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ORIGINAL ARTICLE

Inhibition of epithelial to mesenchymal transition in metastatic prostate cancer cells by the novel proteasome inhibitor, NPI-0052: pivotal roles of Snail repression and RKIP induction

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Metastasis is associated with the loss of epithelial features and the acquisition of mesenchymal characteristics and invasive properties by tumor cells, a process known as epithelial to mesenchymal transition (EMT). Snail expression, through nuclear factor (NF)-κB activation, is an EMT determinant. The proteasome inhibitor, NPI-0052, induces the metastasis tumor suppressor/immune surveillance cancer gene, Raf kinase inhibitor protein (RKIP), via NF-κB inhibition. We hypothesized that NPI-0052 may inhibit Snail expression and, consequently, the metastatic phenotype in DU-145 prostate cancer cells. Cell treatment with NPI-0052 induced E-cadherin and inhibited Snail expression and both tumor cell invasion and migration. Inhibition of Snail inversely correlated with the induction of RKIP. The underlying mechanism of NPI-0052-induced inhibition of the metastatic phenotype was corroborated by: (1) treatment with Snail siRNA in DU-145 inhibited EMT and, in contrast, overexpression of Snail in the nonmetastatic LNCaP cells induced EMT, (2) NPI-0052-induced repression of Snail via inhibition of NF-κB was corroborated by the specific NF-κB inhibitor DHMEQ and (3) RKIP overexpression mimicked NPI-0052 in the inhibition of Snail and EMT. These findings demonstrate, for the first time, the role of NPI-0052 in the regulation of EMT via inhibition of NF-κB and Snail and induction of RKIP.
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Introduction

Metastatic disease is the primary cause of death for most cancer patients. Metastasis is a process that allows many tumors to expand to different areas of the body, involving either multiple mutations or epigenetic changes. Carcinomas arise from glandular or epithelial cells lining different compartments of the body. Most deaths caused by this class of tumor result from the tumor's metastatic characteristics that allow it to spread to different organs (Steeg, 2006; Pantel *et al*., 2008). The epithelial to mesenchymal transition (EMT) process is the principal way through which metastasis occurs, namely, beginning with a disruption of intercellular contacts and the enhancement of cell motility and, thereby, resulting in the release of cells from the parent epithelial tissue. Epithelial cells, therefore, lose their association with epithelial cell sheets and acquire, instead, many of the attributes of mesenchymal cells including acquisition of increased invasiveness and resistance to apoptosis (Shook and Keller, 2003; Condeelis and Pollard, 2006). This transdifferentiation program is regulated by distinct pleitropically acting transcription factors such as Snail, Twists, Slug and Goosecoid (LaBonne and Bronner-Fraser, 2000; Thiery and Sleeman, 2006). At the biochemical level, the EMT program involves the downregulation of epithelial protein expression, notably cytokeratins and the induction of mesenchymal protein expression including vimentin, N-cadherin, fibronectin, platelet-derived growth factor receptor and matrix metalloproteinases, and acquisition of motility and invasiveness (Thiery and Sleeman, 2006). When active in cancer cells, the EMT program enables these cells to complete the initial steps of invasiveness in the metastatic cascade, specifically local invasion, intravasation, survival in the circulation and extravasation.

Snail belongs to the Snail superfamily of zing-finger transcription factors including Twist and SIP1, all considered essential for the induction of EMT in tumor metastasis (Nieto, 2002). Snail was initially identified to play a role in embryonic development, neural differentiation, cell division and cell survival. Snail triggers the induction of metastasis during tumor progression by

promoting the acquisition of invasive and migratory properties by tumor cells via transcriptional repression of metastasis suppressor genes such as E-cadherin (Peinado *et al*., 2004). Snail is transcriptionally regulated by nuclear factor (NF)-κB (Barbera *et al*., 2004; Julien *et al*., 2007) and posttranscriptionally by glycogen synthase kinase (GSK)-3β-mediated phosphorylation that results in its cytoplasmic localization and proteasome degradation (Dominguez *et al*., 2003; Zhou *et al*., 2004). In addition to E-cadherin, Snail downregulates the expression of tight junctions components, such as claudins and occludins and epithelial markers mucin-1 and cytokeratin 18 (Garcia de Herreros, 2001). It also increases the expression of the mesenchymal markers vimentin and fibronectin, proteins involved in cancer invasion such as metalloproteinases 2 and 9 and transcription factors ZEB-1 and LEF-1 (Cano *et al*., 2000; De Craene *et al*., 2005).

Among the survival pathways known to be associated with tumor progression and metastasis are the Raf-1/MEK/ERK and NF-κB pathways (Inoue *et al*., 2007; Granovsky and Rosner, 2008). Raf kinase inhibitor protein (RKIP) is a member of a conserved group of proteins called PEBP (phosphatidylethanolamine-binding protein), which participate in the regulation of growth and survival signaling pathways (Odabaei *et al*., 2004). RKIP has been reported to function by inhibiting the proliferative and survival Raf-1/MEK/ERK and NF-κB signaling pathways (Yeung *et al*., 1999, 2001). The importance of RKIP in metastases was demonstrated by the finding that the restoration of RKIP expression inhibits prostate cancer metastases in a murine model (Fu *et al*., 2003, 2006) and, hence, RKIP was identified as a metastasis suppressor protein. RKIP was recently found to be under the regulation of the transcription repressor Snail (Beach *et al*., 2008). RKIP expression levels correlated inversely with Snail expression in metastatic prostate samples, whereas overexpressing Snail in breast or prostate cell lines downregulated RKIP expression (Beach *et al*., 2008).

The proteasome has been implicated to play a major role in the pathogenesis and survival of cancers, mainly, through regulation of NF-κB activity and of antiapoptotic gene products. The proteasome has also been involved in the inhibition of drug-induced apoptosis in tumor cells and in the development of drug resistance. Hence, proteasome inhibitors have been developed to target the proteasome with the objective to induce cancer cell cytostasis or cell death when used alone or in combination with other cytotoxics (Sterz *et al*., 2008). NPI-0052 (Salinosporamide A), is a novel nonpeptide inhibitor of the 20S proteasome, identified from the marine bacterium *Salinispora tropica*, and exhibits a therapeutic potential against a wide variety of tumors through a poorly understood mechanism (Chauhan *et al*., 2006). The major biological effect of NPI-0052 in tumor cell models is apoptosis induction and osteoclastogenesis suppression through inhibition of constitutive and inductive NF-κB activation and, thus, downmodulation of NF-κB-regulated antiapoptotic gene products (Ahn *et al*., 2007; Miller *et al*., 2007).

Metastasis is regulated by several complex mechanisms, though, it has been reported that both RKIP and Snail levels of expression in tumor cells dictate the metastatic behavior of tumor cells. We have recently reported that the novel proteasome inhibitor, NPI-0052, induces RKIP expression in prostate and lymphoma cell lines (Baritaki *et al*., 2008). As Snail is transcriptionally regulated, in part, by NF-κB and NPI-0052 inhibits NF-κB activity, we hypothesized that NPI-0052 treatment of tumor cells will inhibit Snail expression and, consequently, inhibit Snail-induced regulation of EMT and the initiation of the metastatic cascade. This study was designed to test the above hypothesis. The followings have been addressed: (1) Does NPI-0052 inhibit Snail transcription and expression in the metastatic prostate cancer cell line DU-145? (2) Does NPI-0052-induced Snail suppression correlate with NPI-0052-induced RKIP expression? (3) Does NPI-0052 induced repression of Snail is due, in part, to NPI-0052-mediated inhibition of NF-κB activity and whether a specific NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), mimics NPI-0052-induced repression of Snail and induction of RKIP? (4) Does NPI-0052-induced repression of Snail result in the inhibition of EMT and induction of E-cadherin? (5) Does treatment with small interfering RNA (siRNA) against Snail mimic NPI-0052-mediated effects and result in the inhibition of EMT in the metastatic DU-145 cell line? In contrast, does overexpression of Snail induce EMT in the nonmetastatic LNCaP prostate cancer cell line? and (6) Does overexpression of RKIP mimic NPI-0052-induced inhibition of Snail and EMT in the metastatic DU-145 cell line? The findings reported here support our hypothesis and reveal, for the first time, a novel function for the proteasome inhibitor NPI-0052 in the prevention of metastasis.

Results

One of the key activators of tumor cell resistance to therapy and acquisition of metastatic properties by the tumor cells is the NF-κB pathway. Proteasome inhibitors, including NPI-0052, have been shown to mediate their biological function mainly by suppressing the NF-κB survival pathway and resulting in antitumor effects (Chauhan *et al*., 2005). Two major metastasis suppressor gene products, E-cadherin and RKIP, have been reported to be negatively regulated by the transcription repressor Snail, and Snail is transcriptionally regulated by NF-κB and plays a critical role in the initiation of EMT (Barbera *et al*., 2004; Julien *et al*., 2007; Beach *et al*., 2008). In this report, we hypothesized that NPI-0052 could suppress EMT via inhibition of NF-κB and downstream both the inhibition of Snail and upregulation of RKIP expression. Our hypothesis was tested using the DU-145 human metastatic prostate carcinoma cell model whereas the nonmetastatic human prostate carcinoma cell line LNCaP served as control.

NPI-0052 suppresses the constitutive activity of NF-κB

The inhibitory effect of NPI-0052 on NF-κB in our system was determined by assessing the NF-κB promoter activity and the expression of phospho-IκBα. We initially determined subtoxic NPI-0052 concentrations for DU-145 cells by performing a drug titration using the trypan blue exclusion assay (Figure 1a). NPI-0052 (50 nM) was chosen as ID20 drug concentration to be used in the following experiments. In the NF-κB reporter system, increasing concentrations of NPI-0052 inhibited proportionally the NF-κB promoter activity as shown in Figure 1b, whereas 50 nM of NPI-0052 induced time-dependent accumulation of phospho-IκBα protein due to lack of degradation by the proteasome (Figure 1c).

NPI-0052 inhibits the invasive and migratory tumor cell properties via modulation of the mesenchymal metastatic cell phenotype

As NF-κB plays a crucial role in the induction of metastasis and can be inhibited by NPI-0052, we investigated whether NPI-0052 can also inhibit EMT. NPI-0052 induced morphological changes in the metastatic cell line DU-145. After 24 h treatment with increasing concentrations of NPI-0052, DU-145 cells acquired a more epithelial-like phenotype (see arrows in Figure 2a). In contrast, the nonmetastatic LNCaP cells treated with the same concentrations of NPI-0052 maintained the epithelial phenotype without any significant change.

We then examined the genetic background of the observed NPI-0052-induced cell morphological changes by monitoring the expression patterns of epithelial markers including cytokeratin 18 and E-cadherin as well as the expression of the mesenchymal phenotype-related gene products, fibronectin and vimentin. The protein levels were assessed by western blot and immunofluorescence analysis in both the DU-145 and LNCaP cell lines before and after treatment with NPI-0052. NPI-0052 significantly induced the expression of E-cadherin and cytokeratin 18 mainly in DU-145 cells, where the basal levels of the above epithelial gene products are low (Figures 2b and c). In contrast, NPI-0052 downregulated the levels of fibronectin and blocked almost completely vimentin expression. LNCaP cells have undetectable vimentin expression and their low basal fibronectin level remained unchanged after NPI-0052 treatment (Figures 2b and c). These findings suggest that NPI-0052 modifies, at least at the post-transcriptional level, gene products involved in EMT induction and resulting in inhibition of the mesenchymal cell phenotype.

The functional significance of the NPI-0052-induced changes in the expression profiles of the above metastasis-related gene products was expected to be reflected in the migratory and invasive cell properties. To maximize the effects of NPI-0052 on cell migration and invasion activity, DU-145 cells were stimulated with tumor-necrosis factor (TNF)-α because TNF-α has been shown to induce the expression of many genes involved in tumor metastasis (van de Stolpe *et al*., 1994). To determine tumor cell migratory and invasive properties, the cells were seeded onto the top chamber of the appropriate plates with or without TNF-α in the presence or absence of NPI-0052 and then examined for migration and invasion as described in the Materials and methods. Shown in Figure 3, NPI-0052 suppressed significantly both the tumor cell migratory (Figure 3a) and invasive (Figure 3b) properties. TNF-α-induced tumor cell migration and invasion (augmented by almost 10-fold compared to untreated cells) was also

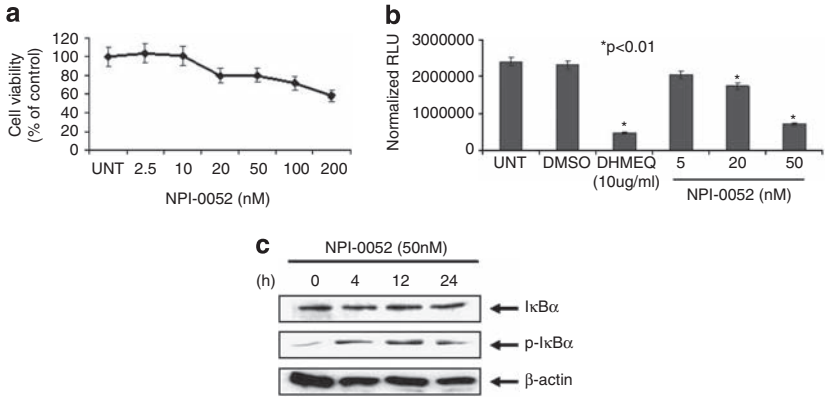

Figure 1 NPI-0052 inhibits nuclear factor (NF)-κB activation. (**a**) Titration of NPI-0052-induced cytotoxicity in DU-145 cells. Cells were treated with different concentrations of NPI-0052 for 24 h. Cell viability was examined microscopically by trypan blue dye exclusion. (**b**) NPI-0052 inhibits NF-κB promoter activity. NF-κB promoter activity was assessed in DU-145 cells treated with various concentrations of NPI-0052 using an NF-κB-luciferase (NF-κB-Luc) reporter plasmid as described in Materials and methods. Values represent the mean ± s.e.m. of three independent experiments and were calculated based on the control value set at 100% (control: untreated cells). Transfected cells treated with 10 μg/ml dehydroxymethylepoxyquinomicin (DHMEQ) served as a positive inhibition control of the NF-κB promoter activity. *P* value: treated vs untreated cells (Mann–Whitney *U*-test). RLU: relative light units. (**c**) NPI-0052 prevents p-IkBα degradation and results in its cytoplasmic accumulation. DU-145 cells were treated with 50 nM NPI-0052 for the indicated time points and western blot analysis was performed with whole cell lysates for the detection of both phosphorylated and total IκBα levels.

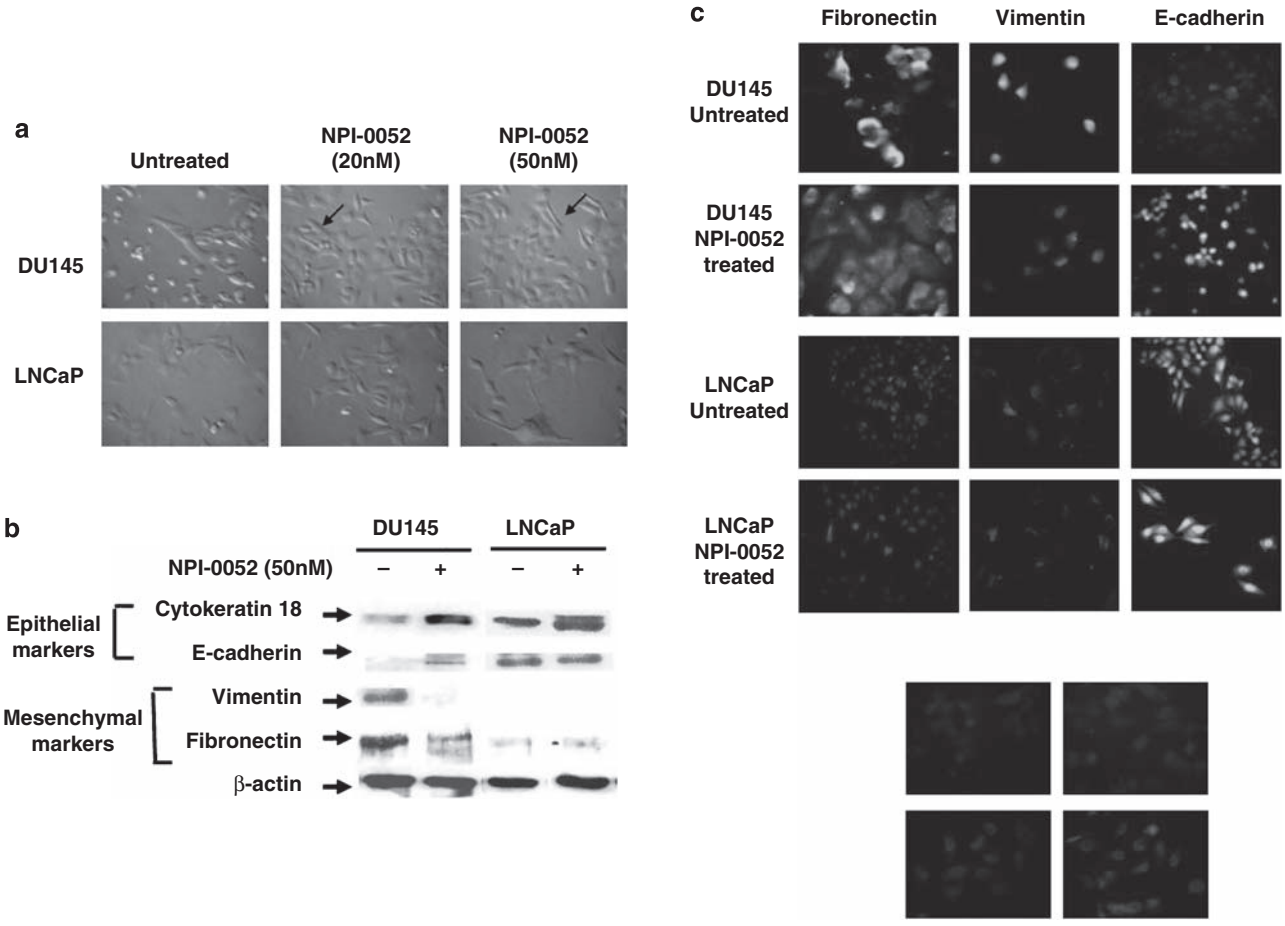

Figure 2 NPI-0052 suppresses the expression of mesenchymal gene products and induces the expression of epithelial gene products in prostate tumor cells. (**a**) Analysis of cell morphology after cell treatment with NPI-0052. NPI-0052 induces changes in the mesenchymal morphology of DU-145 and LNCaP cells when used in concentrations of 20 or 50 nM ($\times$ 40 magnification). (**b**) Total protein lysates derived from DU-145 and LNCaP cells that were treated for 4 h with 50 nM NPI-0052 were subjected to western blot analysis for determination of protein expression of the indicated epithelial and mesenchymal gene markers. The results were compared with the expression profiles of the same gene products in untreated cell lysates. Actin was used as an internal control for loading. (**c**) Immunofluorescent analysis (IFA) images of rabbit anti-fibronectin, rabbit anti-vimentin and mouse anti-E-cadherin stained DU-145 and LNCaP cells before and after treatment with 50 nM of NPI-0052. Cells stained with mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) and rabbit IgG-RPE antibodies were used as negative controls. Images were obtained under an immunofluorescent microscope.

suppressed by NPI-0052. These findings suggest that NPI-0052 can regulate metastasis via modification of EMT-related gene products and resulting in inhibition of tumor cell migratory and invasive properties.

NPI-0052 induces the metastasis suppressor gene product, RKIP, whose overexpression reverses the mesenchymal cell phenotype in DU-145 cells

RKIP has been shown to inhibit NF-κB suppression and suppresses metastasis (Yeung *et al*., 2001; Fu *et al*., 2003). As NPI-0052 also downregulates NF-κB, we expected that the inhibitory effects of NPI-0052 on EMT may coincide with the upregulation of RKIP. We first investigated whether NPI-0052 interferes with the transcriptional regulation of RKIP in DU-145 cells. We determined the RKIP mRNA levels in cells treated with 50 nM NPI-0052 for various time periods (0.5, 1, 2, 3, 4 and 12 h). RKIP mRNA expression showed a constant increase starting at 30 min after treatment up to the final time point of 12 h (Figure 4a). Similarly, RKIP protein levels followed a time-dependent significant increase in the presence of NPI-0052 and starting as early as 4 h after treatment (Figure 4b).

To determine the direct effect of RKIP induction in the regulation of the metastatic phenotype of DU-145 cells, we performed ectopic expression of RKIP using a CMV-HA-RKIP expression vector. Cells overexpressing RKIP were characterized by morphological changes related to a more epithelial-like phenotype as shown in Figure 4c. These changes were reflected at the level of gene expression in which RKIP overexpression correlated with suppression of vimentin and fibronectin and induction of the epithelial gene markers E-cadherin and cytokeratin 18, as assessed by western blot and immunofluorescent analysis (IFA; Figures 4d and e).

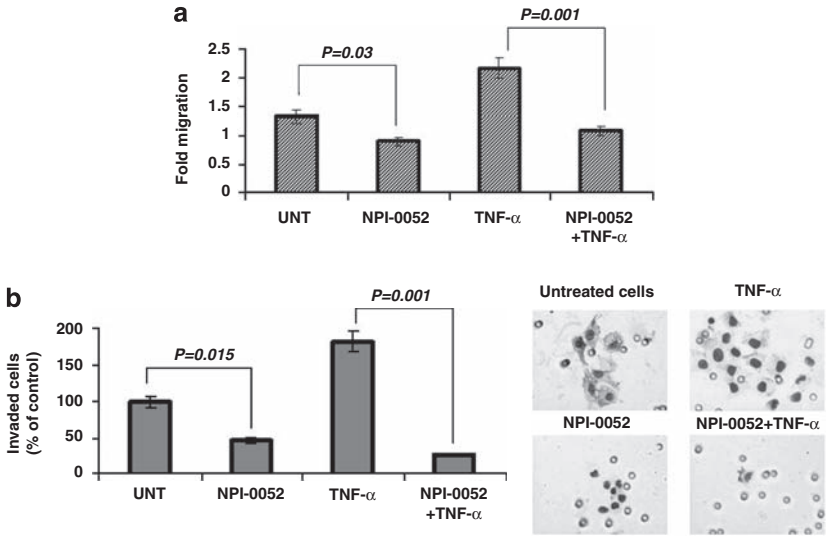

Figure 3 NPI-0052 suppresses the migratory and invasive properties of the metastatic DU-145 cells. (**a**) DU-145 cell migration was inhibited by 50 nM NPI-0052 used alone or in combination with 100 ng/ml of tumor-necrosis factor (TNF)-α. 10^5 cells were seeded in the upper chamber, whereas in the lower chamber RPMI 1640 containing 10% fetal bovine serum (FBS) was added. Cell migration was allowed for 22 h in a humidified incubator at 37 °C with 5% CO_2 and measured in a fluorescence plate reader using a cell postlabeling approach with BD Calcein AM Fluorescent dye. Only those labeled cells that had migrated through the pores of the membrane were detected. The data were expressed as the fold cell migration derived from the mean relative fluorescence units (RFU) of cell migration through the membrane toward FBS divided by the mean RFU of cell migration in the absence of FBS. (**b**) NPI-0052 inhibited the invasive cell properties in both unstimulated DU-145 cells or after cell stimulation with 100 ng/ml TNF-α. 2.5×10^4 cells were seeded in the upper chamber, whereas RPMI 1640 containing 10% FBS was added in the lower chamber. The cells were treated or left untreated with combinations of 50 nM NPI-0052 and 100 ng/ml TNF-α and incubated for 22 h. Invaded cells were counted under the microscope after Matrigel membrane fixation and staining with crystal violet. Data are expressed as the percent invasion through the Matrigel matrix and membrane relative to the migration through the control insert membrane. Right panel: representative pictures of invaded cells (blue staining) under different treatment conditions (× 40 magnification). Control: untreated cells. For both assays *P* values were set significant at the level of 0.05 and were calculated using the Mann–Whitney *U*-test.

Cells transfected with the control cytomegalovirus (CMV) empty vector (CMV-HA-EV) didn't show any significant differences in the expression profiles of the gene products tested above compared to untransfected cells. RKIP overexpression also resulted in decrease of Snail, an important EMT-inducer, as it will be discussed bellow. These above findings suggested that the induction of the metastasis suppressor gene product, RKIP is a crucial factor underlying the mechanism by which NPI-0052 regulates metastasis.

NPI-0052 downregulates Snail expression via NF-κB inhibition and resulting in RKIP upregulation and inhibition of EMT

We have recently shown that RKIP is under the negative transcriptional regulation of Snail whereas Snail has been previously reported to be positively regulated, in part, by NF-κB (Barbera *et al*., 2004; Beach *et al*., 2008). As NPI-0052 inhibits NF-κB, we anticipated that NPI-0052-mediated RKIP induction and inhibition of EMT might be mediated via suppression of Snail. We show that NPI-0052 interferes with Snail expression at both the mRNA and protein levels. Treatment of DU-145 cells with 50 nM of NPI-0052 induced a potent decrease in Snail transcription as early as 1 h after treatment and reaching the lowest level at 4 h after treatment (Figure 5a). Concomitantly, Snail protein levels were significantly reduced at $\geqslant$4 h after treatment with NPI-0052 (Figure 5b). The baseline RKIP and Snail levels in DU-145 cells were inversely correlated with Snail expression to be dominant over RKIP, as expected, according to the high metastatic potential of this cell line. Cell treatment with NPI-0052 maintained the inverse correlation between RKIP and Snail levels of expression.

The direct role of Snail repression by NPI-0052 in the inhibition of EMT was determined by using Snail siRNA-transfected DU-145 cells (high basal levels of Snail). Transfection of DU-145 cells with Snail siRNA mimicked NPI-0052 with respect to morphological changes related to inhibition of the mesenchymal cell phenotype and acquisition of the epithelial-related morphology (Figure 5c). The above changes were reflected by the induction of RKIP, E-cadherin and cytokeratin 18 protein levels and downregulation of vimentin and fibronectin gene expression, as assessed by western blot and IFA (Figures 5d and e). These findings corroborate our earlier observations and suggest the role of Snail inhibition by NPI-0052 in the inhibition of EMT.

Ectopic expression of Snail in LNCaP cells represses RKIP expression and increases their metastatic potential

In contrast to the direct inhibitory effect of Snail silencing or RKIP overexpression by NPI-0052 in the metastatic potential of DU-145 as shown above, we

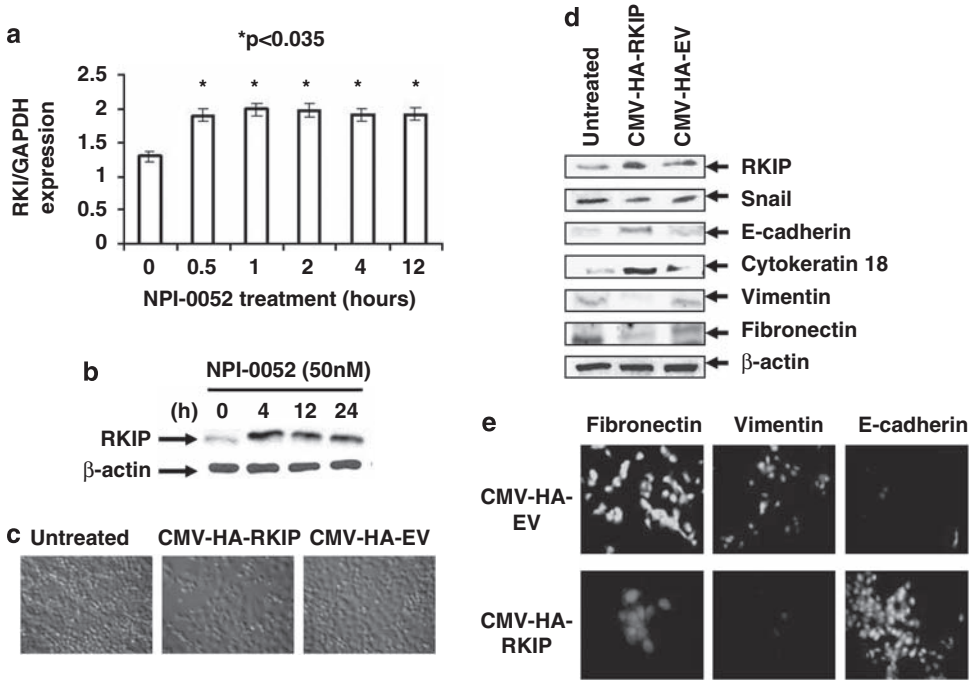

Figure 4 NPI-0052 induces Raf kinase inhibitor protein (RKIP) expression and overexpression of RKIP reverses the mesenchymal cell phenotype in metastatic prostate cells. (**a** and **b**) Time kinetic analysis of RKIP mRNA and protein expression in DU-145 cells treated with 50 nM NPI-0052. (**a**) RKIP transcript levels were determined by reverse transcription (RT)–PCR for each time point tested (0–12 h). Normalized mRNA values were derived by dividing the mRNA value of each target gene with the corresponding quantity of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. Statistical analysis was performed using the Mann–Whitney *U*-test. *P* values: treated vs untreated cells. The data represent the mean values ± s.e.m. from three independent experiments. (**b**) Protein lysates were harvested at the indicated time points and subjected to western blot analysis for RKIP protein determination. Actin was used as an internal control for loading. Blots are representative of three independent and reproducible experiments. (**c**) Changes in DU-145 cell morphology from a mesenchymal to a more epithelial phenotype after transient transfection of cells with a CMV-HA-RKIP expression vector (× 40 magnification). (**d**) Total cell lysates were harvested from DU-145 cells after overexpression of RKIP for 48 h using a CMV-HA-RKIP vector and subjected to western blot analysis for determination of the protein expression of the indicated gene products. A cytomegalovirus (CMV) empty vector (CMV-HA-EV) was used as a control in the transfection assays. Actin was served as an internal control. Blots are representative of three independent and reproducible experiments. (**e**) Immunofluorescent analysis (IFA) images of rabbit anti-fibronectin, rabbit anti-vimentin and mouse anti-E-cadherin stained DU-145 cells transfected with the CMV-HA-RKIP or CMV-HA-EV constructs. The corresponding immunoglobulin G (IgG) controls are shown in Figure 2c.

predicted that overexpression of Snail in the nonmetastatic LNCaP cell line would result in EMT induction and the acquisition of a more mesenchymal phenotype compared to the wild-type cells. We first examined the effect of NPI-0052 on the levels of RKIP and Snail expression in LNCaP cells. Subtoxic NPI-0052 concentrations were assessed using trypan blue exclusion assay (Figure 6a). Compared to DU-145 cells, LNCaP cells exhibited higher RKIP and lower Snail expression. Treatment of LNCaP cells with 50 nM NPI-0052 resulted in slight increase of RKIP protein levels at 4 h after treatment, whereas the baseline low Snail level was completely undetectable at 4 and 12 h after treatment and reappeared at 24 h following treatment (Figure 6b). These results suggest that the observed effect of NPI-0052 on the modification of RKIP and Snail expression could be dependent on the baseline levels of the above gene products in the cell line tested.

As Snail is a highly unstable protein due to aberrant phosphorylation and proteasome degradation (Zhou *et al*., 2004), Snail overexpression in LNCaP cells was performed by using both the unstable wild-type expression vector (CMV-f-Snail) and the stable vector carrying mutations in the phosphorylation sites (CMV-f-Snail S6A; Figure 6c). Ectopic Snail expression in LNCaP cells by CMV-f-Snail was less effective compared to CMV-f-Snail S6A in reducing the basal high level of RKIP as well as E-cadherin and cytokeratin 18 expressions, whereas inducing the expression of vimentin and fibronectin. These findings corroborated the above findings in DU-145 cells and showed that Snail inhibition by NPI-0052 controls, at least in part, the suppressive effect of NPI-0052 on the EMT phenotype.

Direct role of NF-κB inhibition on the regulation of Snail and RKIP expression

The interrelationship among the NF-κB, Snail and RKIP gene products shown above were modified by NPI-0052 and the inhibition of EMT was examined in DU-145 cells that were treated with the NF-κB inhibitor DHMEQ. Concomitant with the effect of NPI-0052 on the expression profiles of Snail and RKIP (Figures 4b

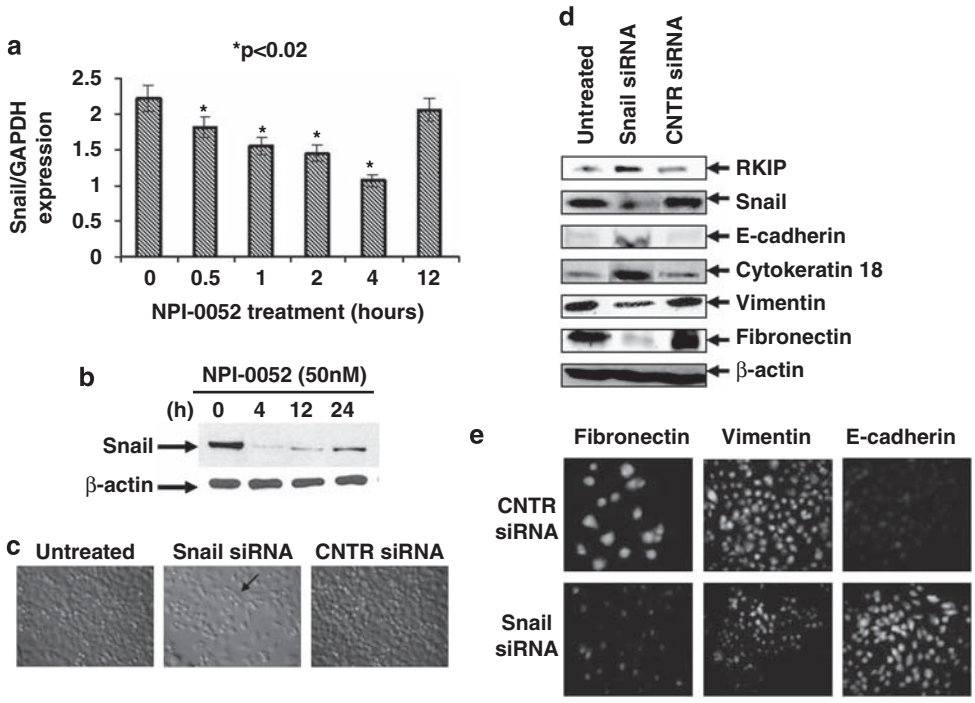

Figure 5 NPI-0052 inhibits Snail expression and Snail silencing reverses the mesenchymal cell phenotype in metastatic DU-145 prostate cells. (**a** and **b**) Time kinetic analysis of Snail mRNA and protein expression in DU-145 cells treated with 50 nM NPI-0052. (**a**) Snail transcript levels were determined by reverse transcription (RT)–PCR for each time point tested (0–12 h). (**b**) Cell lysates were harvested at the indicated time points and subjected to western blot analysis for Snail protein determination. Snail expression was significantly inhibited by NPI-0052. (**c**) Changes in DU-145 cell morphology from a mesenchymal to a more epithelial phenotype after silencing of Snail using Snail small interfering RNA (siRNA; $\times 40$ magnification). (**d**) Total cell lysates were harvested from DU-145 cells after suppression of Snail for 72 h using Snail siRNA (72 h) and subjected to western blot analysis for determination of the protein expression of the indicated gene products. A random nucleotide sequence (CNTR siRNA) was used as a control in the transfection assays. Actin was served as internal control. Blots are representative of three independent and reproducible experiments. (**e**) Immunofluorescent analysis (IFA) images of rabbit anti-fibronectin, rabbit anti-vimentin and mouse anti-E-cadherin stained DU-145 cells transfected with the Snail or control siRNAs. The corresponding immunoglobulin G (IgG) controls are shown in Figure 2c.

and 5b), DHMEQ treatment resulted in Snail suppression and RKIP induction (Figure 6d). These findings suggested the regulation by NPI-0052 of the NF-κB-Snail-RKIP loop and resulting in the inhibition of EMT.

Discussion

Although surgery, chemotherapy and radiation therapies effectively control many cancers at the primary site, the development of metastastic disease signals a poor prognosis with increased morbidity and mortality. Relatively few factors of the metastatic process have been successfully developed as chemotherapeutic targets. New insights into the initial steps of the metastatic process were revealed in developmental genetics by a set of regulators that induce the transdifferentiation program termed 'the epithelial to mesenchymal transition (EMT)'. Among the survival pathways playing a critical role in the regulation of metastasis is the NF-κB signaling pathway. NF-κB contributes to the development and/or progression of malignancy by regulating the expression of genes involved in cell growth and proliferation, apoptosis, angiogenesis and metastasis. As most of the proteasome inhibitors have been shown to mediate their main effects through NF-κB inhibition (Miller *et al*., 2007), we tested whether the proteasome inhibitor, NPI-0052 was able to suppress metastasis. Here, we present evidence for a role of NPI-0052 in suppressing EMT via a mechanism involving inhibition of Snail and induction of the metastasis suppressor gene products RKIP and E-cadherin. We further show that RKIP induction attenuates directly a feedback loop of Snail suppression via NF-κB inhibition in addition to suppression mediated directly by NPI-0052. We also demonstrate that Snail suppression by NPI-0052 also represses the expression of Snail-regulated mesenchymal markers. These modifications result in the reversal of the mesenchymal cell phenotype and inhibition of the migratory and invasive properties of the tumor cells. Overall, we demonstrate, for the first time, the potential therapeutic role of the proteasome inhibitor NPI-0052 in the regulation of EMT and metastasis through the metastasis suppressor factors (RKIP, E-cadherin, epithelial markers) as schematically diagrammed in Figure 7.

The involvement of NPI-0052 in the inhibition of tumor cells invasion was recently proposed by Ahn *et al*. (2007). According to this study, NPI-0052 was able to

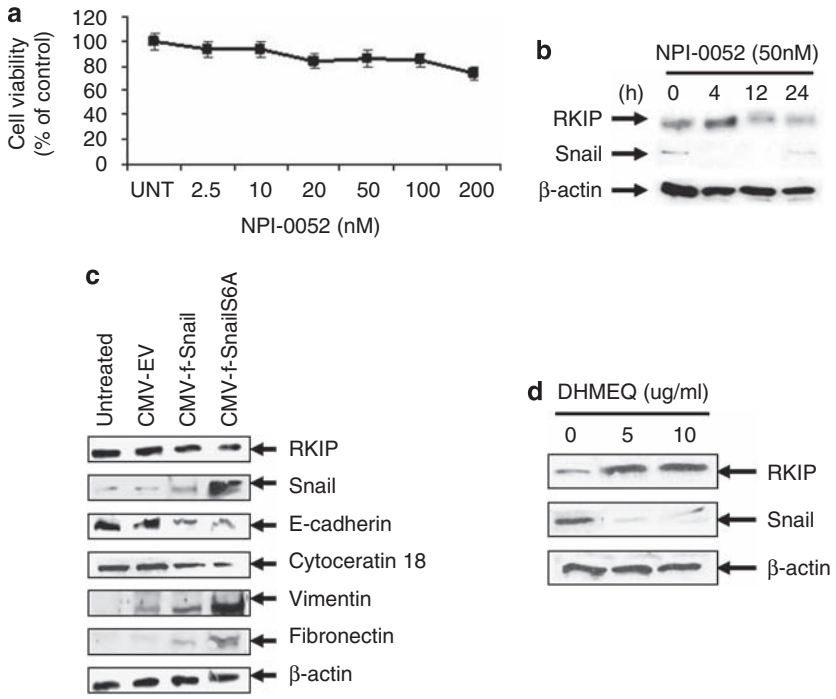

Figure 6 Ectopic expression of Snail in nonmetastatic LNCaP cells represses Raf kinase inhibitor protein (RKIP) expression and induces the metastatic phenotype. (**a**) Titration of NPI-0052-induced cytotoxicity in LNCaP cells. Cells were treated with different concentrations of NPI-0052 for 24 h. Cell viability was examined microscopically by trypan blue dye exclusion. (**b**) Time kinetic analysis of RKIP and Snail expression in LNCaP cells treated with 50 nM NPI-0052. (**c**) Reversal of epithelial phenotype in LNCaP cells by Snail overexpression. LNCaP cells were transfected with cytomegalovirus-flag-Snail expression vectors (CMV-f-Snail) using lipofectamine. Twenty-four hours after transfection, cell lysates were harvested and subjected to immunoblot analysis. To circumvent the possible effect of the highly unstable Snail on RKIP expression or the expression of the other gene products tested, a mutated stable variant (Snail-6SA), which has all its six phosphorylable Ser in the consensus glycogen synthase kinase (GSK)-3β sites mutated to Ala was used. A CMV-EV was used as control. (**d**) Inhibition of nuclear factor (NF)-κB by its chemical inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) inhibits Snail and upregulates RKIP expressions. DU-145 cells were treated with 5 or 10 μg/ml DHMEQ and protein lysates were harvested 24 h after treatment for RKIP and Snail protein determination by western blot. Actin expression was used as an internal loading control in b, c and d panels.

suppress in human adenocarcinoma cell lines TNF-α-induced NF-κB-dependent gene products involved in tumor metastasis including VEGF, MMP-9 and ICAM-1 protein expressions. Our results are in agreement with the reported findings and, in addition, we show that NPI-0052 inhibits EMT in nontreated tumor cells. Our findings also demonstrate that NPI-0052 downregulates mesenchymal and invasive markers such as vimentin and fibronectin and reverses the cell phenotype by inducing a mesenchymal to epithelial transition (MET), a process associated with the reexpression of E-cadherin. Concomitant with these observations are the findings derived, herein, of the *in vitro* tumor cell invasion and migration studies showing that NPI-0052 potentiates significantly the inhibition of both native and TNF-α-induced tumor cells migratory and invasive properties.

RKIP loss or depletion has been associated with metastatic disease in an increasing number of solid tumors. Initially RKIP loss was identified as a prognostic marker for prostate cancer (Fu *et al*., 2003, 2006). Further studies showed that RKIP is depleted in distant metastases for various tumor types, including colorectal and breast carcinoma (Hagan *et al*., 2005; Minoo *et al*., 2007), and could be a prognostic marker for disease-free survival. The antimetastatic properties of RKIP have been attributed to its ability to inhibit survival pathways such as the Raf-1/MEK/ERK and NF-κB pathways (Yeung *et al*., 1999, 2001). A recent report by Dangi-Garimella *et al*. (2009) corroborates and highlights the importance of RKIP as a key metastasis suppressor and potential therapeutic agent. As NF-κB pathway is an additional target for RKIP and NPI-0052 is able to downregulate NF-κB, we hypothesized that the inhibitory effects of EMT by NPI-0052 might be due to RKIP upregulation. NPI-0052 was found to increase both RKIP mRNA and protein levels. Also, RKIP overexpression resulted in the inhibition of EMT-related gene products such as vimentin and fibronectin that are overexpressed in the metastatic DU-145 cell line and the reappearance of epithelial gene products related to metastasis suppression including E-cadherin and cytokeratin 18. The above gene modifications were accompanied with tumor cell morphological changes associated with the acquisition of an epithelial cell phenotype. These findings concur with a previous report demonstrating in a xenograft mouse model for prostate cancer and metastatic prostate cell lines that exogenous RKIP expression suppressed invasion and metastasis *in vivo* and *in vitro*, respectively (Fu *et al*., 2003).

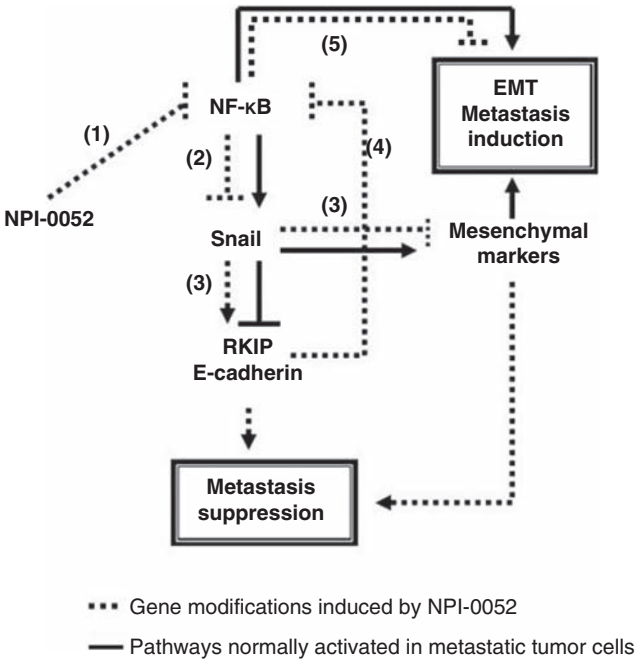

Figure 7 Schematic diagram illustrating the pathways by which the proteasome inhibitor NPI-0052 contributes in the regulation and reversal of the initiation of tumor metastasis. Proteasome inhibitors mediate their biological effect mainly by inhibiting the nuclear factor (NF)-κB pathway and consequently the expression of NF-κB-regulated gene products. The Snail transcription factor, an essential initiator of epithelial to mesenchymal transition (EMT), is under the positive regulation of NF-κB and inhibits the expression of metastasis suppressor genes such as *RKIP* and *E-cadherin*, whereas it induces directly and/or indirectly the expression of mesenchymal markers including vimentin and fibronectin, resulting in the acquisition of a metastatic phenotype by the tumor cells. Based on the present findings, we show that NPI-0052 inhibits NF-κB (1), which in turn inhibits the expression of the transcriptional repressor of Raf kinase inhibitor protein (RKIP), Snail (2), which results in upregulation of the metastasis suppressor RKIP and inhibition of mesenchymal gene products that are responsible for the initiation of EMT (3). RKIP induction by NPI-0052 will further result in additional NF-κB suppression (4) (feedback loop), which in addition to Snail inhibition will downregulate the expression of other gene products that participate in EMT induction (5). Thus, overall we demonstrate that Snail suppression by NPI-0052-induced NF-κB inhibition not only induces metastasis suppression and epithelial gene products, but also represses the expression of Snail-regulated mesenchymal markers, resulting in reversal of the mesenchymal cell phenotype and inhibition of the migratory and invasive properties of the tumor cells.

The direct involvement of NF-κB inhibition by NPI-0052 in the modulation of Snail and RKIP expression was tested by using the NF-κB chemical inhibitor DHMEQ, which inhibits the translocation of NF-κB from cytoplasm to the nucleus (Katsman *et al*., 2007). We showed that DHMEQ was able to suppress significantly the Snail expression, and to induce RKIP suggesting that NF-κB inhibition has a leading role in the modulation of the above gene products and, concomitantly, in the regulation of invasion and metastasis.

We have recently reported in the metastatic prostate cell line, DU-145, that the Snail transcription factor functions as a transcriptional repressor not only of E-cadherin but also of RKIP, whereas in primary and metastatic prostate tumors Snail expression is inversely correlated with RKIP and E-cadherin levels (Beach *et al*., 2008). These observations suggested that Snail might be an appropriate therapeutic target to inhibit the EMT process and, in turn, to block tumor invasion. Julien *et al*. (2007) have recently reported that activation of NF-κB by upregulation of AKT results in downstream upregulation of Snail expression leading to induction of EMT suggesting that the Snail promoter is regulated positively by NF-κB p65. Therefore, in this study, we examined whether NPI-0052-mediated inhibition of NF-κB and induction of RKIP resulted from the suppression of Snail and leading to inhibition of EMT. We showed that NPI-0052 has a significant inhibitory effect on Snail expression and this inhibition was directly associated with reexpression of RKIP and E-cadherin, inhibition of mesenchymal gene markers and induction of an epithelial cell phenotype as assessed by Snail silencing. These results suggested that inhibition of Snail may be pivotal for NPI-0052-mediated RKIP and E-cadherin induction and inhibition of EMT.

Overexpression of Snail contributes directly to EMT, accelerating tumor survival, migration and bad prognosis. This has been shown in different cancer cell lines and tumor biopsies including breast cancer (Blanco *et al*., 2002), gastric cancer (Rosivatz *et al*., 2002), hepatocellular carcinomas (Jiao *et al*., 2002), ovarian carcinoma (Elloul *et al*., 2006), oral squamous cell carcinoma (Yokoyama *et al*., 2001) and head and neck cancer (Yang *et al*., 2007). Overexpression of the stable Snail form, Snail-6SA, in our nonmetastatic prostate cell line LNCaP resulted in acquisition of the EMT phenotype with expression of vimentin and fibronectin. This is in agreement with previous studies showing that, in addition to E-cadherin suppression, Snail transfectants downregulate other epithelial markers such as mucin1 and cytokeratin 18 (Garcia de Herreros, 2001) and upregulate and redistribute mesenchymal markers such as vimentin and fibronectin (Cano *et al*., 2000). In contrast, the shift of EMT markers and consequently the EMT phenotype was reversed by siRNA-mediated repression of Snail expression in DU-145. This finding corroborates previous reports by Yang *et al*. (2007) and Olmeda *et al*. (2007), indicating suppression of tumor growth and invasiveness by Snail silencing.

NPI-0052 activity in tumor cell models has been shown to be mainly involved in induction of apoptosis in combination with other cytotoxics by mechanisms involving NF-κB downregulation (Ahn *et al*., 2007; Miller *et al*., 2007). The antitumor properties of NPI-0052 have been evaluated in a wide range of nonclinical studies including *in vitro* and *in vivo* models for a wide range of solid tumors and hematologic malignancies (Chauhan *et al*., 2005, 2008; Cusack *et al*., 2006; Ruiz *et al*., 2006; Miller *et al*., 2007; Roccaro *et al*., 2008; Sloss *et al*., 2008). NPI-0052 as a single agent has shown advantages compared to conventional proteasome inhibitors such as bortezomib and MG-132 with regards to higher speed and duration of action, wider spectrum of inhibitory effects to the 20S proteasome, greater

suppressive effect on NF-κB activation in many tumor cell models, potent apoptotic activity at low concentrations and ability to reverse tumor resistance to Bortezomib *in vitro* and *in vivo*. (Chauhan *et al*., 2006, 2008; Ruiz *et al*., 2006). In addition, we and others have shown that NPI-0052 as single agent has good tolerance *in vivo* in mice models and limited toxicity in human colony-forming assays (Chauhan *et al*., 2005; Baritaki *et al*., 2008). We recently demonstrated that NPI-0052 is able to reverse prostate tumor cell resistance to TRAIL apoptosis with a mechanism involving induction of TRAIL receptors (DR4 and DR5) and activation of type I and type II apoptotic pathways via Yin Yang 1 (YY1) and NF-κB inhibition (Baritaki *et al*., 2008). Thus, NPI-0052 seems to be an effective anticancer agent with unique pharmacogenic properties that can achieve high levels of proteasome inhibition *in vitro* and *in vivo*.

In summary, we report for the first time the potential beneficial role of NPI-0052 in the regulation of metastasis by modulating the ratio between metastasis suppressor factors (RKIP, E-cadherin, epithelial markers) and metastasis inducer factors (Snail, NF-κB, mesenchymal markers) and propose the therapeutic application of NPI-0052 in the management of metastatic prostate tumors and other cancers. NPI-0052 is currently proceeding through dose escalation in several concurrent Phase I clinical trials for evaluation as a single agent in patients with multiple myeloma, solid tumors or lymphomas. Phase Ib studies are also underway in combination with a specific targeted therapy (Fenical *et al*., 2009) The clinical relevance and significance of inhibiting Snail and restoring RKIP expression by NPI-0052 might correlate with a favorable clinical outcome accompanied by diminution of tumor progression and spread. Moreover, RKIP induction by NPI-0052 may improve the efficacy of antitumor therapies, especially if they are combined with conventional immuno- and/or or chemotherapeutics, as well as the host immune surveillance against cancer as reported by us (Baritaki *et al*., 2007). Also, we propose that RKIP and Snail expression profiles in tumors could be used as potential prognostic biomarkers.

Materials and methods

Cell lines

The prostate carcinoma cell lines DU-145 (metastatic bone-derived human androgen-independent human prostatic adenocarcinoma) and LNCaP (nonmetastatic bone-derived human androgen-dependent human prostatic adenocarcinoma) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Both cell lines were maintained in a humidified 5% CO_2 environment in RPMI 1640 supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 1% nonessential amino acids, 2 mM L-glutamine, 1% sodium pyruvate and 10% fetal bovine serum (FBS; Life Technologies Inc., Grand Island, NY, USA).

Reagents and plasmid constructs

The proteasome inhibitor NPI-0052 (Salinosporamide A) was provided by Nereus Pharmaceuticals (San Diego, CA, USA). The NF-κB inhibitor DHMEQ was kindly provided by Dr Umezawa (Keio University, Japan). Stock solutions of DHMEQ and NPI-0052 were prepared in dimethyl sulfoxide. The recombinant human TNF-α was purchased from Peprotech (Rocky Hill, NJ, USA). Anti-human RKIP, Snail and β-actin antibodies used for western blot analysis were obtained from Zymed (San Francisco, CA, USA), AbCam (Cambridge, MA, USA) and Chemicon (Temecula, CA, USA), respectively. The mouse monoclonal anti-human cytokeratin 18 antibody, as well as the siRNA against Snail and the siRNA transfection reagent were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The anti-human rabbit fibronectin, mouse E-cadherin and goat vimentin antibodies used for western blot analysis were all obtained from Sigma (St Louis, MO, USA). The mouse monoclonal anti-E-cadherin-fluorescein isothiocyanate (FITC) used for IFA was obtained from Pharmingen (BD Biosciences, San Jose, CA, USA). The rabbit anti-fibronectin and anti-vimentin antibodies for IFA were purchased from AbCam, whereas the mouse immunoglobulin G (IgG)-FITC, rabbit IgG-R-phycoerythrin (RPE) and anti-rabbit IgG-RPE antibodies were obtained from Sigma.

The NF-κB activity was determined by using an NF-κB-luciferase reporter plasmid (pNF-κB-Luc) purchased from Invitrogen (Carlsbad, CA, USA). For ectopic RKIP expression, we used a CMV-HA-RKIP expression vector containing the full-length cDNA of RKIP under control of a CMV promoter, as described previously (Chatterjee *et al*., 2004). The relevant empty vector (CMV-HA-EV) was used as negative control. For Snail overexpression in LNCaP cells a CMV-flag-Snail expression construct (CMV-f-Snail) was used. To circumvent the possible effect of the highly unstable Snail on RKIP expression or the expression of the other gene products tested, a mutated stable variant (CMV-f-Snail-6SA), which has all its six phosphorylated serine residues in the consensus GSK-3β sites mutated to Ala was used along with the wild-type Snail vector CMV-f-Snail (Beach *et al*., 2008).

Transient transfections

To determine the effect of NPI-0054 on NF-κB promoter activity, exponentially grown DU-145 cells were transiently transfected in six-well plates with an pNF-κB-Luc reporter as previously described (Baritaki *et al*., 2008). DHMEQ at 10 μg/ml was used as positive control for NF-κB inhibition. Luciferase activity in protein extracts was measured in an analytical luminescence counter according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). Data were normalized to protein concentration levels using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

For overexpression of RKIP, DU-145 cells were transiently transfected with RKIP expression plasmid (CMV-HA-RKIP) or the relevant empty control vector (CMV-HA-EV) as previously described (Baritaki *et al*., 2007). The transfection was performed for 48 h as described above. LNCaP cells were transfected with the Snail expression vectors CMV-f-Snail and CMV-f-Snail-6SA. Transfection was performed in reduced serum medium Opti-MeM (Gibco, Invitrogen Corporation, NY, USA) supplemented with 10 μl of lipofectamine (Invitrogen, Carlsbad, CA, USA) and 2 μg plasmid DNA. Twenty-four hours after transfection, cell lysates were harvested and subjected to immunoblot analysis.

Application of small interfering RNA against Snail

A total of 3×10^5 LNCaP cells/well were plated in a six-well plate 24 h before transfection in an antibiotic-free growth medium. Snail siRNA (4 μl; 0.5 μg) or a relevant amount of a control siRNA solution was mixed with 6 μl of transfection

reagent in OptiMeM and transfection was performed for 48 h according to the manufacturer's protocol (Santa Cruz Biotechnology Inc.). Snail inhibition at the protein level was confirmed using western blot analysis.

Semiquantitative RT–PCR
Total RNA was extracted and purified from 1×10^6 LNCaP and DU-145 cells treated with 50 nM NPI-0052 for different time periods using Trizol reagent (Life Technologies Inc., Carlsbad, CA, USA). cDNA synthesis was performed according to manufacturer's instructions (Promega Corporation). Amplification of 1/20 of these cDNAs by PCR was performed using the following gene-specific primers: Snail (forward): (5′-GCCGTGCCTTCGCTGAC-3′), Snail (reverse): (5′-GC ACACGCCTGGCACTGGTA -3′) (85 bp expected product); RKIP (forward): (5′-AGACCCACCAGCATTTCG-3′), RKIP (reverse): (5′-GCTGATGTCATTGCCCTTC-3′; 148 bp expected product); glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers (as internal control) in a three-step thermal cycling incubations (94 °C/30 s; 60 °C/30 s; 72 °C/45 s for 40 cycles) and a final extension at 72 °C for 10 min, as previously described (Baritaki *et al*., 2007).

Western blot analysis
Whole cell lysates from LNCaP or DU-145 cells derived from each experimental condition were prepared in RIPA lysis buffer as previously described (Baritaki *et al*., 2007). Lysates from untreated or untransfected cells were served as control. Cell extracts (20 μg) were subjected to western blot analysis for determination of RKIP and Snail levels using as primary antibodies an anti-human RKIP or Snail antibodies, respectively, both at 1/1000 dilution. Western blot analyses for vimentin, fibronectin, E-cadherin and cytokeratin 18 expressions were performed in 40 μg protein lysates using specific monoclonal antibodies all at dilution of 1/500. The expression of β-actin was used as an internal control.

In vitro *cell invasion and migration assays*
Migration and invasion studies were performed using the 24-well BD Biocoat 3 μm migration chambers or 8 μm Matrigel invasion chambers (Becton Dickinson Labware, Bedford, MA, USA), respectively, and according to manufacturer's instructions. For both assays the cells were starved for 4–5 h before setting up the assay by adding basal medium supplemented with 0.1% bovine serum albumin (BSA). RPMI 1640 medium containing 10% serum was used as chemoatractant. For single drug treatment 50 nM NPI-0052 or 100 ng/ml TNF-α was added to both chambers, whereas for combinational treatment NPI-0052 was added 4 h before the addition of TNF-α.

Measurement of cell migration was performed by cell postlabeling with BD Calcein AM Fluorescent dye (Becton Dickinson Labware) at 4 μg/ml. Data were expressed as the fold cell migration derived from the mean relative fluorescence units (RFU) of cell migration through the membrane toward FBS divided by the mean RFU of cell migration in the absence of FBS. For the invasion assays, inserts allowing cell migration were used as controls (control inserts). Invaded cells through Matrigel were counted under the microscope at ×40 magnification. Data were expressed as the percent invasion through the Matrigel matrix and membrane relative to the migration through the control insert membrane.

Immunofluorescent analysis
DU-145 or LNCaP cells were grown on glass coverslips and treated according to the followed experimental procedures described above. The cells were rinsed briefly in phosphate-buffered saline (PBS) 1× and fixed with fresh 4% PFA for 10 min in room temperature. The cells were then incubated in 1% BSA/10% normal goat serum/0.3 M glycine in 0.1% PBS-Tween for 1 h at room temperature to permeabilize the cell membranes and block nonspecific protein–protein interactions. After one wash with permeabilization buffer, the cells were incubated with the primary antibodies (rabbit anti-vimentin, rabbit anti-fibronectin, mouse anti-E-cadherin-FITC or with the corresponding IgG control antibodies, mouse IgG-FITC and rabbit IgG-RPE) in 1/100 dilution in incubation buffer (permeabilization buffer and 1% BSA) overnight at 4 °C. After thoroughly washing the primary antibodies, the cells that were stained with anti-vimentin or anti-fibronectin antibodies were further incubated with an anti-rabbit-RPE secondary antibody at a 1/1000 dilution for 1 h at room temperature. The coverslips were then washed as before and mounted with aqueous mounting media (Biomeda Inc., Foster, CA, USA) on slides. The epithelial and mesencymal markers' staining was identified under an immunofluorescent microscope (Olympus BX51, San Diego, CA, USA) using appropriate wavelengths for FITC and RPE.

Determination of cell morphology
The cell morphology and phenotype were determined under microscopic observation at ×40 magnification before and after cell treatment with NPI-0052 or transfection with the various expression vectors.

Statistical analysis
Significant differences between values obtained from DU-145 and LNCaP cells that were treated under different experimental conditions were determined by the Mann–Whitney *U*-test and Kruscal–Wallis H-test. Probability (*P*) was set significant at the level of 0.05. Statistical analyses were performed using the SPSS software.

Abbreviations

DHMEQ, dehydroxymethylepoxyquinomicin; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehydes-3-phosphate dehydrogenase (G-3-PDH); NF-κB, nuclear factor-kB; PBS, phosphate-buffered saline; RFU, relative fluorescence units; RPE, R-phycoerythrin.

Conflict of interest

Dr Michael Palladino is Chief Technology Officer at Nereus Pharmaceutical Inc., San Diego. The other authors have no conflict of interest.

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