

**Keywords:** prostate cancer; iron chelators; NDRG1; PTEN; AKT; SMAD2

# Dp44mT targets the AKT, TGF- $\beta$ and ERK pathways via the metastasis suppressor NDRG1 in normal prostate epithelial cells and prostate cancer cells

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**Background:** Effective treatment of prostate cancer should be based on targeting interactions between tumour cell signalling pathways and key converging downstream effectors. Here, we determined how the tumourigenic phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), tumour-suppressive phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and transforming growth factor- $\beta$  (TGF- $\beta$ ) pathways are integrated via the metastasis suppressor, N-myc downstream-regulated gene-1 (NDRG1). Moreover, we assessed how the novel anti-tumour agent, Dp44mT, may target these integrated pathways by increasing NDRG1 expression.

**Methods:** Protein expression in Dp44mT-treated normal human prostate epithelial cells and prostate cancer cells (PC-3, DU145) was assessed by western blotting. The role of NDRG1 was examined by transfection using an NDRG1 overexpression vector or shRNA.

**Results:** Dp44mT increased levels of tumour-suppressive PTEN, and decreased phosphorylation of ERK1/2 and SMAD2L, which are regulated by oncogenic Ras/MAPK signalling. Importantly, the effects of Dp44mT on NDRG1 and p-SMAD2L expression were more marked in prostate cancer cells than normal prostate epithelial cells. This may partly explain the anti-tumour selectivity of these agents. Silencing NDRG1 expression increased phosphorylation of tumourigenic AKT, ERK1/2 and SMAD2L and decreased PTEN levels, whereas NDRG1 overexpression induced the opposite effect. Furthermore, NDRG1 silencing significantly reduced the ability of Dp44mT to suppress p-SMAD2L and p-ERK1/2 levels.

**Conclusion:** NDRG1 has an important role in mediating the tumour-suppressive effects of Dp44mT in prostate cancer via selective targeting of the PI3K/AKT, TGF- $\beta$  and ERK pathways.

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Prostate cancer is the most frequently diagnosed non-cutaneous cancer in men (Jemal *et al*, 2009). However, effective chemotherapeutic options are limited due to drug resistance and toxicity (Lee *et al*, 2008), and therefore potent and specifically targeted therapies are required. Prostate cancer is a highly heterogeneous disease with many points of disruption in cell signalling (Assinder *et al*, 2009). Three such pathways are the tumourigenic phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), tumour-suppressive phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and transforming growth factor- $\beta$  (TGF- $\beta$ ) pathways (Assinder *et al*, 2008, 2009). Several points of integration appear to occur between these pathways, with N-myc downstream-regulated gene-1 (NDRG1) being a possible common point of cross-talk (Assinder *et al*, 2008, 2009).

NDRG1 has a range of biological functions (Kovacevic and Richardson, 2006), including that NDRG1 upregulation has a critical role in preventing tumour growth and metastasis (Bandyopadhyay *et al*, 2003, 2004a, b). While NDRG1 is widely expressed in normal tissues (Lachat *et al*, 2002), its levels are significantly lower in various cancers (Guang *et al*, 2000; Bandyopadhyay *et al*, 2003, 2004a). In contrast, elevated levels of active (phosphorylated) AKT (p-AKT) are correlated with poor prostate cancer prognosis (Samuels and Ericson, 2006), while in the normal prostate its level is very low (Assinder *et al*, 2009). The major tumour-suppressive activity of PTEN is via AKT pathway antagonism (Cantley and Neel, 1999), with opposite effects on proliferation and survival. Approximately 50% of prostate cancer cases display loss of PTEN (Facher and Law, 1998) and re-expression of normal PTEN in prostate cancer cells induces apoptosis (Davies *et al*, 1999).

PTEN expression can be induced by TGF- $\beta$  through the canonical mothers against decapentaplegic homologue (SMAD)-dependent pathway, involving phosphorylation of SMAD homologue 2 (SMAD2) at the COOH-terminal residues Ser<sup>465/467</sup> (p-SMAD2C) to stimulate a tumour-suppressive response (Chow *et al*, 2007). In addition, SMAD activation suppresses oncogenic c-Myc (Massague *et al*, 2000) and anti-apoptotic Bcl-2 expression (Guo and Kyprianou, 1999), but increases expression of the cyclin-dependent kinase inhibitors: p15, p21 and p27 (Guo and Kyprianou, 1998).

Transforming growth factor- $\beta$ /SMAD signalling is negatively regulated by the Ras/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade, which is induced by epidermal growth factor receptor signalling (Kretzschmar *et al*, 1999). Specifically, Ras signalling inhibits TGF- $\beta$ -induced nuclear accumulation of the SMAD complex by phosphorylating SMAD2 at the linker region at Ser<sup>245/250/255</sup> (p-SMAD2L) (Kretzschmar *et al*, 1999). In normal epithelial cells, TGF- $\beta$  overcomes the proliferative effects of Ras-activating factors (Kretzschmar *et al*, 1999), indicating that Ras-mediated SMAD2 phosphorylation acts to modulate TGF- $\beta$ /SMAD signalling. However, in cancer cells where Ras is hyper-activated by oncogenic mutations, this same mechanism could silence the tumour-suppressive functions of TGF- $\beta$ /SMAD signalling and promote tumour progression (Kretzschmar *et al*, 1999). Thus, inhibiting SMAD2L phosphorylation may result in a novel therapeutic strategy.

Interestingly, NDRG1 is upregulated by PTEN in the PC-3 and DU145 prostate cancer cell lines via suppression of the AKT pathway (Bandyopadhyay *et al*, 2004b). NDRG1 is also markedly upregulated by cellular iron depletion induced by chelators such as desferrioxamine (DFO; Supplementary Figure 1A) (Merlot *et al*, 2012), via hypoxia inducible factor-1 $\alpha$  (HIF1 $\alpha$ )-dependent and -independent pathways (Le and Richardson, 2004). Novel thiosemicarbazone chelators such as those of the dipyriddy thiosemicarbazone (DpT) class (e.g., di-2-pyridyl ketone-4,4-dimethyl-3-thiosemicarbazone, Dp44mT; Supplementary Figure 1B) demonstrate

potent and selective anti-tumour activity (Yuan *et al*, 2004; Whitnall *et al*, 2006; Kovacevic *et al*, 2011a). Their mechanism of action has been demonstrated to be due to the ability to bind cellular iron and form iron and copper complexes that generate cytotoxic radicals (Yuan *et al*, 2004; Richardson *et al*, 2006; Lovejoy *et al*, 2011; Merlot *et al*, 2012). In addition, Dp44mT inhibits topoisomerase (II) $\alpha$  (Rao *et al*, 2009) and upregulates NDRG1 expression (Le and Richardson, 2004), which inhibits metastasis *in vivo* (Liu *et al*, 2012).

Interestingly, iron chelation upregulates TGF- $\beta$  that mediates its anti-proliferative activity through p27<sup>kip1</sup> (Yoon *et al*, 2002) and also inhibits TGF- $\beta$ -induced epithelial-mesenchymal transition via upregulation of NDRG1 (Chen *et al*, 2012). Moreover, NDRG1 upregulates neural precursor cell expressed developmentally downregulated 4-like (NEDD4L), which modulates the TGF- $\beta$  pathway (Kovacevic *et al*, 2012). These latter studies also show that NDRG1 upregulates the tumour suppressors, PTEN and SMAD4 (Kovacevic *et al*, 2012).

We hypothesised that the tumour-suppressive effects of iron chelators in prostate cancer are mediated by the integration of the AKT/PTEN and TGF- $\beta$  cell signalling pathways via NDRG1. To test this idea, we specifically investigated the effects of DFO and Dp44mT on the AKT/PTEN and TGF- $\beta$  pathways in prostate cancer cell lines relative to normal prostate epithelial cells (PrECs). Further, we assessed whether differences in signalling could explain the marked selectivity of these ligands against tumour cells relative to normal cells (Whitnall *et al*, 2006; Kovacevic *et al*, 2011a, 2012; Liu *et al*, 2012).

## MATERIALS AND METHODS

**Cell treatments.** The chelator, Dp44mT, was synthesised using standard methods (Richardson *et al*, 2006), while DFO was purchased from Novartis (Basel, Switzerland). Dp44mT was dissolved in DMSO at 10 mM and then diluted in media containing 10% (v/v) fetal bovine serum (Sigma-Aldrich, New South Wales, Australia) so that the final (DMSO) was  $\leq 0.1\%$  (v/v). Dp44mT was used at a final concentration of 2.5  $\mu\text{M}$  in media, while DFO was used at a concentration of 250  $\mu\text{M}$  for most studies, but also at 50, 100, 150 and 200  $\mu\text{M}$  in dose-response experiments. IGF-1 (BioVision Inc., CA, USA) was utilised at 50 ng ml<sup>-1</sup> and TGF- $\beta$  (R&D Systems, MN, USA) was used at 10 ng ml<sup>-1</sup>.

**Cell lines and culture.** Primary cultures of normal human PrECs (Lonza Australia Pty. Ltd., Victoria, Australia) were grown and maintained in prostate epithelial growth medium (Lonza). The DU145 and PC-3 human prostate cancer cell lines (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 IU ml<sup>-1</sup>), streptomycin (100  $\mu\text{g}$  ml<sup>-1</sup>), glutamine (2 mM), non-essential amino acids (100 mM) and sodium pyruvate (100 mM; all supplements from Life Technologies, Victoria, Australia). The PC-3 cells stably transfected with PTEN (PC-3-PTEN) (Zhao *et al*, 2004) were obtained from Dr D LeRoith (NIH, MD, USA).

**Plasmid construction and transfection.** For NDRG1 overexpression, we used the pCMV-tag2-FLAG-NDRG1 vector (GenHunter, Nashville, TN, USA) and the empty vector (pCMV-tag2-FLAG; Stratagene, Santa Clara, CA, USA) as a control. Both plasmids contained a G418 resistance marker. The shRNA and scrambled control plasmids were from Qiagen (cat. no.: KH02202H; Valencia, CA) and contained a hygromycin resistance marker. All cells were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol. Cells were selected by incubation with G418 (400  $\mu\text{g}$  ml<sup>-1</sup>; Alexis Biochemicals, CA, USA) for overexpression clones or hygromycin (500  $\mu\text{g}$  ml<sup>-1</sup>; Roche Diagnostics,

Mannheim, Germany) for knock-down clones for a period of 4 weeks.

**Flow cytometry.** Cell cycle analysis was performed by flow cytometry using standard techniques (Yao *et al*, 2012).

**Cellular proliferation assay.** Proliferation was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay and confirmed by viable cell counts using Trypan blue (Kovacevic *et al*, 2011b).

**Protein extraction and western analysis.** Western blots were performed using established procedures (Chen *et al*, 2012). Primary antibodies to PTEN (1:1000), p-NDRG1 (Ser<sup>330</sup>; 1:1000), p-NDRG1 (Thr<sup>346</sup>; 1:1000), p-mTOR (Ser<sup>2448</sup>; 1:1000), SMAD2 (1:1000), p-SMAD2L (Ser<sup>245/250/255</sup>; 1:1000), p-SMAD2C (Ser<sup>465/467</sup>; 1:1000), p-ERK1/2 (1:2000), ERK1/2 (1:2000) were from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies to p-AKT<sup>1/2/3</sup> (Ser<sup>473</sup>; 1:400), AKT<sup>1/2/3</sup> (1:400) and cyclin D1 (1:1000) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody to NDRG1 (1:6000) was from Abcam (Cambridge, MA, USA), while the antibody to  $\beta$ -actin (1:10 000) was from Sigma-Aldrich. Protein band densitometry was measured using Image J (NIH, Bethesda, MD, USA).

**Statistical analysis.** Statistical analyses were carried out using Graphpad Prism (GraphPad Software Inc., La Jolla, CA, USA). Significant differences were determined by one-way analysis of variance followed by Dunnett's post-test. Significant differences between treatment groups were determined by analysis of variance followed by the Tukey-Kramer post-test.

## RESULTS

To characterise the integration of the tumourigenic PI3K/AKT and the tumour-suppressive PTEN and TGF- $\beta$  pathways via NDRG1, we compared primary cultures of normal human PrECs with the well-characterised prostate cancer cell lines, PC-3 and DU145. Of relevance, PC-3 and DU145 cells were compared owing to their molecular heterogeneity in these signalling pathways. In fact, PC-3 does not express PTEN (Vlietstra *et al*, 1998), which antagonises p-AKT levels (Assinder *et al*, 2009) and this difference was used to examine the integration between PTEN and p-AKT, as well as the effects of the chelators on these pathways.

Cells were incubated over 24 h at 37 °C with the iron chelators, DFO (250  $\mu$ M) or Dp44mT (2.5  $\mu$ M). Under these conditions, the ligands have been shown to inhibit iron uptake from the iron-binding protein, transferrin, and increase iron release from cells to induce iron deprivation (Richardson *et al*, 1994; Yuan *et al*, 2004). As a positive control for the depletion of cellular iron pools, the effect of the chelators was examined on cell cycle distribution after a 24-h incubation (Supplementary Table 1A). This was done as these compounds are known to induce a G<sub>1</sub>/S arrest upon iron depletion (Noulsri *et al*, 2009). As shown previously, the fraction of PC-3 and DU145 cells in the G<sub>0</sub>/G<sub>1</sub> phase was significantly ( $P < 0.01$ ) increased, while the proportion in S phase significantly ( $P < 0.01-0.05$ ) decreased after incubation with DFO or Dp44mT (Noulsri *et al*, 2009) (Supplementary Table 1A), demonstrating inhibition of cell cycle progression. In clear contrast, no significant alterations to cell cycle distribution were observed in normal PrEC cells following incubation with the chelators (Supplementary Table 1A). Moreover, proliferation assays demonstrated that the IC<sub>50</sub> values for DFO or Dp44mT measured after a 72-h incubation with PrEC cells were markedly and significantly ( $P < 0.001-0.01$ ) higher than the values for PC-3 and DU145 prostate cancer cells (Supplementary Table 1B), which is consistent with studies demonstrating the selective anti-tumour activity of these agents (Whitnall *et al*, 2006).

In all studies herein, a higher concentration of DFO was implemented due to its limited ability to permeate membranes (Richardson *et al*, 1994). The chelator Dp44mT was utilised at a lower concentration because this ligand shows high membrane permeability and demonstrates marked iron chelation efficacy in cultured cells (Yuan *et al*, 2004).

**Iron chelators increase expression of NDRG1 and its phosphorylation.** Initial studies showed that incubation of normal PrECs (Figure 1A) with either DFO or Dp44mT significantly ( $P < 0.01-0.05$ ) increased levels of NDRG1 relative to the control. Notably, the same treatment with DFO or Dp44mT in prostate cancer cells had a more pronounced effect on NDRG1 expression than in PrECs when specifically comparing the treatment and control groups, significantly increasing its levels in PC-3 ( $P < 0.001$ ) and DU145 ( $P < 0.01$ ) cells (Figure 1B and C). Interestingly, one NDRG1 band only (~44 kDa) was observed in PrECs (Figure 1A), while two bands were found for NDRG1 in PC-3 and DU145 cells at ~43 kDa and ~44 kDa (Figure 1B and C). In PC-3 cells, the ~44 kDa band was markedly more abundant and regulated after incubation with iron chelators, with the lower ~43 kDa band being very faint (Figure 1B) or difficult to detect in other blots. In contrast, in DU145 cells, both NDRG1 bands were usually detectable and each was increased after incubation with DFO or Dp44mT (Figure 1C). The exact identity of these two NDRG1 bands remains unclear, but they have been suggested to correspond to the different phosphorylation states of the protein (Sugiki *et al*, 2004). Considering this, the phosphorylation of NDRG1 at Ser<sup>330</sup> and Thr<sup>346</sup> was examined and found also to be significantly ( $P < 0.01-0.05$ ) increased after incubation with DFO or Dp44mT in PrECs (Figure 1A). Both DFO and Dp44mT significantly ( $P < 0.001$ ) increased NDRG1 phosphorylation in PC-3 and DU145 cells to a greater extent than in PrECs (Figure 1A-C). Furthermore, the effect of DFO on increasing NDRG1 and p-NDRG1 levels at Ser<sup>330</sup> and Thr<sup>346</sup> in PrEC, PC-3 and DU145 cells was generally concentration dependent (Supplementary Figure 2A-C).

**Iron chelators increase PTEN levels and influence AKT phosphorylation.** Next, we examined the effects of the iron chelators on PTEN and AKT signalling. Both DFO and Dp44mT significantly ( $P < 0.01-0.05$ ) increased the levels of tumour suppressor PTEN in PrEC and DU145 cells (Figure 1A and C). As expected, PTEN was undetectable in PC-3 cells due to the homozygous deletion of the *PTEN* gene (Vlietstra *et al*, 1998) (Figure 1B). PTEN levels also generally increased as a function of DFO concentration in both PrECs and DU145 cells (Supplementary Figure 3A and C).

Unexpectedly, both DFO and Dp44mT significantly increased p-AKT (Ser<sup>473</sup>) in PrECs and prostate cancer cells ( $P < 0.001$ ; Figure 1A-C), whereas there was no significant ( $P > 0.05$ ) alteration in levels of total AKT (Figure 1A-C). Notably, a higher DFO concentration (250  $\mu$ M) was required to significantly ( $P < 0.05$ ) increase p-AKT levels in PrECs, while concentrations as low as 100  $\mu$ M resulted in increased p-AKT in PC-3 and DU145 cells (Supplementary Figure 3A-C). Hence, the prostate cancer cells were more sensitive to the effects of the chelators than normal PrECs, which correlates to their relative lack of susceptibility to the anti-proliferative activity of these agents (Supplementary Table 1). Total AKT levels remained unaltered regardless of the DFO concentration (Supplementary Figure 3A-C).

**The effects of DFO and Dp44mT on NDRG1, PTEN and p-AKT are reversed by addition of the iron donor, ferric ammonium citrate (FAC).** As these iron chelators have clear anti-proliferative effects in cancer cells and can modify levels of NDRG1, PTEN and p-AKT, we investigated whether the ability of DFO and Dp44mT to increase the levels of these proteins was dependent on their

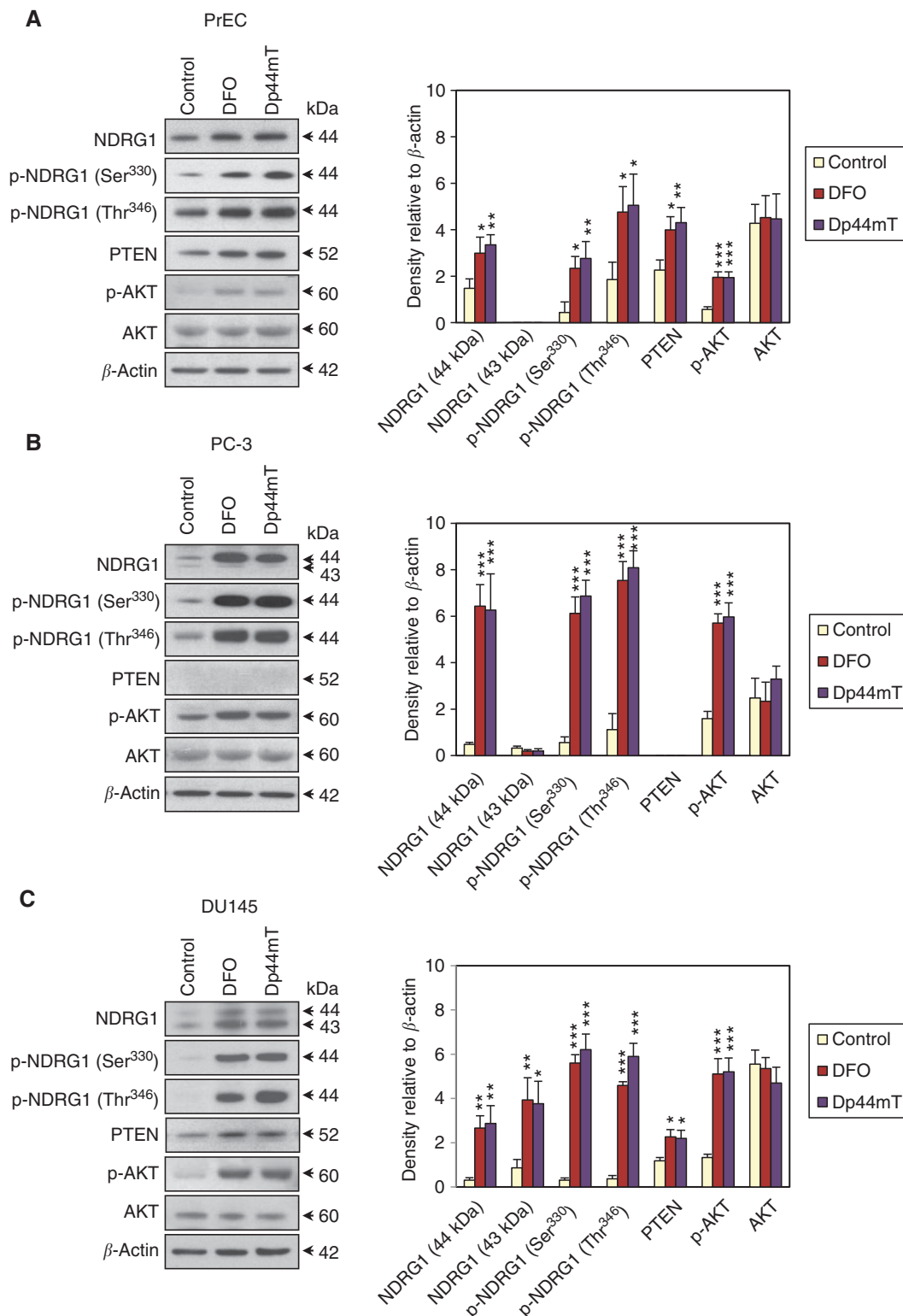
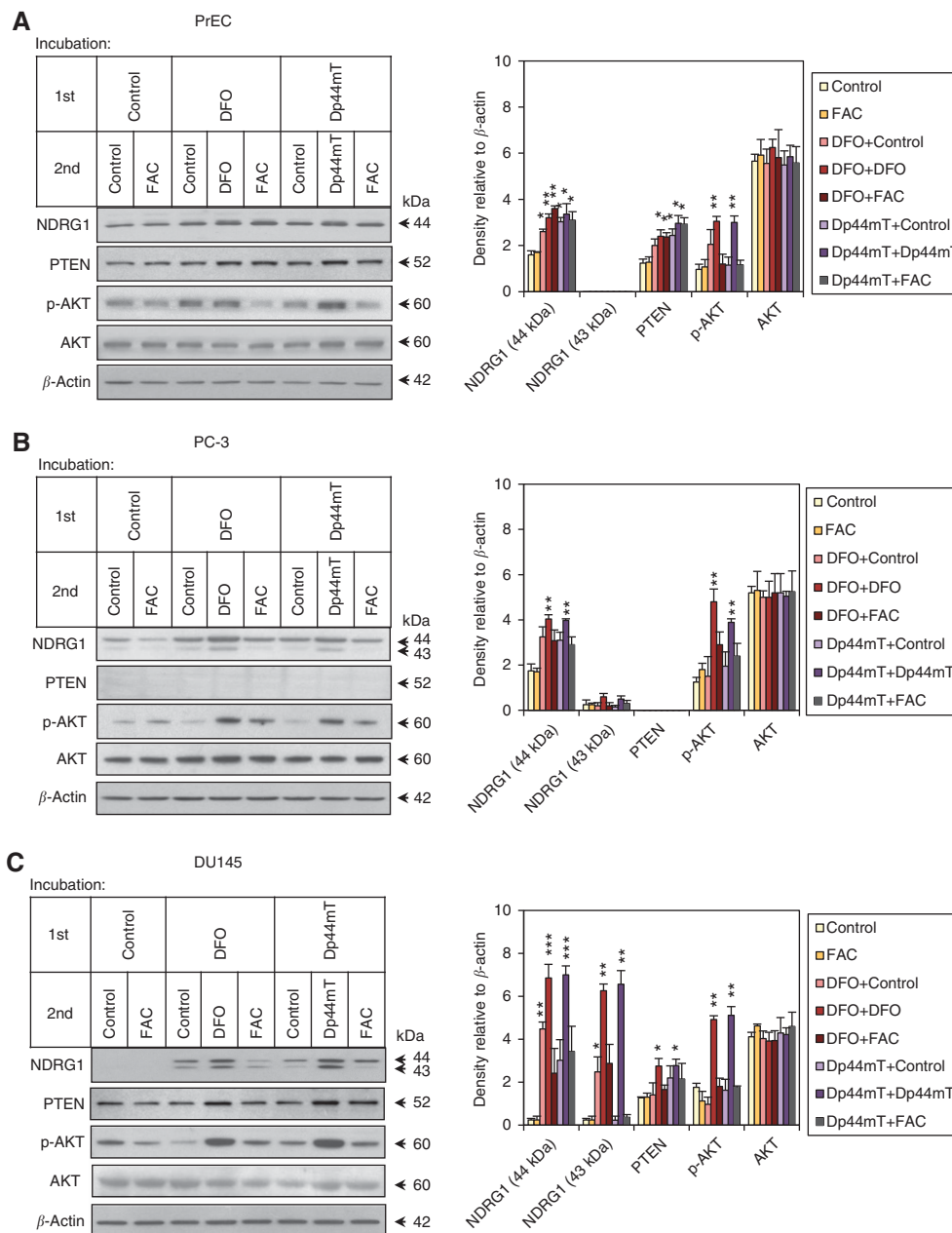


Figure 1. Increase of NDRG1, p-NDRG1 (Ser<sup>330</sup>), p-NDRG1 (Thr<sup>346</sup>), PTEN, p-AKT (Ser<sup>473</sup>) and AKT protein levels in (A) PrEC, (B) PC-3 and (C) DU145 cells after a 24 h incubation at 37 °C with control medium, DFO (250 μM) or Dp44mT (2.5 μM). Western blots are typical of three independent experiments, with densitometric analysis representing mean ± s.d. Relative to control: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

ability to chelate iron. The three cell types were incubated with either growth medium alone, or medium containing DFO or Dp44mT for 14h, followed by a second incubation with either RPMI, DFO, Dp44mT or the iron donor, FAC, for a further 14 h. Ferric ammonium citrate is well known to replenish intracellular iron levels after iron depletion (Le and Richardson, 2004), and

hence, it should reverse the effects of chelators if their activity is due to chelation of cellular iron pools.

Initial experiments examined PrECs and after the first incubation with DFO or Dp44mT followed by a second incubation with these chelators, there was a significant (P<0.01–0.05) increase in NDRG1 and PTEN expression relative to cells



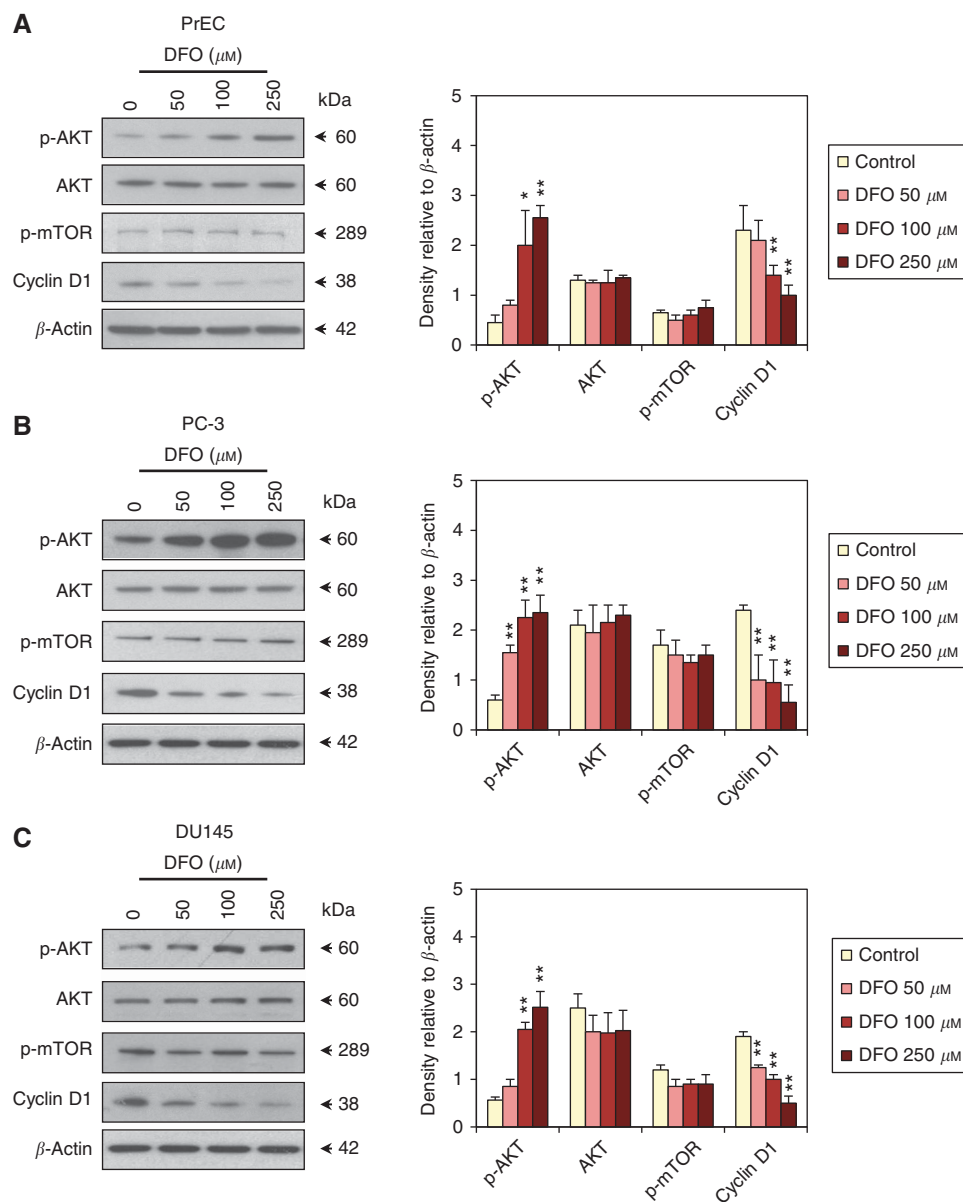
**Figure 2.** The effect of iron supplementation on the DFO- or Dp44mT-induced increase in expression of NDRG1, PTEN and p-AKT (Ser<sup>473</sup>) in (A) PrEC, (B) PC-3 and (C) DU145 cells. Cells were incubated first with either control medium, DFO (250  $\mu\text{M}$ ) or Dp44mT (2.5  $\mu\text{M}$ ) for 14 h at 37 °C and then secondly re-incubated with either media alone, DFO (250  $\mu\text{M}$ ), Dp44mT (2.5  $\mu\text{M}$ ) or the iron donor, FAC (100  $\mu\text{g ml}^{-1}$ ), for a further 14 h at 37 °C. Western blots are typical of three independent experiments, with densitometric analysis representing mean  $\pm$  s.d. Relative to control: \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

incubated with control media in both incubations (Figure 2A). There was no significant ( $P$ >0.05) reversal of the increase in NDRG1 or PTEN expression after re-incubation of chelator-treated PrEC cells with FAC (Figure 2A).

In contrast to PrECs, the significant ( $P$ <0.001–0.01) increase in NDRG1 levels by iron depletion induced after the first incubation of PC-3 and DU145 prostate cancer cells with DFO or Dp44mT was partially or fully reversed by the addition of FAC (Figure 2B and C). This observation agrees with previous studies showing NDRG1 is regulated by intracellular iron levels (Le and Richardson, 2004; Dong *et al*, 2005). The increase in PTEN induced by iron chelators in DU145 cells was also reversed by a subsequent incubation with FAC (Figure 2C). Similarly, the upregulation of p-AKT levels was reduced in all three

chelator-treated cell types when cellular iron levels were subsequently replenished with FAC (Figure 2A–C). For all cell types examined, there was no significant effect on total AKT levels for any of the treatments relative to the controls (Figure 2A–C).

**The DFO-induced increase in p-AKT does not cause activation of its downstream effectors.** To assess whether the increase in p-AKT induced by iron chelators (Figures 1 and 2) resulted in activation of its downstream effectors (e.g., phosphorylation of the mammalian target of rapamycin at Ser<sup>2448</sup> (p-mTOR) and increased expression of cyclin D1) (Assinder *et al*, 2009), cells were incubated for 24 h with increasing DFO concentrations (50, 100 or 250  $\mu\text{M}$ ; Figure 3). As observed above in all three cell types, DFO significantly ( $P$ <0.01–0.05) increased p-AKT levels, without



**Figure 3.** Incubation of cells with DFO (50, 100 and 250  $\mu\text{M}$ ) increases p-AKT (Ser<sup>473</sup>) levels, but in terms of its effect on downstream effectors, has no effect on p-mTOR (Ser<sup>2448</sup>), while decreasing cyclin D1 expression in (A) PrEC, (B) PC-3 and (C) DU145 cells after 24 h at 37 °C. Western blots are typical of three independent experiments, with densitometric analysis representing mean  $\pm$  s.d. Relative to control: \* $P < 0.05$ , \*\* $P < 0.01$ .

altering total AKT (Figure 3A–C). Interestingly, DFO did not significantly increase p-mTOR (Figure 3A–C), and in contrast to the expected increase in cyclin D1 expression, there was a significant ( $P < 0.01$ ) dose-dependent decrease of cyclin D1 in all cell types (Figure 3A–C). Unlike the effect observed with DFO, incubation of cells with the positive control, insulin-like growth factor-1 (IGF-1), led to increased p-AKT and p-mTOR levels (Supplementary Figure 4). This suggested the effect of DFO on p-AKT did not result in downstream activation of the classical AKT pathway.

To investigate whether the DFO-induced increase in p-AKT was dependent on PTEN levels, we incubated PC-3 cells stably transfected with the vector alone (PC-3-Control) or a PTEN construct (PC-3-PTEN) with DFO or Dp44mT (Supplementary Figure 5A and B). While DFO and Dp44mT increased levels of p-AKT in PC-3-PTEN cells by up to 2.5-fold, the proportional increase in p-AKT was smaller compared with the significant ( $P < 0.001$ ) 3.5-fold increase observed in PC-3-Control cells.

**Iron chelators decrease phosphorylation of SMAD2 at a site regulated by oncogenic Ras.** Considering our results demonstrating an effect of the chelators on AKT/PTEN and previous studies indicating cellular iron chelation upregulates TGF- $\beta$  (Yoon *et al*, 2002), we examined the effects of DFO and Dp44mT on TGF- $\beta$ /SMAD signalling. In particular, we focused on SMAD2, as it is a key molecule involved in mediating tumour-suppressive TGF- $\beta$ /SMAD signalling (Ten Dijke *et al*, 2002; Assinder *et al*, 2009). Iron chelators significantly ( $P < 0.05$ ) reduced levels of total SMAD2 in the prostate cancer cell lines, but not in PrECs (Figure 4A–C). However, incubation with chelators did not significantly alter p-SMAD2C levels in all cell types (Figure 4A–C). As p-SMAD2C was barely detected or not observed in DU145 cells after incubation with control medium or chelators, TGF- $\beta$  (10 ng ml<sup>-1</sup>) was used as a positive control and demonstrated that p-SMAD2C was significantly ( $P < 0.01$ ) induced (Figure 4D). Importantly, DFO and Dp44mT significantly ( $P < 0.001$ – $0.05$ ) reduced oncogenic p-SMAD2L levels in all three cell types (Figure 4A–C), indicating a

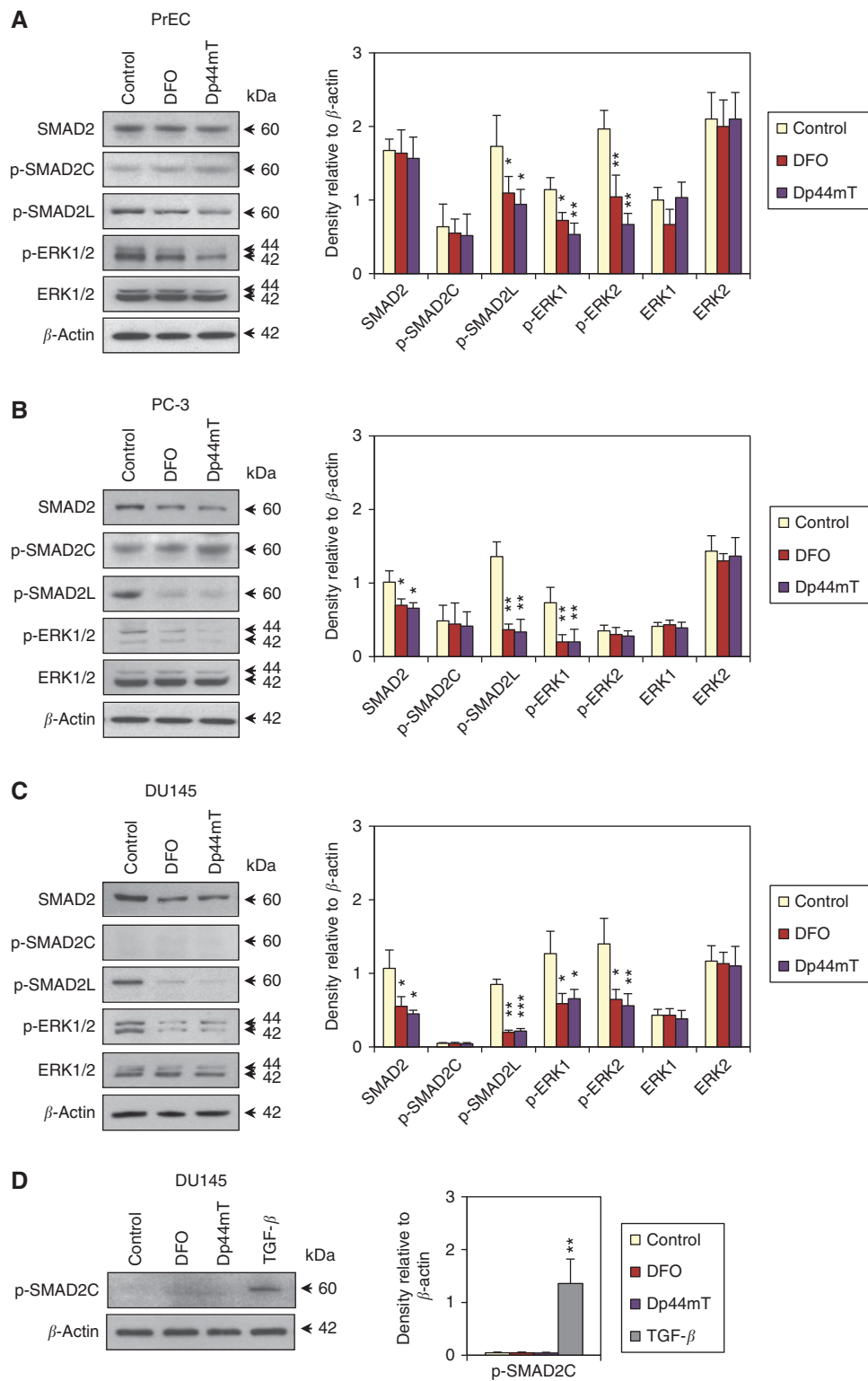


Figure 4. Incubation of cells with DFO (250  $\mu\text{M}$ ) or Dp44mT (2.5  $\mu\text{M}$ ) for 24 h at 37  $^{\circ}\text{C}$  decreases p-SMAD2L and p-ERK1/2 levels, but has no effect on total ERK1/2, or p-SMAD2C levels in (A) PrEC, (B) PC-3 and (C) DU145 cells. After incubation of DU145 cells with control medium or chelators, p-SMAD2C was barely detectable, but its levels were detected after (D) a 24-h incubation at 37  $^{\circ}\text{C}$  with TGF- $\beta$  (10 ng ml $^{-1}$ ), indicating that the protein is inducible in DU145 cells. Western blots are typical of three independent experiments, with densitometric analysis representing mean  $\pm$  s.d. Relative to control: \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

possible mechanism of their anti-tumour activity (Torti and Torti, 2011; Merlot *et al.*, 2012). Notably, the effect of the chelators on reducing p-SMAD2L expression was markedly less pronounced in normal PrECs relative to both prostate cancer cell lines (Figure 4A–C).

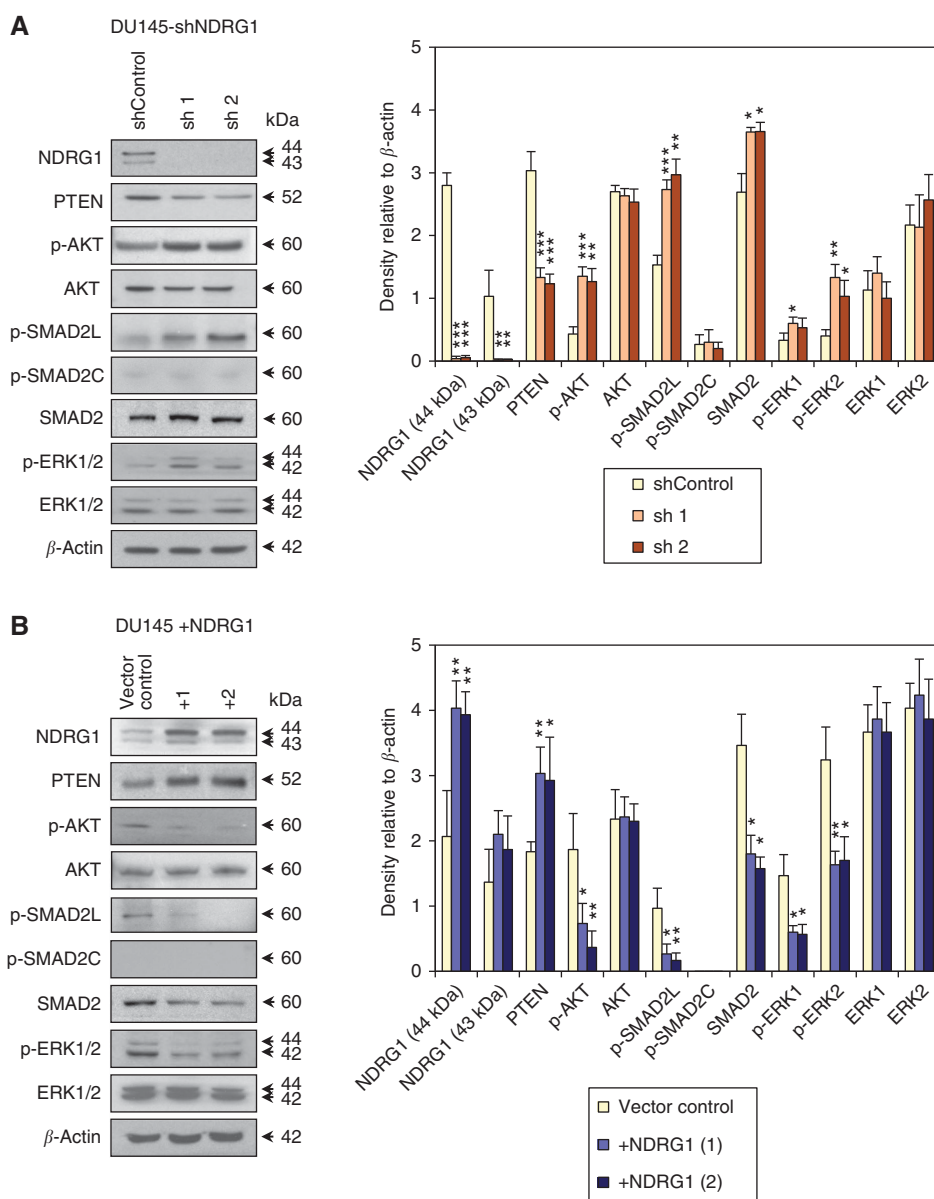
Previous studies have indicated that ERK1/2 is responsible for the phosphorylation of p-SMAD2L (Kretzschmar *et al.*, 1999). Hence, we examined the response of phosphorylated ERK1 at Thr $^{202}$ /Tyr $^{204}$  and ERK2 at Thr $^{185}$ /Tyr $^{187}$  (p-ERK1/2) and total ERK1/2 to incubation with DFO and Dp44mT. As shown in

Figure 4A–C, incubation of cells with DFO or Dp44mT resulted in a significant ( $P < 0.01–0.05$ ) decrease in phosphorylation of ERK1 at 44 kDa (Thr<sup>202</sup>/Tyr<sup>204</sup>) in PrEC, PC-3 and DU145 cells. For p-ERK2 at 42 kDa (Thr<sup>185</sup>/Tyr<sup>187</sup>), incubation with chelators led to a significant ( $P < 0.01–0.05$ ) decrease in phosphorylation for PrEC and DU145 cells, whereas no significant effect was observed in PC-3 cells. No significant alterations in total ERK were observed in any of the cell types studied under all conditions (Figure 4A–C). These studies indicated that the decrease in p-ERK could have a role in the reduced p-SMAD2L observed after incubation with chelators.

**Silencing or overexpression of NDRG1 modulates levels of p-AKT, PTEN, p-SMAD2L and p-ERK1/2.** We then investigated how NDRG1 could be involved in the effects of DFO and Dp44mT on PTEN, p-AKT and p-SMAD2L. To this end, we silenced NDRG1 expression in DU145 cells using shRNA (DU145-shNDRG1) implementing two constructs (sh 1 and sh 2) and the scrambled control and observed the subsequent effects on these

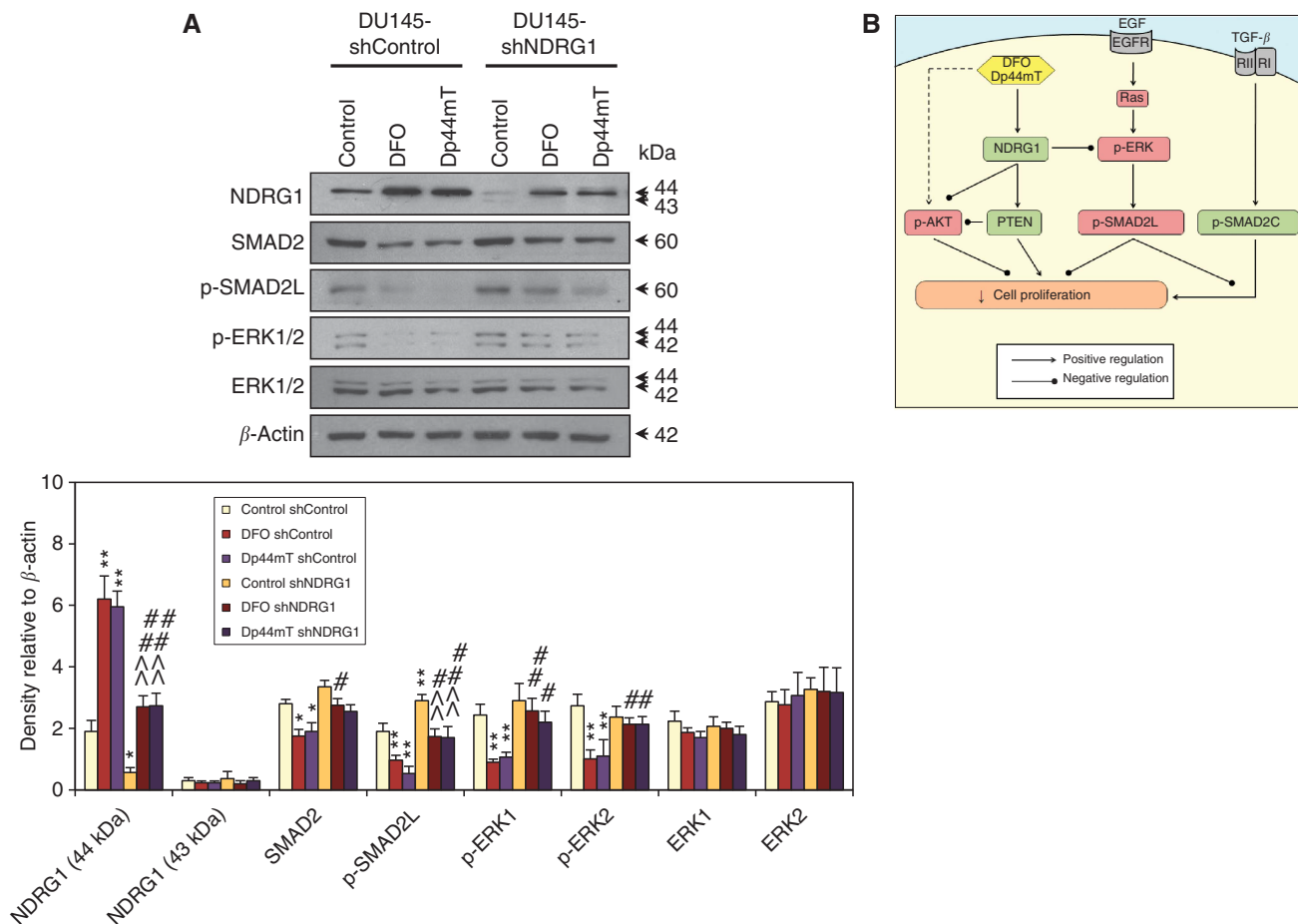
proteins (Figure 5A). The DU145 cell line was chosen for these studies due to the relatively high expression of the proteins of interest in control cells (Figures 1, 3 and 4). Silencing NDRG1 resulted in significantly ( $P < 0.001$ ) decreased PTEN expression, while significantly ( $P < 0.001–0.05$ ) increasing p-AKT, p-SMAD2L, SMAD2 and p-ERK1/2. In contrast, there was no significant change in total AKT and p-SMAD2C levels (Figure 5A). Conversely, NDRG1 overexpression in DU145 cells (using two clones: +1 and +2) resulted in significantly ( $P < 0.01–0.05$ ) increased PTEN expression, whereas levels of p-AKT, p-SMAD2L, SMAD2 and p-ERK1/2 were significantly reduced ( $P < 0.01–0.05$ ; Figure 5B). No significant alteration in the level of total AKT was observed, and as described previously in Figure 4C and D, p-SMAD2C expression was barely detectable (Figure 5B).

To determine if the effects of the chelators were dependent on NDRG1 activity, we used DU145-shNDRG1 cells (Figure 6A). Both DU145-shControl (i.e., scrambled construct) and DU145-shNDRG1 cells were incubated with DFO or Dp44mT for 24 h and



**Figure 5.** Modulation of NDRG1 expression using (A) two shRNA constructs (sh 1 or sh 2) and the scrambled control, or (B) an overexpression construct (two separate clones: +1 and +2) and the vector control leads to alterations in the levels of PTEN, p-AKT (Ser<sup>473</sup>), p-SMAD2L, SMAD2 and p-ERK1/2 in DU145 cells compared with the respective controls. Western blots are typical of three independent experiments, with densitometric analysis representing mean  $\pm$  s.d. Relative to control: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .





**Figure 6.** (A) Decreasing NDRG1 expression via shRNA reduces the DFO- or Dp44mT-induced decrease in p-SMAD2L, SMAD2 and p-ERK1/2 levels in DU145 cells. Cells with and without silenced NDRG1 (shNDRG1) were incubated for 24 h at 37 °C with DFO (250  $\mu$ M) or Dp44mT (2.5  $\mu$ M). Western blots are typical of three independent experiments, with densitometric analysis representing mean  $\pm$  s.d. \* $P$ <0.05, \*\* $P$ <0.01 significantly different to non-silenced control; ^^ $P$ <0.01 significantly different to silenced control; # $P$ <0.05, ## $P$ <0.01 significantly different to respective chelator (DFO or Dp44mT)-treated non-silenced control. (B) Schematic illustrating the effect of the chelators DFO and Dp44mT on key cell signalling pathways in prostate cancer. The studies herein demonstrate that the chelators upregulate NDRG1, which is required for the increase in the expression of the tumour suppressor, PTEN, leading to decreased cell proliferation. While the chelators also initially increase p-AKT, the NDRG1-mediated increase in PTEN may subsequently decrease p-AKT levels. The chelator-mediated increase in NDRG1 expression also reduces levels of oncogenic p-ERK and its downstream target, p-SMAD2L, preventing proliferation and accounting, in part, for the anti-tumour activity of these agents.

the effects on protein expression assessed. Interestingly, NDRG1 silencing almost ablated the expression of the protein in control cells (DU145-shNDRG1) and significantly ( $P$ <0.01) reduced chelator-mediated upregulation of NDRG1 compared with non-silenced DU145 cells (Figure 6A). The partial NDRG1 silencing in chelator-treated cells reduced the ability of these agents to decrease SMAD2, and to a greater extent, p-SMAD2L and p-ERK1/2 levels relative to the non-silenced cells. The silencing of NDRG1 or treatment with chelators had no significant effect on levels of total ERK1/2. In summary, these studies show that upregulation of NDRG1 by DFO or Dp44mT has a role in the downregulation of SMAD2, p-SMAD2L and p-ERK1/2.

## DISCUSSION

A key aim in the development of specific anti-cancer therapies is to restore lost tumour-suppressive functions and disrupt those essential signalling pathways crucial for tumour growth and metastasis. Here, we aimed to apply this principle to prostate cancer therapy by investigating how novel iron chelators target the

complex relationship between the tumourigenic PI3K/AKT and tumour-suppressive PTEN and TGF- $\beta$  pathways via NDRG1.

**Iron chelators increase NDRG1 and its phosphorylation at Ser<sup>330</sup> and Thr<sup>346</sup>.** In this investigation, for the first time, we show that iron chelation increases phosphorylation of NDRG1 at Ser<sup>330</sup> and Thr<sup>346</sup> in normal human PrECs and prostate cancer cell lines (Figure 1). In recent studies by Murakami *et al* (2010), NDRG1 phosphorylation at Ser<sup>330</sup> and Thr<sup>346</sup> in pancreatic cancer cells was essential for its tumour-suppressive action. This indicates that in addition to upregulation of total NDRG1 levels, phosphorylation of NDRG1 at Ser<sup>330</sup> and Thr<sup>346</sup> by chelators may be important for their activity in prostate cells.

Previous studies have demonstrated that some chelators such as thiosemicarbazones show substantial selectivity against tumour cells (Whitnall *et al*, 2006; Yu *et al*, 2012). An important aspect of this study was to assess the differential anti-tumour activity of these agents using PrECs and the PC-3 and DU145 prostate tumour cell lines. Although the chelators significantly increased NDRG1 levels and its phosphorylation in PrECs, the extent of the upregulation was markedly greater in prostate cancer cells. Moreover, the chelators more effectively decreased oncogenic p-SMAD2L in the

prostate cancer cell lines relative to PrECs. These effects may have a role in the selective anti-tumour activity of these compounds.

**NDRG1 attenuates p-AKT levels independently of PTEN.** To further investigate the molecular targets of chelators and their integration, their effect on the PI3K/AKT pathway was assessed. The chelators not only increased expression of the tumour-suppressive molecules, NDRG1 and PTEN, but also increased phosphorylation of oncogenic AKT. This latter effect was unexpected, given the well-documented anti-proliferative effects of iron chelators (Buss *et al*, 2004; Torti and Torti, 2011; Merlot *et al*, 2012) and our observation that upregulation of NDRG1 suppresses p-AKT levels (Figure 5). These somewhat paradoxical observations may be explained by the chelators inducing an initial pro-survival response that increases p-AKT. We found the elevated p-AKT expression induced by iron chelators did not result in increased levels of its downstream targets, p-mTOR and cyclin D1 (Figure 3). In fact, cyclin D1 expression was significantly decreased by DFO, which is consistent with previous reports (Nurtjahja-Tjendraputra *et al*, 2007). These results indicate that increased p-AKT in prostate cells induced by DFO does not correlate with activation of its classical targets, p-mTOR and cyclin D1. Indeed, activation of mTOR by the PI3K/AKT pathway is well known to occur during growth-factor signalling (Assinder *et al*, 2009), but this did not occur after incubation with chelators. However, it is notable that DFO and Dp44mT affect multiple signalling pathways within cells (Chen *et al*, 2012), and it is possible that other processes are affecting the activity of p-AKT, mTOR and other downstream targets of p-AKT in a way that differs from its classical signalling. In fact, Ohyashiki *et al* (2009) demonstrated that incubation with the iron chelator, deferasirox, repressed signalling through mTOR in leukaemia cells via REDD1. A number of studies have also reported increased p-AKT levels in response to DFO in cancer cell lines (Alvarez-Tejado *et al*, 2001; Box *et al*, 2008; Dongiovanni *et al*, 2008). Taken together, the increased p-AKT induced by chelators may be a short-term stress-induced response before their anti-proliferative effects overcome the initial pro-survival response to these agents.

Using DU145 cells, we showed that silencing NDRG1 significantly increased p-AKT levels and decreased PTEN levels, indicating that NDRG1 attenuated oncogenic p-AKT activity. Conversely, NDRG1 overexpression significantly reduced p-AKT levels and increased PTEN. These data demonstrate an important role for NDRG1 in inhibiting the oncogenic PI3K/AKT pathway (Figure 5) and leads to an NDRG1-dependent mechanism by which iron chelators induce their anti-proliferative effects (Figure 6B).

Furthermore, we showed that NDRG1 overexpression in PTEN-null PC-3 cells attenuated p-AKT levels (Supplementary Figure 6), which mirrors our observations in PTEN-positive DU145 cells (Figure 5). A similar effect was also shown by others, where NDRG1 overexpression in the PC-3MM and MCF7 cell lines decreased p-AKT levels (Liu *et al*, 2012). Collectively, the reduction in p-AKT mediated by NDRG1 occurs independently of PTEN. Hence, the lack of PTEN expression in ~50% of prostate cancer patients (Facher and Law, 1998) is unlikely to abrogate the effects of NDRG1 on p-AKT.

**Iron chelators decrease levels of p-ERK1/2 and its downstream target, p-SMAD2L.** Considering that the PI3K/AKT and TGF- $\beta$  pathways are integrated via NDRG1 (Assinder *et al*, 2009), we examined the effect of the chelators and NDRG1 on TGF- $\beta$  signalling. Iron chelators did not significantly alter p-SMAD2C levels, which is involved in activation of the canonical anti-proliferative TGF- $\beta$  pathway in all three prostate cell types. We report that incubation of these cell types with chelators significantly decreased p-SMAD2L levels. Thus, this effect alters the ratio of p-SMAD2L to p-SMAD2C, therefore promoting the tumour-suppressive effects of p-SMAD2C. This effect could be

mediated, in part, by the suppression of p-ERK1/2 levels (Figure 4), as ERK1/2 mediates phosphorylation of SMAD2 at the linker region (Kretzschmar *et al*, 1999).

To further investigate the mechanisms behind this observation, we silenced NDRG1 and observed increased p-ERK1/2 and p-SMAD2L in DU145 cells, whereas overexpression of NDRG1 had the opposite effect. This demonstrates a role for NDRG1 in the attenuation of ERK1/2 signalling and its downstream effects on p-SMAD2L. Moreover, incubation of DU145-shNDRG1 cells with chelators led to a less marked reduction in p-ERK1/2 and p-SMAD2L compared with vector control cells (Figure 6A). This indicated NDRG1 was at least partly responsible for the chelator-mediated decrease in p-ERK1/2 and p-SMAD2L. Thus, chelators exert their anti-proliferative activity, specifically by suppressing oncogenic p-ERK1/2 signalling via NDRG1 and its downstream effects on p-SMAD2L (Figure 6B).

In conclusion, the chelators used herein exploit tumour-suppressive functions (i.e., NDRG1 and PTEN) and disrupt tumorigenic effectors (i.e., p-ERK1/2 and p-SMAD2L) in cancer cells and represent a new advance in targeting signalling pathways. Importantly, the effects of DFO and Dp44mT on NDRG1, p-SMAD2L and SMAD2 expression were more marked in prostate cancer cells than normal PrECs, which may, in part, explain their selective anti-tumour activity. Indeed, the decrease in oncogenic p-SMAD2L is hypothesised to enhance tumour-suppressive SMAD-dependent TGF- $\beta$  signalling. Finally, while the mechanisms of integration of these pathways are highly complex, this study has demonstrated the potential for specific targeting of these pathways with novel pharmacological agents.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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