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Review

Expanding horizons in iron chelation and the treatment of cancer: Role of iron in the regulation of ER stress and the epithelial–mesenchymal transition

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ABSTRACT

Cancer is a major public health issue and, despite recent advances, effective clinical management remains elusive due to intra-tumoural heterogeneity and therapeutic resistance. Iron is a trace element integral to a multitude of metabolic processes, including DNA synthesis and energy transduction. Due to their generally heightened proliferative potential, cancer cells have a greater metabolic demand for iron than normal cells. As such, iron metabolism represents an important "Achilles' heel" for cancer that can be targeted by ligands that bind and sequester intracellular iron. Indeed, novel thiosemicarbazone chelators that act by a "double punch" mechanism to both bind intracellular iron and promote redox cycling reactions demonstrate marked potency and selectivity *in vitro* and *in vivo* against a range of tumours. The general mechanisms by which iron chelators selectively target tumour cells through the sequestration of intracellular iron fall into the following categories: *(1)* inhibition of cellular iron uptake/promotion of iron mobilisation; *(2)* inhibition of ribonucleotide reductase, the rate-limiting, iron-containing enzyme for DNA synthesis; *(3)* induction of cell cycle arrest; *(4)* promotion of localised and cytotoxic reactive oxygen species production by copper and iron complexes of thiosemicarbazones (*e.g.*, Triapine® and Dp44mT); and *(5)* induction of metastasis and tumour suppressors (*e.g.*, NDRG1 and p53, respectively). Emerging evidence indicates that chelators can further undermine the cancer phenotype *via* inhibiting the epithelial–mesenchymal transition that is critical for metastasis and by modulating ER stress. This review explores the "expanding horizons" for iron chelators in selectively targeting cancer cells.

Contents

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; Bcl-2, B-cell lymphoma 2; BiP, binding immunoglobulin protein; bZIP, basic leucine zipper; CDK, cyclin-dependent kinase; CDKI, CDK inhibitors; CHOP, C/EBP homologous protein; DFO, desferrioxamine; dNTP, deoxyribonucleotide triphosphate; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3- thiosemicarbazone; eIF2α, eukaryotic initiation factor 2α; EMT, epithelial–mesenchymal transition; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GADD34, growth arrest DNA-damage inducible gene 34; GSK-3, glycogen synthase kinase 3; HIF, hypoxia inducible factor; IRE1, inositol-requiring enzyme 1; JNK, c-Jun *N*-terminal kinase; MEFs, mouse embryonic fibroblasts; MET, mesenchymal–epithelial transition; NDRG1, N-myc down-stream regulated gene 1; NF-κB, nuclear factor of κ light polypeptide gene enhancer in B-cells; p53, tumour suppressor protein 53; p53R2, p53-inducible RR; $p58^{IPK}$, protein 58 inhibitor protein kinase; PERK, protein kinase-like ER kinase; Rb, retinoblastoma protein; RR, ribonucleotide reductase; R1, RR subunit 1; R2, RR subunit 2; ROS, reactive oxygen species; siRNA, small interfering RNA; SMAD, mothers against decapentaplegic homolog; TGF-β, transforming growth factor β; TNF, tumour necrosis factor; TRAF2, TNF-receptor associated factor 2; UPR, unfolded protein response; XBP1, X box binding protein-1

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1. Introduction

Cancer is a debilitating disease that, despite years of research, remains a leading cause of death in developed countries [1]. While "cancer" is an umbrella term covering a broad group of diseases, which typically have different aetiologies, outcomes and mechanisms of propagation, all cancers are characterised by uncontrolled and/or abnormal cellular proliferation. Estimates made in 2008 indicate that 12.7 million new cancer cases were diagnosed and 7.6 million cancer deaths occurred worldwide [1]. The continuing growth and ageing of the world's population will result in an ever-increasing global cancer burden. For example, it has been estimated that the number of new cancer cases will increase to 22.2 million by 2030, thereby becoming the global leading cause of death, regardless of region or socio-economic status [2]. While this predicted rise in cancer burden will largely be due population expansion and ageing, the limitations of our current therapeutic arsenal will only serve to exacerbate this looming problem.

Current cancer treatments consist of surgery, chemotherapy, radiotherapy and immunotherapy, either alone or in combination. The main limitation of these therapeutic modalities is the inability to differentiate between normal, healthy cells and cancerous cells. As a result, severe side effects, such as nausea, vomiting, loss of appetite and alopecia are inherent, particularly with chemotherapeutics [3]. In addition, the DNA damage that some anti-cancer drugs induce can lead to carcinogenicity [4], potentially turning healthy cells malignant or increasing the malignancy of already cancerous cells.

Recent research into iron chelation therapy has demonstrated promising novel anti-cancer mechanisms [5]. Iron plays a major role in many crucial biological systems, in particular DNA synthesis, cell growth and proliferation, making it an effective target for anti-cancer chemotherapeutics [6,7]. Recent advances in iron chelation have demonstrated significant metastasis suppression mechanisms, many of which putatively operate through an up-regulation of the iron-regulated metastasis suppressor, N-myc down-stream regulated gene 1 (NDRG1) [8]. As will be discussed further in this review, the molecular targeting of this regulatory protein has greatly extended our understanding of the effect of iron chelators into numerous oncogenic pathways, including NDRG1-dependent modulation of proliferative signals, endoplasmic reticulum (ER) stress pathways and the inhibition of key initiating steps of metastasis [9–11]. This is of particular interest, especially since it is the metastases, not the primary tumour, that accounts for 90% of cancer deaths [12].

2. Iron and cellular function—cancer's Achilles' heel

Iron is an essential nutrient that plays an important role in almost all cellular processes, making it critical for virtually all cells [13]. Primarily, iron functions as a co-factor, or it is a constituent of co-factors, within the active sites of numerous proteins and enzymes (*e.g.*, hemoproteins and iron–sulphur cluster proteins) that play roles in cellular energy metabolism, DNA synthesis, cell growth and proliferation [7,13]. Indeed, iron is crucially involved in cellular respiration and energy transduction *via* oxidative phosphorylation and oxygen transport [14]. However, the molecular utility of iron's promiscuous redox activity means that high and/or improperly sequestered iron can result in cellular toxicity, predominantly resulting in the production of reactive oxygen species (ROS) that cause cellular dysfunction [14]. Consequently, it is important to monitor and maintain iron levels, in order to regulate normal cellular function and evade iron overload or deficiency.

2.1. Iron and ribonucleotide reductase

The necessity for a constant source of cellular iron is exemplified by the iron-dependence of the enzyme, ribonucleotide reductase (RR). Notably, RR is the rate-limiting enzyme for the *de novo* synthesis of all four 2′-deoxyribonucleotides from their 5′-ribonucleotide counterparts that are required as precursors for DNA synthesis and repair [6]. In addition to decreasing cellular iron uptake and increasing cellular iron mobilisation, this activity has long been believed to be the primary target of iron chelators in inhibiting cell growth and exerting their anti-cancer activity [15]. As with other higher organisms, mammals express only class Ia RR, which is a tetrameric enzyme ($\alpha_2\beta_2$) consisting of two homodimeric subunits, R1 and R2. R1 is the larger of the two subunits and contains the active sites enabling it to bind and reduce 5′-ribonucleotide substrates, while the R2 subunit has a dimeric iron binding site in each polypeptide chain [6,16]. In the active form, the R2 proteins contain a stable tyrosyl radical close to the iron centres, which occur in a high-spin ferric state and are anti-ferromagnetically coupled through a μ-oxo bridge [6]. The bound iron ions in each subunit of R2 redox cycle to abstract the hydrogen atom from the phenolic OH of a vicinal tyrosine to produce a tyrosyl radical that is essential for the catalytic activity of R1 [6]. In the absence of iron-binding to R2, the production of 2′-deoxyribonucleotides is inhibited [6]. Therefore, iron depletion potently inhibits RR activity and consequently inhibits DNA synthesis, ultimately leading to cell cycle arrest and apoptosis [17]. Specifically, the siRNA-mediated knockdown of R2 in HCT-116 human colon cancer

cells leads to cell cycle arrest at the G_1/S phase, as well as enhanced apoptosis, in response to DNA damage and replication stress [18].

While RR is responsible for maintaining deoxyribonucleotide triphosphate (dNTP) pools during cellular proliferation, a more recently discovered stress-inducible RR subunit, protein 53 (p53)-inducible RR (p53R2), has been proposed to aid in DNA repair mechanisms [19,20]. Activation of p53R2 can only occur *via* association with the tumour suppressor, p53, which is itself activated following the detection of DNA damage, suggesting it is important for the supply of a readily available pool of dNTPs for DNA repair following the initiation of cellular stress [19]. Guittet et al. [21] suggest that the mechanism of action of p53R2 is very similar to that of R2 despite the different pathways of activation. In this previous study, p53R2 was shown to be able to substitute for R2 to form a complex with R1 and function normally [21]. Additionally, sequence comparisons revealed that the iron coordination sites are highly conserved between the two subunits [20], indicating a similar dependence on iron for tyrosyl radical generation.

Generally, cancer cells undergo proliferation at a greater rate than normal cells and, consequently, greater RR activity is needed to sustain adequate DNA requirements [22]. As such, increased RR activity has been observed to occur concomitantly with tumour growth and malignant transformation [23]. RR inhibition through iron chelator-mediated iron depletion results in a significant reduction in all four dNTP pools, and subsequently, inhibition of DNA production and cell growth [24].

2.2. Iron in cell cycle progression

Iron repletion facilitates cell cycle progression through the regulation of a number of essential molecules including cyclins, cyclin-dependent kinases (CDKs), p53, retinoblastoma protein (Rb), and CDK inhibitors (CDKI) [25–27]. In contrast, iron depletion results in cell cycle arrest in the G_1/S phase *via* the regulation of these molecules [15]. The cell cycle itself is divided into four phases: G_1, S, G_2 and M, consisting of cell growth, followed by DNA replication and cell preparation for mitotic division [27]. Cell cycle progression is tightly regulated by CDKs and their associated cyclins in order to prevent and/or repair DNA damage.

Iron depletion has been demonstrated to alter the activity of CDKs in human T lymphocytes, with a reduction in iron levels resulting in the decrease of cyclin A and its associated kinase, CDK2 [28]. This leads to the stalling of the cell cycle in S phase, preventing the cell from proliferating. Similarly, the cyclin D family, which is responsible for the transition from phase G_1 to S [29], was also shown to be affected by iron depletion [30–32]. In fact, iron depletion in cells in culture caused a significant decrease in cyclin D1, D2 and D3 levels, as well as reduced CDK2 expression [31]. A later study suggested that the mechanism of cyclin D1 regulation by iron depletion involved decreasing the protein's already relatively short half-life from 80 ± 15 to 38 ± 3 min *via* an ubiquitin-independent proteasomal pathway [30]. Additionally, Rb protein is also important in the progression of cells through the cell cycle, specifically playing a role at regulating the restriction point at the G_1/S phase transition [33]. Normal progression occurs following the CDK2, CDK4 and CDK6-dependent phosphorylation of Rb [15]. As previously mentioned, CDKs 4 and 6 are activated by the cyclin D family, and consequently, iron depletion also leads to hypo-phosphorylation of Rb [31]. This is further accentuated by the decrease in CDK2 expression, which normally combines with cyclin E to further phosphorylate Rb that is essential for G_1/S progression [15]. Hence, the decrease in CDK2 expression could also play a role in the inhibition of the G_1/S progression observed after iron depletion [31].

2.3. Iron and reactive oxygen species—a "Double-Edged Sword"

The molecular properties of iron that allow it to participate as a cofactor in a wide variety of redox enzymes also endow it with the ability to damage cells [7,13]. This "dark side" of iron predominantly results from the propensity of improperly sequestered iron to catalyse the production of highly noxious ROS (*e.g.*, the hydroxyl radical). As iron is able to readily redox cycle between the Fe(II) and Fe(III) states in an aqueous solution, the celebrated Haber–Weiss cycle, as well as qualitatively similar reactions, enable catalytic amounts of redox-active iron to facilitate the production of hydroxyl radicals in the presence of hydrogen peroxide and a reductant such as superoxide or ascorbate [13,34]. The excessive production of hydroxyl radicals can stimulate apoptosis *via* DNA oxidation, mitochondrial damage, and/or the peroxidation of membrane lipids [35].

Oxidative reactions such as these ultimately lead to the tissue and organ damage present in diseases leading to cellular iron loading (*e.g.*, hereditary hemochromatosis, β-thalassemia and Friedreich's ataxia [13,36–38]). Oxidative DNA lesions caused by ROS can result in DNA modifications through single or double strand breaks or even in the modification of nucleotide bases [39]. In this way, iron-induced oxidative stress can play a role in initially promoting tumourigenesis through DNA mutations and activation of pro-oncogenic signalling pathways. However, somewhat paradoxically, as cancer cells show increased susceptibility to oxidative stress [40], the redox activity of iron can potentially be turned against the cancer cell as an anti-cancer strategy. Indeed, as discussed further below, this can be achieved by novel ligands that bind cellular iron and copper in a highly redox-active form [7,41].

3. Iron chelators—modes of action in cancer treatment

Depletion of intracellular iron by chelators impacts a range of different molecules that regulate the cell cycle, angiogenesis and metastasis suppression [42] (Fig. 1). In particular, the depletion of cellular iron by chelators in breast cancer, leukaemia, Kaposi's sarcoma and neuroepithelioma cells, affects the expression and activation of RR, cyclins, CDKs and the CDKI, protein 21 (p21), ultimately resulting in the inhibition of cancer cell proliferation [15,20]. Iron chelators are ligands that bind cellular iron and may promote its mobilisation out of the cell and/or inhibit its uptake from the serum iron transport protein, transferrin, resulting in cellular iron depletion [43,44].

Another mechanism of action of iron chelators is to mimic hypoxia, in part, *via* the activation of hypoxia-inducible factor (HIF)-dependent transcription of HIF-target genes, including NDRG1 [8]. This enigmatic protein, which will be discussed in depth later in this review, is a known metastasis suppressor in multiple cancers [45]. NDRG1 is also involved in embryogenesis and development, cell growth and differentiation, lipid biosynthesis and myelination, stress responses and immunity [46]. In addition to its putative role as a metastasis suppressor, NDRG1 can also influence other stages of carcinogenesis such as angiogenesis and primary tumour growth [45–48]. NDRG1 is regulated by multiple effectors in normal and neoplastic cells, including N-myc, histone acetylation, hypoxia, cellular iron levels, intracellular calcium [46], cell density and ascorbate [49].

Therefore, through their ability to chelate cellular iron, iron chelators can negatively impact cancer cell biology by, among other mechanisms, inhibiting RR activity, decreasing expression of cyclins A, B and D, increasing levels of the CDK inhibitor, $p27^{Kip1}$, decreasing the expression of CDK2 [31,32], promoting Rb hypo-phosphorylation [15,20] and up-regulating p53 and NDRG1 [45]. Interestingly, the advent of novel chelators that bind iron in a form that is capable of redox cycling within the tumour microenvironment (discussed further below) has significantly expanded the repertoire of modalities by which iron chelators can exert their anti-cancer activity [17].

3.1. The canonical iron chelator, desferrioxamine

The naturally occurring chelator, desferrioxamine (DFO) (Fig. 2A), which is a hexadentate bacterial siderophore produced by the actinobacterium, *Streptomyces pilosus*, was the initial focus of iron-chelation-based anti-cancer studies [7,50]. It functions by binding Fe(III) and forming a complex that is both metabolically and redox

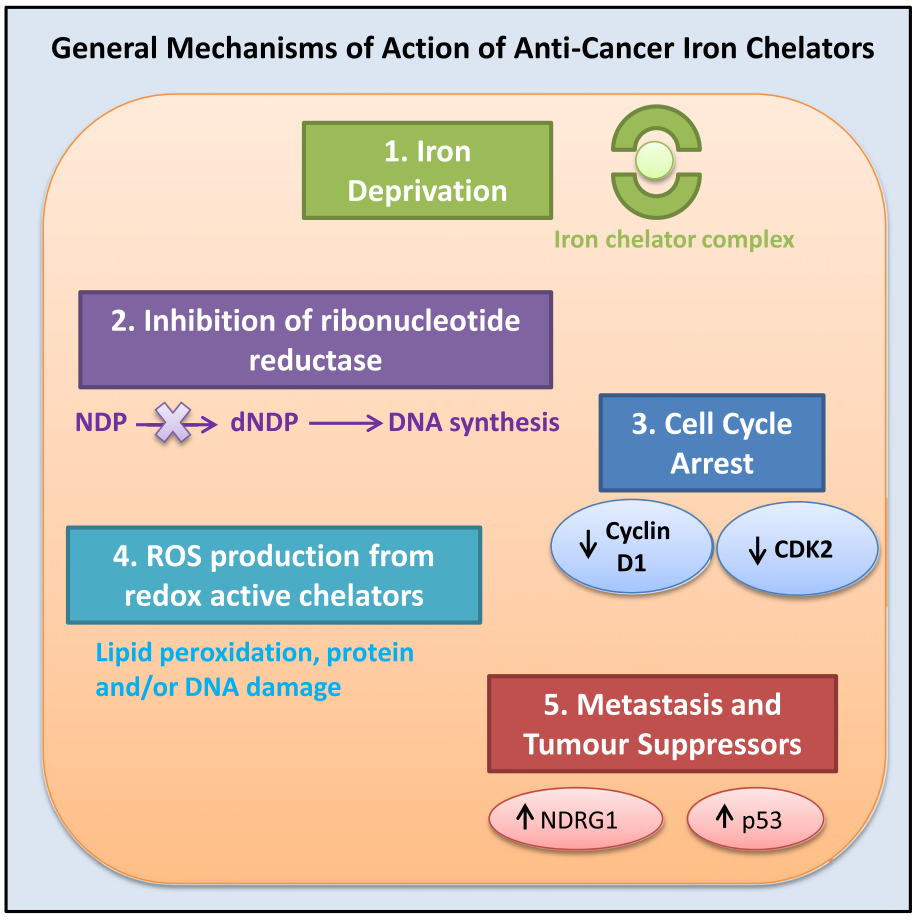

Fig. 1. General mechanisms of action of iron chelators that are responsible for their anti-tumour activity. Iron chelators have multiple molecular targets, with the following being important in terms of their mechanism of action: (1) Iron deprivation mediated by inhibition of iron uptake from transferrin (Tf) and increased mobilisation of intracellular iron; (2) Inhibition of ribonucleotide reductase activity; (3) Cell cycle arrest mediated by decreased expression of cyclin D1 and cyclin-dependent kinase 2 (CDK2); (4) Generation of reactive oxygen species (ROS) after the formation of redox active iron or copper complexes, leading to lipid peroxidation, protein and/or DNA damage; and (5) Induction of the expression of the iron-regulated metastasis suppressor, N-myc downstream regulated gene-1 (NDRG1), and the tumour suppressor protein, p53.

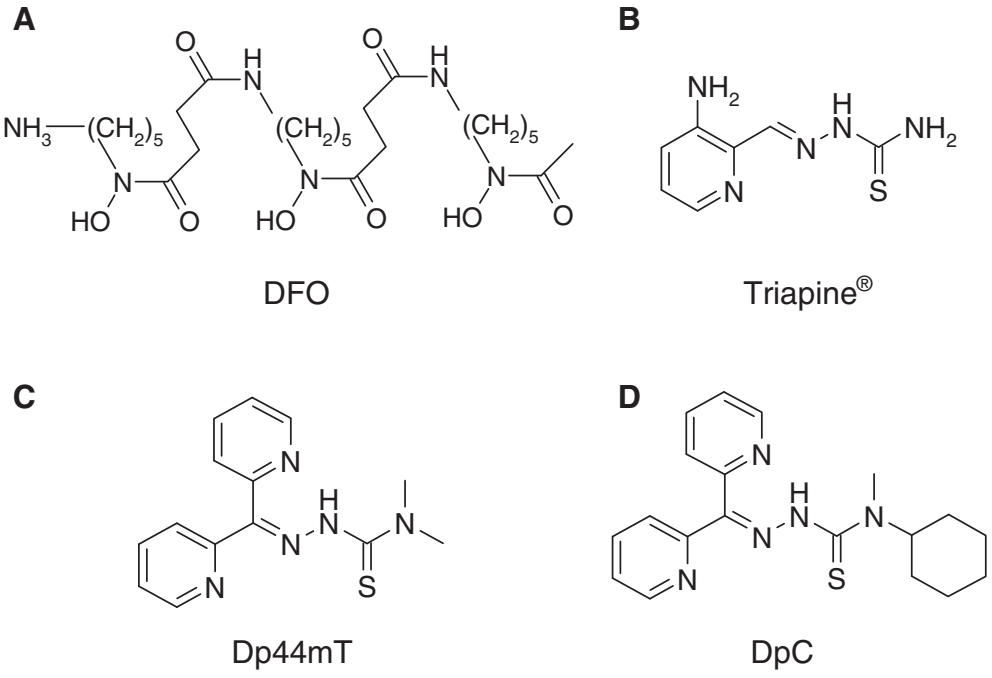

Fig. 2. Line drawings of the iron chelators: (A) DFO; (B) Triapine®; (C) Dp44mT and (D) DpC.

inactive [7]. Primarily used as a treatment for iron overload diseases such as β-thalassaemia [17], DFO has also demonstrated anti-tumour activity *in vitro* and in clinical trials for the highly-aggressive cancer, neuroblastoma [51]. For instance, continuous exposure of NB100, SMS-KCNR and SH-SY5Y neuroblastoma cell lines to DFO in culture led to cell cycle arrest at the G_1 phase [52]. Additionally, two neuroblastoma cell lines incubated *in vitro* for 72 h demonstrated that 60 μM DFO resulted in greater than an 80% reduction in cell viability compared to several non-neuroblastoma cell lines [53]. However, for similar effects to be observed *in vivo*, treatment typically requires continuous high doses of DFO due to its rapid metabolism, as well as its hydrophilicity that limits its ability to permeate cell membranes and access intracellular iron pools [54]. These limitations have prompted the development of synthetic chelators with greater membrane permeability, longer pharmacokinetic half-lives and markedly improved anti-cancer activity [5].

3.2. Anti-cancer chelators: thiosemicarbazones

Thiosemicarbazones are a class of synthetic tridentate chelators designed specifically for anti-cancer therapy and have demonstrated potent anti-proliferative efficacy *in vitro* and *in vivo* [5,17]. In fact, the thiosemicarbazone, Triapine® (Fig. 2B), demonstrates potent anti-cancer activity and has progressed through greater than twenty Phase I and Phase II clinical trials in patients with a range of cancers [41,55–65]. In the case of metastatic renal carcinoma, the study was terminated before completion due to adverse effects such as fatigue, nausea, neutropenia, methemoglobinemia, hypoxia and hypotension [55]. However, more recent studies examining gynaecological tumours have been more promising, particularly when Triapine® was combined with radiation or other cytotoxics [56].

Thiosemicarbazones bind intracellular iron in both the Fe(II) and Fe(III) states and can potently induce cellular iron depletion (Fig. 3)

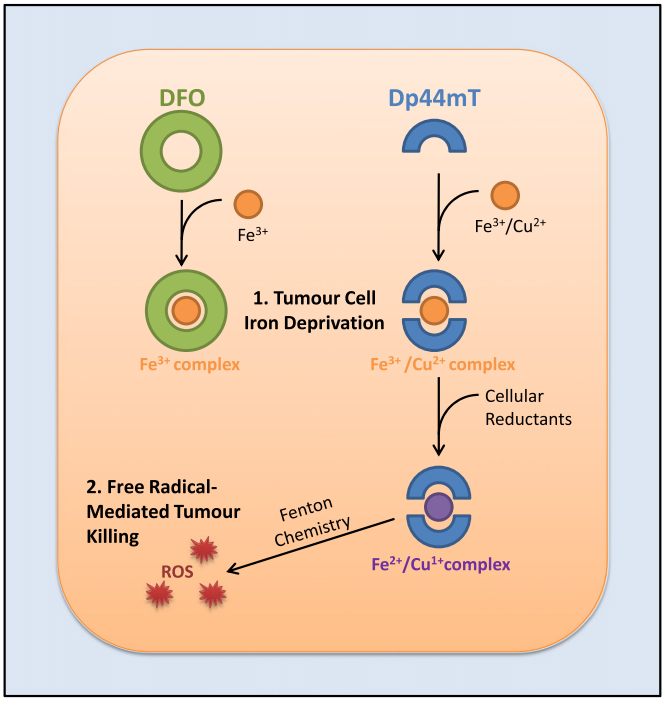

Fig. 3. Differences in the mechanisms of anti-cancer action of DFO and Dp44mT. Iron chelators enter tumour cells and bind intracellular iron, causing iron deprivation. DFO is a hexadentate chelator that binds Fe^{3+} with high affinity forming a 1:1 complex. In contrast, Dp44mT forms a complex with Fe^{3+} in a 2:1 ratio. Moreover, unlike DFO, Dp44mT forms redox-active iron and copper complexes, inducing the generation of ROS and greater cytotoxicity *via* Fenton chemistry.

[32]. However, in addition to chelating iron, this class of chelators can also bind copper [66], enabling the formation of redox-active iron and copper complexes that accumulate and produce ROS in subcellular compartments such as the lysosome [67].

The dysregulated production of ROS is a potent cellular stressor that can cause cytotoxicity and cell death, and ROS are well-known to exert anti-proliferative activity in cancer cells [68]. For example, studies in melanoma and breast cancer cells demonstrated that ROS may be largely responsible for the ability of the thiosemicarbazone chelator, Triapine®, to inactivate RR [69]. An advantage of thiosemicarbazones over DFO lies in the greater pharmacokinetic half-life and membrane permeability, which allows for administration at much lower doses. As with other iron chelators, thiosemicarbazones probably target cancer cells over their non-cancerous counterparts, in part, due to the greater requirement for iron-dependent DNA synthesis [17]. A number of highly promising thiosemicarbazones have shown to be particularly effective in this regard, including di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 2C) and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC; Fig. 2D) [68,70].

3.2.1. Dp44mT

Dp44mT was shown to significantly inhibit the growth and metastatic spread of tumours, both *in vitro* and *in vivo* [68,70]. Dp44mT treated mice showed marked reduction in tumour growth of M109 lung carcinoma cells with a 47% decrease in tumour weight [68]. This was demonstrated to be p53-independent, which is advantageous considering that approximately 50% of tumours contain a mutation in the *p53* gene [70]. Furthermore, systemic iron deficiency was not observed in association with Dp44mT administration [70]. In fact, despite the ability of Dp44mT to reduce iron uptake from transferrin into cells in culture [68], Dp44mT did not lead to overall iron depletion in tumours *in vivo* [70]. This observation suggested that the production of ROS is the more prominent anti-tumour mechanism of this class of chelators [70], as has been suggested for Triapine® in its ability to inhibit RR activity [69].

Intriguingly, Dp44mT has been shown to act, in part, by becoming trapped within the acidic lysosomal compartment due to its ionisation properties [67]. This results from the ligand gaining a net positive charge at the acidic pH associated with the lysosome (*e.g.*, pH 5), which prevents it from being released from the organelle. The lysosome plays a critical role in autophagy and protein catabolism, including the degradation of iron and copper containing proteins [71]. As such, Dp44mT has been shown to bind iron and copper, forming highly redox active complexes that then lead to lysosomal membrane permeabilisation and subsequently apoptosis [67].

While Dp44mT demonstrated pronounced activity both *in vitro* and *in vivo*, high non-optimal intravenous doses led to some cardiac fibrosis in nude mice [70]. This observation led to the development of a second generation of DpT analogues, of which the ligand, DpC (Fig. 2D), is the lead compound for clinical development [72].

3.2.2. DpC

Of the second generation of DpT analogues, DpC showed marked and selective anti-proliferative activity in pancreatic and lung tumours *in vivo* [72,73]. Important advantages of DpC over Dp44mT include: (1) DpC does not induce cardiotoxicity, even at markedly higher doses; (2) DpC demonstrates marked activity when administered by both the oral and intravenous routes, while Dp44mT is toxic *via* the oral route [74]; (3) DpC shows greater activity than Dp44mT at inhibiting pancreatic cancer growth *in vivo* [73]; and (4) in contrast to Dp44mT, DpC does not markedly induce oxidation of oxyhaemoglobin in red blood cells *in vitro* and *in vivo* [75]. Similarly to Dp44mT, DpC has multiple molecular targets and has been shown to markedly up-regulate the metastasis suppressor, NDRG1, leading to the inhibition of ROCK1/MLC2 signalling that is important for cellular migration [76]. Again, like Dp44mT, DpC acts to up-regulate the CDKI, p21, and down-

regulate the expression of cyclin D1 [73], and inhibit ribonucleotide reductase activity (D.R. Richardson, unpublished results), all of which act to inhibit cell cycle progression. Further studies examining the development of DpC are currently underway to ensure its entrance into clinical trials.

While the molecular mechanisms of action of Dp44mT and DpC have been deciphered in some detail, their effects and the influence of other iron chelators on other critical processes important for inducing cellular stress (*e.g.*, ER stress; see below) remain unknown. Such details are important to determine, not only from the point of view of understanding the cytotoxic mechanisms of action of iron chelators, but also for elucidating the cellular response to iron depletion.

4. Endoplasmic reticulum (ER) stress and the role of iron

The ER is an elaborate eukaryotic organelle that has a diverse range of roles, from protein folding and secretion, to detoxification and calcium homeostasis [77]. Indeed, it is the primary site for the folding of membrane-bound, secreted and many organelle-targeted proteins. The intricate web of reactions and interactions required for proper protein folding within the ER require, among other things, a ready supply of ATP, chaperone proteins, glycosylating enzymes, high calcium levels and oxidising conditions [78]. Unsurprisingly, protein folding within this multifaceted organelle is highly sensitive to perturbations in the cellular milieu, including stressors such as hypoxia, hypoglycaemia and the accumulation of redox-active species [79]. Any disruption to normal protein folding can result in an accumulation of unfolded or misfolded proteins within the ER: a condition known as ER stress [77]. If left unresolved this can completely disrupt ER and cellular function, inducing apoptosis, which is a pathological process underpinning some neurodegenerative diseases, diabetes and cancer [80]. To overcome these effects of ER stress, the cell can initially activate a set of protective signalling pathways, known as the unfolded protein response (UPR), to return normal functioning to the ER [78]. However, if the original stressor is not removed and the ER stress is unresolved, apoptosis will ensue. As discussed below, emerging evidence indicates that ER stress can be modulated by changes in intracellular iron metabolism [81]. Conversely, ER stress can also modulate cellular and systemic iron homeostasis through effects on the expression of the hormone of iron metabolism, hepcidin [82].

4.1. The unfolded protein response

The UPR is primarily a pro-survival response that aims to normalise ER functioning by removing the accumulation of unfolded or misfolded proteins (Fig. 4). The molecular "gatekeepers" of the response are three

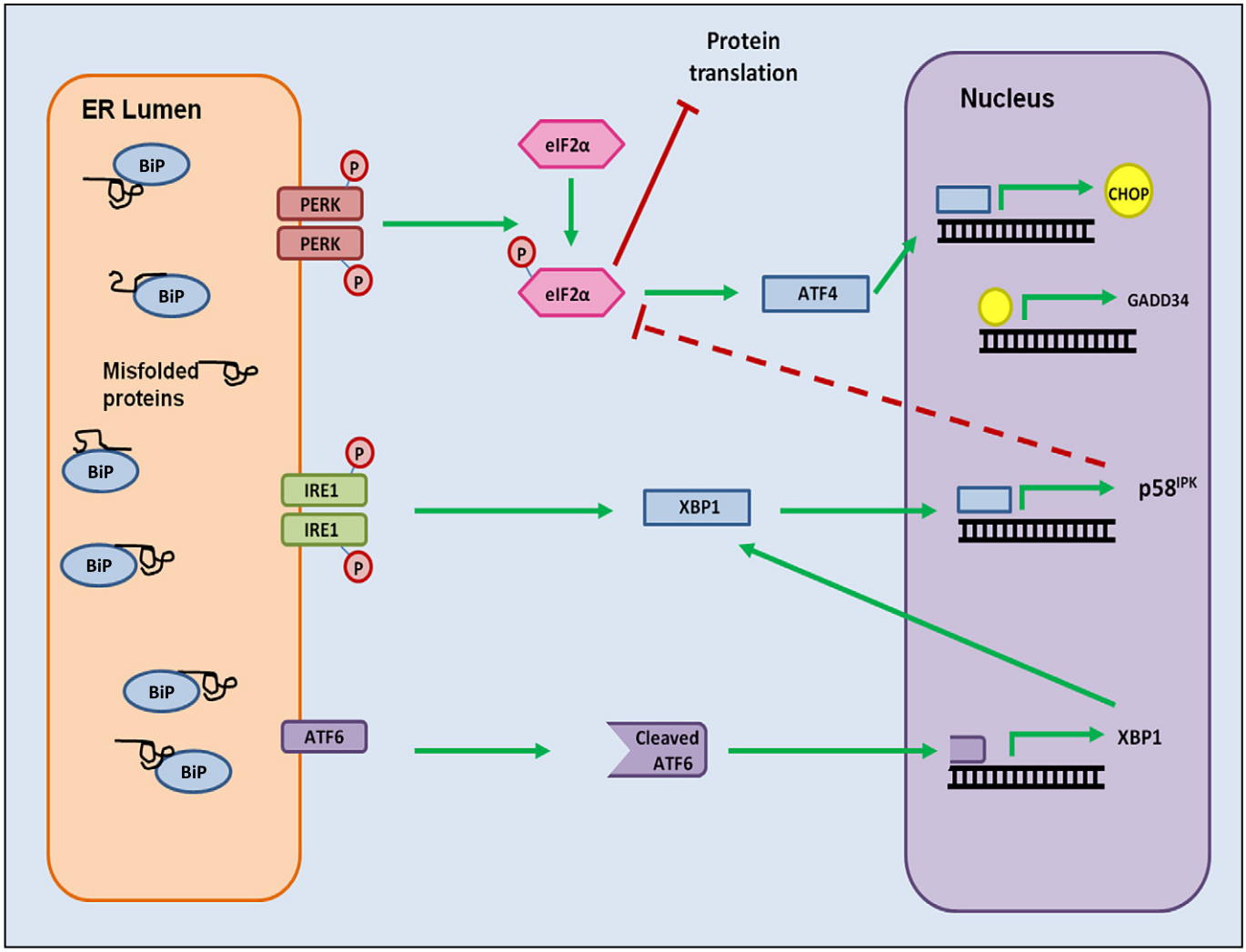

Fig. 4. The UPR is mediated by three ER stress sensors. Binding of unfolded proteins to BiP within the ER lumen activates PERK, ATF6 and IRE1. PERK phosphorylates eIF2α, inhibiting cap-dependent translation, while also activating CHOP transcription *via* ATF4. Activation and cleavage of ATF6 initiates *XBP1* transcription, while IRE1 catalyses the alternate splicing of *XBP1* mRNA, leading to the expression of the active XBP1 transcription factor. One of the targets of XBP1 is $p58^{IPK}$ which inhibits eIF2α.

ER transmembrane receptors: protein kinase-like ER kinase (PERK/EIF2AK3), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1/ERN1) [78]. In a normal, non-stressed cell, all three receptors remain in their inactive state through an association with binding immunoglobulin protein (BiP; also known as glucose-regulated protein 78) to their luminal domains. BiP is a member of the HSP70 family of heat shock proteins and is an ER chaperone that also aids in the folding and assembly of nascent proteins [77]. Importantly, BiP stabilises the unfolded protein in a state that is favourable to folding, rather than directly participating in the folding of the protein itself [78]. This protein also targets terminally misfolded proteins for ER-associated degradation (ERAD) by recognising their characteristic hydrophobic regions [78]. Therefore, when there is an accumulation of misfolded proteins, such as during ER stress, the unbound BiP pool is depleted, resulting in a dissociation of the protein from its transmembrane receptor binding partners (*i.e.*, PERK, ATF6 or IRE1), activating PERK, ATF6 and IRE1 [77]. The down-stream effects of activation of the UPR include the inhibition of cap-dependent translation, up-regulation of ER chaperones to assist protein folding, increased proteolytic degradation of misfolded proteins *via* ERAD, and, in the case of prolonged or severe stress, the initiation of apoptotic pathways [83].

4.2. PERK

As previously stated, the activation of PERK is dependent on the dissociation of BiP from the intralumenal domain of the transmembrane receptor, which results in its homo-dimerisation, auto-phosphorylation and activation [77]. Once PERK is activated, it can then phosphorylate the α-subunit of the eukaryotic initiation factor 2 (eIF2α). The phosphorylation of eIF2α is a convergence point for the so-called "integrated stress response" [84], which can be activated by numerous cellular stressors (including cellular iron depletion [85]), and leads to an inhibition of cap-dependent translation and general protein synthesis in order to reduce the load of nascent proteins entering the ER [86]. Many proteins vital for proliferation, such as cyclin D, decay rapidly and are unable to be replaced, resulting in cell cycle arrest, which ultimately aids recovery [87]. The dependence of this process on PERK was demonstrated in a study in which *PERK*$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibited no translational inhibition when ER stress was induced, leading to increased cell death [88]. The regulation of this translation during stress occurs within stress granules, which are dynamic cytoplasmic aggregates containing stalled pre-initiation complexes that function as mRNA triage centres [89,90].

Notably, when PERK is activated and eIF2α is phosphorylated, not all translation is inhibited, with some specific mRNAs able to bypass the block [77,89,90]. These particular mRNAs carry specific regulatory sequences in their 5′ untranslated regions that allow them to evade this blockade and even be translated at an increased rate [78]. The ability of certain transcripts, particularly those encoding key stress-response proteins, to be translated in the face of a global suppression of translation is typical of the integrated stress response [89]. For example, the translation of activating transcription factor 4 (ATF4) is actually up-regulated by eIF2α phosphorylation [78]. This allows ATF4 to act as a transcription factor for down-stream stress-response/apoptosis genes such as C/EBP homologous protein (CHOP/DDIT3/GADD153) [91].

CHOP is a pro-apoptotic transcription factor that activates many genes to induce cell death by multiple mechanisms [92,93]. Indeed, *Chop*$^{-/-}$ MEFs demonstrate significantly reduced apoptosis when ER stress is induced [94]. Primarily, CHOP regulates the balance between the anti- and pro-apoptotic members of the BCL-2 family in favour of the latter [95,96]. CHOP appears to act in part through induction of ER oxidase 1 alpha (ERO1α), which hyper-oxidises the ER lumen and activates the ER calcium-release channel inositol 1,4,5-triphosphate (IP3) receptor (IP3R) to release calcium and thereby trigger apoptosis [97]. Further apoptotic signals are sent *via* the CHOP-activated death receptor 5 (DR5) [98].

The second function of CHOP is to re-establish protein translation imposed by phosphorylated eIF2α through induction of growth arrest DNA-damage inducible gene 34 (GADD34) [77,87]. GADD34 expression resumes normal translation by dephosphorylating eIF2α, but it has also been shown to exhibit apoptotic functions by activating p53 and inducing cell cycle arrest *via* association with p21 [99]. While the overall effect of CHOP and its target genes is to activate a pro-apoptotic pathway, it is currently unclear how CHOP activity is fine-tuned during UPR activation to ensure that the apoptosis is not initiated prematurely. This may be a result of protein 58 inhibitor protein kinase (p58^{IPK}) which has been shown to bind and inhibit the PERK kinase domain, stalling PERK activity [100]. More recent studies suggest that p58^{IPK} acts as a co-chaperone with BiP within the ER to reduce the accumulation of misfolded proteins [101]. In either scenario, this protein is regulated down-stream of the IRE1 pathway and appears to be part of a negative feedback mechanism, limiting the PERK branch of the ER stress response [77,87].

4.3. ATF6

In the case of ATF6, the dissociation of BiP from the ATF6 ER lumenal domain reveals Golgi localisation sequences that trigger ATF6 to translocate from the ER to the Golgi [77,78]. The receptor is then cleaved in the lumenal and transmembrane domains by resident site-1 and site-2 proteases (S1P and S2P), respectively, to release an active basic leucine zipper (bZIP) transcription factor that resides in its cytosolic domain [102]. Once activated, the bZIP domain of ATF6 translocates to the nucleus to activate transcription by binding to the ER stress response element of target genes such as, *BiP*, *CHOP* and *X box binding protein-1* (*XBP1*) [103]. XBP1 is a key regulator in IRE1 signalling [77,78,87] (see below for further discussion).

The expression of BiP in response to ATF6 activation initiates a negative feedback loop that not only aids in protein folding in the ER, but also in the inactivation of the ER stress receptors [77,87]. Notably, BiP has also exhibited behaviour as an apoptotic regulator by inhibiting caspase activation and caspase-mediated cell death [104]. It can translocate from the ER lumen to the cytosol and ER transmembrane where it has been shown to form a complex with caspase-7 and caspase-12, inhibiting capsase-12 release from the ER and blocking apoptotic signalling [104]. While this process suggests a pro-survival role for ATF6, it can also induce *CHOP* transcription if over-expressed, whereas *ATF6* knockout models show inhibited CHOP expression [103]. This suggests that short periods of mild stress stimulate a pro-survival response, while a prolonged activation of ATF6 due to excessive stress leads to cell death. However, unfortunately, the underlying intricacies of the involved mechanisms remain unclear.

4.4. IRE1

IRE1 is a molecule that functions as both an endonuclease and a kinase (Fig. 5). Activation is similar to that of PERK with the dissociation of BiP from IRE1 resulting in homo-dimerisation and auto-phosphorylation [77]. Once activated, the endonuclease domain of IRE1 removes a 26 nucleotide intron from the unspliced *XBP1* mRNA transcript that is transcribed in response to the nuclear translocation and transcription activity of the bZIP domain of ATF6 [105]. This newly generated splice variant (*XBP1s*) encodes the XBP1 protein that itself acts as a transcription factor, controlling genes involved in protein folding and ERAD, such as BiP and p58^{IPK} [106]. As previously mentioned, p58^{IPK} feeds back and negatively regulates the UPR, suggesting a possible pro-survival function of IRE1. This is supported by a study showing that sustained *XBP1* splicing, due to experimentally prolonged IRE1 signalling independent of ER stress, promoted cell survival [107].

In contrast, the kinase activity of IRE1 induces apoptotic signalling *via* tumour necrosis factor (TNF) receptor associated factor 2 (TRAF2) recruitment, leading to apoptosis signal-regulating kinase 1 (ASK1)

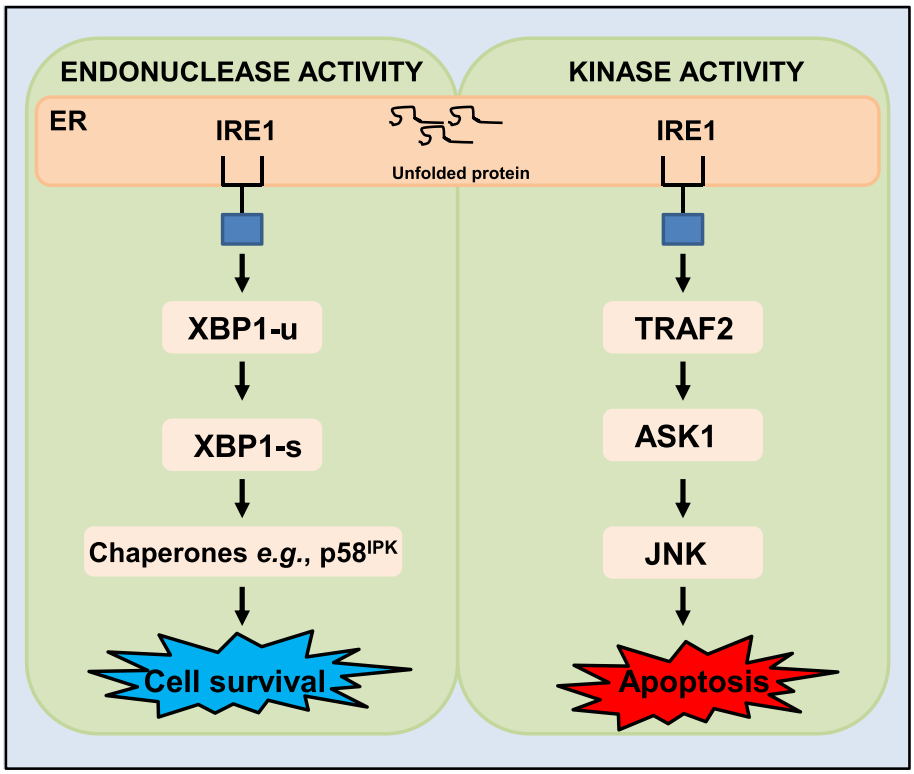

Fig. 5. The UPR can be triggered by the activation of a combined nuclease and kinase called IRE1 (inositol-requiring enzyme-1). Upon activation, the endonuclease domain of IRE1 splices *XBP1-u*, producing *XBP1-s* and subsequently XBP1 protein. XBP1 acts as a transcription factor, regulating the expression of genes concerned in protein folding and ER-associated degradation *e.g.*, $p58^{IPK}$, making this pathway pro-survival. After prolonged or extensive ER stress, the kinase activity of IRE1 is stimulated, activating the IRE1–TRAF2–JNK pathway and promoting apoptosis.

and c-Jun *N*-terminal kinase (JNK) activation [108]. Inhibition of ASK1 activity has been shown to protect cells against ER stress-induced apoptosis [109], indicating the significance of the IRE1/JNK pathway in stress related cell death. The down-stream effects of JNK are thought to be a modulation of the BCL-2 family, possibly similar to that of CHOP. JNK can phosphorylate and inactivate the pro-survival protein B-cell lymphoma 2 (BCL-2), while indirectly activating the pro-apoptotic proteins, BAK and BAX ultimately leading to activation of the intrinsic (*i.e.*, apoptotic) machinery [110]. There also appears to be some evidence of a TRAF2-mediated extrinsic apoptotic pathway. An interesting study indicated that TRAF2 enables the activation of nuclear factor of κ light polypeptide gene enhancer in B-cells (NF-κB)-dependent TNF production, in turn activating the extrinsic death receptor pathway [111].

Interestingly, BAK and BAX have also been found to regulate IRE1 activity by directly binding to the stress receptor and acting as a negative feedback signal. Double knockout mice, lacking both BAK and BAX, present with extensive tissue damage as well as a reduction in XBP1 and its target genes, when exposed to chemically-induced stress [112]. This could indicate that BAK and BAX are responsible for the switch from cell survival to apoptotic signalling. However, recent studies have implicated a large number of proteins involved in a complex that regulates IRE1, known as the UPRosome, making IRE1 the most regulated of the three stress receptors [113].

5. ER stress, cancer and iron

The major barrier to the survival of cancer cells that have overcome cell cycle and apoptotic checkpoints is limited oxygen and nutrients [22]. Consequently, subtle changes in the cell microenvironment, such as hypoxia, activate the UPR pathways making it a novel target for anti-cancer therapy. Throughout the early stages of tumourigenesis, the ER stress pathways can benefit neoplasia and cell survival by ATF4-dependent up-regulation of the pro-angiogenic glycoprotein, vascular endothelial growth factor [114]. Similarly, ATF4 and IRE1-dependent expression of XBP1 has been shown to promote tumour growth, with $XBP1^{-/-}$ tumour xenografts in nude mice having a significantly lower volume than wild-type xenografts [115]. Alternatively, activation of the PERK pathway has been show to inhibit transcription of cyclin D1 *via* phosphorylated eIF2α [116], leading to cell cycle arrest at the G_1 phase. PERK-dependent p53 transcription can also induce G_2 phase arrest [117] resulting in tumour cells becoming dormant. However, dormancy can also protect cancerous cells from apoptosis [118].

The signalling between the various UPR pathways determines whether cancer cell death, dormancy or aggressive growth will take place. Currently, the exact mechanism behind this is unclear. However, it has been suggested it is due to different periods of activation of stress receptors during prolonged ER stress. Human kidney cells exposed to persistent stress showed continued PERK/CHOP activation, while IRE1 and ATF6 signalling was attenuated, resulting in apoptosis [119]. Notably, this latter study fails to explain the down-stream apoptotic signalling of the IRE1/TRAF2 pathway and was done using high doses of pharmacological stress inducers. The effect of prolonged stress *in vivo* is still unclear, as are the correct mechanisms that modulate the conversion from cyto-protective to apoptotic pathways. Further research in this area would aid in the development of chemotherapeutics that could target these pathways.

Intriguingly, there have been some important recent advances in understanding the interplay between iron and ER stress in cancer and other disease states. For instance, it was recently demonstrated that the chelation and depletion of intracellular iron in MCF7 breast cancer cells leads to the formation of stress granules as well as the up-regulation of certain putative stress-response proteins, including NDRG1 [120]. Moreover, it was shown that cellular iron depletion in PANC1 pancreatic cancer cells using either DFO or the novel chelator, Dp44mT, resulted in the activation of the PERK/eIF2α axis of ER stress

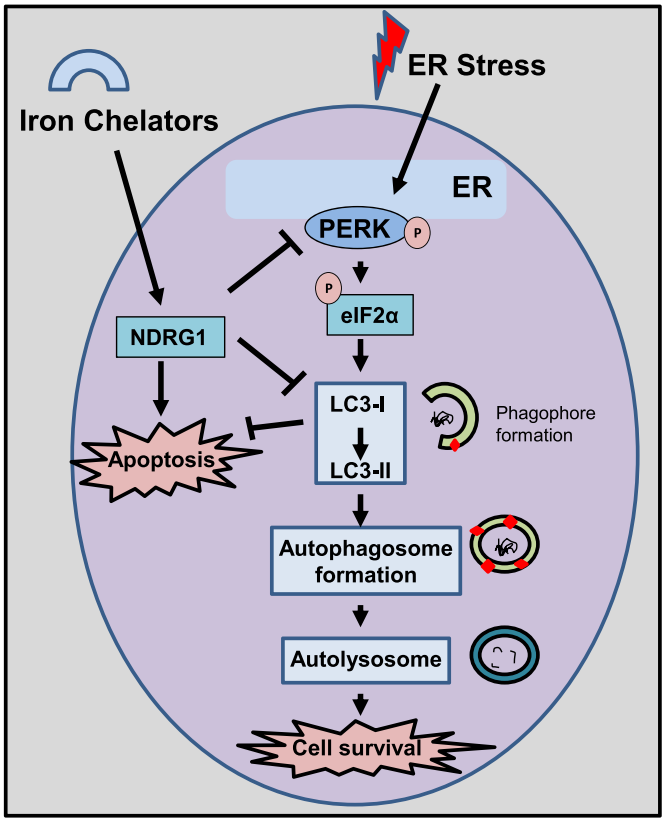

Fig. 6. Modulation of UPR signalling and autophagy pathways by iron chelators and NDRG1 in human cancer cells. ER stress leads to the activation of the PERK/eIF2α pathway, which induces the conversion of LC3-I into LC3-II, leading to autophagy. Iron chelators effectively cause ER stress activating the PERK/eIF2α pathway and increasing LC3-II conversion. However, iron chelators also induce the expression of NDRG1. NDRG1 blocks the phosphorylation of PERK and its downstream effector, eIF2α. Moreover, NDRG1 suppresses LC3-II expression and the formation of autophagosomes, inhibiting the pro-survival UPR activation and initiating apoptosis.

and autophagy in a manner that was negatively regulated by NDRG1 [85] (Fig. 6). In addition, the conditional deletion of frataxin, a key mitochondrial iron metabolism protein whose deficiency causes the debilitating cardio- and neurodegenerative disease, Friedreich's ataxia, leads to early and sustained activation of eIF2α phosphorylation, autophagy and apoptosis [81]. These observations suggest that ER stress can be activated by alterations in cellular iron levels and/or metabolism. Reciprocal regulation also seems to occur in that ER stress induced by the classical stressors brefeldin A, the ionophore A23187, or tunicamycin, can induce the expression of hepcidin, which causes hypoferremia and spleen iron sequestration in mice through its effects on the iron exporter, ferroportin [82]. The precise mechanisms by which alterations in cellular iron levels and/or metabolism affect the induction of ER stress are yet to be determined.

5.1. Metastasis

Metastasis is a highly complex process involving a series of gene deregulations and subsequent tumour–host interactions [121]. This results in a set of specific signalling pathways not present in generic tumour cells, enabling a behavioural and phenotypic change [122]. The key initiating steps of the metastatic cascade involve the disruption of adhesive interactions with adjacent cells and the adoption of a more motile, mesenchymal morphology through a process known as the epithelial–mesenchymal transition (EMT) [122–124]. This process is highly conserved and allows for increased invasiveness and motility [122]. The invasive capacity of cancer cells constitutes their ability to breakdown tissue barriers, in particular the basement membrane and extracellular matrix [121]. This involves the up-regulation of matrix metalloproteinases which proteolytically degrade tissue barriers to gain access to blood or lymphatic circulation [125].

Metastatic cells can then spread to distant organs *via* the circulation, extravasate into surrounding tissue and colonise, thereby forming secondary tumours (Fig. 7). To successfully accomplish this, these cells must be able to evade host immune responses, induce angiogenesis and respond to organ specific growth factors [126]. Heterogeneity exists between metastatic and non-metastatic cells, with gene-expression profiles of adenocarcinoma metastases and cells of the primary tumour showing comparatively distinct gene signatures between the two [127]. Additionally, recent studies reveal metastatic progression to be independent of primary tumour progression [128]. While it is unclear exactly what determines which cells become metastatic, the initiating events in the EMT have been identified [122–124,129].

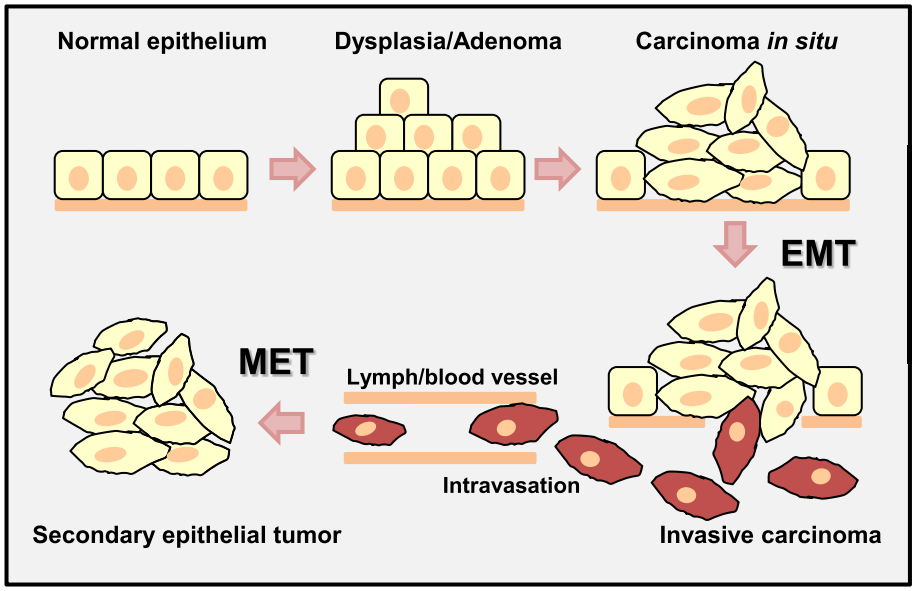

Fig. 7. Progression from normal epithelium to invasive carcinoma. *In situ* carcinoma derived from normal epithelium must undergo the epithelial mesenchymal transition (EMT) in order to invade surrounding tissue and move into the blood or lymphatic circulation. Once in the new tissue, the metastatic cells colonise by undergoing the mesenchymal epithelial transition (MET) and reverting back to epithelial cells.

5.2. The epithelial–mesenchymal transition

Most organs and tissues in adults are comprised of highly specialised epithelial cells with unique morphologic properties. They maintain an apical-basal axis of polarity through tight adherens junctions with adjacent cells, enabling intercellular communication, preservation of differentiation, allowing cells to work as a cohesive unit [130]. In contrast, mesenchymal cells are loosely arranged in the extracellular matrix, facilitating a greater migratory capacity, invasiveness and enhanced resistance to apoptosis [131]. The conversion from an epithelial to mesenchymal phenotype, which occurs during EMT, is crucial for embryonic development as well as wound healing [122]. However, throughout the progression of cancer, in particular carcinomas, malignant cancer cells often present with a significant down-regulation of epithelial markers, reduced cellular differentiation and polarity, loss of cell–cell adhesion, and increased mesenchymal cell motility and invasiveness. These phenotypic alterations can be traced back to the loss of intercellular adherens junctions [132].

The necessity for cancer cells to undergo EMT in order to metastasise has been questioned due to the lack of a full EMT phenotype in clinical metastasised carcinomas [133]. However, numerous theories accommodate these observations, including the concept that EMT may only occur within a small sub-population of cells within the tumour (*e.g.*, cancer stem cells [129]). A clinical study observing cells at the invasive front of the cancer showed a lack of the intercellular adhesion molecule, E-cadherin, while it was maintained in the core of the tumour [134]. Secondly, following the migration and invasion into distant tissues, metastatic cells can re-establish epithelial morphology through a process known as the mesenchymal–epithelial transition (MET) [130], allowing them to acquire growth signals specific for the new location. Additionally, it is important to note that some cancers may not require a full mesenchymal phenotype in order to metastasise. It is thought that an intermediate process, known as "partial EMT", takes place, resulting in the expression of only some mesenchymal characteristics, while retaining some features of the epithelium [135]. This process is normally seen in wound healing and suggests it may only be necessary for a cell to focus on a few EMT pathways rather than a whole mesenchymal conversion. With the ability to detach and migrate away from adjacent cells being the most important attribute in metastasising cells, pathways leading to the loss of adherens complexes would be present in both full and partial EMTs, making it a crucial topic of research.

5.3. Loss of the adherens junction

The adherens junction is a structure that maintains the epithelial phenotype [136,137]. Not only does it bind adjacent cells together, but it enables cell–cell communication and maintains cellular polarity and epithelial morphology [136,137]. The junction itself is a complex consisting of the transmembrane protein, E-cadherin, and stabilising catenins, in particular β-catenin [138]. Inhibition of either of these proteins results in the destabilisation of the entire complex, and E-cadherin knockout studies have associated loss of this gene with higher invasive and metastatic potentials [139]. Therefore, this is a primary target for any cell in order to become metastatic.

5.4. β-Catenin

β-Catenin is predominantly regulated through the Wnt signalling pathway, playing an important role in both the EMT and tumour progression [140]. There are normally two pools of β-catenin: one is bound to E-cadherin in the adherens complex, and another occurs in the cytosol [140]. In the absence of Wnt signalling, glycogen synthase kinase 3β (GSK-3β) phosphorylates cytosolic β-catenin, marking it for proteasomal degradation [141]. When the signalling molecule, "Wnt ligand", binds to its receptor, frizzled, B-cell lymphoma 9 (BCL-9) removes β-catenin from the adherens junction [142]. This leads to the destabilisation of the latter [142]. Furthermore, the binding of Wnt ligand to frizzled receptors also results in the inactivation of GSK-3β and consequently prevents β-catenin degradation [143,144]. This allows β-catenin to accumulate and translocate to the nucleus, where it binds to transcription factor 7/lymphoid enhancer-binding factor 1 (TCF/LEF1) to form an oncogenic transcription factor complex [144] (Fig. 8) that is able to up-regulate N-*myc*, c-*myc* and cyclin D1 to aid in cell cycle progression [145]. Mutations in a variety of β-catenin regulatory sequences, as well as the aberrant nuclear expression of this molecule, have been found in a number of cancers as well as experimentally induced tumours [146,147] highlighting its oncogenic potential.

5.5. E-cadherin

Loss of E-cadherin expression is one of the key indicators of EMT and is associated with elevated metastasis in breast, gastric, pancreatic and lung carcinomas [148]. Expression of this protein is regulated during the EMT through the repression of the E-cadherin promoter, either directly or indirectly (Fig. 8). For example, the Snail family of transcription factors, consisting of Snail and Slug, were shown to bind directly to the E-box consensus sequences in the promoter region, repressing E-cadherin expression [149]. While Snail and Slug both act *via* very similar mechanisms, Slug is a much less potent inhibitor of E-cadherin expression and has fewer target genes than Snail [150]. In particular, Snail has been reported to interact with nuclear β-catenin [151], promoting activation of Wnt target genes and further driving EMT. Therefore, the regulation of E-cadherin, and subsequently the EMT, is largely affected by the regulation of these transcriptional repressors.

Transcriptional regulation of Snail and Slug is a result of transforming growth factor β (TGF-β) signalling [152–155]. TGF-β mediated phosphorylation of mothers against decapentaplegic homolog 2 and 3 (SMAD2/3) enables complex formation with SMAD4 [156]. This complex can then translocate into the nucleus and act as a *Snail* transcription factor [157]. However, it is thought that Snail binds and represses its own promoter [158], creating a negative feedback loop. It has also been demonstrated that Snail expression is increased by NF-κB [159], which is then up-regulated by Snail [160], producing a self-stimulatory, amplification loop

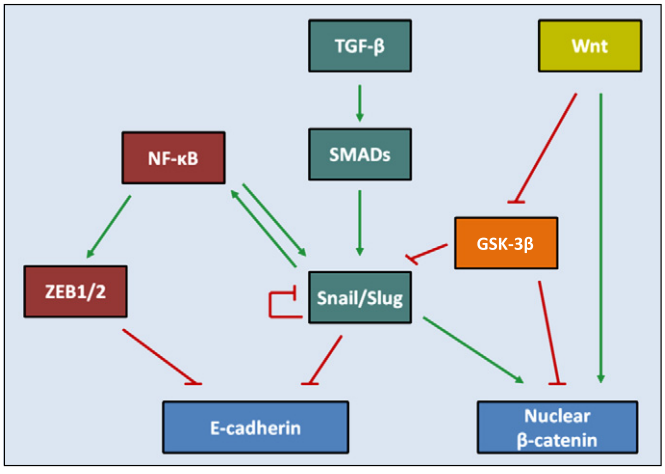

Fig. 8. Pathways involved in loss of adherens complex. Wnt signalling dissociates β-catenin from the adherens complex, allowing its translocation into the nucleus to act as an oncogenic factor. TGF-β signalling initiates the SMAD complex, enabling transcriptional activation of Snail and Slug, which repress E-cadherin transcription, while Snail also enhances nuclear β-catenin expression. GSK-3β phosphorylation prevents nuclear localisation of both Snail and β-catenin, while NF- B regulates the auto-inhibition of Snail. NF-κB also up-regulates ZEB1 and 2, which inhibit E-cadherin transcription.

of E-cadherin repression. NF-κB has also demonstrated the ability to increase expression of Zeb1 and Zeb2, both of which are transcription factors that repress E-cadherin expression [161].

The activity of Snail and Slug can also be regulated through post-translational modifications, predominantly their phosphorylation state, which determines their nuclear localisation or degradation [155]. Phosphorylation of Snail by GSK-3β on Ser 104 and 107 facilitates translocation out of the nucleus and subsequent degradation [162], whereas *C*-terminus phosphorylation by p21 protein-activated kinase 1 retains nuclear localisation [163]. Similarly, Slug degradation is triggered by the complex formation of p53, the ubiquitin ligase Mdm2, and Slug itself [164]. Ultimately, the dys-regulation of these transcriptional repressors initiates the loss of adherens complexes, leading to an oncogenic progression into metastasis.

6. The iron-regulated metastasis suppressor, NDRG1

As previously mentioned, NDRG1 is a metastasis suppressor that is ubiquitously expressed, predominantly in the epithelial tissue of the brain, gastro-intestinal tract, kidneys and prostate [9]. It was originally shown to be markedly up-regulated during colon epithelial cell differentiation [165]. However, NDRG1 was also down-regulated in colorectal cancer cell lines *in vitro* [165] and later studies indicate a negative correlation between NDRG1 expression and metastasis, both *in vitro* and *in vivo* [45,46]. Expression of NDRG1 in severely immunodeficient mouse models completely inhibited highly metastatic prostate cancer cells colonising the lung, yet it had no effect on the growth of the primary tumour [166]. Similarly, *NDRG1* mRNA levels in colon cancer metastases were significantly lower than their primary cancer cells, suggesting that the loss of the *NDRG1* gene allowed these cells to metastasise [167]. In addition, there seems to be a negative correlation between NDRG1 expression and the Gleason grading of prostate tumours and patient survival [166]. Analysis of tumour specimens from prostate and breast cancer patients supported this correlation, with reduced NDRG1 expression associated with lower cancer survival [168]. This indicates the potential use of NDRG1 as a predictive marker for tumour progression.

Notably, NDRG1 up-regulation has been reported in hepatic, pancreatic and kidney cancers, while it is reduced in prostate, breast, colon and oesophageal cancers [169]. The specific mechanisms for these observations are unclear, but there are a range of different factors that regulate NDRG1 expression and could be the cause of such variations. For example, hypoxia, ER stress, DNA damage and iron chelation are known to alter *NDRG1* transcription and translation [8,9], while HIF-1α, p53, EGR1, androgens, c-*myc* and N-*myc* can bind the NDRG1 promoter region to directly modulate its expression [170–172].

6.1. HIF-1-dependent and -independent regulation of NDRG1

NDRG1 expression is strongly up-regulated in a hypoxic cellular environment, in part, *via* HIF-1, with HIF-1-binding sites on the promoter region of the *NDRG1* gene [8,173] (Fig. 9). HIF-1 has been observed to play a regulatory role in NDRG1-mediated cellular differentiation, with normal myeloid cell differentiation into monocytes correlating with high HIF-1 accumulation [174]. Additionally, both mRNA and protein expression of NDRG1 were significantly higher in the leukaemia cell line U973 during elevated HIF-1 levels [175]. NDRG1 expression is also thought to aid in the survival of normal cells during exposure to hypoxic stress [169]. However, NDRG1 regulation has also been revealed to be HIF-1-dependent in cancer cells exposed to hypoxia [176]. Furthermore, Le and Richardson [8] demonstrated that iron chelators mimic hypoxia, resulting in a marked increase in NDRG1 expression *via* HIF-1α-dependent mechanisms. The increase in NDRG1 expression was then shown to be reversed through iron-repletion [8], elucidating a promising target for anti-cancer therapies. The suggested mechanism through which this regulation occurs involves association of the constitutively

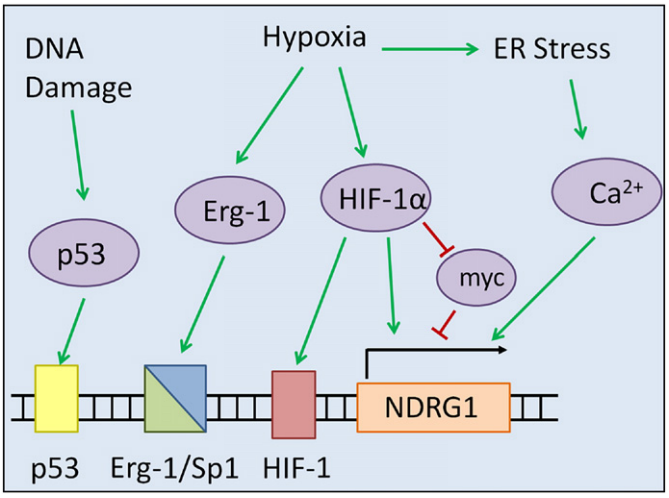

Fig. 9. Transcriptional regulation of NDRG1. Various environmental stressors activate transcription factors that enhance mRNA and protein levels of NDRG1 by acting on both the promoter and upstream of the promoter. For instance, p53, which is induced by DNA damage, can bind to the NDRG1 promoter and enhance its expression. In addition, Erg-1 and HIF-1α, both of which are up-regulated in response to hypoxia, can also bind to the NDRG1 promoter to up-regulate its expression. HIF-1α also functions to inhibit a suppressor of NDRG1, namely the myc family of proteins. ER stress pathways, through their ability to increase calcium (Ca^{2+}) levels, also promote NDRG1 expression.

expressed HIF-1β subunit and a hypoxia-regulated HIF-1α subunit, which dimerise in the presence of low oxygen concentrations or so-called hypoxia mimetics [177]. The assembled HIF-1 complex can then bind to the hypoxia-response element in the *NDRG1* promoter region and activate its transcription [173]. This was supported by studies of *HIF-1α*$^{-/-}$ MEFs, in which there was no induction of NDRG1 by hypoxia [178]. Importantly, the activity of the *myc* family of NDRG1 suppressors were, in turn, suppressed by HIF-1α [179,180] (Fig. 9).

However, high NDRG1 expression does not always correspond with elevated HIF-1α. NDRG1 levels remain raised significantly longer upon the return to normoxic conditions, compared to HIF-1α [173] and could simply suggest NDRG1 is a more stable protein. However, significantly higher expression of NDRG1 has been reported in some cancer cells compared to normal cells, while no such difference exists for HIF-1α [173], suggesting there are HIF-1 independent pathways. Epithelial growth response-1 (Egr-1) is another hypoxia regulated transcription factor that binds to an overlapping *Egr-1/Sp1* binding site in the NDRG1 promoter [170] (Fig. 9). Egr-1 over-expression in MEFs positively regulates the *NDRG1* gene [170]. Similarly, p53 also has a binding site in the promoter region which may increase NDRG1 expression in response to DNA damage [171]. A rise in intracellular Ca^{2+} induced by Ni^{2+} compounds, and the resulting oxidative stress, has also been suggested to activate *NDRG1* transcription [181]. However, despite this range of effectors, NDRG1 expression is down-regulated in multiple cancers, particularly in prostate, breast, colon and oesophageal cancer [169]. This is primarily achieved through the up-regulation of oncogenes N-*myc* and c-*myc* in some cancers leading to the suppression of NDRG1 activity and expression [182]. Significantly, these cancer cells have exhibited increased metastatic potential [182].

While the previous regulators have all impacted expression at a transcriptional level, a recent study by Lane et al. [120] has identified a mechanism of NDRG1 translational regulation *via* eIF3a. Cellular stress, induced by conditions such as hypoxia and redox-active species, result in the formation of stress granules in the cytoplasm that act to prioritise the translation of specific proteins [183]. Under iron chelator-induced cellular stress, eIF3a was shown to prioritise the translation of *NDRG1* mRNA, while in the absence of eIF3a, *NDRG1* was still translated, but at a greatly reduced rate [120]. Hence, NDRG1 expression is regulated by a number of distinct molecular mechanisms.

6.2. Cellular proliferation and NDRG1

NDRG1 has been shown to be an effective regulator of cellular proliferation, both in the cell cycle and in up-stream oncogenic signalling pathways [11,184,185]. Studies have shown NDRG1 to be a microtubule-associated protein that maintains spindle structure during cell division in a p53-dependent manner, ensuring an appropriate number of chromosomes in each daughter cell [186]. Expression of NDRG1 throughout the cell cycle is biphasic, with NDRG1 levels peaking in the G_1 and G_2/M phases, while being lowest in the S phase [184]. However, inhibited proliferation and slower growth rates were observed at G_1 and G_2 phases in human cervical cancer cells transfected to over-express NDRG1 [184], highlighting a novel role of NDRG1 as a potential cell cycle inhibitor. In contrast, in other cell-types, NDRG1 expression had no effect on cell cycle distribution [187].

Recently, NDRG1 has been shown to regulate a number of growth pathways, often utilised by cancer cells to induce proliferation. Of particular note is the Ras pathway, which has been found to be constitutively active in up to 25% of all human cancers and up to 90% in pancreatic cancer [188]. NDRG1 up-regulation in prostate and pancreatic cancer cells was shown to interfere with this pathway by inhibiting the phosphorylation, and activation, of extracellular-signal-regulated kinase (ERK) [11,185]. Additionally, NDRG1 has been shown to up-regulate the tumour-suppressive phosphatase and tensin homologue deleted on chromosome 10 (PTEN) which, in turn, inhibits phosphorylated protein kinase B (pAKT), a well-known apoptotic inhibitor [11,185]. The integration of NDRG1 in a number of pathways involved in cell survival and proliferation makes it a promising target in anti-cancer therapies.

6.3. Cellular differentiation and NDRG1

NDRG1 was initially identified as a gene involved in differentiation which was later supported by a number of studies [189]. Increased NDRG1 expression has been observed during keratinocyte differentiation, as well as embryonic organ formation and maturation [166,189]. Increased NDRG1 expression has also been shown to induce cellular differentiation in metastatic prostate and colon cancers [166,167], with this function now being correlated with its suppressive effects on migration and invasion [45,46]. NDRG1-induced cellular differentiation has demonstrated an ability to reverse morphological changes of EMT in the initial stages of metastasis [10]. Similarly, induced differentiation in rat peripheral glioma cells resulted in the halting of cellular proliferation, while simultaneously, N-*myc* expression was reduced and NDRG1 expression was significantly increased [190]. This highlights the potential for NDRG1 to be a novel target for anti-cancer therapeutics as an inducer of differentiation.

6.4. The EMT and NDRG1

As previously stated, EMT is an important initiating step in cancer migration and invasion. Correctly functioning intercellular E-cadherin-β-catenin adhesion complexes are crucial for the suppression of this metastatic process and have been shown to be maintained by NDRG1 (Fig. 10). In fact, NDRG1-silenced metastatic cells show a corresponding failure to express E-cadherin, while NDRG1 over-expression results in high E-cadherin levels [10]. This is, in part, due to the maintenance of E-cadherin through NDRG1 acting as a Ras-related protein Rab-4A effector protein, preventing E-cadherin endosomal degradation [191]. NDRG1 also preserves E-cadherin expression through regulation of the TGF-β/SMAD EMT pathway with NDRG1 over-expression in prostate cancer cells having exhibited a significant inhibition of this pathway *via* a reduction in SMAD2 and p-SMAD3 expression [10]. Conversely, NDRG1 silenced cancer cells had increased SMAD2, pSMAD3 and SMAD4 expression resulting in down-stream E-cadherin promoter repression *via* Slug/Snail [10].

Interestingly, it has been recently shown that the chelators, DFO and Dp44mT, inhibit the TGF-β-induced EMT by maintaining E-cadherin and β-catenin at the cell membrane *via* the up-regulation of NDRG1 [10]. Overexpression of NDRG1 maintained membrane E-cadherin and β-catenin and inhibited TGF-β-stimulated cell migration and invasion [10]. Conversely, NDRG1 knock-down induced the EMT and enhanced TGF-β effects [10]. Furthermore, the TGF-β/SMAD and Wnt pathways were implicated in NDRG1 regulation of E-cadherin and β-catenin expression [10,128]. Hence, chelators inhibit the TGF-β-induced EMT *via* NDRG1 up-regulation, providing a therapeutic modality to inhibit

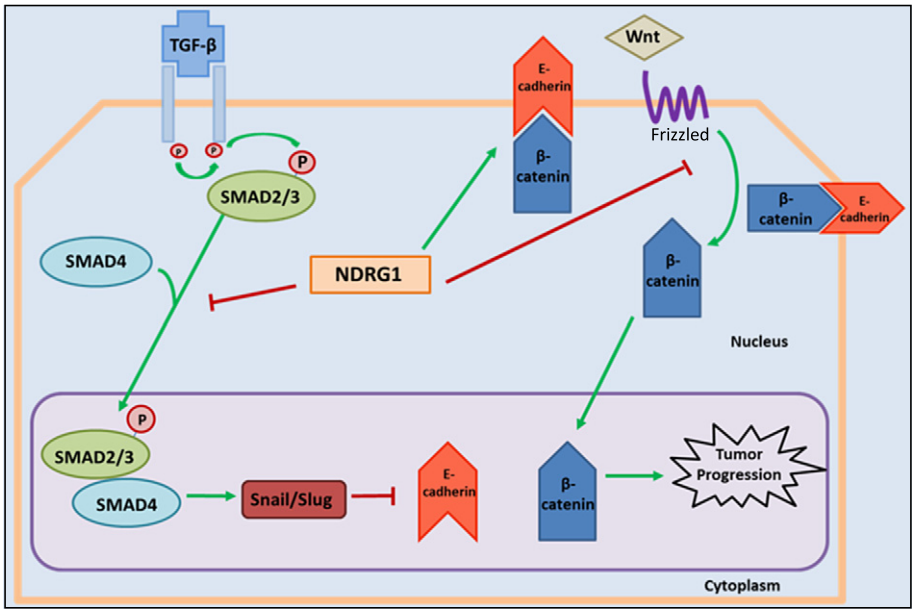

Fig. 10. NDRG1 prevents EMT by inhibiting the loss of the adherens complex. NDRG1 expression inhibits the TGF-β/SMAD pathway, preventing Snail/Slug-dependent repression of E-cadherin. Moreover, NDRG1 can associate with the Wnt co-receptor, low density lipoprotein receptor-related protein (LRP6), inhibiting Wnt signalling, thereby preventing the translocation of β-catenin to the nucleus where it stimulates cell cycle progression. Considering Wnt suppression also stabilises the E-cadherin–β-catenin complex, NDRG1-mediated Wnt signalling inhibition also maintains membrane β-catenin, preventing β-catenin dissociation and EMT development.

metastasis [10], with studies by others *in vivo* confirming this observation [128].

A recent study by Sun et al. [76] showed NDRG1 is also able to inhibit stress fibre assembly, which is an integral part of cancer cell motility following metastatic activation. NDRG1 over-expressing cancer cells have shown impaired activity in the Rho-associated, coiled-coil containing protein kinase 1/phosphorylated-myosin light chain pathway that is key to stress fibre formation. In contrast to the epithelial morphology maintained by E-cadherin, these stress fibres promote the mesenchymal morphology important for metastasis [192].

NDRG1 is also a key regulator of Wnt signalling during metastatic events such as extravasation, invasion and colonisation. NDRG1 can associate with the Wnt co-receptor, low density lipoprotein receptor-related protein (LRP6), effectively suppressing Wnt signalling, and inhibiting Wnt/β-catenin metastatic progression [128]. Since Wnt suppression enables the stability of the E-cadherin–β-catenin complex [143], this pathway acts as another target for NDRG1 to inhibit EMT.

7. Conclusions

Iron is essential for normal cellular function and, due to their rapid cell growth and proliferation, neoplastic cells have iron demands that exceed that for most non-malignant cells. It is apparent that "single-target" cancer therapeutics will always be faced with the spectre of the development of therapeutic resistance. Thus, the targeting of cancer cell iron metabolism with rationally designed iron-binding ligands represents a potentially more robust anti-cancer strategy. This results from the fact that targeting cellular iron generates an effective multitude of down-stream molecular targets. Indeed, while classical iron chelators are restricted to the depletion of cellular iron, novel thiosemicarbazone iron chelators that act by a "double punch" mechanism to both bind intracellular iron and promote intracellular redox cycling reactions hold much therapeutic promise as anti-neoplastic agents. In addition to cellular iron depletion and induction of oxidative stress, recent data indicate that these novel chelators can act to inhibit tumour growth by: (1) regulating the PERK/eIF2α axis of the ER-stress response that either leads to the UPR and/or apoptosis in response to cellular stressors [85]; and (2) inhibiting the epithelial–mesenchymal transition [10], which is necessary for metastasis and possibly the formation of cancer stem cells [193]. The ability of iron chelators to regulate these processes appears to be at least partially dependent on NDRG1, which may act as a master-regulator of numerous signalling pathways that are involved in tumour progression. Obtaining a better understanding of this increasingly intricate web of interactions will undoubtedly enhance our ability to refine the structures of novel iron-binding therapeutics in order to more potently target keystone molecules such as NDRG1 and p53.

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