic protein encoded by the ubiquitously expressed *N-myc down-stream regulated gene 1* (*NDRG1*) [24,40]. The molecule has been referred to under a number of different designations, including reducing agent and tunicamycin-responsive protein (RTP) [40], differentiation-related gene (Drg)-1 [24], reduced in tumours, 42-kDa protein (Rit42) [41], Ca^{2+}-associated protein 43 (Cap43) [42] and protein regulated by oxygen (PROXY)-1 [43]. The current designation, "NDRG1", has been agreed upon by a consortium of NDRG1 researchers and is the currently accepted gene symbol assigned by the HUGO Gene Nomenclature Committee [44]. Orthologues of *NDRG1* have been identified in the mouse, as *Ndr1* [45] and *TDD5* [46], and the common sunflower (*Helianthus annuus L.*), as *sf21* [47]. Therefore, *NDRG1* appears to be highly conserved across a wide range of organisms, indicating its important function in the cell.

2. Molecular structure of NDRG1 and its interactions

NDRG1 is a member of the NDRG family [48], currently consisting of *NDRG1*, *NDRG2*, *NDRG3* and *NDRG4* [49,50]. Notably, phylogenetic analysis indicates that each NDRG family member forms a separate homology cluster across multiple species with possibly specific and functionally divergent roles relative to the other family members (Fig. 1A) [51]. This group of genes is subdivided into two subfamilies, one of which is composed of *NDRG1* and *NDRG3* and the other is composed of *NDRG2* and *NDRG4* [50]. Proteins encoded by this family share approximately 57–65% amino acid identities with each other (Fig. 1B, C) [49,50] and are characterised by an NDR protein domain, consisting of an esterase-/lipase-/thioesterase-active-site serine and an α/β hydrolase fold of approximately 220 amino acids [50].

Although NDRG proteins do not possess a catalytic triad consisting of nucleophile–acid–histidine residues that is required for function as a hydrolase [48,52], the proteins belong to the α/β hydrolase superfamily [48]. The various *NDRG* family member genes may have arisen from the convergent evolution for this specific molecular architecture, but with distinct conformations and functions [53]. In fact, analyses indicate that the *NDRG* genes arose from multiple duplication events during evolution [51].

NDRG1 is mapped to the long arm of chromosome 8 [54], specifically to the locus 8q24.2 [55] or, in agreement with the AceView database [56], 8q24.3 (Fig. 2A) [41]. Spanning 60,085-bp, *NDRG1* contains 16 exons and 15 introns, and it encodes a 2997-bp mRNA, 1182 bp of which are coding (Fig. 2B) [24,55]. *NDRG1* mRNA is translated into a deduced 42,835-Da, 394-amino acid protein with a predicted pI of 5.7 [24,40,42,49]. The chromosomal region in which *NDRG1* is located frequently undergoes genetic alteration in cancers, such as those of the prostate [57], and can be amplified in other tumours [44]. The proto-oncogene, *c-myc,* is also located in close proximity to *NDRG1*, at the locus 8q24.21 [58] and is also frequently amplified in tumours [59,60]. Interestingly, c-myc, like N-myc, can repress *NDRG1* transcription and is implicated in causing a more metastatic phenotype [44,61].

Human NDRG1 differs from other NDRG proteins by the inclusion of not one but three decapeptide tandem repeats [49], each of which consists of the residues GTRSRSHTSE (Fig. 2C) [40]. In addition, other NDRG proteins also lack the *C*-terminal two residues, Ser and Glu [62]. The *C*-terminal domain of NDRG1 is unique amongst the NDRG proteins, as it can act as a substrate for phosphorylation by serum- and glucocorticoid-induced kinase (SGK)-1, which primes NDRG1 for phosphorylation by glycogen synthase kinase (GSK)-3β [63,64]. Specifically, SGK-1 has been confirmed to phosphorylate NDRG1 at Thr^{328}, Ser^{330}, Thr^{346}, Thr^{356} and Thr^{366} [65,66], while GSK-3β subsequently phosphorylates NDRG1 at Ser^{342}, Ser^{352} and Ser^{362} [63]. Thus far, enhanced phosphorylation *via* increased SGK-1 activity has been associated with severe Alzheimer's disease, but the underlying mechanisms have not been elucidated [67]. Moreover, phosphorylation of NDRG1 is a reversible process that has been suggested to influence the localisation of NDRG1 [68,69]. Further, phosphorylation of NDRG1 is putatively associated with the multiple physiological roles of NDRG1, including cell differentiation [70], centromere function during mitosis [71] and partitioning of daughter cells during telophase [71].

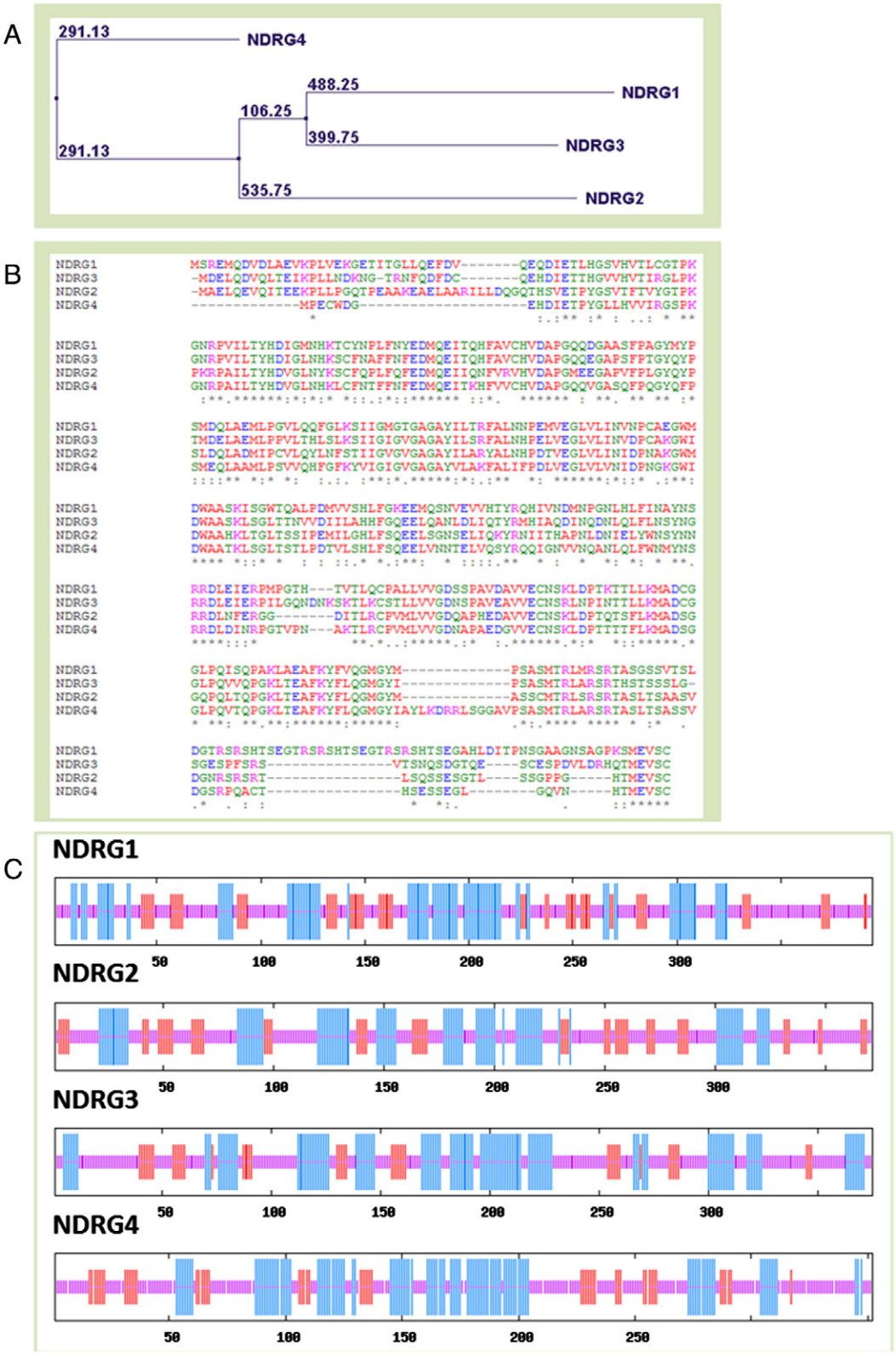

Fig. 1. *Predicted structural relationship amongst NDRG proteins.* (A) The phylogenetic tree of NDRG genes. The phylogenetic tree is visualised using TreeView [263]. Relative evolutionary distances by the sum of horizontal branch lengths are indicated. (B) The multiple sequence alignment of NDRG proteins. Protein sequences of NDRG family members were obtained from the UniProtKB/Swiss-Prot database [264,265] and aligned using the MUSCLE programme [266,267]. (C) The predicted protein secondary structure of NDRG family members. These were generated using the MLRC method [268]. In this figure, helices, extended strands and random coils are shown in blue, red and pink, respectively. Adapted from [53].

In the context of cancer, phosphorylation of NDRG1 by SGK-1 at Ser^{330} and Thr^{346} has been shown to be crucial for the suppression of the nuclear factor (NF)-κB signalling pathway and expression of CXC cytokines [72]. The CXC cytokines are known to mediate tumourigenesis and processes critical to metastasis, such as proliferation, adhesion and resistance to chemotherapeutic agents [73,74]. Hence, the phosphorylation of NDRG1 may play a critical role in its anti-metastatic activity.

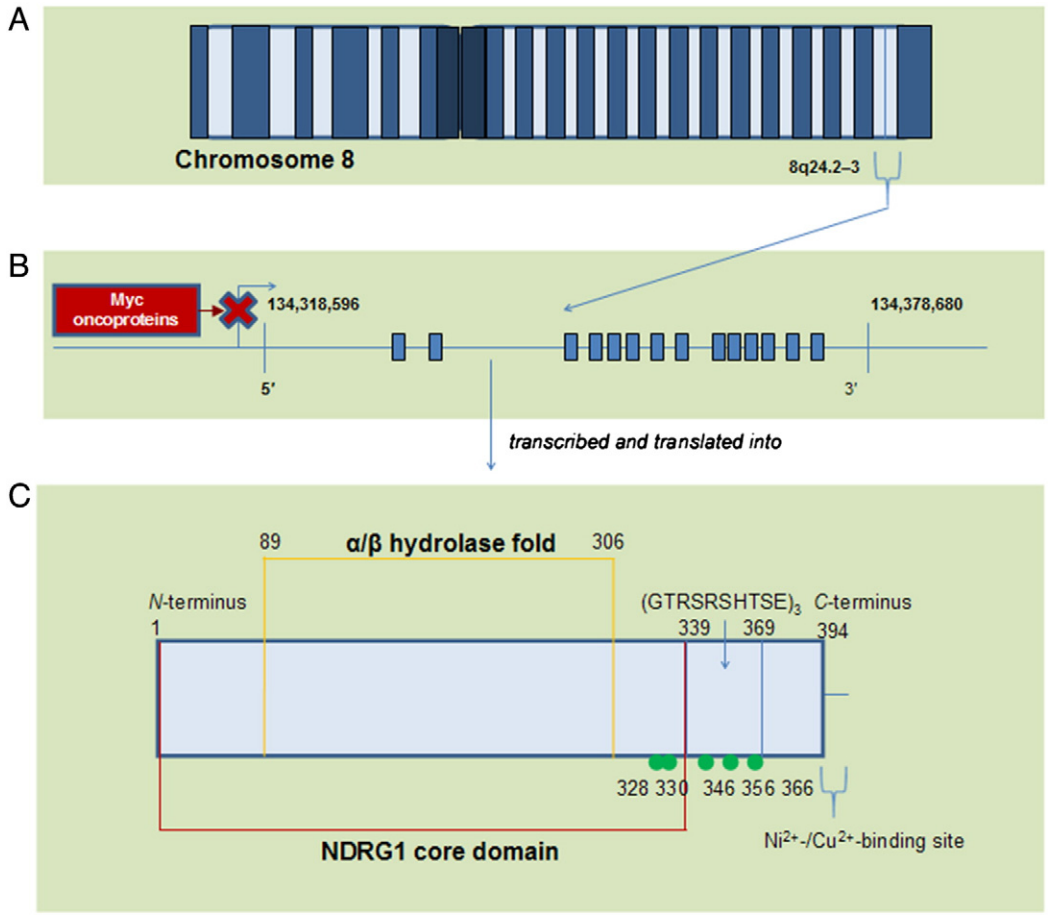

Fig. 2. *Structure of NDRG1 gene and NDRG1 protein.* (A) Chromosomal location of the *NDRG1* gene. (B) Organisation of *NDRG1* gene. *NDRG1* contains 16 exons. Myc oncoproteins, such as N-myc and c-myc, can repress *NDRG1* at the transcription level, albeit not *via* direct binding with the proximal promoter of *NDRG1*. The exons are shown as blue boxes. (C) Structure of the NDRG1 protein. Sites of post-translational modification and attachment are indicated. PKA, protein kinase A phosphorylation site; PKC, protein kinase C phosphorylation site; CK-II, casein kinase II site; CAMK, calmodulin kinase II site; P, SGK1 phosphorylation site.

The decapeptide tandem repeats contained in the *C*-terminus domain of NDRG1 were originally speculated to be significant for the binding of divalent metals (*e.g.*, Ni^{2+} and Cu^{2+}) to the *C*-terminus domain and the formation of coordination complexes [75]. Indeed, subsequent studies showed that the *C*-terminal domain effectively binds Ni^{2+} [76,77] and Cu^{2+} [78]. Currently, the effect of this activity is unclear, but it is suggested that metal-binding is important in regulating NDRG1 expression and its potential function in detoxification processes [30,79,80]. However, unlike Ni^{2+}, it has been reported that Cu^{2+} does not markedly induce *NDRG1* expression [42].

Structure prediction programs [81] do not suggest the presence of any trans-membrane domains [82] or amphipathic in-plane membrane anchors in NDRG1. While NDRG1 does not appear to possess a trans-membrane domain, signal sequence, or endoplasmic reticulum retention sequence [40], it possesses at least seven phosphorylation sites [83,84], with five calmodulin kinase II phosphorylation sites, a casein kinase II site, five myristoylation sites, a phosphopantotheine attachment site and a tyrosine phosphorylation site [85]. The significance of these particular phosphorylation sites on the activity of NDRG1 has been suggested to affect its function in mast cells, which will be further discussed in Section 4.2.5 below.

In multiple cancers [24,25,41,55], the oncoprotein N-myc represses *NDRG1* expression at the transcriptional level [61] *via* a mechanism independent of N-myc directly binding to the *NDRG1* proximal promoter [45]. In fact, the 5′ end of *NDRG1* contains a CpG island, to which myc oncoproteins preferentially bind [86]. The binding of myc oncoproteins to CpG islands suggests a potential role for methylation in the repression of *NDRG1* expression [25,87] and for hyper-methylation in the silencing of *NDRG1* expression [58]. In fact, *NDRG1* was found to be hyper-methylated in breast [88], gastric [89], colon [90] and pancreatic [58] cancer cells, leading to suppressed NDRG levels. Importantly, promoter methylation of *NDRG1* correlated with metastasis, lymph node invasion and poor histological grade in breast cancer patients [88].

Certain evolutionary characteristics are conserved across, or shared amongst, the NDRG family and, by way of analogy, may be of utility in speculating upon the molecular structure of NDRG1 and its function. Although the crystal structure of NDRG1 remains to be elucidated, the crystal structure of another member of the NDRG family, NDRG2, a putative tumour-suppressive protein, has recently been solved [62]. Notably, the predicted protein secondary and tertiary structures of NDRG1 and NDRG2 demonstrate their similarity (Fig. 3). NDRG2 is composed of an α/β hydrolase fold domain and a cap-like domain [62]. The α/β hydrolase fold domain consists of a β-hairpin structure (β1–β2) and a six-stranded, parallel sheet (β3–β8) surrounded by an outer solvent-exposed layer composed of eight α-helices (α1–α5 and α11–α13) and two 3_{10} helices, whereas the cap-like domain consists of five α-helices (α6–α10) [62]. Consistent with a previous study [48], structural similarity search analysis of NDRG2 suggests that it belongs to the α/β hydrolase superfamily due to significant similarity between NDRG2 and multiple α/β hydrolases [62]. However, compared to other α/β hydrolases, in NDRG2 Ser^{96} is substituted with Gly^{132}; His^{247} with Gly^{279}; and Asp^{219} with Ala^{251} [62]. The sequence, Ser^{96}–His^{247}–Asp^{251} normally constitutes the catalytic triad conserved amongst α/β hydrolases, but this is absent in NDRG proteins and no apparent catalytic activity is consequently observed [62].

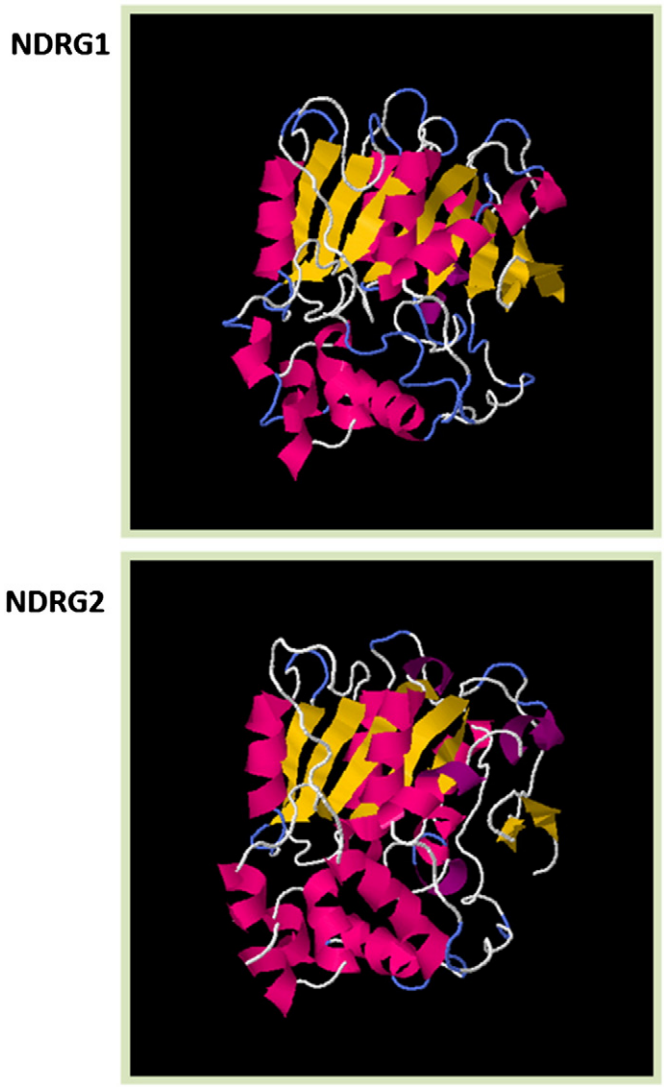

Fig. 3. *Predicted protein tertiary structures of NDRG1 and NDRG2*. Protein sequences of NDRG1 and NDRG2 were obtained from the UniProtKB/Swiss-Prot database [264,265] and modelled using Geno3D [269]. The models were visualised using Jmol [270,271]. In this figure, α-helices, 3_{10} helices, β-strands, β-turns and other secondary structures are shown in light pink, dark pink, blue and white, respectively.

Other critical structural differences between NDRG proteins and non-NDRG α/β hydrolases include the presence of His^{186} and the different residue distribution of helix α6 in NDRG proteins [62]. His^{186} prevents the formation of a substrate-binding site *via* extension of helix α7 into the region where other α/β hydrolases possess Lys^{187} and a substrate-binding site [62]. A different residue-distribution within helix α6, in which hydrophilic residues are inward-facing and hydrophobic residues are outward-facing and exposed to solvents, is responsible for the absence of a hydrophobic substrate-binding site [62]. Thus, instead of possessing enzymatic activity, NDRG2 may engage in binding interactions, as observed with other non-enzymatic α/β hydrolases [91]. In fact, as helix α6 extends from the main body of NDRG proteins, the region may more easily interact with potential binding targets [62]. Indeed, the point mutation L172D in NDRG2 has been shown to result in a reduction in the ability of helix α6 to interact with β-catenin and abolition of T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) signalling [62]. This point mutation disrupts the molecular architecture of helix α6 at its middle and where it is fully exposed to solvents [62]. Following this, it is clear that subtle differences in the molecular architecture of NDRG proteins may elicit significant differences in their biological activities.

Table 1
Summary of NDRG1 band number after immunoblotting studies using anti-NDRG1 antibody.

Cell Line	Type	No. of bands	Approx. masses (kDa)	Reference(s)
SK-N-MC	Brain cancer	2 or 3	43, 44 or 43, 44, 45	[97]
MCF7	Breast cancer	1	1	
		2	43, 44	[152]
MDA		2		[272]
SK-BR-3		2		[272]
HeLa	Cervical cancer	2		[63]
HT29	Colon cancer	2	43, 44	[94]
U973	Leukaemia	1	43	[145]
DMS-53	Lung cancer	2	43, 44	[97]
H1299		2	43, 43	[183]
MIAPaCa-2	Pancreatic cancer	2	43, 44	[96]
PANC-1		2	43, 44	[96]
CAPAN-2		2	43, 44	[96]
CFPAC-1		2	43, 44	[96]
PrEC	Prostate epithelium	1	43	[95]
		1	46	[93]
DU145	Prostate cancer	3	43, 43, 46	[183]
		2	43, 44	[94]
		2	43, 44	[95]
		2	41, 46	[93]
LNCaP		2	41, 46	[93]
PC-3MM		2	43, 43	[183]
		2	43, 44	[97]
		2	41, 46	[93]

2.1. Isoforms of NDRG1 and the potential role of trypsins in NDRG1 cleavage

As with other proteins, the structure of NDRG1 is critical to its function, and structural alterations may result in significant changes in functionality. In support of this hypothesis, isoforms of NDRG1 have been identified in diseases, such as hereditary motor and sensory neuropathy type-Lom (HMSNL) [92] and cancer [93–97]. In addition, in *v-ets avian erythroblastosis virus E26 oncogene homologue-1* (*ERG*)-overexpressing prostate cancers, *NDRG1* has been shown to be fused to the coding region of *ERG-1*, which is suggested to disrupt *NDRG1* and its protein product and may result in an increased risk of metastasis [98].

Recent immunoblotting studies using specific anti-NDRG1 antibodies have revealed the presence of additional NDRG1-immunoreactive bands in multiple cancer cell lines and other biological samples, under different conditions (Table 1). These additional bands indicate the presence of isoforms of NDRG1 that may alter the function of this protein [93]. Multiple immunoreactive forms of NDRG1 have also been detected during rat brain and kidney postnatal development [99]. In addition to a prominent band at approximately 43-kDa in brain and kidney tissue, additional bands were detected at approximately 129-kDa and 172-kDa in brain tissue and at approximately 215-kDa in kidney tissue, particularly prior to postnatal day 14 [99]. These higher molecular weight species may correspond to a trimer, tetramer or pentamer composed of NDRG1, respectively, and it was suggested that NDRG1 forms polymers to function during postnatal development *in vivo* [99].

In subsequent studies, multiple immunoreactive bands have been detected with anti-NDRG1 antibodies in numerous cell lines (Table 1). It has been suggested that these bands correspond to different phosphorylation states of NDRG1 or other post-translational modifications of this protein [63]. Notably, NDRG1 has been shown to be a multi-phosphorylated protein [63,83,84], and its phosphorylation has been reported to be associated with its tumour-suppressive activity as described in Section 2 above [71,72]. In addition to the tabulated observations (Table 1), it is of interest that the bands of greater molecular mass, such as the 44-kDa and 45-kDa bands in SK-N-MC cells [97] and the

44–46-kDa band in DU145 cells [93,95], were more intense after incubation with agents that induce NDRG1 expression. As these bands may correspond to different phosphorylation states of NDRG1, it has been suggested that these phosphorylation states could be involved in modulating NDRG1 activity [72,96].

Further studies examining the multiple NDRG1 bands in immunoblots indicated that the higher M_r band was not a phosphorylated or glycosylated isoform of NDRG1 [93]. In fact, assessing DU145, LNCaP and PC-3MM prostate cancer cells, a *C*-terminal antibody against NDRG1 yielded two bands, while the *N*-terminal antibody yielded only one NDRG1 band [93]. In these three prostate cancer cell lines, the band of greater mass, migrating at approximately 46-kDa, was shown to be full-length, unmodified NDRG1, which suggests that the lower 41-kDa band may be a truncated isoform of NDRG1 that lacks the *N*-terminal region [93]. In addition, using primary cultures of normal prostate epithelial cells (PrECs), only one NDRG1 band migrating at approximately 46-kDa was identified, and this corresponded to the higher M_r band in the three prostate cancer cell lines [93]. Hence, this further suggests that NDRG1 is truncated in prostate cancer cells when compared to normal prostate cells where this protein is intact. It was speculated that truncated isoforms of NDRG1 may be implicated in the altered function of NDRG1 in metastasis suppression and carcinogenesis [93].

2.1.1. Cancer-associated trypsin-like proteases

Protein sequence analysis using PSORT II [100] has predicted a potential cleavage site within NDRG1 between Cys^{49} and Gly^{50} [93]. Further, a cleavage event at this site was predicted to yield a 5-kDa *N*-terminal by-product [93], which is consistent with the difference in mass between the two immunoreactive bands detected using an antibody raised to the NDRG1 *C*-terminal. At this juncture, the proteins that cleave NDRG1 at this predicted cleavage site remain unknown. However, protein sequence analysis using the ExPASy PeptideCutter tool [101,102] has predicted that NDRG1 can be cleaved by trypsins, such as chymotrypsin, mesotrypsin, pseudotrypsin and trypsin [93]. As a link between trypsin expression and tumours has been established [103–109], it may be speculated that trypsins cleave NDRG1 in cancers, thereby potentially attenuating or altering its functionality and metastasis-suppressing activities [110–112]. The trypsin-like proteases, including mesotrypsin and tumour-associated trypsinogen (TAT), that may be involved in NDRG1 cleavage are briefly discussed below.

It is well known that mesotrypsin (also known as protease, serine, 3; PRSS3) promotes tumour growth and metastasis of human pancreatic, breast and prostate cancers [109,113,115]. Moreover, PRSS3 expression in primary prostate cancer is prognostic of systemic progression after prostatectomy [109]. Importantly, a novel and potent inhibitor of mesotrypsin, BPTI-K15R/R17G, was demonstrated to inhibit prostate cancer cell invasion to a similar extent as PRSS3 gene silencing [109]. In fact, the high levels of mesotrypsin in tumours can be attributed to the pathological trypsinogen activator, cathepsin B, which is often highly expressed in tumours [114] and activates mesotrypsinogen [109,113,115,116]. The carcinogenic effects of mesotrypsin are mediated *via* its substrates, which include protease activated receptor (PAR)-1 in pancreatic cancer and CD109 in breast cancer cells [113,115].

Another trypsin-like protease that is often highly expressed in cancer cells is tumour-associated trypsinogen-2 (TAT-2) [117]. TAT-2 is the predominant isoform shown to be expressed in ovarian neoplasms [118], pancreatic cancer [119], colon carcinoma, fibrosarcoma, leukaemia [120], colorectal carcinoma [121] and lung neoplasms [122]. The level of TAT-2 has been shown to correlate with the malignant phenotype and cell invasive potential of tumours [123]. Hence, the effect of proteases such as mesotrypsin and TAT-2 on NDRG1 may affect NDRG1 function and is an intriguing possibility that requires further investigation.

3. Tissue and cellular distribution of NDRG1

NDRG1 mRNA is ubiquitously expressed in human tissues [24,40,124–126]. However, higher mRNA levels are detectable in the brain, kidney, placenta, prostate and small intestine than in other tissues [24,40,124]. Interestingly, *NDRG2* mRNA is highly expressed in brain, salivary gland, skeletal muscle and spinal cord; *NDRG3* mRNA is highly expressed in testis and *NDRG4* mRNA is highly expressed in heart [49,50]. Although structurally similar, the distinct mRNA expression patterns of *NDRG* genes suggest that corresponding proteins possess different but related functions in tissues or organs [50].

Further to this, three NDRG4 isoforms, NDRG4-B, NDRG4-B^{var} and NDRG4-H, all of which are encoded by the *NDRG4* gene, have been identified [49]. NDRG4-B and NDRG4-B^{var} are produced by alternative splicing of the *NDRG4* gene [49]. These NDRG4 isoforms exhibit distinct mRNA and protein expression patterns, possibly due to alternative promoter usage [49]. For instance, *NDRG4-B* mRNA is only detectable in brain and *NDRG4-B^{var}* mRNA level is higher in heart than in brain [49].

In contrast to its ubiquitous mRNA expression, human NDRG1 protein (UniProt entry: Q92597) is predominantly expressed in epithelial cells [41], although it is absent in specific tissues, such as blood vessels, connective tissue, muscle and a majority of the nervous system, suggesting roles for NDRG1 other than as a housekeeping protein [124]. Indeed, the precise subcellular localisation of NDRG1 varies amongst cell types [124]. For instance, NDRG1 is predominantly localised in the nucleus of prostate epithelial cells (PrECs), the plasma membrane of intestinal and lactating breast epithelial cells and the mitochondrial inner membrane of renal proximal tubule cells [124,127]. In mast cells, NDRG1 exhibits a punctate distribution in the cytoplasm, particularly around secretory granules [127,128]. However, NDRG1 also translocates from the cytoplasm to the nucleus after IgE/Ag activation of mast cells, suggesting possible nuclear regulatory functions in activated cells [128]. Also in mast cells, NDRG1 has been shown to interact with the 70-kDa heat shock cognate protein (Hsc70), a chaperone that regulates the translocation of NDRG1 between the cytoplasm and nucleus and is implicated in ATP-dependent protein folding, vesicular transport and exocytosis [129–131].

The program PSORT II [100] predicts that NDRG1 is localised in the cytoplasm, nucleus and mitochondrion, at probabilities of 47.8%, 26.1% and 8.7%, respectively. Further, PSORT II predicts that NDRG1 is localised in the cytoskeleton, peroxisome, plasma membrane and vacuoles, at probabilities of 4.3% each. Interestingly, during embryonic development, *NDRG1* mRNA is detectable in brain [45,132] and during postnatal development in rat, NDRG1 is detectable in brain and kidney [99]. Despite NDRG1 being observed to localise in the nucleus of certain cell types [124], no nuclear sequence is predicted by WoLF PSORT [133] or is identifiable from its amino acid sequence [124]. The nuclear localisation of NDRG1 is speculated to involve phosphorylation or interaction with a nuclear protein, such as Hsc70, as described above [124]. Indeed, in response to DNA damage following low concentrations of actinomycin D, NDRG1 translocates from the cytoplasm to the nucleus, where it may inhibit cell growth and promote DNA repair mechanisms [41]. Hence, it is suggested that NDRG1 acts as a stress response gene [41] or potentially as a transcription factor [134].

Consistent with the translocation of NDRG1 between the cytoplasm, cell membrane and nucleus, NDRG1 has been detected in these subcellular compartments and perinuclear regions [135]. Moreover, NDRG1 has been observed to associate with cell membranes, adherens junctions, desmosomes and intermediate and microfilament bundles that insert into adherens junctions, strongly suggesting a role for NDRG1 in cell adhesion [124]. Indeed, the maintenance of membrane-bound E-cadherin and β-catenin by over-expression of *NDRG1* supports the hypothesis that NDRG1 is involved in increasing cell adhesion *via* stabilisation of the adherens junction, which is composed of these two membrane proteins [25,94].

Table 2
Induction of NDRG1 and its putative functions.

Induced by	Function(s)	Reference(s)
Terminal differentiation	Loss of cell proliferative potential	[25]
Cell differentiation and maturation	Promotion of immature cells to become mature cells	[128,170]
Cell stress	Cell growth arrest	[24,40,42]
DNA damage and p53	Cell growth arrest; euploidy maintenance; possible cell cycle regulation; p53-dependent apoptosis; down-regulation of angiogenesis	[41,79,148–150]
High cell density and nutritional starvation	Hypoxia-like response	[162]
Hypoxia, HIF-1α, hypoxia mimetics and iron chelators	Hypoxia response	[43,94,124,140,141,143,151,152,158,273–275]
Androgens	Cell growth arrest; cell differentiation	[125]
AP-1	Hypoxia response	[158]
Intracellular Ca^{2+}	HIF-1α-independent hypoxia response	[42,139,158,276,277]
Co^{2+} and Ni^{2+}	Possible hypoxia-like response	[42,140,141,273,278]
DNA methylation inhibitors and histone deacetylation inhibitors	Housekeeping function(s); possible cell differentiation	[25]
E2a-Pbx1	Possible apoptotic response	[149]
Free-radical nitric oxide ($NO^{\bullet}$)	Unknown	[159]
Lipophilic agents	Loss of cell proliferative potential	[70,145]
Phorbol myristate acetate	Unknown	[145]
PTEN	Down-regulation of angiogenesis; down-regulation of metastasis (*e.g.*, *MMP-1*, *-2*, *-13*)	[23,43,147]
Reducing agents	Possible ischaemic disease prevention	[40,42]

Through an understanding of the expression and distribution of NDRG1, it is apparent that NDRG1 may be involved in DNA repair and cell adhesion, both of which are important to tumourigenesis and metastasis. Therefore, isoforms of NDRG1 may thus have significant consequences on these processes crucial to cancer development and progression.

4. Biological functions and regulation of NDRG1

The precise role of NDRG1 remains unclear, but it appears that this protein is multi-functional (Tables 2 and 3). Putative functions include roles in cell growth, differentiation, development and stress responses. Recent proteomic studies have suggested a link between *NDRG1* and genes associated with immune responses [136] and with carcinogenesis [137]. Further, the expression of *NDRG1* is induced, repressed and regulated by a diverse range of effectors (Fig. 4) [138].

4.1. Regulation of NDRG1

In addition to the diverse mechanisms by which *NDRG1* is regulated, as outlined above in Section 2, DNA methylation inhibitors (*e.g.*, 5′-aza-2′-deoxycytidine) and histone deacetylase inhibitors (*e.g.*, trichostatin A), promote *NDRG1* expression, indicating a role for DNA methylation and histone acetylation in regulating its levels [25]. Multiple other agents, including Ca^{2+} [139] and heavy metal ions (*e.g.*, Co^{2+} [140], Ni^{2+} [42,140]), androgens [68,125], DNA-damaging agents (*e.g.*, actinomycin D), forskolin [65], lysophosphatidylcholine [30], okadaic acid [42,141], sulfhydryl reducing agents and tunicamycin [40], ligands of the peroxisome proliferator-activated receptor (PPAR)-γ and retinoid X receptor (RXR) [25], iron chelators [96,111,142,143] and vitamins A and D_3 [70,144,145] also promote *NDRG1* expression [49,146]. Further, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [23,43,147], p53 [41,79,148–150] and the von Hippel–Lindau protein (pVHL) [32] have been reported to promote *NDRG1* expression.

Table 3
Down-regulation of NDRG1 expression and its putative functions.

Downregulated by	Function(s)	Reference(s)
Myc oncoproteins	Cell differentiation	[45]
Reoxygenation	Cell migration	[231]
Camptothecin analogues	Apoptosis	[200]
Digoxin	Unknown	[279]
Oestradiol	Unknown	[280]
von Hippel–Lindau protein (pVHL)	Hypoxia response	[32]

Three hypoxia-inducible factor (HIF)-1-binding sites have been identified in the non-coding sequence of *NDRG1*, of which one is located in the promoter region and two are located in the 3′ untranslated region [151]. Moreover, putative HIF-response elements identified upstream of the *NDRG1* promoter, at −1370-bp and −7503-bp, are involved in its regulation [143]. Indeed, studies have shown that hypoxia promotes *NDRG1* expression *via* HIF-1α-dependent and –independent mechanisms [140], the latter of which may involve eukaryotic initiation factor 3a (eIF3a) and its ability to regulate the balance between translation and translational suppression *via* the formation of stress granules [152], Sir2-like protein 1 (SIRT1), p53-dependent mechanisms [153] and by the transcription factor c-Jun/AP-1/JUN in response to the iron chelator, L-mimosine [154].

Incubation with iron chelators, which mimic hypoxia, results in induction of *NDRG1*, decreased proliferation and the induction of G_1/S arrest and apoptosis in normal and neoplastic cells [143,155–157]. Further, hypoxic responses *via* HIF-1α-independent mechanisms involve intracellular Ca^{2+}, which itself can induce *NDRG1* [158]. Cellular stress due to iron depletion has also been shown to positively regulate NDRG1 at the message and protein levels *via* an eIF3a-dependent mechanism [152]. This activity of eIF3a could be due to its recruitment to stress granules under conditions of hypoxia and iron depletion and/or its ability to differentially regulate mRNA translation during cellular stress [152].

In a recent study, the free radical messenger molecule nitric oxide (•NO) was shown to up-regulate NDRG1 [159]. Interestingly, •NO has a very strong affinity for iron [160], and it is known to complex intracellular free iron and also prevent degradation of the HIF-1α protein [161]. This study further demonstrated that concomitant up-regulation of NDRG1 and HIF-1α by •NO was required for •NO-mediated migration suppression [159].

The presence of functional binding sites for early growth response protein (EGR)-1 and specificity protein (Sp)-1 that overlap the *NDRG1* promoter indicates their potential roles in regulating *NDRG1* expression [80]. In fact, this was confirmed by the concomitant transcriptional activation of the *NDRG1* promoter after specific binding of EGR1 to the binding sites for EGR1 and Sp-1, and this interaction can mediate hypoxia-induced transcription of *NDRG1* [80]. Other transcription factors, such as activator protein (AP)-1, [158] and v-ets avian erythroblastosis virus E26 oncogene homologue 2 (ETS) [162], are also known to regulate *NDRG1* expression.

The effect of protein kinases on NDRG1 expression has also been evaluated, but only to a limited extent. For instance, incubation of cells with a protein kinase C (PKC) inhibitor led to an increase in NDRG1

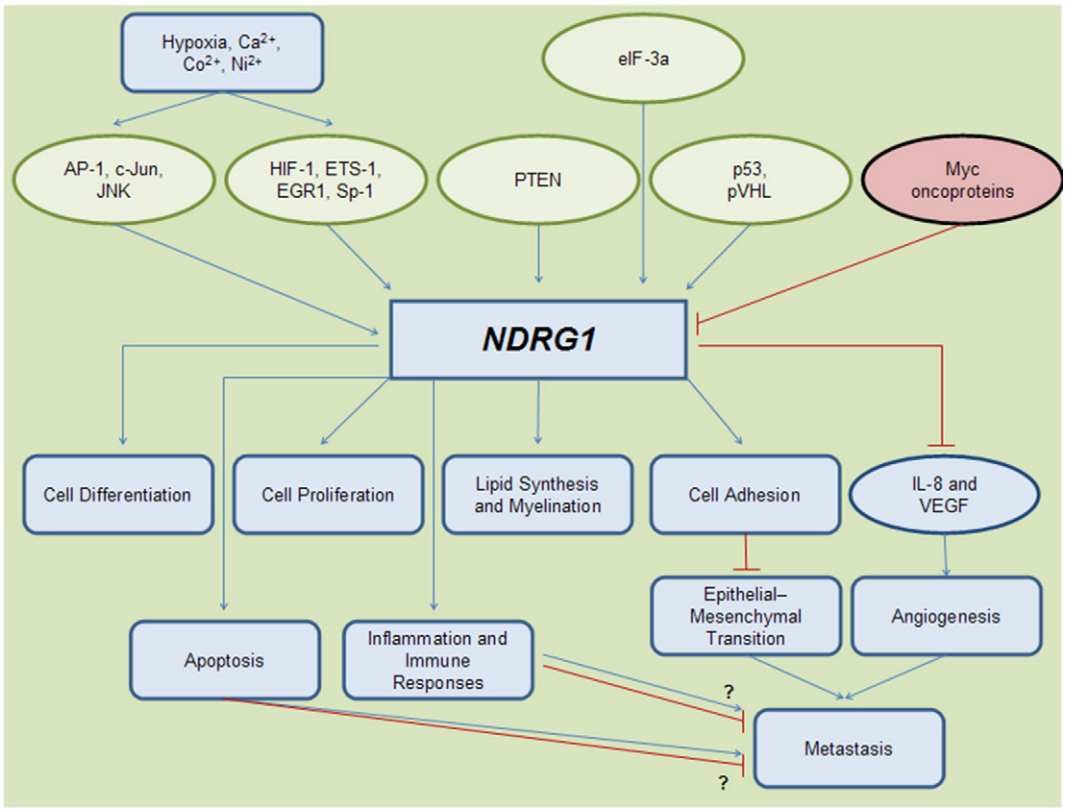

Fig. 4. *Biological functions and regulation of NDRG1*. Transcription factors, such as AP-1/c-Jun, JNK, HIF-1, ETS-1, Egr-1 and Sp-1, and other proteins, such as PTEN, p53 and pVHL, can up-regulate *NDRG1* expression, whereas myc oncoproteins, such as N-myc and c-myc, can down-regulate *NDRG1*. In certain cancers, the activity of these regulators may be altered. *NDRG1* can promote apoptosis, cell differentiation, cell proliferation, inflammation and other immune responses, lipid biosynthesis, myelination and cell adhesion, while inhibiting interleukin (IL)-8 and vascular endothelial growth factor (VEGF) signalling. Through the interplay of its multiple functions, NDRG1 inhibits metastasis in certain cancers.

levels, whereas this was not observed after treatment with either a protein kinase A (PKA) inhibitor or protein tyrosine kinase inhibitor [69]. Therefore, it has been suggested that activation of the PKC pathway is also involved in NDRG1 expression.

4.2. Biological functions of NDRG1

4.2.1. Embryogenesis and development in early life

NDRG1 expression is correlated with multiple stages of differentiation, including placentation [69], trophoblast differentiation [153] and organogenesis and organ morphogenesis [163]. After treatment with forskolin, which elevates cAMP levels and activates PKA, NDRG1 and β-human chorionic gonadotropin, a trophoblastic differentiation marker, were induced in the BeWo human choriocarcinoma cell line [69]. Indeed, NDRG1 is predominantly expressed in syncytiotrophoblasts and in the intermediate trophoblasts of the basal plate during the second- and third-trimester placenta [69]. Further, as NDRG1 is elevated in trophoblasts cultured under hypoxic conditions and in placental villi of pregnancy with growth-restricted foetuses, NDRG1 may be involved in the response to hypoxic injury or other stresses in trophoblasts [164], as in intrauterine growth restriction or preeclampsia [165].

Furthermore, NDRG1 is implicated during the development of the urinary and reproductive organs with its expression being tightly regulated during this process [163]. Whereas silencing of *NDRG1* is associated with failure of pronephric morphogenesis, over-expression is associated with disrupted formation of pronephric ducts, reduced size of pronephric tubules and somite disorganisation in the African clawed frog (*Xenopus laevis*) [163]. In addition, NDRG1 has been shown to promote specification of oesophagus, pancreas, stomach and duodenum progenitor cells *via* the repression of Wnt/B-catenin signalling in *Xenopus* spp. embryos [166].

Immunohistochemistry (IHC) studies demonstrated that NDRG1 was highly expressed in the rat renal proximal convoluted tubules during the first two weeks of postnatal life, reflecting the morphological and functional differentiation of the proximal convoluted tubules [99]. IHC studies also revealed immunoreactivity in the pyramidal neurons of the hippocampus, with a peak in immunoreactivity after 8 to 10 days of postnatal life, reflecting the metabolic changes in the developing brain [99]. Moreover, western blot studies revealed an NDRG1-immunoreactive band at approximately 215-kDa in rat early postnatal life brain and kidney samples [99]. This protein was shown to disappear, while other smaller bands were shown to appear and become more pronounced from the second week of postnatal life [99]. Considering this, it was suggested that during postnatal development, NDRG1 may be processed leading to alterations in its molecular mass [99]. While the aforementioned investigations underscore a developmental role for NDRG1, there is an apparent lack of studies on its role in human organogenesis and organ morphogenesis.

4.2.2. Cell growth and differentiation

NDRG1 is suggested to be involved in cell growth and differentiation. In fact, *NDRG1* expression is shown to be biphasic, peaking during the G_1 and G_2/M phases and being lowest during the S phase [41]. In the kidney, over-expression of the polycystic kidney disease gene 2 (PKD2) induces NDRG1 *via* the EGR1 transcription factor [167]. Under the influence of this gene, NDRG1 appears to play a pro-proliferative role where it can be induced by PKD2 *via* its effects on the transcription factor, EGR1 [167]. Further, NDRG1 can associate with the microtubule and localise in centrosomes and spindle esters and it is required to maintain spindle structure in a p53-dependent manner during cell division [168]. NDRG1 knockdown is associated with a reduction in α-tubulin acetylation, disappearance of α-tubulin, changes in microtubule structure and a failure to form dividing spindle fibres [168]. These latter

studies indicate a role for NDRG1 in ensuring fidelity of cell division and suppression of genomic instability. Conversely, reduced NDRG1 expression may result in genomic instability and tumourigenesis [168].

NDRG1 is induced during cellular differentiation [24,55], a role that is corroborated by multiple studies [25,145,169,170]. For instance, *NDRG1* has been identified as a gene that is up-regulated when myelomonocytic cells cease to proliferate, suggesting a role for NDRG1 in promoting terminal differentiation [145]. Consistent with this, *NDRG1* over-expression is associated with up-regulation of epithelial cell differentiation markers in metastatic colon cancer [25] and epidermal keratinocyte cell lines [134]. Furthermore, ligands of nuclear transcription factors involved in cellular differentiation, such as PPAR-γ [25], vitamin D [70,144,145] as well as differentiation-inducing isobutylmethyl xanthine [44], have all been shown to induce *NDRG1* expression. NDRG1 has also been to shown to down-regulate a number of genes involved in suppression of cellular differentiation (*e.g.*, Hod, S100a10, connective tissue growth factor [Ctgf], ectonucleotide pyrophosphatase/phosphodiesterase family member 3 [Enpp3] and secreted phosphoprotein 1 [Spp1]) [142]. Down-regulation of these genes by NDRG1 resulted in increased cellular differentiation, indicating the important role of NDRG1 in this process [142].

4.2.3. Lipid biosynthesis and myelination

NDRG1 is implicated in lipid synthesis and myelination, with its expression being integral to axonal survival [171]. Although its expression is not detectable in sensory or motor neurons or their axons, NDRG1 is highly expressed in peripheral nerves [92], Schwann cells [92] and oligodendrocytes [172]. Consistent with the presence of a putative phosphopantetheine attachment site in its structure, this expression pattern suggests a potential role for NDRG1 in lipid biosynthesis, myelination and axonal survival [171]. In fact, truncated isoforms of NDRG1 are associated with demyelinating diseases. HMSNL, which is also known as Charcot–Marie–Tooth disease, type 4D (CMT4D) [173], is an early-onset, autosomal recessive form of Charcot–Marie–Tooth disease, characterised by demyelination of peripheral nerves [54]. HMSNL progresses to severe morbidity and disability in adulthood and its signs and symptoms include muscle weakness, gait abnormality, myopia, bowel dysfunction, deafness and scoliosis [174–177]. A truncating nonsense mutation in *NDRG1*, namely R148X, has been identified as causative or characteristic of the disease phenotype and suggests a critical role for NDRG1 in myelination and axonal survival [54,171]. Interestingly, mutated *NDRG1* is also associated with polyneuropathies in Alaskan Malamutes [178] and Greyhound dogs [179].

Additional support for the role for NDRG1 in myelination came from studies using *NDRG1*$^{-/-}$ mice. In fact, these animals retained complicated motor skills, although they exhibited muscle weakness, particularly in distal limbs and progressive demyelination of nerves, such as the sciatic nerve [180]. Consistent with previous studies, NDRG1 was shown to be highly expressed in the cytoplasm of Schwann cells, which further suggests a role for NDRG1 in both the physiological function of Schwann cells, as well as the production and maintenance of the myelin sheath [180]. Recently, another mechanism leading to Schwann cell dysfunction has been suggested to occur after NDRG1 down-regulation [181]. In these studies, *NDRG1* silencing led to decreased uptake of low-density lipoprotein (LDL) due to reduced LDL receptor expression [181]. In addition, there was down-regulation of oligodendrocyte differentiation factor, *Olig2* [181]. Both LDL and *Olig2* are implicated in multivesicular body formation, endosomal LDL receptor trafficking, lipid processing and differentiation of Schwann cells [181]. Together, these studies furnish evidence in favour of a role for NDRG1 in myelination.

In addition to its putative role in myelination and axonal survival, NDRG1 has been associated with other lipid biosynthetic pathways. NDRG1 interacts with apolipoprotein A-I and -II, two lipoprotein constituents of high-density lipoprotein cholesterol (HDL-C) [135,181]. As HDL-C is an established preventative factor for atherosclerosis [182], NDRG1 may play a role in atherogenesis [53,135,137,146,183]. In fact, HMSNL sufferers, in whom *NDRG1* contains the truncating R148X mutation, have been identified to possess decreased HDL-C levels and an increased total cholesterol/HDL-C ratio [135], which is associated with an increased risk for atherosclerosis and other cardiovascular diseases [184].

4.2.4. Stress response

Another role for NDRG1 has been identified in terms of the response to cellular stress. For instance, elevated homocysteine levels which can be cytotoxic are associated with *NDRG1* up-regulation [40]. Hence, NDRG1 is speculated to possess cyto-protective functions. Consistent with putative cytoprotective activities, an association between NDRG1 and responses to hypoxia have also been identified [140,151]. During hypoxia, NDRG1 is up-regulated [140] (see Section 4.1) and attenuates p53 expression, which is crucial in the induction of apoptosis in hypoxic trophoblasts [153]. Thus, from these studies, NDRG1 is suggested to possess a pro-survival function. In contrast, NDRG1 was found to sensitise cells to p53-mediated apoptosis and caspase activation in colon cancer cells [79]. Hence, the effect of NDRG1 on cell survival may be tissue- or cell-type specific or pleiotropic [53,112].

4.2.5. Immunity

Recently, studies have shown that NDRG is an important mediator of the immune system and is involved in allergy and anaphylaxis, defence against bacterial pathogens and bacterial clearance, inflammation and wound healing [51,136,138]. Given the centrality of the immune system, NDRG1 may be implicated in pathologies other than those in which its expression is dysregulated or otherwise abnormal [185]. In fact, chronic inflammation is now regarded as an enabling characteristic of cancer and contributes to the pathogenesis of this condition [186–191].

Expression of *NDRG1* mRNA is induced during the maturation of primitive mouse bone marrow-derived mast cells into their mature counterparts in connective tissue [170]. Interestingly, the expression of NDRG1 allows these cells to degranulate more rapidly and enhance exocytosis in response to extracellular stimuli [170]. Notably, *NDRG1*$^{-/-}$ mice, or mice transfected with mutant *NDRG1* where the three decapeptide tandem repeats are deleted, exhibit markedly reduced immune responses when compared to wild-type mice, indicating that the *C*-terminal domain is required for NDRG1 function in mast cells [170]. Further, *NDRG1*$^{-/-}$ mice exhibit decreased numbers of mast cells that possess impaired degranulation, resulting in an attenuated immune response to antigens [128]. Finally, relevant to its role in immune function, NDRG1 expression has been correlated with neutrophil differentiation [192]. Collectively, these studies provide evidence in support of the differentiation-associated and immunomodulatory activities of NDRG1.

In vitro, NDRG1 has been shown to undergo phosphorylation at multiple Ser and Thr residues by calmodulin kinase II, PKA and PKC

Table 4
Expression and putative functions of NDRG1 in reproductive cancers.

Tissue	Expression and function(s)
Breast	Low expression in cancer [22,23,281] Expression may be lower due to aberrant methylation [88] Expression higher in invasive breast cancer than in non-invasive cancer [140,282] Expression is associated with cell differentiation [272], reduced vascular invasion *in vitro* [22,23,283,284], SPARC down-regulation [285]
Cervical	Low expression in cancer [39] Expression higher in invasive cervical cancer than in carcinoma [38] Expression may be associated with angiogenesis [39]
Endometrial	High expression in cancer [214] Silencing enhances cell proliferation, colony formation, cell migration and invasion [232]
Prostate	Low expression in cancer [23,31] Expression appears to be associated with favourable clinical outcomes Expression is associated with reduced vascular invasion [31] and expression of E-cadherin [286]

Table 5
Expression and putative functions of NDRG1 in gastrointestinal cancers.

Tissue	Expression and function(s)
Colorectal	Low expression in cancer [24–28,55,287–289] Expression appears to be associated with favourable clinical outcomes [24–28,55,287–289] Expression may be associated with angiogenesis *via* JNK activation and an autocrine loop of IL-1 signalling [247]
Gallbladder	High expression in cancer [290] Expression higher in advanced tumours than in early tumours [290]
Liver	High expression in cancer [34,140,291] High expression is associated with vascular invasion, metastasis, late tumour stage, large tumour size and high histological grade [34,35] Silencing reduced cell proliferation, apoptosis and vascular invasion *in vitro* and inhibited tumour growth *in vivo* [33]
Oesophageal	Expression lower in advanced tumours (0–I vs. II–IV) and local tumour invasion (T1–2 vs. T3–4) [29]
Oral	High expression in cancer [36,292]
Pancreatic	Expression may be altered in cancer by epigenetic regulation [58] Expression appears to be associated with favourable clinical outcomes Expression is associated with cell differentiation [293] and reduced tumour growth *in vitro* and *in vivo* [228]; reduced production of IL-8 and VEGF-A, microvascular density and vascular invasion [30]; reduced production of CXC cytokines, decreased inhibitor of κB signalling and induced nuclear factor-κB signalling [245]
Stomach	Low expression in cancer [229] High expression in cancer [199,235] Expression higher in cytoplasm in local tumours and in nucleus in advanced, invasive tumours [199] Expression is associated with reduced stromal invasion, lymph node metastasis and pathological stage [229]; aberrant expression of p53, cell differentiation and tumour growth [199]; and angiogenesis [235]

[83]. Phosphorylation at these residues are associated with marked mast cell degranulation and exocytosis [83]. Deletion of the *C*-terminus domain results in increased phosphorylation by PKA and PKC, but not by calmodulin kinase II, suggesting that the *C*-terminus domain masks the PKA and PKC phosphorylation sites [83]. NDRG1 has also been reported to interact with other proteins involved in mediating immune responses and carcinogenesis, including Hsc70 [131] and hepatitis C virus protein NS5A [193]. Together, these studies suggest NDRG1 plays a potential immunomodulatory role that is cell- or tissue-specific.

5. NDRG1 and cancer

Studies utilising a variety of methodologies have revealed an association between NDRG1 expression and cancer, with NDRG1 levels being higher in certain cancers and lower in others (Tables 4–6). Hence, it is clear that NDRG1 plays an important role in the promotion or inhibition of carcinogenesis depending on factors such as the cell-type affected. In fact, a number of studies revealed a negative correlation between NDRG1 expression and severity of cancer, which suggests that NDRG1 may have a role as a tumour suppressor, metastasis suppressor, or both [51,53,138]. However, some studies also revealed a positive correlation between NDRG1 expression and cancer stage [32,35,37,38,89], which suggests that this protein plays pleiotropic roles, with its activity being context-dependent. In addition, recent studies have identified NDRG1 isoforms [54,94–97,171], which may be relevant to alterations in its activity and subsequent promotion or suppression of tumourigenesis or carcinogenesis.

Table 6
Expression and putative functions of NDRG1 in non-gastrointestinal and non-reproductive cancers.

Tissue	Expression and function(s)
Brain	Low expression in cancer [21,140] High expression in central neurocytoma [294] and grade IV–V glioma [21,140] Expression higher in glioblastoma than in low-grade astrocytoma [234]
Lung	High expression in cancer [140,295–297] High expression is associated with production of IL-8 and VEGF-A, high microvascular density and vascular invasion, [295,296]
Renal	High expression in cancer [32,140] Expression higher in nucleus in progression-free survival [298]
Skin	High expression in cancer [37,140]
Thyroid	Low expression in cancer [299] High expression in cancer [300]

5.1. Regulatory pathways of NDRG1 in cancer

In addition to the regulatory mechanisms outlined above in Section 4.1, *NDRG1* is regulated at the transcriptional and translational levels by pathways involving transcription factors and proteins, such as p53, PTEN and myc oncoproteins [194]. Moreover, growing evidence demonstrates that NDRG1 expression is epigenetically regulated in cancer [58,88,90,195]. These mechanisms are described in detail below.

5.1.1. NDRG1 and p53

The tumour suppressor protein, p53, has been reported to induce *NDRG1* expression by binding to the *NDRG1* promoter [41,79]. DNA damage also appears to induce *NDRG1* expression in a p53-dependent manner [41,79]. However, conflicting studies in different cell lines and tissues have not demonstrated a consistent association between DNA damage and *NDRG1* expression, though p53 expression may be up-regulated [85,143]. Further, using p53-null H1299 lung cancer cells [143] or DLD-1 colorectal cancer cells expressing a mutant, inactive form of p53 [79], it was shown that while p53 expression could be up-regulated, NDRG1 expression did not occur. As these observations were made in different cell lines, it is possible that p53 regulates *NDRG1* expression in a cell- or tissue-specific manner. Interestingly, p53 expression was shown to be down-regulated by NDRG1 in human placental trophoblasts [153], possibly *via* up-regulation of Mdm2 [196,197]. However, the involvement of NDRG1 in such an autoregulatory pathway may be limited to only normal human placental trophoblasts and not to cancer cells.

Although p53 appears to be a positive regulator of *NDRG1*, it is notable that NDRG1 is still expressed in $p53^{-/-}$ mice, suggesting that other effectors up-regulate *NDRG1* [41]. Indeed, it is notable that other mechanisms have been identified which induce NDRG1 expression, such as HIF-1 [140,198], EGR1 [146,199–202] and PTEN [85,111].

5.1.2. NDRG1 and PTEN

Like p53 [203], PTEN is a commonly inactivated or mutated tumour suppressor protein in cancer that is implicated to inhibit oncogenesis *via* NDRG1 [204]. Both PTEN and NDRG1 have been demonstrated to be more highly expressed in normal breast and prostate tissue than in matched neoplastic breast and prostate tissue [23]. Moreover, PTEN expression is associated with decreased lymph node metastasis in mice with prostate cancer [205], while down-regulation of PTEN is correlated with vascular invasion and metastasis *in vitro* and *in vivo* [23,205,206]. The tumour-suppressive activities of PTEN are mediated through its inhibition of the phosphoinositide 3-kinase (PI3K) pathway [207], with which it is coupled in normal physiology [207–210]. The PI3K and PTEN pathways constitute a negative feedback loop that is normally tightly regulated [207–210]. However, in cancer, the pathways are uncoupled and pro-oncogenic PI3K signalling overwhelms and reduces tumour-suppressive PTEN signalling [207–210].

PTEN is a tumour suppressor protein that induces *NDRG1* [25], and immunohistochemistry studies have demonstrated similar expression patterns for PTEN and NDRG1 in breast and prostate cancer tissue [23]. PTEN appears to be a mediator of *NDRG1* expression, as silencing of PTEN expression has been shown to down-regulate *NDRG1* levels, whereas over-expression of PTEN up-regulates *NDRG1* in a dose- and time-dependent manner [23]. Although no PTEN-binding sequence can be identified in the promoter region of *NDRG1* [211,212], a potential binding site has been identified for BPOZ, a protein involved in the PTEN signalling pathway [147], in the promoter region of *NDRG1* [211,212], indicating a possible mechanism by which PTEN regulates *NDRG1* [53].

In addition, up-regulation of *NDRG1* by PTEN has been demonstrated to involve PTEN phosphatase activity, which decreases phosphatidylinositol-3,4,5-triphosphate and results in suppression of the PI3K/protein kinase B (Akt)/mTOR pathway [23]. Conversely, Akt activation was suggested to inhibit *NDRG1* expression [213].

Interestingly, loss or down-regulation of *PTEN* has been associated with enhanced expression of NDRG1 in endometrial cancer, possibly *via* the PI3K/Akt/mTOR pathway [214,215]. Although the loss or down-regulation of *PTEN* is consistent with previous studies [216–218], the up-regulation of NDRG1 is unexpected and may suggest a possible cell- or tissue-specific, pro-oncogenic role in endometrial tissue [215]. The precise mechanisms underlying the association between PTEN and NDRG1 remain to be elucidated, but it is suggested that other regulatory mechanisms may be involved and further investigation is necessary.

Recently, NDRG1 has been demonstrated to induce *PTEN* expression in pancreatic cancer cells [111], indicating that NDRG1 and PTEN constitute a positive feedback loop. In fact, when PI3K signalling overwhelms PTEN signalling, up-regulation of NDRG1 restores the coupling of the PI3K and PTEN pathways *via* up-regulation of PTEN and down-regulation of PI3K [111], thereby resulting in reduced phosphorylated Akt levels and suppressed tumourigenesis [201].

5.1.3. NDRG1 and myc oncoproteins

The myc family consists of three oncoproteins, c-myc, L-myc and N-myc [87]. Myc oncoproteins are commonly over-expressed in cancer [219,220]. Indeed, dysregulation of myc oncoproteins is strongly correlated with carcinogenesis, as they activate and interact with a variety of oncogenic pathways [221] and transcriptionally repress *NDRG1* expression [44,45]. Interestingly, *N-myc* expression itself may be down-regulated by Ni^{2+} and retinoids, thereby resulting in the up-regulation of *NDRG1* expression [44]. In neuroblastoma cell lines with amplified *N-myc* and neuroepithelioma cell lines with amplified *c-myc*, over-expression of c-myc and of N-myc were both shown to strongly repress *NDRG1* expression [44]. Indeed, as the expression of these myc oncoproteins decreases during differentiation, the resultant up-regulation of NDRG1 may be required for the initiation of cellular differentiation [44].

The repressive effect of c-myc and N-myc on *NDRG1* is mediated *via* the promoter region of *NDRG1* at a site 52-bp from the transcription start site of *NDRG1* [45] and involves hetero-dimerisation of myc oncoprotein with myc-associated factor X (MAX) [222–226]. However, the repression mediated by the myc-MAX heterodimer does not appear to be due to direct binding [45] and may instead depend on an interaction of the myc-MAX complex with general transcriptional machinery [45].

5.2. Functions and functional pathways of NDRG1 in cancer

In contrast to normal cells and tissues in which NDRG1 is widely and highly expressed [124], neoplastic cells and tissues have been demonstrated to express low levels of NDRG1 [51]. Hence, the functions of NDRG1 in primary and metastatic tumour growth as well as the associated processes of angiogenesis, attachment and adhesion have been intensely investigated and are described in the following sections.

5.2.1. NDRG1 and primary tumour growth

NDRG1 has been demonstrated to inhibit primary tumour growth *in vitro* and *in vivo* [41]. *In vitro*, after over-expression of NDRG1, the proliferation rate of MCF7 breast and EJ bladder cancer cell lines decreased by approximately 70% and colonies on soft agar of these cell lines were observed to be smaller than the relative control [41]. Further, *in vivo*, mice injected with EJ bladder cancer cells that over-expressed *NDRG1* were observed to exhibit smaller tumours compared to those injected with control EJ bladder cancer cells [41]. Subsequent studies reported a reduction in tumour microvascular density, invasion depth and histopathological grading, with a corresponding increase in overall survival rates for patients with higher levels of NDRG1 expression *in vivo* [30]. In this latter study, although NDRG1 over-expression affected cell growth *in vivo*, it did not affect cell growth *in vitro*, potentially due to *in vivo* modulatory factors such as those associated with the stroma and angiogenesis [30].

Further evidence for the role of NDRG1 in cell proliferation comes from a microarray study, which showed NDRG1 dependent up-regulation of thiamine triphosphatase (Thtpa) protein levels in prostate and pancreatic cancer cells over-expressing NDRG1 [142]. Thtpa is involved in hydrolyzing thiamine triphosphate, which is an important energy currency molecule in cells [227]. Thus, increased NDRG1 levels may lead to a decrease in the energy state of the cell followed by diminished cell growth.

Recently, it has been suggested that NDRG1 expression in pancreatic cancer may inhibit tumour growth *via* increasing apoptosis [228]. Expression of NDRG1 was associated with decreased tumour growth *in vitro* and *in vivo*, and NDRG1 was demonstrated to completely inhibit tumour growth in anchorage-independent assays [228]. These observations indicate a role for NDRG1 as a tumour suppressor, but the underlying mechanisms responsible for this activity remain to be investigated.

5.2.2. NDRG1 and metastasis

As described in Section 4.2.2, NDRG1 is involved in cell differentiation and its expression has been negatively associated with tumour metastasis [22,25–27,31,229–231]. Indeed, the role of NDRG1 as a metastasis suppressor has been demonstrated *in vitro* [31] and *in vivo* [142]. NDRG1 was shown to inhibit metastasis by reducing cell–cell and cell–matrix adhesion in AT6.1 rat prostate cancer cells [142] and to inhibit metastasis to lungs without affecting primary tumour growth in a SCID mouse model [31]. NDRG1 expression was also found to inhibit cell proliferation in metastatic cell lines, but not in non-metastatic cell lines, indicating its differential functions and activities [80,138]. Further, suppression of NDRG1 was demonstrated to significantly enhance cell proliferation, migration and invasion in Ishikawa endometrial cancer cells [232]. In contrast, over-expression of NDRG1 was shown to inhibit cell proliferation and invasion of this latter cell line [232].

In support of a role of NDRG1 in metastasis suppression, NDRG1 expression has been demonstrated to be higher in organ-confined tumours compared to bone or lymph node metastases [31]. In fact, it has been suggested that NDRG1 could be used as a prognostic biomarker for gastric cancer, as strong evidence indicates that it can reasonably predict metastasis and early invasion [229], in addition to discerning hypoxic regions within tumours [233,234]. On the other hand, higher expression has been reported in the cytoplasm in local tumours and in the nucleus in advanced metastatic cancers, and NDRG1 expression has been associated with aberrant expression of p53, cell differentiation, tumour growth and angiogenesis [199,235]. These conflicting observations may be due to a multitude of factors, including disparities between stage of cancer, sample selection, or experimental protocols. Hence, it appears that the efficacy of NDRG1 as a prognostic biomarker for gastric cancer is currently limited and requires further study.

Recently, NDRG1 has been shown to suppress metastasis by a mechanism involving the modulation of the structural protein actin [236]. In cancer cells, actin is polymerised to form stress fibres that are required for cell migration [237]. NDRG1 has been demonstrated to inhibit the Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)/phosphorylated myosin light chain 2 (pMLC2) pathway [236], which would therefore result in suppression of the assembly and rearrangement of stress fibres from actin [238]. As this pathway has also been implicated in diverse processes, including adhesion, cell motility, proliferation, differentiation, apoptosis, production of proteolytic enzymes and invasion [239–241], inhibition of the ROCK1/pMLC2 pathway suggests another mechanism by which NDRG1 suppresses cancer cell metastasis [236].

5.2.3. NDRG1 and angiogenesis

Angiogenesis is the process by which new blood vessels are formed from pre-existing vessels and it is well known to play an important role in multiple diseases, including cancer [242,243]. In metastasis, angiogenesis is required for tumours to grow and invade [244] and NDRG1 over-expression has been associated with decreased production of pro-angiogenic factors, such as IL-8, MMP-9 and VEGF1, leading to decreased angiogenesis in pancreatic cancer [30]. In addition, NDRG1 has been demonstrated in pancreatic tumours to suppress the production of CXC cytokines [72,245], which are known to mediate processes critical to metastasis, such as proliferation, adhesion, resistance to chemotherapeutic agents and inhibition of apoptosis [73,74]. Moreover, NDRG1 has been shown to suppress angiogenesis *via* attenuating the expression and phosphorylation of the inhibitor of κB kinase (IκBα) and also NF-κB signalling [245].

Another putative mechanism by which NDRG1 modulates angiogenesis has been recently proposed. In prostate cancer, the PI3K/PTEN/AKT pathway has been shown to modulate angiogenesis *via* regulation of HIF-1 and VEGF expression and that PTEN, which inhibits PI3K and AKT, reduces angiogenesis and tumour growth [246]. Considering that NDRG1 promotes PTEN expression in prostate cancer [95], this further supports an anti-angiogenic role for NDRG1 in this neoplasm. In contrast, expression of NDRG1 has been associated with angiogenesis and low survival rates for patients with cervical cancer [39] and gastric cancer [235]. In cervical cancer, high NDRG1 expression is correlated with high VEGF1 expression and high microvascular density [39]. In support of this role for NDRG1 as a pro-angiogenic protein, angiogenic activity and mRNA levels of angiogenesis-related proteins, including CXC cytokines, IL-1β, IL-6 receptor, MMP-1 and VEGF1, were shown to be higher in gastric cancer cells that over-expressed *NDRG1* than in control cancer cells [247]. The mechanism by which NDRG1 promoted angiogenesis was demonstrated to involve IL-1α and activation of JNK/AP-1 [247]. Together, these studies again indicate that NDRG1 possesses pleiotropic effects and that its activities depend upon the tumour-type. The differences in pro- and anti-angiogenic activity may, in part, be due to involvement of the complex microenvironment in which these tumours develop [51,53,142].

5.2.4. NDRG1, attachment and adhesion

In the last decade, emerging evidence has suggested that NDRG1 modulates metastasis *via* proteins including MMPs, which degrade extracellular matrix, and adhesion molecules, such as β-catenin and E-cadherin that form the adherens junction at the cell membrane [25,94,248]. In agreement with these latter studies, NDRG1 has also been demonstrated to up-regulate β-catenin in breast cancer cells [25]. Further, NDRG1 has also been shown to inhibit TGF-β-induced epithelial–mesenchymal transition and to restore β-catenin and E-cadherin levels, which are suppressed by TGF-β in cancer cells [94]. Hence, these observations indicate that NDRG1 promotes the formation of the adherens junction, which is critical for cell–cell adhesion, and therefore reduces neoplastic cell migration and invasion. However, in gastric cancer, down-regulation of NDRG1 led to the induction of MMP-2 *via* MT1-MMP [249], while expression of NDRG1 was associated with down-regulation of E-cadherin, vimentin and Snail [250]. These observations, as for other activities of NDRG1, reflect the cell- or tissue-specific activities of NDRG1 [53].

5.2.5. The controversial role of NDRG1 in oncogenesis

Although the majority of studies examining NDRG1 report a tumour suppressive role for this protein, there are also a number of studies that contradict this view and demonstrate a tumourigenic role for NDRG1. In fact, in some studies, androgen receptor positive prostate cancers shows higher levels of NDRG1 compared with normal tissue [125]. These studies claim the decreased level of NDRG1 observed in other studies with prostate cancer is due to loss of hormone-dependence [53]. Furthermore, another study examining colorectal cancer found that NDRG1 expression was higher in more advanced lesions, leading to speculation that *NDRG1* is a metastasis promoter gene [35]. In more recent studies, NDRG1 was shown to be up-regulated in a variety of tumours including those of the liver, cutaneous squamous cell carcinoma, oral squamous cell carcinoma, cervical cancer and renal carcinoma [32,35–38]. In fact, silencing NDRG1 in liver cancer reduced proliferation, invasion and apoptosis *in vitro* and inhibited tumour growth *in vivo* [35].

However, the observed increase of NDRG1 in certain cancers can be attributed to the hypoxic conditions within tumours [251], as hypoxia also up-regulates NDRG1 levels [143]. In fact, a clinical study involving 223 prostate cancer specimens found no conclusive correlation of NDRG1 with tumour stage and grading, this being due to the differential response to hypoxia and androgens in the prostate epithelium of different patients [252]. These results again indicate the tissue-specific effects of NDRG1 and outline the importance of considering the tumour microenvironment and hormonal factors when assessing NDRG1 function.

5.3. NDRG1 and chemotherapy

NDRG1 has been suggested to be involved in modulating sensitivity and resistance of cancer cells to chemotherapeutic agents, such as irinotecan (CPT-11), a topoisomerase I inhibitor [253]. Specifically, over-expression of *NDRG1* in the SW620 colon cancer cell line is associated with resistance to CPT-11 and repression of *NDRG1* in the HCT116 colon cancer cell line increased the sensitivity of cells to undergo apoptosis after treatment with CPT-11 [253]. In support of this, tumours established from xenografted HCT116 and SW620 colon cancer cells that over-express *NDRG1* were more resistant to CPT-11 compared to the vector-transfected controls in mice, indicating a role for NDRG1 in resistance to chemotherapeutic agents [253]. Moreover, patients with low *NDRG1* expression consistently appeared to remain on CPT-11-based chemotherapy for longer durations than patients with high *NDRG1* expression [26]. Further studies are required to elucidate the precise role and mechanism by which NDRG1 modulates sensitivity of cancer cells to chemotherapeutic agents [26].

5.3.1. Targeting NDRG1 by binding cellular iron

Iron is a trace element integral to multiple metabolic processes, including DNA synthesis [157] and the metabolic demand for iron is greater in neoplastic than in normal cells [254]. Indeed, intracellular iron depletion is known to result in anti-tumour activity, prompting the development of chelating agents that possess marked anti-proliferative efficacy [157,255,256]. Intracellular iron depletion by iron chelators, such as desferrioxamine (DFO) [143], 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone (NIH) [143], di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) [143,257,258], Triapine [257] and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) [96], more significantly affects neoplastic cells than normal cells [254].

A number of iron chelators have been shown *in vitro* [143] and *in vivo* [257] to exhibit their potent, selective anti-tumoural activity, in part, by the up-regulation of *NDRG1* [143]. Further, up-regulation of *NDRG1 via* iron chelators involves depletion of intracellular iron pools and the up-regulation of HIF-1α-dependent and -independent mechanisms [143,152]. Notably, the potent anti-proliferative efficacy of chelators is due to their effects on a variety of critical molecular targets in addition to NDRG1, including $p21^{CIP1/WAF1}$ [96], cyclin D1 [96] and the rate-limiting enzyme of DNA synthesis, ribonucleotide reductase [259], all of which are involved in regulating proliferation and/or metastasis.

A variety of chelators have been developed [255,260], with ligands of the di-2-pyridylketone thiosemicarbazone (DpT; Fig. 5) class being a highly active series that demonstrate marked potency and selectivity *in vitro* and *in vivo* against tumour cells [257,261]. Unlike conventional iron chelators, such as desferrioxamine, these compounds do not cause whole body iron depletion at optimal doses [262]. In fact, these agents work *via* a "double-punch" mechanism in which they: *(1)* bind

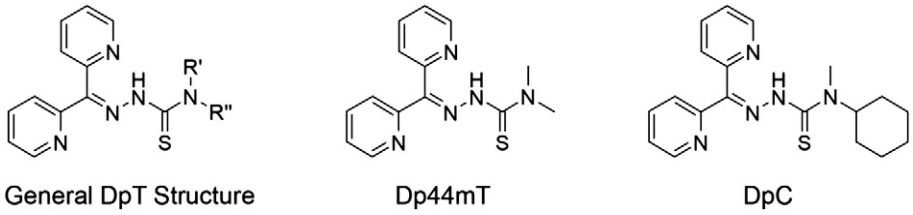

Fig. 5. Chemical structures of di-2-pyridylketone thiosemicarbazone iron chelators. Line drawings of the general structure of the di-2-pyridylketone thiosemicarbazone ligands, together with the structures of di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC).

and sequester iron, and *(2)* form redox active metal complexes, leading to generation of reactive oxygen species (ROS), which further potentiates their anti-cancer activity [262].

Of these agents, a second-generation DpT analogue, DpC (Fig. 5), demonstrates marked and selective anti-tumour activity *in vivo* using models of pancreatic [96] and lung cancer [262]. In fact, DpC possesses greater anti-tumoural efficacy *in vivo* than the current gold-standard chemotherapeutic agents against pancreatic cancer, namely gemcitabine [96]. Moreover, unlike the former lead compound of the DpT series of ligands, namely Dp44mT (Fig. 5) [257], DpC does not cause cardiac cardiotoxicity [96,262]. Notably, DpC was shown to markedly increase NDRG1 expression and phosphorylation in pancreatic cancer cells and it is probable that its effect on this molecular target, as well as others (*e.g.*, cyclin D1 and p21), is critical for its anti-tumour activity [96]. More recent studies have demonstrated that DpC inhibits ROCK1/pMLC2-modulated actin-filament polymerization, stress fibre assembly and formation that are critical for the migration and metastasis of tumour cells *via* a mechanism involving NDRG1 [236]. Currently, DpC is undergoing intensive preclinical toxicological assessment in a variety of animal models that is a pre-requisite for its entrance into future clinical trials in humans as a new anti-tumour/anti-metastatic therapeutic.

A particularly interesting aspect of the activity of these new ligands is their ability to inhibit metastasis *in vivo* [97] and prevent the epithelial–mesenchymal transition *via* their specific ability to up-regulate NDRG1 [94]. Notably, a recent study demonstrated the important role of NDRG1 in the anti-metastatic effects of DpT iron chelators *in vivo* [258]. These studies demonstrated a marked reduction in the ability of Dp44mT to inhibit metastasis in xenografts of NDRG1-knockdown MDA-MB-231-BoM cells compared to MDA-MB-231 breast cancer cells. These results further demonstrate the importance of NDRG1 as a therapeutic target in anti-metastatic chemotherapy.

6. Conclusions and future directions

NDRG1 plays important pleiotropic roles depending upon the tissue examined. It is well established that its role as a metastasis suppressor is important in numerous cancers including those of the prostate, breast, pancreas and colon. In the future, further studies need to be devoted to understanding the structural motifs in this protein that are responsible for its anti-metastatic activity. This is particularly critical considering a preliminary recent study demonstrating that NDRG1 is cleaved in cancer cells, but not normal cells [93]. Further, does this cleavage event prevent the anti-metastatic role of NDRG1 or could it actually promote this process? Additionally, is this cleavage regulated and by what mechanism does it occur?

Another interesting aspect of the activity of NDRG1 is its propensity of molecular targets that may suggest that the molecule could be involved in phosphorylation/de-phosphorylation as part of its cellular signalling roles. Associated with this function could be the direct binding of NDRG1 to other molecules to mediate its activity and the formation of complex, multi-protein metabolic units, namely, metabolons, that could facilitate its function as a metastasis suppressor.

Finally, the critical function of NDRG1 in terms of inhibiting the EMT is vital to assess in detail. Further understanding of the role of NDRG1 may lead to the development of new anti-cancer therapeutics such as the DpT group of thiosemicarbazones that markedly up-regulate this protein to inhibit metastasis *in vivo* [258].

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