Biochimica et Biophysica Acta 1783 (2008) 1981–1992

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

The iron-regulated metastasis suppressor, Ndrg-1: Identification of novel molecular targets

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ARTICLE INFO

Article history:
Received 12 March 2008
Received in revised form 5 May 2008
Accepted 15 May 2008
Available online 26 June 2008

Keywords:
Iron chelator
Iron
Desferioxamine
Ndrg-1
Metastasis suppressor

ABSTRACT

A recently identified metastasis suppressor, *N-myc downstream regulated gene-1* (*Ndrg-1*), has been shown to reduce the invasion and metastasis of breast, colon, prostate and pancreatic cancer. Among its many functions, Ndrg-1 is involved in modulating differentiation, proliferation and angiogenesis. However, knowledge of the molecular targets of Ndrg-1 is limited. The current study has focused on examining the functions of Ndrg-1 in a number of different cancer cell models including prostate, colon, lung and pancreatic cancer to elucidate the known pleiotropic nature of this protein. Furthermore, the potential gene targets of Ndrg-1 were analyzed using whole genome gene array revealing a substantial number of genes whose expression was affected by this metastasis suppressor. Significantly, Ndrg-1 up-regulated *thiamine triphosphatase* (*Thtpa*) expression in three of the four cell models. Thtpa is known to decrease the levels of the energy currency molecule, thiamine triphosphate, suggesting a potential pathway for the anti-proliferative effects of Ndrg-1. Furthermore, Ndrg-1 reduced the protein levels of cathepsin C which plays a role in invasion, indicating a potential mechanism of its anti-metastatic role in pancreatic cancer cells. These findings provide a potential link between the observed functions of Ndrg-1 and its molecular targets, further demonstrating its anti-metastatic effect.

1. Introduction

The ability of a tumor to metastasize is a significant clinical problem responsible for poor patient prognosis [1]. Therefore, it is important to develop treatments that target the metastatic ability of tumors. A recently identified group of metastasis suppressor proteins has provided new insight into the treatment of cancer [2].

One such protein, N-myc downstream regulated gene-1 (Ndrg-1), has been identified as a metastasis suppressor [3–6]. Several studies have demonstrated that Ndrg-1 levels are lower in tumors compared to adjacent normal tissue [3–6]. Furthermore, forced Ndrg-1 expression reduced metastasis [3–6]. In fact, in human prostate cancer, Ndrg-1 expression was much higher in organ-confined tumors compared to lymph node or bone metastasis, suggesting that Ndrg-1 was negatively correlated with metastasis [4]. Studies examining cancer patient survival rates found that tumors with high Ndrg-1 levels lead to improved survival compared to matched patients with lower Ndrg-1 expression [4–6].

Ndrg-1 is a member of the NDRG family which consists of three other genes, namely *Ndrg-2*, *Ndrg-3* and *Ndrg-4* [7,8]. A distinguishing feature of *Ndrg-1* is the three tandem repeats of GTRSRSHTSE in its C-terminal region, suggesting a unique function [8]. *Ndrg-1* has been mapped to chromosome 8q24.3, where it encodes a 3.0 kb mRNA that is translated into a 43 kDa protein [9–11].

Although Ndrg-1 function is unclear, its expression affects many cellular processes ranging from myelin sheath maintenance to differentiation [11–17]. In fact, Ndrg-1 appears to function in a pleiotropic manner dependent on cell type [17,18]. The molecular targets of Ndrg-1 have yet to be assessed in a range of tumor cells. A recent study found Ndrg-1 down-regulates activating transcription factor 3 (ATF3), which plays a role in cell cycle progression and apoptosis [19]. However, no complete gene array data have been presented. Therefore, it was important to examine the potential molecular targets of Ndrg-1 in a variety of tumor cells to elucidate its cell-specific role.

We identified that *Ndrg-1* is up-regulated in cells following iron (Fe)-depletion using Fe chelators *via* hypoxia inducible factor-1α (HIF-1α)-dependent and -independent mechanisms [20]. Further studies by others using Fe chelators, mimosine and desferrioxamine (DFO), have extended this [21,22]. Iron chelators are very effective in reducing tumor growth in a selective manner [23,24] and one agent, Triapine®, is in clinical trials [25]. The anti-tumor effects of Fe chelators may be partly due to Ndrg-1 up-regulation, which reduces cancer cell growth and metastasis [3–6,26]. Recently, it was found that Ndrg-1 was activated by hypoxic stress, where it played a protective role [27,28]. Considering that Fe chelators mimic hypoxia and lead to Ndrg-1 up-regulation [20], it was important to assess whether this leads to resistance to these agents. In fact, high Ndrg-1 expression was

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doi:10.1016/j.bbamcr.2008.05.016

found to mediate resistance to the topoisomerase inhibitor, CPT-11 [29].

In this investigation, we examined the role of Ndrg-1 in cellular adhesion, proliferation, and the resistance to anti-proliferative activity mediated by the Fe chelator, DFO. Furthermore, to investigate the molecular targets of Ndrg-1, we demonstrate using whole genome microarray that Ndrg-1 modulates the expression of a variety of genes including *thiamine triphosphatase* (*Thtpa*) and *cathepsin C* (*Ctsc*). These findings are important in terms of understanding the anti-metastatic function of Ndrg-1.

2. Materials and methods

2.1. Cell culture

Rat metastatic prostate cancer cells (AT6.1) were a gift from Dr. K. Watabe (Southern Illinois University School of Medicine, USA) [4]. These cells were stably transfected with the pcDNA3 vector containing *Ndrg-1* cDNA (#7 and #10 clones) or the pcDNA3 vector alone (# 9 clone) [4]. The human MIAPaCa-2 cells transfected with a pIRESneo2-*Ndrg-1* vector and vector control cells were obtained from Dr. Michihiko Kuwano (Research Centre for Innovative Cancer Therapy, Fukuoka, Japan) [6]. Human metastatic lung cancer cells, H1299, and non-metastatic human colorectal adenocarcinoma cells, DLD-1, transfected with the tetracycline-inducible human *Ndrg-1* vector, pBI-*Ndrg-1*-EGFP, in a Tet-off system [15] were purchased from GenHunter (Nashville, TN, USA).

2.2. Western blot and RNA analysis

Western analysis was performed *via* established protocols [30]. The primary antibodies used were against Ndrg-1 (Zymed, CA, USA), Thtpa (Abnova Corporation, Taiwan), Ctsc (Abs: L14 and T17; Santa Cruz, California, USA) and Kifc3 (ProteinTech Group Inc. IL, USA).

Isolation of mRNA was performed using TRIzol® (Invitrogen, Melbourne, Australia) by standard procedures [31]. RT-PCR was carried out by established methodology [31] using the primers in Table 1.

2.3. Adhesion assays

2.3.1. Cell–Matrix adhesion assay

The ability of cells to adhere to plastic and collagen was analyzed using standard methods [32,33]. Briefly, 96-well plates were coated with collagen and allowed to dry. They were then washed in 0.1% BSA RPMI and blocked with 0.5% BSA RPMI for 1 h, before being washed again. Wells not coated with collagen were not blocked. Cells were seeded at 50,000 cells/well and allowed to adhere for 15 min to 1 h at 37°C before being washed twice with PBS. To determine cell number, the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, MO, USA) assay was used [34].

2.3.2. Cell–Cell adhesion assay

Cell–Cell adhesion assays were performed according to established techniques [35]. Cells were harvested using 1 mM EDTA in Ca(II)/Mg(II) free PBS and washed twice with Hank's balanced salt solution (HBSS) containing 1% BSA. The cells were then seeded in 24-well plates (blocked with HBSS containing 2% BSA/3 h/37°C) at a density of 5000 cells/well. As a positive control, 1 mM $CaCl_2$ was added to cells to promote cell–cell adhesion [35]. The cells were incubated in a gyrating shaker (37°C/90 rpm/1–2 h) and the reaction terminated by 0.5 mL of 25% formaldehyde/well. Aggregation was determined as described [35].

2.4. Microarray analysis

AT6.1 cells transfected with human Ndrg-1 (#7 clone) and the vector control cells (#9 clone) were used for microarray analysis. RNA was isolated and microarray analysis was carried out by the Australian Genome Research Facility (Victoria, Australia) [36].

A two-phase strategy was used to identify differentially expressed genes. First, genome-wide screening was performed using Affymetrix GeneChips® (Millenium Sciences, Victoria, Australia). The empirical Bayes procedure [37] was applied to detect genes most likely to be differentially expressed between the #7 and #9 clones. Individual p values were then adjusted using the Holm step-down procedure to reduce false positives [38]. Further analysis produced a list of statistically significant ($p<0.05$) genes with a $\log_2$ value >2. Definitive

Table 1
Primer sequences used to amplify RNA extracted from human and rat cell lines

Primer name	Accession no.	Oligonucleotides (5′–3′)				Product size (bp)
		Forward	Priming sites	Reverse	Priming sites	
hβ-actin	X00351	CCCGCCGCCAGCTCACCATGG	25–46	AAGGTCTCAAACATGATCTGGGTC	421–397	397
rβ-actin	NM_031144	GAGGGAAATCGTGCGTGAC	696–715	CGTACTCCTGCTTGCTGATC	1168–1148	473
hNdrg-1	NM_006096	TCACCCAGCACTTTGCCGTCT	394–415	GCCACAGTCCGCCATCTT	1010–992	617
rNdrg-1	AY500369.1	GGATCAGTTGGCTGAAAT	446–464	ATCTTGAGTAGGGTGGTCTT	958–938	513
rHod	NM_133621	AGCAGACGCAGAAATGGT	250–268	ATCTGTCGCTGCTAAACG	897–879	648
rKifc3	XM_240978	CTGTGACCTTTGACCCTGAT	1658–1678	TCAGCAAACTTGAGGGAGTAC	2543–2521	886
rATP6v0e2	NM_001002253	GACAGCCCATTCCTTTGC	75–93	TTTCACAGCAGGGCATCT	368–349	294
rCtsc	NM_017097	GCCCAAACCTGCCCCGATAA	696–716	AGTAGTCTAACCCAGTGACTGGATC	1321–1295	626
rScd1	NM_139192	TCCTACACGACCACCACTACC	136–157	ACGGCGGGAGTTGTGAGGG	624–605	489
rCryl1	NM_175757	TTATGTCCCGCTGGTTGA	516–534	AAGGAGCAGGGGCATTAC	1048–1030	533
rFmo3	NM_053433	TCGGAGCAAAGCCCAATA	1392–1410	TATTGTCGGTGACTCGCAGACATTC	1706–1681	315
rThtpa	NM_001007682	CAGGGCTTGATTGAGGTG	369–387	CCACGGCATAACCGAAAT	828–810	460
rCol15a1	XM_216399	CCACCTTCCGAGCGTTTC	3874–3892	TGCCATGTCCGTGGTTCG	4175–4157	302
rBtg3	NM_019290	TTCCAATGTGGCACCCTT	589–607	AATAAGCCTGATGGTTTTGTC	917–896	329
hFmo3	NM_006894	TGCCATTCCCACAGTTGA	1207–1225	TCTGAAGTCTCCCGACCA	1595–1577	389
hHod	NM_032495	ATGTCGGCGGAGACCGCGAGC	283–304	TCTGTGACGGATCTGCAC	500–482	218
hThtpa	NM_024328	TTGAGGTGGAGCGAAAGT	753–771	AGCCAAAGTCGGCTGTAT	1193–1175	441
hCol15a	NM_001855	CCAAACCCTATTTCAAGTGC	3819–3839	CGCCATGCTTCACAGTAGTT	4234–4214	416
hCtsc	NM_001814	CAGACCCCAATCCTAAGCC	933–952	AACAATCCAGTAATCCATCCC	1373–1352	441
hATP6v0e2	NM_145230	ATAAGAATGCCGCGTGAC	764–782	CCAGAAGAGGTAACAGCAGAC	1254–1233	491
hKifc3	NM_005550	CGGCTGAAAGGGAACATC	1518–1536	TTGGTGAACTCGGTCGTG	2113–2095	596
hCryl1	NM_015974	AGTTGTTTGCTGGCTTGG	468–486	CCTTCGCTGTATCTGTCGC	852–833	385
hScd1	NM_005063	GCGATATGCTGTGGTGCT	1246–1264	CATAGGCCAGACCGAGGG	1493–1475	248
hBtg3	NM_006806	AAATTGCTGCCGTTGTCTT	164–183	CACATTGGAAGAGGTGGAAA	707–687	544

evidence of differential expression was obtained from RT-PCR of samples used for microarray analysis and three other RNA samples.

2.4.1. Annotation

Functional annotation of genes was assigned through Gene Ontology (http://www.geneontology.org) and classifications obtained using NetAffx (http://www.affymetrix.com/analysis/index.affx) and DAVID (http://david.abcc.ncifcrf.gov).

2.4.2. Data availability

The complete microarray data set can be accessed on the Gene Expression Omnibus (accession number GSE9076; http://www.ncbi.nlm.nih.gov/geo/).

2.5. Statistics

Data are presented as mean ± standard deviation (number of experiments) and were compared using the Student's *t*-test. Results were considered significant when $p<0.05$.

3. Results

Considering that Ndrg-1 has important roles in metastasis [4,19,39], understanding its function is important for developing new treatments. To this end, we investigated Ndrg-1 function in a variety of cancer cells and its effect on the anti-proliferative activity of DFO. This was performed using cell lines hyper-expressing *Ndrg-1*,

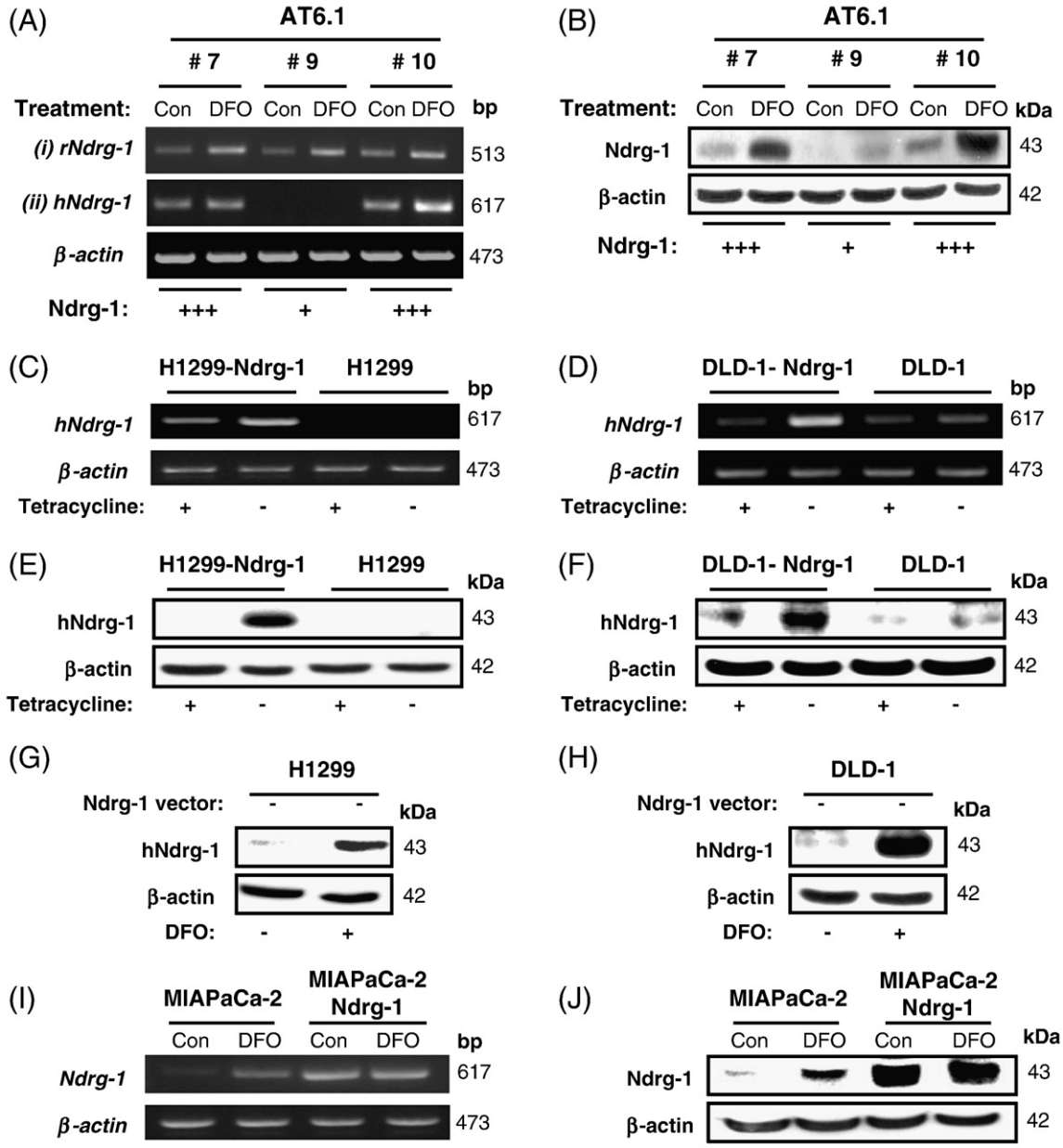


Fig. 1. Ndrg-1 is up-regulated in all cells transfected with human *Ndrg-1* and the chelator, desferrioxamine (DFO), up-regulates endogenous rat Ndrg-1 expression. Cells were incubated with control medium (Con) or this medium containing DFO (250 μM) for 24 h at 37°C. (A) *Ndrg-1* mRNA expression in the #7, #9 and #10 clones showing: *(i)* that DFO up-regulates endogenous rat Ndrg-1 mRNA levels in each clone; *(ii)* that human *Ndrg-1* mRNA is only expressed in the #7 and #10 clones but not the vector control clone. (B) Ndrg-1 protein expression in the #7, #9 and #10 clones showing higher Ndrg-1 levels in #7 and #10 clones and very low levels in the #9 vector control clone as well as the up-regulation of Ndrg-1 protein in each clone upon incubation with DFO. H1299 and DLD-1 cells transfected with human *Ndrg-1* under the control of a tetracycline-responsive promoter (tet-off system) over-express *Ndrg-1* mRNA in the absence of tetracycline (C and D, respectively). Ndrg-1 protein levels are markedly up-regulated in the absence of tetracycline in H1299-Ndrg-1 and DLD-1-Ndrg-1 cells (E and F, respectively). Ndrg-1 protein expression is up-regulated by DFO in H1299 and DLD-1 vector control cells (G and H, respectively). MIAPaCa-2-Ndrg-1 cells show increased Ndrg-1 mRNA and protein expression when transfected to over-express Ndrg-1 (I and J, respectively). MIAPaCa-2 vector control cells show increased Ndrg-1 mRNA and protein levels upon incubation with DFO (I and J, respectively). Results are from a typical experiment of 3 performed.

including AT6.1 rat prostate cancer, H1299 lung carcinoma, DLD-1 colon carcinoma and MIAPaCa-2 pancreatic cancer cells. These cell types were chosen as they have all been shown to be susceptible to the effects of Ndrg-1 [4,6,15]. Furthermore, Ndrg-1 over-expressing cell models were used, as the basal levels of this gene are generally low and its over-expression has been previously shown to have an impact on each of the cell lines used [4,6,15].

Initial experiments using this range of cell types examined Ndrg-1 levels and the effect of DFO on the expression of this gene. This was critical to ensure their suitability for the functional studies described below.

3.1. Ndrg-1 expression in each cell model and its up-regulation upon Fe chelator treatment

Rat AT6.1 prostate cancer cells transfected with human *Ndrg-1* (*hNdrg-1*) have been shown to be a good model of the metastasis suppressor role of Ndrg-1 *in vivo* [4]. We used three clones of AT6.1 cells, two transfected with a pcDNA3 vector containing *hNdrg-1* (clones: #7 and #10) and one vector control (clone: #9) [4]. As this model consists of rat cells transfected with *hNdrg-1* cDNA, the expression of both *hNdrg-1* and rat *Ndrg-1* (*rNdrg-1*) mRNA (Fig. 1A) was assessed. Since *Ndrg-1* is up-regulated by Fe chelators [20–22], we also examined the effect of DFO on *Ndrg-1* mRNA expression (Fig. 1A).

The AT6.1 clones were incubated with DFO (250 μM) for 24 h at 37°C and RT-PCR was performed to test endogenous *rNdrg-1* mRNA and its response to Fe chelation. This chelator concentration up-regulated endogenous Ndrg-1 without marked cytotoxicity (>80% viability), as the incubation was only for 24 h. In each clone, DFO increased *rNdrg-1* mRNA to 2- to 3-fold greater than the control (Fig. 1A*i*). Since the AT6.1 clones #7 and #10 were transfected with *hNdrg-1*, they were also assessed by RT-PCR for *hNdrg-1* mRNA. As anticipated, *hNdrg-1* mRNA was only detected in the #7 and #10 clones (Fig. 1A*ii*). An unexpected finding was that DFO increased *hNdrg-1* mRNA levels in the transfected cells. The reason for this was unclear, as *hNdrg-1* is within a vector regulated by the pcDNA3 promoter.

To determine whether Ndrg-1 protein expression was proportional to its mRNA levels in AT6.1 cells, Western blots were performed (Fig. 1B). The primary anti-Ndrg-1 antibody used was against hNdrg-1, although it also detected rNdrg-1 in the #9 vector control (Fig. 1B). Hence, this antibody detected both hNdrg-1 and rNdrg-1 in a single band (Fig. 1B). The Western blot results showed significantly ($p<0.05$) higher Ndrg-1 in #7 and #10 cells, which are transfected with *hNdrg-1*, compared to the #9 vector control that has only rNdrg-1 (Fig. 1B).

We also assessed Ndrg-1 expression in human lung cancer (H1299) and colorectal adenocarcinoma (DLD-1) cells transfected with *hNdrg-1* under the control of a tetracycline-responsive promoter (Fig. 1C–F). These cells over-express Ndrg-1 in the absence of tetracycline (tet-off system), while Ndrg-1 expression should be suppressed to endogenous levels when tetracycline is present [15]. To ensure that the tetracycline-regulated Ndrg-1 system was functional, the mRNA and protein levels of hNdrg-1 in the H1299 and DLD-1 cells were analyzed by RT-PCR (Fig. 1C–D) and Western analysis (Fig. 1E–F) in the presence and absence of tetracycline. As shown in Fig. 1C–D, *Ndrg-1* was regulated by tetracycline, with a marked increase in expression only being found when tetracycline was absent. Endogenous Ndrg-1 protein in H1299 cells was virtually undetectable in the vector control samples (Fig. 1E). However, H1299 cells transfected with *hNdrg-1* (H1299-Ndrg-1) showed significant ($p<0.001$) Ndrg-1 protein induction upon tetracycline removal (Fig. 1E). DLD-1 cells endogenously express low Ndrg-1 protein, as shown in the vector control samples, which were not significantly affected by tetracycline (Fig. 1F). In the absence of tetracycline, DLD-1-Ndrg-1 cells transfected with the tetracycline-regulated *hNdrg-1* vector also showed significantly ($p<0.001$) increased Ndrg-1 (Fig. 1F).

Further work examined whether endogenous Ndrg-1 was up-regulated by DFO in H1299 and DLD-1 cells. The H1299 and DLD-1 vector control cells were treated with 250 μM DFO for 24 h at 37°C and Ndrg-1 protein examined (Fig. 1G–H). Upon DFO treatment, both cell types showed a marked increase in Ndrg-1 protein (Fig. 1G–H).

The MIAPaCa-2 pancreatic cell type transfected with Ndrg-1 compared to its vector control was also assessed for Ndrg-1 expression in the presence and absence of DFO. These studies confirmed that MIAPaCa-2 cells transfected with *hNdrg-1* have significantly ($p<0.001$) higher *Ndrg-1* mRNA and protein levels when compared to vector controls (Fig. 1I–J). Furthermore, a 24 h incubation at 37°C with 250 μM DFO markedly increased Ndrg-1 in the vector controls (Fig. 1I–J). However, the effect of DFO on Ndrg-1 was not observed in MIAPaCa-2-Ndrg-1 cells, as these extensively over-express Ndrg-1.

3.2. The role of Ndrg-1 in cell–matrix and cell–cell adhesion

3.2.1. Cell–Matrix adhesion

Cell–Matrix adhesion is a mediator of tumor progression [40]. For tumor cell migration to occur, cell–matrix adhesion needs to be reduced [40]. On the other hand, cell–matrix adhesion is a component of invasion, with some tumors exhibiting increased expression of cell–matrix adhesion molecules [40]. Furthermore, Ndrg-1 has been suggested to modulate cell adhesion [3,41] and was important to assess in the 4 cancer models of Ndrg-1 expression (Fig. 1).

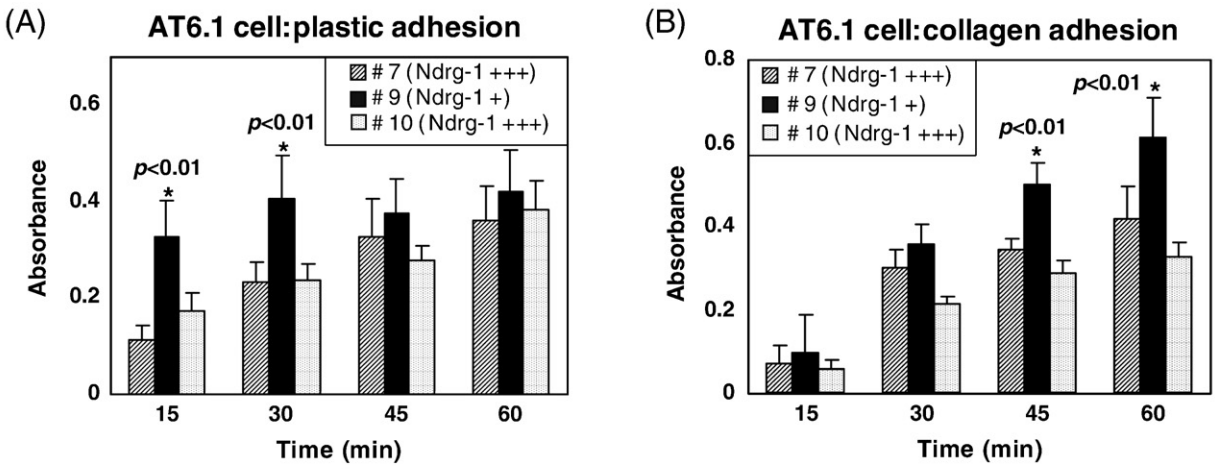
We assessed the role Ndrg-1 plays in cell–matrix adhesion on plastic and also collagen coated plates over a 15–60 min incubation as an indication of whether this contributes to its metastasis suppressor function. Adhesion of AT6.1 cells with high Ndrg-1 levels (#7 and #10) was compared to the vector control (#9). These results showed that clones #7 and #10 had significantly ($p<0.01$) lower cell–matrix adhesion than #9 control cells after incubations of 15 and 30 min on plastic (Fig. 2A) and at 45 and 60 min on collagen (Fig. 2B). This indicates that AT6.1 cells with high Ndrg-1 take longer to adhere, which may affect their ability to seed and invade tissues. The MIAPaCa-2, H1299 and DLD-1 cells over-expressing Ndrg-1 were also assayed for adhesion. However, Ndrg-1 levels were found to have no effect on cell–matrix adhesion in these cells (data not shown). These results may be rationalized considering the pleiotropic roles of Ndrg-1 [17,18,42].

3.2.2. Cell–Cell adhesion

Since Ndrg-1 modulates cell–matrix adhesion in AT6.1 cells (Fig. 2A and B), but not the other cell types, we examined its effect on cell–cell adhesion in all four models. This was done as cell–matrix adhesion is a separate process to cell–cell adhesion [43] and Ndrg-1 could affect each differently. Cells expressing high Ndrg-1 were compared to the vector controls in an *in vitro* cell–cell adhesion assay. These results revealed that AT6.1 cells with high Ndrg-1 expression (clones #7 and #10; Fig. 1B) formed smaller numbers of aggregates when compared to control cells (clone #9), which were able to form a large number of aggregates (Fig. 2C). In fact, while there was 72% aggregation in the control cells, only 8% and 47% of #7 and #10 cells, respectively, were able to aggregate (Fig. 2C). While there was a difference in the aggregation of AT6.1 clones (#7 and #10) with high Ndrg-1 expression, both were significantly ($p<0.03$) lower than the control. Positive controls treated with $CaCl_2$ resulted in even aggregation across all three clones, demonstrating their equal ability to form cell–cell adhesions (data not shown). This suggested in AT6.1 cells that Ndrg-1 affects cell–cell adhesion *in vitro* as well as cell–matrix adhesion. In contrast, H1299, DLD-1 and MIAPaCa-2 cells showed no significant difference in cell–cell adhesion between control cells and those hyper-expressing Ndrg-1 (data not shown).

3.3. High Ndrg-1 expression induces alterations in morphology and proliferation in rat prostate cancer cells

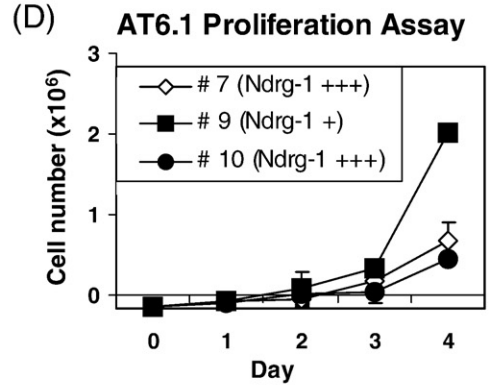
Ndrg-1 reduces proliferation of certain cancer cells [15,26] which may be due to its ability to induce differentiation [3,10,42,44].

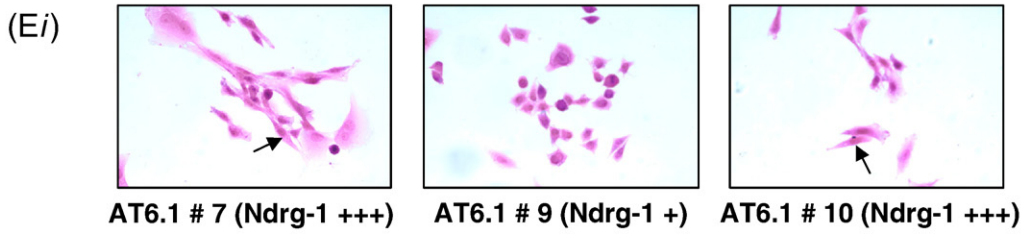


(C) **Cell-cell adhesion assay**

AT6.1 clone	Cell #	Particle # *	%Agg.	*p*-value
#7(+++ Ndrg-1)	1513	128	8	$p<0.001$
#9(+ Ndrg-1)	1520	1104	72	-
#10(+++Ndrg-1)	1474	690	47	$p<0.03$

***Particle #** refers to clumps containing >3 cells





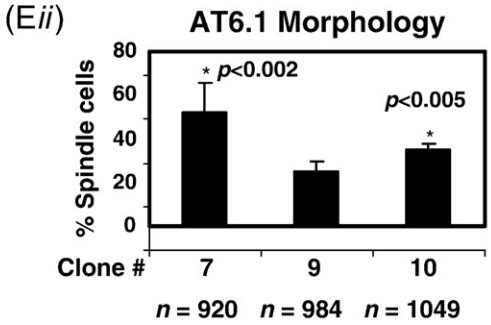


Fig. 2. Ndrg-1 expression alters the morphology, cell adhesion and proliferation in some cancer cell types. Cell–Plastic (A) and cell–collagen (B) adhesion assay of AT6.1 cells showing that the #7 and #10 clones with high Ndrg-1 expression have significantly (*$p<0.01$) lower cell to matrix adhesion at the 15 and 30 min time points on plastic and at 45 and 60 min on collagen. (C) Table showing that #7 and #10 clones have significantly lower ($p<0.03$) cell–cell adhesion compared to #9 controls as determined by calculating the percentage of aggregation. (D) An AT6.1 proliferation assay showing that cells transfected with Ndrg-1 (#7 and #10 clones) have significantly ($p<0.05$) lower proliferation rates than the vector control #9 clone after 5 days. (E*i*) Photomicrograph (200×) of the morphology of AT6.1 cells showing that cells with high Ndrg-1 (#7 and #10) expression have a greater proportion of spindle-shaped cells than the vector control clone (#9). (E*ii*) Quantification by cell counts demonstrating that the proportion of spindle cells in the clone #9 population with low Ndrg-1 expression is significantly (*$p<0.002$; $p<0.005$) less than that found for clone #7 and clone #10 that hyper-express Ndrg-1. Results in (A), (B) and (D) are mean ±+ SD from 3 experiments.

Moreover, the ability of Ndrg-1 to reduce proliferation and induce differentiation may contribute to its anti-metastatic effects [3,10]. Considering this, we assessed the effect of Ndrg-1 on proliferation using prostate, lung, colon and pancreatic cancer cells. The Ndrg-1 over-expressing clones of AT6.1 cells (#7 and #10 clones), as well as the #9 vector controls, were seeded and their growth examined by

viable cell counts. The #7 and #10 clones, which have the highest Ndrg-1 (Fig. 1B), had significantly lower proliferation ($p<0.01$) when compared to the #9 control clone at day 5 (Fig. 2D).

A comparison of the phenotypes displayed by AT6.1 cells revealed that cells with high Ndrg-1 appeared more differentiated, displaying a significantly ($p<0.005$) higher number of spindle-shaped cells (see arrows Fig. 2E*i*–E*ii*). Such morphology has been linked to greater differentiation in a variety of cell types [45,46]. Moreover, Ndrg-1 has been associated with differentiation in several cancers [3,4,10]. Previous studies showed that Ndrg-1 hyper-expression leads to colon cancer cells with spindle-shaped morphology [3], whereas these cells with low Ndrg-1 were rounded, smaller and clumped [3]. These findings agree with this study, where prostate cancer cells with low Ndrg-1 were small, spherical and had a tendency to aggregate (Fig. 2E*i*). This suggested that Ndrg-1 reduces AT6.1 cell proliferation *via* induction of differentiation.

The proliferation of lung cancer (H1299), colon adenocarcinoma (DLD-1) and MIAPaCa-2 pancreatic carcinoma cells was also analyzed. In all cases, cells with high Ndrg-1 showed no significant difference in growth or morphology compared to controls (data not shown).

3.4. Ndrg-1 does not modulate cellular sensitivity to DFO

Ndrg-1 is involved in generating resistance to CPT-11 [14,29]. Interestingly, *Ndrg-1* was identified as a stress response gene which protects cells from insults, e.g., hypoxia [20,27,28] which may be responsible for the resistance observed to CPT-11. It was shown previously [20–22] and in this investigation (Fig. 1) that Fe chelators up-regulate Ndrg-1. Considering the development of chelators for cancer treatment [23–25,47], it was important to assess if Ndrg-1 modulated sensitivity to DFO.

We analyzed proliferation of AT6.1, H1299, DLD-1 and MIAPaCa-2 cells with high or low Ndrg-1 at different DFO concentrations (3.125, 6.25, 12.5 or 25 μM) over 6 days (Fig. 3A–D). For all cell types, DFO decreased proliferation as a function of concentration (Fig. 3A–D). However, cells over-expressing Ndrg-1 showed no significant difference in susceptibility to the anti-proliferative effects of DFO compared to their controls indicating that the difference in expression did not alter the response.

3.5. Gene array reveals that Ndrg-1 affects expression of a range of gene groups

As suggested by the functional analyses of Ndrg-1 above and by others, the role of Ndrg-1 is pleiotropic [3–6,13–15,26], suggesting it is involved in multiple pathways and has numerous targets depending on the cell type. To elucidate the molecular targets of Ndrg-1, we performed an Affymetrix whole genome microarray examining differential gene expression in AT6.1 cells expressing high *Ndrg-1* (#7) compared to vector control cells (#9). This cell type was chosen considering the clear effects of Ndrg-1 hyper-expression on proliferation, cell–matrix and cell–cell adhesion (Fig. 2A–E). Also, studies using AT6.1 cells hyper-expressing *Ndrg-1* demonstrate that this molecule inhibits metastasis *in vivo* [4]. Hence, these cells represent a good model to identify molecular targets of Ndrg-1.

The statistically significant ($p<0.05$) differentially expressed genes in AT6.1 cells were grouped into 9 functional categories (Fig. 4A–B). These included genes involved in ribosome and protein synthesis, cell motility, stress response, proliferation, cell metabolism, lipid metabolism, the immune response, apoptosis and cell communication (Fig. 4A–B).

The Affymetrix® microarray revealed the differential expression of numerous genes between AT6.1 cells hyper-expressing Ndrg-1 and control cells. The top statistically significant ($p<0.05$) up- and down-regulated genes with a $\log_2$ value greater than 2 are shown in Table 2. To validate the microarray, mRNA expression was examined using RT-PCR. Due to the large number of significantly altered

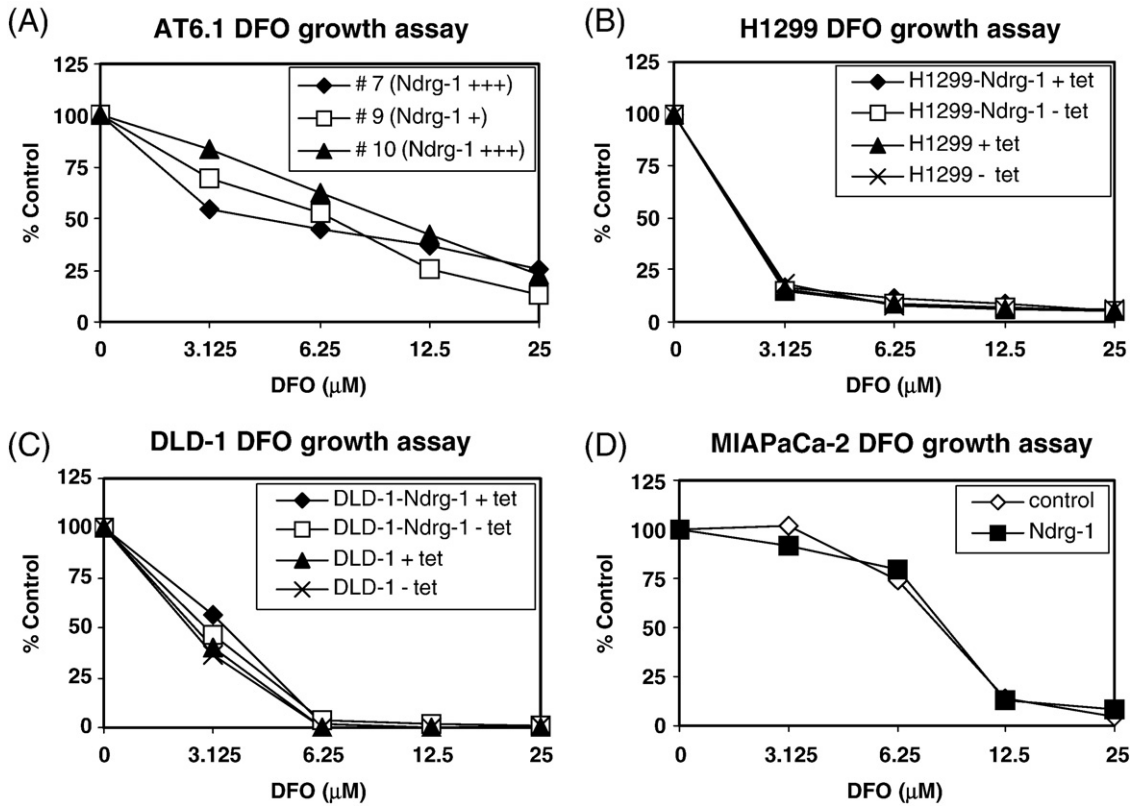


Fig. 3. Ndrg-1 over-expression in (A) AT6.1, (B) H1299, (C) DLD-1 and (D) MIAPaCa-2 cells does not alter their sensitivity to DFO. Cells were incubated with increasing concentrations of DFO (3.125–25 μM) for 6 days at 37°C and then cellular density measured by the MTT assay. Results are mean ± SD (3 experiments).

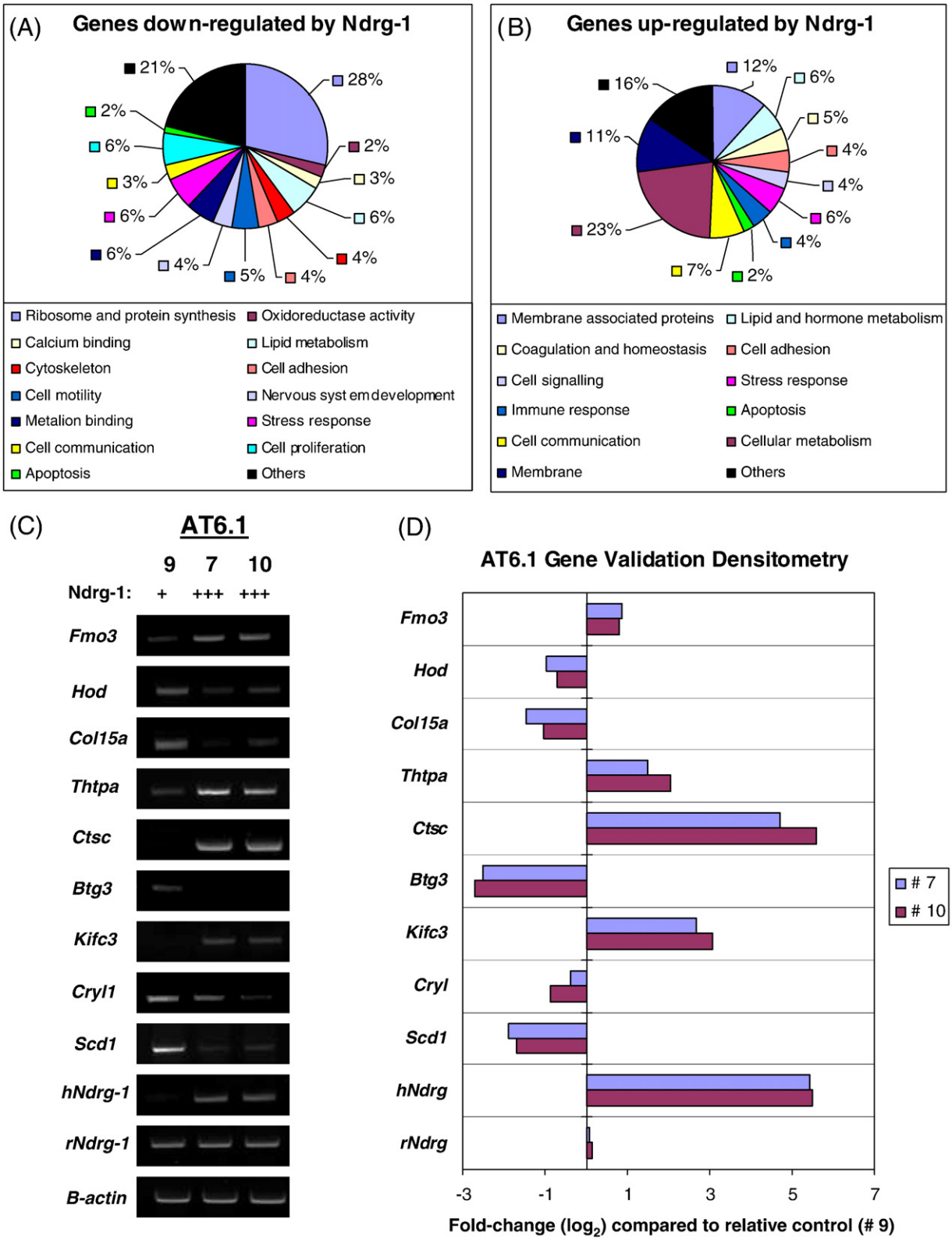


Fig. 4. Microarray analysis of AT6.1 cells reveals multiple gene targets. AT6.1 cells with high Ndrg-1 expression (#7) and the vector control cells (#9) with low Ndrg-1 levels were analyzed by whole genome microarray (Affymetrix Rat Expression Array 230 2.0 chip®) for differential gene expression. The statistically significant ($p<0.05$) genes were analyzed further to determine the functional groups of genes found to be: (A) down-regulated or (B) up-regulated in AT6.1 cells with high Ndrg-1 (#7) relative to low Ndrg-1 expression (#9). Functional analysis was examined using the comprehensive functional annotation software (DAVID, http://david.abcc.ncifcrf.gov). (C) RT-PCR data validating the expression of statistically significant alterations in gene expression in AT6.1 cells with high Ndrg-1 (#7 and #10) relative to low Ndrg-1 expression (#9). (D) Densitometric assessment of the RT-PCR data shown in (C). Results in (C) and (D) are from a typical experiment of 3–5 performed.

genes, it was not possible to analyze each using this method. Therefore, ten of the most statistically significant genes were chosen based on fold change and function. To ensure the alterations in gene expression were not simply due to clonal variation, two clones of Ndrg-1 over-expressing AT6.1 cells (clones #7 and #10) were used in the validation and compared to the control (#9) clone. Furthermore, each gene was tested

Table 2
List of statistically significant ($p<0.05$) differentially expressed genes in AT6.1 cells with a $\log_2$ value greater than 2

Affymetrix ID	Gene title	Gene symbol	$\log_2$ ratio	*p Value*
1374778_at	Cathepsin C	*Ctsc*	7.03	0.000
1369503_at	Amylase 2, pancreatic	*Amy2*	5.76	0.023
1391456_at	Junctional adhesion molecule 3	*Jam3*	4.52	0.000
1377457_a_at	Sortilin-related receptor, LDLR class A repeats-containing	*Sorl1*	4.47	0.006
1367899_at	Coagulation factor II (thrombin) receptor	*F2r*	4.28	0.000
1368829_at	Fibrillin 1	*Fbn1*	4.20	0.025
1388713_at	Thiamine triphosphatase	*Thtpa*	3.93	0.000
1368304_at	Flavin containing monooxygenase 3	*Fmo3*	3.86	0.000
1372639_at	Tripartite motif-containing 54	*Trim54*	3.77	0.036
1389374_at	Kinesin family member C3	*Kifc3*	3.57	0.002
1370831_at	Monoglyceride lipase	*Mgll*	3.37	0.000
1375951_at	Thrombomodulin	*Thbd*	3.18	0.000
1369679_a_at	Nuclear factor I/A	*Nfia*	3.16	0.004
1368655_at	Proteoglycan peptide core protein	*Pgsg*	3.03	0.000
1388395_at	G0/G1 switch gene 2	*G0s2*	2.82	0.000
1368247_at	Heat shock 70 kDa protein 1A	*Hspa1a*	2.80	0.000
1375043_at	FBJ murine osteosarcoma viral oncogene homolog	*Fos*	2.80	0.000
1370987_at	Sialophorin	*Spn*	2.77	0.000
1372439_at	Procollagen, type IV, alpha 1	*Col4a1*	2.76	0.000
1384180_at	Interferon-induced protein with tetratricopeptide repeats 2	*Ifit2*	2.72	0.000
1383575_at	Catenin (cadherin-associated protein), delta 2	*Ctnnd2*	2.68	0.003
1388798_at	Ubiquitin-conjugating enzyme E2E 2	*Ube2e2*	2.46	0.036
1372835_at	Ras homolog gene family, member J	*Rhoj*	2.44	0.001
1371824_at	Adenylate kinase 3-like 1	*Ak3l1*	2.42	0.003
1373245_at	Procollagen, type IV, alpha 1	*Col4a1*	2.41	0.000
1367554_at	Secretoglobin, family 2A, member 1	*Scgb2a1*	2.33	0.000
1390195_at	Pleckstrin and Sec7 domain containing 4	*Psd4*	2.32	0.014
1387988_at	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	*Hsd3b1*	2.29	0.001
1373351_at	Ankyrin 2, neuronal	*Ank2*	2.29	0.000
1383119_at	Opioid growth factor receptor-like 1	*Ogfrl1*	2.25	0.000
1387018_at	Arg/Abl-interacting protein ArgBP2	*Argbp2*	2.19	0.000
1373577_at	Neuropilin 1	*Nrp1*	2.05	0.000
1377497_at	2¢–5′ oligoadenylate synthetase-like 1	*Oasl1*	2.03	0.008
1389067_at	Solute carrier organic anion transporter family, member 4a1	*Slco4a1*	2.01	0.007
1368322_at	Superoxide dismutase 3, extracellular	*Sod3*	−2.01	0.000
1367923_at	Acyl-CoA synthetase bubblegum family member 1	*Acsbg1*	−2.05	0.004
1367816_at	Homeobox only domain	*Hod*	−2.10	0.000
1372649_at	Heat shock 27 kDa protein family, member 7 (cardiovascular)	*Hspb7*	−2.15	0.000
1377599_at	Lipin 1	*Lpin1*	−2.22	0.008
1370158_at	Myosin, heavy polypeptide 10, non-muscle	*Myh10*	−2.29	0.000
1367949_at	Proenkephalin 1	*Penk1*	−2.32	0.000
1367905_at	Ectonucleotide pyrophosphatase/phosphodiesterase 3	*Enpp3*	−2.37	0.002
1391052_at	DOM-3 homolog Z	*Dom3z*	−2.73	0.000
1391442_at	EH-domain containing 3	*Ehd3*	−2.74	0.000
1368072_at	B-cell translocation gene 3	*Btg3*	−2.80	0.000
1388939_at	Procollagen, type XV	*Col15a1*	−2.94	0.015
1386899_at	Cathepsin H	*Ctsh*	−3.20	0.000
1394022_at	Inhibitor of DNA binding 4	*Id4*	−3.51	0.038
1368080_at	Response gene to complement 32	*Rgc32*	−3.54	0.000
1376051_at	Crystallin, lamda 1	*Cryl1*	−3.65	0.000
1370355_at	Stearoyl-Coenzyme A desaturase 1	*Scd1*	−3.92	0.000
1368633_at	Cysteine-rich secretory protein 1	*Crisp1*	−4.15	0.000
1398245_at	Synuclein, gamma	*Sncg*	−4.34	0.000
1389362_at	Protein tyrosine phosphatase, non-receptor type 3	*Ptpn3*	−4.43	0.000
1387029_at	Complement component factor H	*Cfh*	−5.13	0.000
1372907_at	ATPase, H+ transporting, V0 subunit E isoform 2	*Atp6v0e2*	−5.65	0.005

Data obtained from using Affymetrix GeneChips®.

by RT-PCR at least 3 times in different lysates to confirm the result. Nine of the ten genes assessed were validated by RT-PCR. Indeed, the down-regulation of *Hod*, *Col15a1*, *Btg3*, *Cryl1* and *Scd1* and the up-regulation of *Fmo3*, *Thtpa*, *Ctsc* and *Kifc3* were confirmed in each of the Ndrg-1 over-expressing clones (Fig. 4C–D).

Further studies then examined the expression of these confirmed genes in control and Ndrg-1 hyper-expressing H1299, DLD-1 and MIAPaCa-2 cells. Considering the reported pleiotropic effects of Ndrg-1, these studies were performed to investigate if Ndrg-1 targets the same genes in other cell types. However, most of the genes affected by Ndrg-1 in AT6.1 cells were not differentially expressed in H1299, DLD-1 and MIAPaCa-2 cells (data not shown). Together, these observations confirm the difference in the phenotype of AT6.1 cells compared to H1299, DLD-1 and MIAPaCa-2 cells observed when assessing cell–matrix and cell–cell adhesion as well as proliferation.

3.6. Ndrg-1 hyper-expression up-regulates Thtpa in H1299, DLD-1 and MIAPaCa-2 cells and down-regulates cathepsin C in MIAPaCa-2 cells

Considering that eight genes were confirmed to be differentially expressed at the mRNA level in two hyper-expression clones of AT6.1 cells (Fig. 4C–D), we examined their protein expression. Antibodies were available or found suitable for Western analysis for three of the eight differentially expressed molecules, namely Thtpa, Ctsc and Kifc3. Hence, the expression of these molecules was then examined.

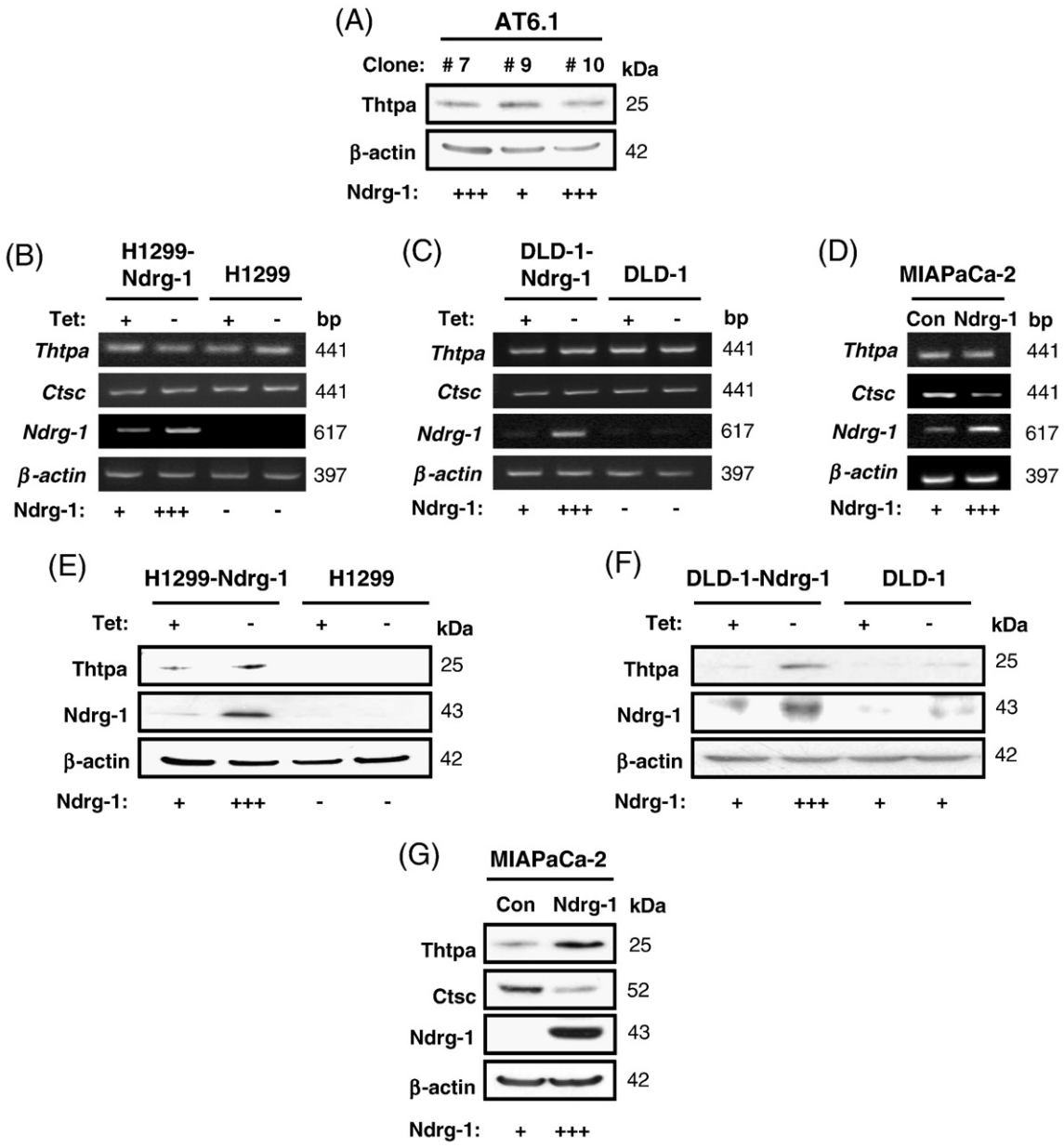


Fig. 5. Assessment of Ndrg-1 target genes identified from whole genome gene array demonstrates that Thtpa and Ctsc are modulated by Ndrg-1. (A) Thtpa protein expression in AT6.1 clones #7, #9 and #10 showing no significant difference regardless of Ndrg-1 levels. (B) *Thtpa* and *Ctsc* mRNA expression in H1299 and H1299-Ndrg-1 cells is not modulated by alterations in *Ndrg-1* expression. (C) *Thtpa* and *Ctsc* mRNA expression in DLD-1 and DLD-1-Ndrg-1 cells is not modulated by alterations in *Ndrg-1* expression. (D) *Thtpa* mRNA levels are not altered in MIAPaCa-2 cells with high or low *Ndrg-1* expression, while *Ctsc* mRNA expression is down-regulated in cells with high *Ndrg-1* expression. (E) Thtpa protein levels in H1299 cells are increased in cells with high Ndrg-1 expression. (F) Thtpa protein expression is up-regulated in DLD-1 cells with high Ndrg-1 expression. (G) Thtpa protein expression is up-regulated in MIAPaCa-2 cells with high Ndrg-1 expression, while Ctsc protein expression is down-regulated in cells with high Ndrg-1 levels. Results are representative of 3 separate experiments.

Since *Thtpa* mRNA was up-regulated in AT6.1 cells with high Ndrg-1 (Fig. 4C–D), we examined the effect of Ndrg-1 over-expression on Thtpa protein levels. However, we found no significant difference in Thtpa protein over 3 experiments regardless of Ndrg-1 levels in AT6.1 cells (Fig. 5A). We then assessed whether Ndrg-1 affected Thtpa expression in all other cell types at both the mRNA and protein levels. Interestingly, *Thtpa* mRNA was not significantly modulated by Ndrg-1 in any of these cells (Fig. 5B–D). However, examining Thtpa protein expression (Fig. 5E–G), this was found to be significantly ($p<0.05$) up-regulated in the DLD-1-Ndrg-1 cells (Fig. 5F) and MIAPaCa-2 (Fig. 5G) cell types over 3 experiments.

The gene identified to be most up-regulated at the mRNA level by Ndrg-1 in AT6.1 cells by microarray and RT-PCR was *Ctsc* (Table 2; Fig. 4C–D). Surprisingly, further studies examining Ctsc protein expression in AT6.1 cells showed that it was not affected by Ndrg-1 expression (data not shown). In fact, Western analysis using two different Ctsc antibodies gave the same negative result. Furthermore, Ctsc expression in H1299 and DLD-1 cells was also unaffected at the mRNA (Fig. 5B–C) and protein levels (data not shown). However, examining MIAPaCa-2 pancreatic cancer cells, *Ctsc* mRNA (Fig. 5D) and protein levels (Fig. 5G) were significantly ($p<0.001$) down-regulated by high Ndrg-1 expression.

Interestingly, Kifc3, which is involved in microtubule dynamics [48] and shown to be significantly up-regulated by Ndrg-1 in AT6.1 cells at the mRNA level (Fig. 4C–D), was unaffected at the protein level in this cell type (data not shown). Furthermore, Kifc3 was not differentially expressed in H1299, DLD-1 or MIAPaCa-2 cells at the mRNA or protein levels (data not shown), indicating that Ndrg-1 does not modulate the expression of this protein in these cell lines.

In summary, the gene expression studies above demonstrate that Ndrg-1 up-regulated Thtpa expression in three of the four models at the protein level. Furthermore, Ctsc protein levels were reduced by Ndrg-1 in MIAPaCa-2 cells.

4. Discussion

Tumor metastasis leads to significant clinical problems, contributing to poor prognosis [1]. Recently, strategies to prevent cancer metastasis have focused on a new group of proteins, the metastasis suppressors [2,49]. The recently discovered metastasis suppressor, Ndrg-1, has been demonstrated to function by affecting differentiation, proliferation and invasion [3–6,14,15,39]. Although numerous potential functions for Ndrg-1 have been described, its precise molecular function remains unclear. The current study has further examined Ndrg-1 function in a number of different cancer models and assessed its role in the inhibition of proliferation mediated by Fe chelators, a potential new form of chemotherapy [23–25]. Furthermore, to our knowledge, this is the first time that a full gene array analysis has been reported which identifies molecular targets of Ndrg-1 which may be responsible for its anti-metastatic effects.

An important component of tumor metastasis is the adhesive properties of cancer cells, and hence, this property could be a target of the metastasis suppressor function of Ndrg-1. To examine whether Ndrg-1 reduces metastasis *via* cell adhesion, we performed cell–matrix and cell–cell adhesion assays on all four cell models. We found that Ndrg-1 reduced cell–matrix (Fig. 2A and B) and cell–cell adhesion (Fig. 2C) in AT6.1 cells, there being no effect in other cell types examined. Considering this, reduced cell adhesion can be either pro- or anti-metastatic [40]. In a primary tumor mass, cells with reduced cell adhesion may have a greater metastatic potential, as they are more likely to detach and metastasize [40]. On the other hand, decreased cell adhesion may impair the ability of tumor cells to adhere and invade potential sites of metastasis [40]. The ability of Ndrg-1 to reduce cell adhesion in AT6.1 cells may suggest an anti-metastatic role, since high Ndrg-1 expression in this cell type reduces lung metastasis *in vivo* [4]. In addition, it has also been reported in prostate cancer cells, that reduced cell adhesion contributed to decreased cell migration and metastasis [50]. Others have reported a link between Ndrg-1 and a key adhesion molecule, E-cadherin, suggesting that Ndrg-1 up-regulates E-cadherin expression and its re-cycling [3,41]. However, we observed no alteration in E-cadherin in AT6.1 cells with high or low Ndrg-1 expression (data not shown), and thus, this could not explain the alterations in adhesion in this cell type.

Numerous investigations have found that Ndrg-1 modulates differentiation leading to reduced proliferation [3,44]. Following our observation that Ndrg-1 over-expression changes the morphological phenotype of AT6.1 cells in a manner consistent with increased differentiation (Fig. 2E*i*, *ii*), it was imperative to examine whether this reduces proliferation. Over-expression of Ndrg-1 significantly reduced AT6.1 proliferation (Fig. 2D), while having no effect on growth of other cell types. This suggests that in AT6.1 cells, the effect of Ndrg-1 on reducing proliferation may be due to its ability to induce differentiation. Further evidence supporting the role of Ndrg-1 in AT6.1 differentiation comes from our gene array analysis. Among the top 200 statistically significant differentially expressed genes, 5 play significant roles in cell differentiation. One of these is *Hod*, a gene found to be reduced by Ndrg-1 expression (Fig. 4C–D) which belongs to a family of homeobox genes shown to be up-regulated in prostate cancer leading to reduced differentiation [51]. The others include *S100a10*, *Ctgf*, *Enpp3* and *Spp1*, all of which are down-regulated by Ndrg-1 and have been shown to negatively regulate cell differentiation [52–55].

Our results clearly indicate that Ndrg-1 has different effects depending on the cell type in which it is expressed. Numerous studies have also demonstrated the pleiotropic nature of Ndrg-1 in terms of its function between different cell types. Clearly, the AT6.1 cell represents a prostate cancer model, while the other lines represent lung, colon and pancreatic tumor lines. Our gene array analysis demonstrated that most of the Ndrg-1 target genes in AT6.1 were not affected in the other cell lines. Furthermore, *Ctsc* expression was up-regulated in AT6.1 cells, while it was down-regulated in the MIAPaCa-2 line (Figs. 4 C–D and 5D, G). Thus, the alterations in target gene expression in AT6.1 relative to the other cell types will probably lead to different functional behaviour.

Up-regulation of Ndrg-1 may contribute to reducing metastasis and improving patient prognosis [4,5,14]. One promising new class of anti-cancer agents, namely Fe chelators [23–25], were found to up-regulate Ndrg-1 in many different cancer cell types [20–22] (Fig. 1). Following reports that high Ndrg-1 expression leads to increased resistance to the anti-cancer drug CPT-11 [14,29], it was important to assess whether Ndrg-1 would affect cancer cell sensitivity to Fe chelators. For each cell type tested, the proliferation rate declined with increasing DFO concentration (Fig. 3A–D). However, there was no significant difference in DFO mediated inhibition of proliferation between cells with high and low Ndrg-1 expression, suggesting that Ndrg-1 does not mediate increased resistance to Fe chelators. It was surprising that upon incubation with DFO, cells with higher Ndrg-1 did not show decreased proliferation relative to controls. This indicates that other mechanisms are also important for inhibiting proliferation upon Fe-depletion, including inhibition of ribonucleotide reductase [56], decreased cyclin D1 expression [57] and down-regulation of p21 protein [31] that can induce apoptosis (for review see [58]).

The results of our functional studies of Ndrg-1 prompted exploration of its molecular targets in different cells to explain its cell-specific effects observed here and by others [17,18,42]. Hence, we carried out a whole genome microarray analysis of AT6.1 cells transfected with *Ndrg-1* (#7) compared to its vector control (#9) to examine its potential molecular targets. This cell type was chosen because increased Ndrg-1 expression altered its phenotype leading to decreased adhesion and proliferation. Moreover, previous studies demonstrated that AT6.1 clone #7 led to reduced metastasis to the lung relative to clone #9 [4]. Thus, the AT6.1 cell line was an appropriate model to examine Ndrg-1 molecular targets. Our gene array demonstrated that Ndrg-1 modulates the expression of a range of genes reflecting the numerous functions associated with this protein [17]. These results revealed that 28% of all genes down-regulated by Ndrg-1 were involved in protein synthesis, with most being members of the Rpl and Rps families, which are structural components of ribosomes [59]. Moreover, a proteomic study examining molecular targets of Ndrg-1 in human prostate cancer cells also revealed a large number of ribosomal proteins modulated by Ndrg-1 [60]. This suggests that Ndrg-1 may affect translation, which could contribute to the reduced proliferation observed in AT6.1 cells (Fig. 2D).

The genes most significantly up-regulated by Ndrg-1 included *Fmo3*, *Ctsc*, *Thtpa* and *Kifc3* which are involved in xenobiotic metabolism [61], proteolysis [62], energy metabolism [63] and microtubule movement [48], respectively. On the other hand, the most significantly down-regulated genes by Ndrg-1 included *Hod*, *Col15a1*, *Btg3*, *Cryl1* and *Scd1*, that are involved in the regulation of transcription [64], cell adhesion [65], proliferation and cell cycle progression [66], fatty acid metabolism [67] and lipid metabolism [68], respectively. Surprisingly, when these genes were examined in Ndrg-1 over-expressing H1299, DLD-1 and MIAPaCa-2 cells, there was no difference in expression between cells over-expressing Ndrg-1 and their relevant controls. This is another example of the cell-specific effects of Ndrg-1, showing for the first time that this protein targets different molecules depending on the cell type it is expressed in. Moreover, the differential expression of these genes in the AT6.1 cells was found to be consistent in two different Ndrg-1 over-expressing clones (#7 and #10) when compared to the control clone (#9). This not only further validates the microarray result, but it also suggests that the result was not due to clonal variation.

A further analysis of the Ndrg-1 targets at the protein level revealed that Thtpa was up-regulated significantly in DLD-1 and MIAPaCa-2 cells where Ndrg-1 was over-expressed (Fig. 5E–G). Previous studies have shown that Thtpa is involved in hydrolyzing thiamine triphosphate, which is an important energy currency molecule [69]. Therefore, Thtpa up-regulation by Ndrg-1 may decrease the available energy as thiamine triphosphate, leading to reduced growth and metastasis suppression. Indeed, for MIAPaCa-2 cells, others have observed a reduction in growth and angiogenesis *in vivo* following the up-regulation of Ndrg-1 [6]. However, as demonstrated by the AT6.1 microarray analysis, Ndrg-1 has numerous molecular targets any of which, alone or in combination with other molecules, may contribute to its anti-metastatic effect.

The most markedly up-regulated gene in AT6.1 cells by Ndrg-1 was *Ctsc*, which was increased 380-fold compared to control cells (Fig. 4C–D), while there was no change in its protein expression. A possible reason for this observation may be that *Ctsc* mRNA was not translated or the protein was rapidly degraded. A similar phenomenon under other experimental conditions has been observed for p21, which was up-regulated at the mRNA level, but down-regulated at the protein level [31]. Hence, Ndrg-1 may also induce post-transcriptional alterations resulting in reduced protein synthesis of *Ctsc*. As previously described, our current results and those of others [60] have revealed that Ndrg-1 down-regulates structural components of the ribosome, which may contribute to this effect.

Interestingly, in contrast to the observation in AT6.1 cells, Ctsc was found to be down-regulated in response to Ndrg-1 over-expression in MIAPaCa-2 cells (Fig. 5D, G). Considering this, Ctsc is a cysteine proteinase that plays a role in pancreatic cancer progression [62,70] and Ndrg-1 has been shown to reduce angiogenesis and tumor growth in pancreatic cancer [6]. Thus, the link between Ndrg-1 and Ctsc could potentially explain the anti-metastatic function of the former in pancreatic cancer.

Overall, this study identified numerous genes which were altered when Ndrg-1 was over-expressed. While Ndrg-1 is not a transcription factor itself, it could potentially change gene expression by other mechanisms, such as protein interactions with other transcription factors. Indeed, examination of the top 200 significantly altered genes derived from our gene array analysis revealed Ndrg-1 altered expression of at least 6 different transcription factors (see Gene Expression Omnibus; accession number GSE9076; http://www.ncbi.nlm.nih.gov/geo/). One of these was Hod, which has been confirmed by RT-PCR to be down-regulated in two Ndrg-1 over-expressing AT6.1 clones (Fig. 4C, D). Hence, these transcription factors may be responsible for the altered gene expression mediated by Ndrg-1. As demonstrated in this study (Fig. 4A) and by others [60], another mechanism by which Ndrg-1 influences gene expression may involve its effect on reducing expression of many genes involved in ribosome and protein synthesis.

In conclusion, this study examined the effect of Ndrg-1 over-expression in four cancer cell models and our results suggest that it has specific functions which may depend on the cell type. The pleiotropic nature of Ndrg-1 has been suggested previously, but has not been extensively examined until now. The potential gene targets of Ndrg-1 in rat prostate cancer cells were examined, revealing multiple molecules that may be responsible for the functions of this protein, including Thtpa and Ctsc.

Acknowledgments

This project was supported by a fellowship and project grants from the National Health and Medical Research Council of Australia, Australian Research Council Discovery Grant, University of Sydney Cancer Fund, Cure Cancer Australia Foundation and grants from Australian Rotary Health Research Fund to D.R.R. Z.K. thanks the Australian Rotary Health Research Fund (Dural Rotary Club) and the Cancer Institute of New South Wales for a PhD Scholarship. The authors wish to thank Dr. David Lovejoy, Dr. Erika Becker, Dr. Maggie Lok, Dr. Yohan Suryo Rahmanto, Dr. Rosei Siafakas and Dr. Danuta Kalinowski for carefully reviewing the paper prior to submission. Finally, we gratefully acknowledge the excellent advice from Dr. Yohan Suryo Rahmanto regarding the analysis of the gene array data.

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