Targeting the Metastasis Suppressor, NDRG1, Using Novel Iron Chelators: Regulation of Stress Fiber-Mediated Tumor Cell Migration via Modulation of the ROCK1/pMLC2 Signaling Pathway $^\text{a}$

Jing Sun, Daohai Zhang, Ying Zheng, Qian Zhao, Minhua Zheng, Zaklina Kovacevic, and Des R. Richardson

Department of General Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China (J.S., M.Z.); Department of Pathology, University of Sydney, New South Wales, Australia (D.Z., Z.K., D.R.R.); and Department of Pathophysiology, Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China (Y.Z., Q.Z.)

Received October 21, 2012; accepted November 27, 2012

ABSTRACT

The iron-regulated metastasis suppressor, N-myc downstream-regulated gene 1 (NDRG1), is up-regulated by cellular iron depletion mediated by iron chelators and can inhibit cancer cell migration. However, the mechanism of how NDRG1 achieves this effect remains unclear. In this study, we implemented established and newly constructed NDRG1 overexpression and knockdown models using the DU145, HT29, and HCT116 cancer cell lines to investigate the molecular basis by which NDRG1 exerts its inhibitory effect on cell migration. Using these models, we demonstrated that NDRG1 overexpression inhibits cell migration by preventing actin-filament polymerization, stress fiber assembly, and formation. In contrast, NDRG1 knockdown had the opposite effect. Moreover, we identified that NDRG1 inhibited an important regulatory pathway mediated by the Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)/phosphorylated myosin light chain 2 (pMLC2) pathway that modulates stress fiber assembly. The phosphorylation of MLC2 is a key process in inducing stress fiber contraction, and this was shown to be markedly decreased or increased by NDRG1 overexpression or knockdown, respectively. The mechanism involved in the inhibition of MLC2 phosphorylation by NDRG1 was mediated by a significant ($P < 0.001$) decrease in ROCK1 expression that is a key kinase involved in MLC2 phosphorylation. Considering that NDRG1 is up-regulated after cellular iron depletion, novel thiosemicarbazone iron chelators (e.g., di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone) were demonstrated to inhibit ROCK1/pMLC2-modulated actin-filament polymerization, stress fiber assembly, and formation via a mechanism involving NDRG1. These results highlight the role of the ROCK1/pMLC2 pathway in the NDRG1-mediated anti-metastatic signaling network and the therapeutic potential of iron chelators at inhibiting metastasis.

Introduction

Despite breakthroughs in therapy, prostate and colorectal cancers are major causes of cancer-related mortality (Siegel et al., 2012). Thus, new approaches to prostate and colorectal cancer treatment have focused on therapeutically targeting crucial molecules involved in disease progression (Fu et al., 2012; Tsujii, 2012). One newly identified molecular target, namely the metastasis suppressor, N-myc downstream regulated gene-1 (NDRG1), has been reported to inhibit prostate and colorectal cancer progression (Guan et al., 2000; Liu et al., 2011). In addition, NDRG1 mRNA and protein levels were decreased in the primary tumor and metastases when compared with normal tissues (Kurdistani et al., 1998). Overexpression of NDRG1 in cancer cells not only induces differentiation ( Fotovati et al., 2011) but also reduces invasion and metastasis (Chen et al., 2012). Several studies report a positive correlation between NDRG1 expression and patient survival, indicating NDRG1 may be a prognostic biomarker in prostate (Liu et al., 2011) and colorectal cancer (Guan et al.,

This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: DAPI, 4',6-diamidino-2-phenylindole; DFO, desferrioxamine; DMSO, dimethylsulfoxide; Dp2mT, di-2-pyridylketone 2-methyl-3-thiosemicarbazone; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; DpC, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IOD, integrated optical density; MLC2, myosin light chain 2; NDRG1, N-myc downstream-regulated gene 1; pMLC2, phosphorylated myosin light chain 2; RhoA, ras homolog family member A; ROCK1, Rho-associated, coiled-coil-containing protein kinase 1; shRNAs, small hairpin RNA; siRNAs, small interfering RNA; Y27632, (R)-(−)-trans-4-(1-aminomethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.
Although NDRG1 is largely considered to be a metastasis suppressor, several studies have also found this latter protein to be associated with increased metastasis and poor prognosis in certain neoplasms, such as hepatocellular carcinoma (Ellen et al., 2008). However, notably, the majority of studies have reported a tumor/metastasis suppressive role of NDRG1 (Ellen et al., 2008). The molecular pathways that NDRG1 affects are still unclear and require exploration, as this metastasis suppressor may be a useful therapeutic target.

Studies from our laboratory (Le and Richardson, 2004; Whitnall et al., 2006; Lovejoy et al., 2011; Chen et al., 2012; Kovacevic et al., 2012; Lovejoy et al., 2012; Yu et al., 2012) and others (Rao et al., 2009; Tian et al., 2010; Liu et al., 2012; Rao et al., 2011) have shown that novel iron-binding agents [e.g., di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) and di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC); Fig. 1] possess marked and selective antitumor and antimetastatic activity by up-regulating NDRG1. The mechanism involved occurs through the depletion of cellular iron via pathways dependent and independent of hypoxia-inducible factor-1α (Le and Richardson, 2004). Importantly, NDRG1 up-regulation by cellular iron depletion inhibits the epithelial-mesenchymal transition that is crucial for metastasis (Chen et al., 2012). Apart from iron chelators, NDRG1 was also found to be up-regulated in response to other anticancer agents, including hydroxyurea, cisplatin, and doxorubicin (Mandal et al., 2004; Delgado-Canedo et al., 2005), further implicating its role in suppressing tumor progression.

Cell migration plays a crucial role in cancer cell metastasis. Considering the mechanism of cancer cell migration, actin participates in a range of events in cellular locomotion, including cell motility, cell polarity, cell junction function, and chemotaxis (Dominguez and Holmes, 2011; Lai et al., 2012). Actin exists in the cytoplasm in two different forms, namely the actin monomer (G-actin) and the actin filament (F-actin). When G-actin exchanges bound ADP for ATP, it is activated and polymerizes into F-actin bundles, also known as stress fibers (Dominguez and Holmes, 2011). When cancer cells make stable associations to a substrate, or when metastasis signaling pathways are triggered, stress fibers are rearranged and extend within the cell (Lai et al., 2012). Myosin interacts with F-actin to drive the movement of actin filaments past one another, and the consequent fiber contraction causes alterations in cell shape and cellular migration (Tojkander et al., 2011).

It is reported that a key factor in stress fiber formation is the phosphorylation of myosin light chain 2 (MLC2) at Thr18/Ser19 (Katoh et al., 2011). Cells have a complicated signaling network modulating MLC2 phosphorylation, among which the Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1) pathway is one of the most crucial mechanisms in stimulating cross-linking of actin by myosin, leading to enhanced cancer cell contractility (Chaturvedi et al., 2011). Therefore, targeting the ROCK1/phosphorylated myosin light chain 2 (pMLC2) pathway to inhibit cancer cell migration may become a therapeutic strategy.

Considering the potent antimetastatic effect of NDRG1 in various cancer cell types and the role of the ROCK1/pMLC2 pathway in activating stress fiber-mediated cell motility and migration, we examined whether the ROCK1/pMLC2 pathway played a critical role in NDRG1-mediated inhibition of cell migration. Our studies demonstrate a significant role of NDRG1 expression in inhibiting the ROCK1/pMLC2 pathway and provide novel insights into the mechanisms underlying the ability of NDRG1 to inhibit cell migration. Furthermore, this investigation illustrates that targeting NDRG1 by using potent thiosemicarbazone chelators could be an important new approach to cancer treatment.
Materials and Methods

Cell Culture. The human prostate cancer cell line, DU145, and the human colon cancer cell lines, HT29 and HCT116, were from the American Type Culture Collection (Manassas, VA) and were grown under established conditions (Chen et al., 2012).

Chelators and ROCK1 Inhibitor. Desferoxamine (DFO; Fig. 1A) was purchased from Novartis (Basel, Switzerland). The chelators, Dp44mT and DpC (Fig. 1, B and C), and the negative control compound, di-2-pyridylketone 2-methyl-3-thiosemicarbazone (Dp2mT; Fig. 1D) (Yuan et al., 2004), were synthesized and characterized using standard methods (Yuan et al., 2004; Lovejoy et al., 2012). The ROCK1 inhibitor, (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydricloride (Y27632) (Kuwahara et al., 1999), was from Abcam (Cambridge, UK).

Plasmid Construction/Transfection. NDRG1 overexpression and knockdown clones in DU145 and HT29 cell lines were established as described previously (Chen et al., 2012). The plasmids and small hairpin RNAs (shRNAs) used for transfection of NDRG1 in HCT116 cells were pBABE-3×-Flag-NDRG1 (from G. Chen, Shanghai Jiao Tong University, China) and pSIREN-shRNA (Clontech, Mountain View, CA), respectively. To overexpress NDRG1, HCT116 cells were transfected using the pBABE-3×-Flag-NDRG1 plasmid, whereas the pSIREN-shRNA plasmid containing NDRG1-specific shRNA (Stein et al., 2004; Chen et al., 2006; Zheng et al., 2009) was used to knockdown endogenous NDRG1 expression. All cDNAs were sequenced by BGI-Tech Solutions Co., Ltd. (Shanghai, China) to ensure identity. Viral supernatants were produced in HEK293T cells co-transfected with the pBABE-3×-Flag-control or pBABE-3×-Flag-NDRG1 constructs and packaging vectors GAG-POL and VSV-G (Clontech). The HCT116 cells were transfected using the retrovirus system. Puromycin (2.5 μg/mL, Sigma-Aldrich, St. Louis, MO) was used to select stable single clones.

Transwell Migration and Wound-Healing Assay. The transwell migration assay was performed using the CytoSelect 24-Well Cell Migration Assay kit (Cell Biolabs, San Diego, CA) (Chen et al., 2012). For the wound-healing assay, the cell monolayer was scraped using a Gilson p200 pipette tip (Middleton, WI). After a 14- to 36-hour incubation, the area covered by migrating cells was analyzed by NIH Image J software (Bethesda, Maryland).

Gene Silencing by Small Interfering RNA. Knockdown of ROCK1 expression using ROCK1 small interfering RNA (siRNA) was performed following the manufacturer’s instructions. Briefly, at ∼60%–70% confluence, NDRG1-knockdown and sh-Control cells were transfected with either ROCK1 Trilencer-27 siRNA duplexes (OriGene, Rockville, MD) or the scrambled control siRNA at 10 nM for 24 hours, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Protein Extraction and Immunoblot Analysis. Preparation of cell lysates and immunoblot analysis were performed via established protocols (Kovacevic et al., 2008). Primary antibodies used include: NDRG1 (catalog number ab37897) from Abcam; MLC2 (catalog number 3672) and pMLC2 (catalog number 3675) from Cell Signaling Technology (Beverly, MA); ROCK1 (catalog number WH0006093M1) and β-actin (catalog number A1978) from Sigma-Aldrich; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (catalog number sc-32233) from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies implemented include horseradish peroxidase–conjugated anti-goat (catalog number A5420), anti-rabbit (catalog number A6154), and anti-mouse (catalog number A4416) antibodies from Sigma-Aldrich.

Fractionation and Quantification Analysis of F-Actin. F-actin extraction and fractionation were performed via established methods (Pan et al., 2011). Briefly, cells were harvested and homogenized using 27-gauge syringes in 500 μL of lysis and F-actin stabilization buffer (50 mM 1,4-piperazinediethanesulfonic acid, 50 mM NaCl, 5 mM MgCl2, 5 mM ethylene glycol tetra-acetic acid, 5% glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, and 0.1% β-mercaptoethanol, pH 6.9) at 37°C. The F-actin was then separated by ultracentrifugation at 100,000g for 60 minutes at 37°C. The pellets were resuspended in 500 μL ice-cold G-buffer (2 mM Tris-HCl pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, and 0.5 mM dithiothreitol) and incubated on ice for 1 hour (F-actin depolymerization). The dissociated F-actin was centrifuged at 14,000g for 10 minutes at 4°C. F-actin expression was then examined by immunoblots using a monoclonal anti-β-actin antibody (1:10,000). GAPDH was used as the loading control.

Immunofluorescence. Immunofluorescence was performed as previously described (Chen et al., 2012). Fluorescence quantification was achieved by comparing the integrated optical density (IOD)/area value of each protein of interest to the IOD/area value of the nucleus [4,6-diamidino-2-phenylindole (DAPI)] in the same image. Images were captured using a Leica SPEII confocal microscope (magnification, 63×; Leica, Heerbrugg, Switzerland). Raw images were analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Co-localization analysis was performed using Imaris 7.3 (BITPLANE, Zurich, Switzerland).

Statistical Analysis. Data are expressed as mean ± S.D. of ≥3 independent experiments and were statistically analyzed using Student’s t-test. Results were considered significant when P < 0.05.

Results

NDRG1 Suppresses Cell Migration. The molecular effectors involved in the metastasis suppressor function of NDRG1 remain uncertain. To understand the role of NDRG1 in cancer cell migration, which is a key characteristic of tumor metastasis, we used established and newly generated cell models that constitutively and stably overexpress exogenous human NDRG1 in DU145 prostate cancer cells, as well as in HT29 and HCT116 colorectal cancer cells (Fig. 2A). The DU145 prostate cancer cell line and HT29 colon cancer cell line are representative cell models of the two tumor types in which NDRG1 has been shown to play an important antimetastatic role. These two cell models have been well characterized, and the role of NDRG1 in the inhibition of cell migration and the epithelial-mesenchymal transition was clearly demonstrated in our previous studies (Chen et al., 2012). The third model, HCT116, is different from epithelial HT29 cells in terms of its cell phenotype and aggressiveness, and was introduced to assess if the response is consistent between cell lines.

In the 3 cell-types, exogenous expression of Flag-tagged NDRG1 was confirmed by immunoblots that demonstrated a band at ∼45 kDa (Fig. 2A). In addition, the endogenously expressed NDRG1 was observed at ∼43 and ∼44 kDa, indicating possible phosphorylation or other post-translational modification (Murray et al., 2004; Kovacevic et al., 2011a). Considering this, it is notable that the densitometric analysis shown throughout this investigation represents the total of all NDRG1 bands. Clearly, the selected NDRG1-overexpressing clones generated from DU145, HT29, and HCT116 cells showed a significant (P < 0.001) increase of NDRG1 expression compared with their respective empty vector-transfected control (Vector Control) cells (Fig. 2A).

To further understand the molecular effects of endogenous NDRG1, we also generated NDRG1-knockdown clones in these cancer cell lines. As indicated in Fig. 2A, compared with NDRG1 expression in control cells transfected with scrambled shRNA (sh-Control), the NDRG1-knockdown clones showed a significant (P < 0.001) ∼5- to 20-fold reduction of total endogenous NDRG1 expression in DU145, HT29, and HCT116 cells. Since cancer cell migration is one of the key factors deciding metastasis, and because NDRG1 is a metastasis suppressor...
we investigated the effect of NDRG1 on cell migration using both the transwell (Fig. 2B) and wound healing assays (Supplemental Fig. 1; see Materials and Methods). Densitometric analysis is expressed relative to the loading control, β-actin. (B) DU145, HT29, and HCT116 NDRG1-overexpressing and NDRG1-knockdown cells as well as their respective control cells were seeded at 100,000 cells/well and incubated for 24 hours at 37°C. Migratory cells on the bottom of the polycarbonate membrane were stained with crystal violet, and images were then captured. Migration was quantified by colorimetric determination at 560 nm. Scale bars: 200 μm. Results are typical of 3–5 experiments. and the histogram values are mean ± S.D. (3–5 experiments). ***P < 0.001, relative to respective control cells.

Fig. 2. NDRG1 suppresses cell motility and migration of prostate and colorectal cancer cells. The DU145, HT29, and HCT116 cancer cell lines were stably transfected with NDRG1-overexpressing and sh-NDRG1 knockdown vectors. (A) Whole-cell lysates were extracted, and immunoblotting was performed to assess NDRG1 overexpression and knockdown compared with their relative control cells (Vector Control and sh-Control, respectively). Densitometric analysis is expressed relative to the loading control, β-actin. (B) DU145, HT29, and HCT116 NDRG1-overexpressing and NDRG1-knockdown cells as well as their relative control cells were seeded at 100,000 cells/well and incubated for 24 hours at 37°C. Migratory cells on the bottom of the polycarbonate membrane were stained with crystal violet, and images were then captured. Migration was quantified by colorimetric determination at 560 nm. Scale bars: 200 μm. Results are typical of 3–5 experiments. and the histogram values are mean ± S.D. (3–5 experiments). ***P < 0.001, relative to respective control cells.

NDRG1 Inhibits F-Actin Polymerization and Stress Fiber Formation. The actin cytoskeleton is an important determinant of cell shape and migration (Suetsugu and Takenawa, 2003). Indeed, reorganization of the actin cytoskeleton plays a key role in driving migration and metastasis of cancer cells (Yamaguchi and Condeelis, 2007). Since NDRG1 acts to inhibit migration (Fig. 2B, Supplemental Fig. 1), we further investigated its effects on actin filament polymerization and stress fiber formation. The total F-actin was fractionated from the whole cell protein pool using well characterized procedures and detected using a β-actin antibody (Fig. 3A) (Kake et al., 1995; Kim et al., 2008; Pan et al., 2011). Hence, in these studies, GAPDH was used as a loading control. As shown in Fig. 3A, overexpression of NDRG1 in DU145, HT29, and HCT116 cells significantly (P < 0.001–0.05) decreased the levels of F-actin, whereas knockdown of endogenous NDRG1 in these cells led to a significant (P < 0.001) 2.7- to 3.8-fold increase of F-actin compared with their respective sh-Control cells. Therefore, for the first time, these results indicate an important inhibitory function of NDRG1 in F-actin synthesis.

We then examined whether NDRG1 could remodel the actin cytoskeleton and affect the distribution of F-actin in these cell models. In agreement with the results from immunoblot
analysis (Fig. 3A), immunofluorescence staining of F-actin with rhodamine-phalloidin showed a significant ($P < 0.01$–0.001) decrease or increase in staining intensity and stress fiber formation in the NDRG1-overexpressing or knockdown cells, respectively, relative to their controls (Fig. 3, B–E).

In addition to the inhibitory effect of NDRG1 overexpression on F-actin synthesis, we also observed that NDRG1 overexpression or knockdown in these cell models affected cellular morphology and F-actin distribution (Fig. 3, B–D). In DU145, HT29, and HCT116 Vector Control or sh-Control

Fig. 3. NDRG1 expression inhibits F-actin polymerization and stress fiber formation in prostate and colorectal cancer cells. (A) F-actin was fractionated and quantified by immunoblot in NDRG1-overexpressing and NDRG1-knockdown DU145, HT29, and HCT116 cells relative to their control cells (Vector Control and sh-Control, respectively) using a well-established technique (see Materials and Methods). Densitometric analyses are expressed relative to the loading control, GAPDH. Data are typical of 3–5 experiments, and the histogram values are mean ± S.D. (3–5 experiments). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, relative to respective control cells. (B–D) Merged immunofluorescence images demonstrating F-actin (red) stained with rhodamine phalloidin and cell nucleus (blue) stained with DAPI in NDRG1-over-expressing and NDRG1-knockdown DU145, HT29, and HCT116 cells relative to the Vector Control and sh-Control cells, respectively. White arrows indicate stress fibers. Scale bar: 25 μm. (E) Fluorescence quantification was performed by comparing the IOD/area value of F-actin to the IOD/area value of the nucleus (DAPI) in the same image. Results are typical of 3–5 images from different visual fields, and the histogram values are mean ± S.D. (3–5 images). **$P < 0.01$; ***$P < 0.001$, relative to the respective control cells.
cells, F-actin was relatively evenly distributed between both the cytoplasm and at the circumference of the cell (so-called "cortical distribution"; see arrows in Fig. 3, B–D). Notably, in DU145 cells, NDRG1 overexpression led to: 1) an increase in the size of the cells, and 2) an appearance of more epithelial-like phenotype (Fig. 3B). However, NDRG1 overexpression resulted in no marked alteration in the morphology of HCT116 or HT29 cells (Fig. 3, C and D). Nevertheless, the overexpression of NDRG1 in all three cell lines led to a general decrease ($P < 0.001–0.01$) in the intensity of F-actin staining (Fig. 3E).

The knockdown of NDRG1 in these three cell-types induced a significant remodeling of actin filaments, leading to stress fiber formation (see white arrows), and a morphologic change to a more aggressive phenotype (e.g., fibroblast-like cells, especially for DU145) relative to sh-Control cells (Fig. 3, B–D). These phenotypic alterations are in agreement with our previous studies demonstrating the effect of NDRG1 expression on maintaining an epithelial phenotype in cancer cells (Chen et al., 2012). In fact, the observed epithelial-like phenotype in the NDRG1-overexpressing DU145 cells was consistent with the enhanced expression and membrane localization of the epithelial cell markers, E-cadherin and $\beta$-catenin, as demonstrated in our previous study (Chen et al., 2012). Collectively, these morphologic characteristics indicate that NDRG1 expression modulates cellular phenotype and stress fiber formation.

**NDRG1 Inhibits the ROCK1/pMLC2 Pathway.** Stress fibers form when a cancer cell makes stable connections to a substrate (Tojkander et al., 2012), or when metastasis signaling is triggered by activators (Tanner et al., 2010; Singh et al., 2011). Actin filaments are generally oriented toward the cell surface through their plus end and may overlap in more interior regions of the cell in antiparallel arrays (Tojkander et al., 2012). During contraction of stress fibers, myosin mediates sliding of antiparallel actin filaments and plays a key role in stress fiber stability and cell motility (Hotulainen and Lappalainen, 2006). Notably, phosphorylation of MLC2 has been demonstrated to be a vital motor involved in motility (Chaturvedi et al., 2011; Katoh et al., 2011). Therefore, we investigated the expression and phosphorylation of MLC2 in our NDRG1 overexpression and knockdown models.

Immunoblots showed that the basal level of MLC2 was not significantly ($P > 0.05$) altered when NDRG1 was overexpressed or knocked down in these cells (Fig. 4, A–C). On the contrary, the relative MLC2 phosphorylation (i.e., pMLC2/MLC2 ratio) was significantly ($P < 0.001–0.05$) decreased in NDRG1-overexpressing DU145, HT29, and HCT116 cells (Fig. 4, A–C). Moreover, sh-NDRG1 markedly ($P < 0.001$) increased the pMLC2/MLC2 ratio by 2.4- to 3.7-fold over their respective sh-Control cells in all three cell-types (Fig. 4, A–C).

Given that NDRG1 modulates cytoskeleton reorganization (Fig. 3, B–D) and MLC2 phosphorylation (Fig. 4), we then examined the mechanism behind these effects. We demonstrated that the level of one of the upstream regulators of MLC2 phosphorylation, ROCK1 (Wyckoff et al., 2006), was significantly ($P < 0.001$) reduced by NDRG1 overexpression in our NDRG1 overexpression and knockdown models. To further substantiate the inhibition of ROCK1 by NDRG1, the three NDRG1-knockdown (sh-NDRG1) cell models and their respective sh-Control cells were treated with ROCK1-specific siRNA (si-ROCK1) to transiently reduce endogenous ROCK1 expression (Fig. 7). Transient treatment of these cells with ROCK1-specific siRNA significantly ($P < 0.001$) reduced ROCK1 expression. Similarly to the results observed with cells treated with Y27632 (Fig. 6), incubation with si-ROCK1 did not significantly affect NDRG1 expression (Fig. 7A–C). Instead, while basal MLC2 expression was not significantly changed by decreased ROCK1 expression, the pMLC2/MLC2 ratio was significantly ($P < 0.001–0.01$) attenuated compared with their relative control cells treated with the si-Control.

Previous studies have established a close association of pMLC2 with stress fiber formation (Tojkander et al., 2012). In support of this, we also observed that pMLC2 was predominantly located in the cytoplasm and in proximity with the plasma membrane (Fig. 5, A–C) and was co-localized with F-actin to form filament bundles (see white arrows, Supplemental Fig. 2) when NDRG1 was knocked down. Consistent with the immunoblot results (Fig. 4, A–C), the fluorescence intensity of pMLC2 was significantly ($P < 0.001–0.01$) decreased by 36%–48% in NDRG1 overexpressing DU145, HT29, and HCT116 cells compared with Vector Control cells (Fig. 5D). Conversely, NDRG1 knockdown led to a significant ($P < 0.001–0.05$) increase of pMLC2 expression by 1.6- to 2.8-fold compared with sh-Control cells (Fig. 5D). Taken together, these data further support that the ROCK1/pMLC2 pathway plays an important role in NDRG1-mediated stress fiber formation and actin reorganization.

**NDRG1 Regulates pMLC2 through Modulating ROCK1 Expression.** The aforementioned studies showed that NDRG1 overexpression inhibited, whereas NDRG1 knockdown enhanced, the ROCK1/pMLC2 pathway (Figs. 4 and 5). However, it is uncertain whether NDRG1 can directly regulate MLC2 phosphorylation or if it exerts its function through inhibiting its kinase, ROCK1. To investigate this, further studies using NDRG1-knockdown cell models aimed to examine the effects of the well characterized ROCK1 inhibitor (Y27632; Fig. 6) or transient ROCK1 knockdown with ROCK1 siRNA (Fig. 7).

Since the ROCK1 inhibitor, Y27632, efficiently inhibits ROCK1 protein expression (Tan et al., 2012) and activity (Uehata et al., 1997), the NDRG1 knockdown cells and the sh-Control cells were incubated with Y27632 at the previously established dose of 0.25 $\mu$M (Uehata et al., 1997; Tan et al., 2012) for 48 hours. ROCK1 expression and the pMLC2/MLC2 ratio were then examined (Fig. 6). We showed that Y27632 had no significant effect on NDRG1 expression in these cell models, thus excluding any nonspecific effects of Y27632 on NDRG1 levels (Fig. 6, A–C). However, after inhibition of ROCK1 activity using Y27632, the pMLC2/MLC2 ratio was also significantly ($P < 0.001$) reduced in these cell models (Fig. 6, A–C). Regardless of NDRG1 expression, suppression of MLC2 phosphorylation occurred in accordance with ROCK1 inhibition. Our results suggest that Y27632 was active in inhibiting ROCK1 protein expression and activity under the conditions used in our investigation, in good agreement with previous studies (Uehata et al., 1997; Tan et al., 2012). Furthermore, analysis of immunofluorescence intensity showed significant ($P < 0.001$) inhibition of MLC2 phosphorylation and F-actin polymerization by Y27632 (Supplemental Fig. 3).

To further substantiate the inhibition of pMLC2 by ROCK1, we used in our investigation, in good agreement with previous studies (Uehata et al., 1997; Tan et al., 2012). Furthermore, analysis of immunofluorescence intensity showed significant ($P < 0.001$) inhibition of MLC2 phosphorylation and F-actin polymerization by Y27632 (Supplemental Fig. 3).

To further substantiate the inhibition of pMLC2 by ROCK1, we used in our investigation, in good agreement with previous studies (Uehata et al., 1997; Tan et al., 2012). Furthermore, analysis of immunofluorescence intensity showed significant ($P < 0.001$) inhibition of MLC2 phosphorylation and F-actin polymerization by Y27632 (Supplemental Fig. 3).
Notably, treatment with si-ROCK1 resulted in at least a 35% reduction of ROCK1 expression in all cell types. This reduction led to at least a 55% decrease of phosphorylation of MLC2. These results indicate that phosphorylation of MLC2 is sensitive to ROCK1 kinase. Taken together, the previously described results (Figs. 4–7) reveal that NDRG1 regulates MLC2 phosphorylation via modulating ROCK1 expression and, thus, its activity.

**Novel Thiosemicarbazone Iron Chelators Modulate ROCK1/pMLC2.** Our previous studies (Le and Richardson, 2004; Whitnall et al., 2006; Kovacevic et al., 2008; Kovacevic et al., 2011a; Kovacevic et al., 2012) and those of others (Liu et al., 2011; Liu et al., 2012) have demonstrated that novel thiosemicarbazone chelators (e.g., Dp44mT and DpC) markedly up-regulate NDRG1 in tumor cells in vitro and in vivo. Furthermore, NDRG1 up-regulation in cancer cells is considered to be one of the mechanisms underlying the chelator-induced inhibition of growth and metastasis (Le and Richardson, 2004; Whitnall et al., 2006; Kovacevic et al., 2012; Liu et al., 2012). Because NDRG1 plays an important role in actin filament remodeling and stress fiber formation via the ROCK1/pMLC2 pathway, we then examined whether chelators have similar effects when these cell models were incubated with these agents. In these studies, Dp2mT was used as an appropriate negative control as it has a similar chemical structure to Dp44mT (Fig. 1) but has been designed to not bind iron or up-regulate NDRG1 (Le and Richardson, 2004; Yuan et al., 2004). The well-characterized “gold-standard” iron chelator, DFO (Chaston et al., 2003), was also implemented in this study as a positive control for cellular iron-depletion and NDRG1 up-regulation (Le and Richardson, 2004). Furthermore, NDRG1-overexpressing DU145, HT29, and HCT116 cells were included as positive controls (see lane labeled “NDRG1”; Fig. 8, A–C).
The DU145, HT29, and HCT116 parental cells were incubated with DFO (100 μM), Dp44mT (10 μM), or DpC (10 μM) for 24 hours, and the levels of NDRG1, ROCK1, MLC2, and pMLC2 were examined by immunoblots. Notably, the higher concentration of DFO was used due to its poor membrane permeability relative to the lipophilic and highly membrane-permeable ligands, Dp44mT and DpC, whereas all agents were used at doses that were previously found to be effective at both mobilizing intracellular iron and inducing NDRG1 expression (Richardson et al., 1994; Yuan et al., 2004; Kovacevic et al., 2011a; Lovejoy et al., 2012). As shown in Fig. 8, NDRG1 expression was markedly (P < 0.001) increased after incubation with DFO, Dp44mT, or DpC relative to cells treated with the vehicle control and the negative control, Dp2mT (10 μM).

We also observed that ROCK1 expression was significantly (P < 0.001) decreased in these chelator-treated cells relative to cells incubated with the vehicle control or Dp2mT (Fig. 8, A–C). Furthermore, incubation with DFO, Dp44mT, or DpC did not result in a significant change in basal MLC2 expression (Fig. 8, A–C), but the pMLC2/MLC2 ratio was significantly (P < 0.001–0.01) reduced by these chelators compared with...
cells treated with vehicle or Dp2mT (Fig. 8, A–C). In line with the inhibition of ROCK1/pMLC2 observed in NDRG1-overexpressing cells (Fig. 4), the decrease in ROCK1/pMLC2 levels after incubation with DFO, Dp44mT, or DpC was consistent with that mediated by NDRG1 up-regulation.

Novel Iron Chelators Modulate the ROCK1/pMLC2 Pathway via Up-Regulation of NDRG1. Since the ROCK1/pMLC2 pathway could be inhibited by NDRG1 overexpression (Figs. 4 and 5) or chelator treatment (Fig. 8, Supplemental Figs. 4 and 6), we next explored whether NDRG1 up-regulation is the essential mediator of the ability of chelators to inhibit the ROCK1/pMLC2 pathway (Fig. 9, A–C). To this end, the NDRG1-knockdown clones (sh-NDRG1) and their respective sh-Control cells were incubated with chelators (i.e., 100 μM DFO, 10 μM Dp44mT, or 10 μM DpC), the negative control (10 μM Dp2mT), or the vehicle (0.1% dimethylsulfoxide (DMSO)/
medium] for 24 hours. Nontransfected parental cells (labeled as “Control”) were also used for each cell line as a comparison between the sh-Control and sh-NDRG1 transfections. As demonstrated in Fig. 9, A–C, assessing the DU145, HT29, and HCT116 sh-Control cells, chelator treatment (i.e., DFO, Dp44mT, or DpC) resulted in a pronounced (P < 0.001) increase of NDRG1 expression by ∼4- to 10-fold over cells incubated with the vehicle or Dp2mT. In all three cell-types, ROCK1-specific siRNA inhibits the ROCK1/pMLC2 pathway via an NDRG1-independent mechanism. (A–C) The sh-NDRG1 knockdown and sh-Control DU145, HT29, and HCT116 cells were incubated with scrambled control siRNA (si-Control) or ROCK1-specific siRNA (si-ROCK1), and the levels of NDRG1, ROCK1, pMLC2 and MLC2 were examined by immunoblot. Densitometric analysis for NDRG1 and ROCK1 are expressed relative to the loading control, β-actin, whereas that for MLC2 phosphorylation is expressed as the pMLC2/MLC2 ratio. Results are typical of 3–5 experiments, and the histograms in (A–C) represent mean ± S.D. (3–5 experiments). **P < 0.01; ***P < 0.001, relative to the respective si-Control cells.
upon the chelator-induced up-regulation of NDRG1, ROCK1 expression was significantly ($P < 0.001$) decreased by up to 60–90% in these sh-Control cells, compared with cells incubated with the vehicle or Dp2mT (Fig. 9, A–C). Accordingly, the pMLC2/MLC2 ratio was also markedly reduced by 60–80% ($P < 0.001$) when these cells were treated with chelators (Fig. 9, A–C). Notably, when these cell models were treated with chelators (i.e., DFO, Dp44mT, or DpC), F-actin and pMLC2 were significantly ($P < 0.001$) decreased as assessed by the quantitative analysis of immunofluorescence intensity, relative to cells treated with the vehicle control or Dp2mT (Supplemental Figs. 4–6). Collectively, the effect of chelators on

Fig. 8. Iron chelators modulate ROCK1/pMLC2 levels in prostate and colorectal cancer cells. (A) DU145, (B) HT29, and (C) HCT116 parental cells were treated with the chelators DFO (100 μM), Dp44mT (10 μM), or DpC (10 μM) for 24 hours, and the levels of NDRG1, ROCK1, pMLC2, and MLC2 were examined by immunoblot. Vehicle (0.1% DMSO/medium) or Dp2mT (10 μM) were used as negative controls. Cells transfected with an NDRG1-expression vector were used as a positive control (see lane labeled as “NDRG1”). Densitometric analyses for NDRG1 and ROCK1 are expressed relative to the loading control, β-actin, whereas that for MLC2 phosphorylation is expressed as the pMLC2/MLC2 ratio. Results are typical of 3–5 experiments, and the histograms in (A–C) represent mean ± S.D. (3–5 experiments). **$P < 0.01$; ***$P < 0.001$, relative to the cells treated with Vehicle or Dp2mT.
ROCK1, F-actin, and pMLC2 levels is consistent with the ability of these agents to up-regulate NDRG1. When the NDRG1-knockdown (sh-NDRG1) DU145 and HCT116 cells were treated with DFO, Dp44mT, or DpC, the increase in NDRG1 expression was less pronounced than that observed in the chelator-treated sh-Control cells (Fig. 9, A and C). In fact, sh-NDRG1 HCT116 cells showed no increase in NDRG1 expression following treatment with the iron chelators when compared with the vehicle and Dp2mT controls (Fig. 9C). However, in contrast, the up-regulation of NDRG1 induced by Dp44mT and DpC was similar in the sh-NDRG1 and sh-Control HT29 cells, whereas DFO did not increase NDRG1 expression in sh-NDRG1 cells as much as in sh-Control cells (Fig. 9B). This observed variation in response to chelators after NDRG1-knockdown between cell types may be related to differences in efficacy of shRNA at suppressing chelator-induced NDRG1 expression.

We also demonstrated that NDRG1 knockdown affected the chelator-inhibited ROCK1/pMLC2 pathway. Incubation of sh-Control DU145 cells with chelators (i.e., DFO, Dp44mT, and DpC) decreased ROCK1 expression by 60–90% and the pMLC2/MLC2 ratio by ~75% relative to vehicle- and

---

**Fig. 9.** Iron chelators modulate ROCK1/pMLC2 in prostate and colorectal cancer cells via up-regulation of NDRG1. The NDRG1-knockdown clones and their respective sh-Control DU145, HT29, and HCT116 cells were incubated with: Vehicle (0.1% DMSO/medium), Dp2mT (10 μM), DFO (100 μM), Dp44mT (10 μM), or DpC (10 μM) for 24 hours. Nontransfected parental cells were included as additional controls to allow comparison between the sh-Control and sh-NDRG1 (see lane labeled as "Control"). Results are typical of 3–5 experiments, and the values in histograms in (A–C) represent mean ± S.D. (3–5 experiments). ***P < 0.001, relative to cells treated with Vehicle or Dp2mT; ##P < 0.01; ###P < 0.001, relative to cells with the same treatment in the sh-Control group.
Dp2mT-treated cells (Fig. 9A). In comparison, incubation of NDRG1-knockdown DU145 cells with these chelators resulted in a 50–65% reduction of ROCK1 expression and a 40–45% decrease of the pMLC2/MLC2 ratio. These results suggest that the lower level of chelator-induced NDRG1 in the NDRG1-knockdown DU145 cells versus the sh-Control DU145 cells exerts significantly ($P < 0.001$) less inhibitory effect on ROCK1 expression and the pMLC2/MLC2 ratio (Fig. 9A).

In NDRG1-knockdown and sh-Control HT29 cells in which chelator treatments induced a similar level of NDRG1 expression, ROCK1 expression was reduced ($P < 0.001$) by chelators by 55–80% in the sh-NDRG1 cells and by 65–70% in the sh-Control cells, whereas the pMLC2/MLC2 ratio was decreased ($P < 0.001$) by 70–78% in the NDRG1 knockdown cells and by ~80% in the sh-Control cells (Fig. 9B). Hence, the similar effect of chelators on NDRG1 expression in the sh-Control and sh-NDRG1 HT29 cells resulted in similar responses in ROCK1 levels and the pMLC2/MLC2 ratio. In agreement with these studies, immunofluorescence experiments examining F-actin and pMLC levels in DU145 and HT29 cells demonstrated that the chelators significantly ($P < 0.001$) reduced their levels in both sh-Control and sh-NDRG1 cells (Supplemental Fig. 4, B and C; Supplemental Fig. 5, B and C).

Contrary to the results obtained with HCT116 sh-Control cells in which DFO, Dp44mT, or DpC caused a pronounced decrease in ROCK1 expression and the pMLC2/MLC2 ratio (Fig. 9C), these chelators were unable to exert a similar effect on the ROCK1/pMLC2 pathway in the sh-NDRG1 HCT116 cells. This was probably due to the absence of chelator-induced NDRG1 up-regulation in sh-NDRG1 HCT116 clones (Fig. 9C). Hence, in this cell line, sh-NDRG1 showed the most pronounced effect at reducing NDRG1 expression among the three cell types tested (Fig. 9, A–C). These observations were further confirmed using immunofluorescence to examine F-actin and pMLC2 levels in HCT116 sh-Control and sh-NDRG1 cells, where the chelators markedly inhibited stress fiber formation in sh-Control cells while having little effect in sh-NDRG1 cells (Supplemental Fig. 6, B–C). Failure of NDRG1 induction by chelators in the sh-NDRG1 HCT116 clones may result from the high level of internalized siRNA transcribed from the sh-NDRG1 construct, and this could be a reason for the lack of inhibition of the ROCK1/pMLC2 pathway in this cell type.

Critically, one could find it difficult to understand why sh-NDRG1 knockdown did not markedly inhibit induction of NDRG1 by chelators in DU145 cells and HT29 cells while effectively preventing induction of NDRG1 expression in HCT116 (Fig. 9, A–C). Considering the variation in response, it is notable that the chelators markedly induce NDRG1 expression, and the level of inhibition provided by the sh-NDRG1 construct is probably relatively lower in DU145 and HT29 cells (Fig. 9, A and B). Hence, it failed to markedly antagonize chelator-induced NDRG1 expression. However, in sh-NDRG1 HCT116 cells, treatment with chelators was unable to induce NDRG1 expression, demonstrating effective inhibition by shNDRG1 (Fig. 9C). Clearly, a cell type–dependent factor is affecting the efficiency of sh-NDRG1 to reduce NDRG1 expression.

Collectively, suppression of endogenous NDRG1 expression by sh-NDRG1 in two cell lines interrupts the ability of chelators to induce NDRG1 expression relative to the sh-Control, leading to partial (DU145) or total (HCT116) loss of inhibition of ROCK1 expression and MLC2 phosphorylation. These data indicate that NDRG1 plays an important role in the effector function of novel chelators, such as DpC, on the ROCK1/pMLC2 pathway.

**Discussion**

The metastasis suppressor NDRG1 has been identified as an important molecular target for new therapeutics and can modulate multiple pathways during tumorigenesis and metastatic progression (Kovacevic et al., 2011b; Chen et al., 2012; Kovacevic et al., 2012; Liu et al., 2012). Herein, we identify a novel mechanism by which NDRG1 exerts its antimetastatic effects in prostate and colorectal cancer cells. We demonstrate that NDRG1 can suppress cell migration, F-actin polymerization, and stress fiber formation via inhibiting the ROCK1/pMLC2 pathway in prostate and colorectal cancer cells. Interestingly, although this effect was significant in all three cell models, the changes in F-actin, ROCK1, and pMLC2 in DU145 prostate cancer cells were less marked when compared with the HT29 and HCT116 cells, and this may be due to the genetic heterogeneity between these cancer cells.

The actin cytoskeleton has a fundamental role in tumor cell migration and movement (O'Neill, 2009). This is achieved by two mechanisms: 1) actin polymerization against the cellular membrane at the leading edge provides force (Pollard and Cooper, 2009), and 2) actin filaments together with myosin II filaments form stress fibers composed of contractile actomyosin structures, which play key roles in cancer cell motility (Tojkander et al., 2012). Although the mechanisms of actin filament networks and signaling pathways are well understood, the molecular mechanisms underlying the assembly of stress fibers remain to be elucidated (Michelot and Drubin, 2011). Considering this, we investigated the effects of NDRG1 on actin filaments in prostate and colorectal cancer cells. We showed that NDRG1 inhibits cell migration via its effects on regulating actin filament polymerization and stress fiber formation (Figs. 3 and 5, Supplemental Figs. 2–6).

It has been well-established that there are cellular signaling networks that regulate stress fiber assembly and contraction (Tojkander et al., 2012). Among these pathways, the small GTPase RhoA, as well as its effector, ROCK, directly promote stress fiber assembly (Leung et al., 1996). In addition, stress fiber contractility is regulated by MLC2 phosphorylation (Somlyo and Somlyo, 2000). Furthermore, MLC2 phosphorylation-mediated contractility of stress fibers is also dependent on the ras homolog (Rho) (Katoh et al., 2001). The ras homolog family member A (RhoA)/ROCK cascade results in actomyosin activity through direct phosphorylation of MLC2, generating contractile responses (Amano et al., 1996). Rho-kinases, such as ROCK, have also been demonstrated to regulate MLC2 phosphorylation in the invading margin of tumor cells, indicating its important role in modulating cancer cell migration, invasion, and metastasis (Wyckoff et al., 2006) (Fig. 10). In our investigation, we showed that Rho-kinase ROCK1 promotes MLC2 phosphorylation and that NDRG1 inhibits MLC2 phosphorylation through down-regulating ROCK1. Thus, our studies establish a novel function of NDRG1 in modulating the ROCK1/pMLC2 pathway (Fig. 10).

Considering that NDRG1 plays an important role in regulating actin filament reorganization and stress fiber...
formation through modulating the ROCK1/pMLC2 pathway, we then examined the novel thiosemicarbazone iron chelators, Dp44mT and DpC, and the clinically used iron chelator, DFO, which can have selective anticancer activity and up-regulate NDRG1 via mechanisms both dependent and independent of hypoxia-inducible factor-1α (Le and Richardson, 2004; Yuan et al., 2004; Whitnall et al., 2006; Chen et al., 2012; Kovacevic et al., 2011a; Kovacevic et al., 2012; Lovejoy et al., 2012). Upon chelator-mediated up-regulation of NDRG1, the ROCK1/pMLC2 pathway was markedly suppressed and stress fiber formation was significantly reduced. This indicates the potential general effect of iron chelators on actin filament reorganization and stress fiber formation in cancer cells.

In agreement with our study, it has been reported in rat pulmonary artery endothelial cells that F-actin polymerization and cellular remodeling can be induced by iron supplementation (Gorbunov et al., 2012). Furthermore, iron-induced rearrangements of the cytoskeleton can be partially eliminated in the presence of the iron chelator, $N,N^\prime$-bis-(2-hydroxybenzyl)-ethylenediamine-$N,N^\prime$-diacetic acid (Gorbunov et al., 2012). However, these latter investigations did not dissect the molecular mechanisms involved, nor did they elucidate any role for NDRG1 in the processes observed. In contrast, our mechanistic studies showed that iron chelators inhibit cytoskeleton remodeling and cell migration through targeting the NDRG1/ROCK1/pMLC2 pathway.

Of the thiosemicarbazones used in the current study, it is notable that DpC shows marked and selective antitumor activity both in vitro and in vivo (Kovacevic et al., 2011a; Kovacevic et al., 2012; Lovejoy et al., 2012). Indeed, this ligand has recently been shown to be active via both the oral and intravenous routes in vivo using pancreatic and lung cancer xenograft models (Kovacevic et al., 2011a; Kovacevic et al., 2012; Lovejoy et al., 2012). An important pharmacologic advantage of DpC relative to its predecessor, Dp44mT (Whitnall et al., 2006), is that the agent does not induce cardiotoxicity even when administered at substantially higher doses (Lovejoy et al., 2012). Furthermore, unlike Dp44mT, which produces toxicity when administered by the oral route (Yu et al., 2012), DpC demonstrates marked

---

**Fig. 10.** Schematic illustrating the mechanism of how the metastasis suppressor, NDRG1, affects cellular migration through its ability to inhibit actin filament polymerization, stress fiber assembly, and cell migration via repressing the ROCK1/pMLC2 pathway in prostate cancer and colorectal cancer cells.
antitumor efficacy and tolerability when given via gavage (Lovejoy et al., 2012). Apart from these important pharmacologic properties, the efficacy of Dpc in up-regulating NDRG1 and its effects on other key molecular targets [e.g., cyclin D1 (Kovacevic et al., 2011a)], indicate the marked potential of this chelator as a novel anticancer agent.

In summary, this study demonstrates that NDRG1 is able to inhibit actin filament polymerization, stress fiber assembly, and cell migration through modulating the ROCK1/pMlc2 pathway (see schematic in Fig. 10). Furthermore, novel iron chelators inhibit the ROCK1/pMlc2 pathway via up-regulation of NDRG1. Although the effect of chelators is, in part, to induce NDRG1 expression that results in inhibition of stress fiber formation, the mechanism may not be as straightforward as depicted in Fig. 10. However, this schematic provides an instructive summary of the current studies and is an appropriate working model for further experimentation. In fact, our current results elucidate the underlying mechanism of the anti-metastatic effects of NDRG1 mediated through inhibition of cellular migration and further identify novel iron chelators that target NDRG1 as a promising therapy.

Acknowledgements

The authors thank Professor G. Chen (Shanghai Jiao Tong University, Shanghai, P.R.China) for providing the pBabe-3·Flag plasmid to construct the NDRG1 overexpression cell models. The authors also thank Dr. Louise Cole (University of Sydney, NSW, Australia) for technical support in using the advanced microscope facilities and software of the Bosch Institute (University of Sydney).

Authorship Contributions

Participated in research design: Sun, Zhang, Kovacevic, Richardson.

Conducted experiments: Sun.

Conducted new reagents or analytic tools: Sun, Richardson.

Performed data analysis: Sun, Zhang, Kovacevic, Richardson.

Wrote or contributed to the writing of the manuscript: Sun, Zhang, Y. Zheng, M. Zheng, Zhao, Kovacevic, Richardson.

References


Sun et al.

Sun et al.


Address correspondence to: Dr. D. R. Richardson, Iron Metabolism and Chelation Program, Department of Pathology and Bosch Institute, University of Sydney, Sydney, New South Wales, 2006 Australia. E-mail: d.richardson@med.usyd.edu.au