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Id-1 Induces Cell Invasiveness in Immortalized Epithelial Cells by Regulating Cadherin Switching and Rho GTPases

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ABSTRACT

Epithelial-mesenchymal transition (EMT), characterized by cadherin switching, contributes to cancer metastasis. Our recent study showed that Id-1 (inhibitor of differentiation-1) promotes metastasis in esophageal cancer cells, but whether the invasive and metastatic dynamics can be induced early in the carcinogenesis process is still unclear. Immortalization is regarded as the initial stage in the malignant transformation of normal cells. In this study, we investigated the role and mechanisms of Id-1 in inducing EMT and cell invasiveness in immortalized esophageal epithelial cells. We found that immortalized epithelial cells expressed higher endogenous levels of Id-1 compared with normal cells. Ectopic Id-1 expression inhibited the differentiation of immortalized esophageal epithelial cells and promoted cadherin switching, which was accompanied by increased adhesiveness to extracellular matrix, cell motility, migratory potential and matrix metalloproteinase-dependent invasiveness. GTPase activity assays showed that over-expression or short-hairpin RNA knockdown of Id-1 led to corresponding changes in Rac1 activity, whereas RhoA activity was significantly decreased with Id-1 depletion. Inhibitors targeting Rac1, RhoA, and Rho kinase suppressed the invasiveness of Id-1-expressing NE2-hTERT cells. Knockdown of N-cadherin in Id-1-over-expressing cells inhibited cell invasiveness and down-regulated RhoA activity. These data suggest that the Id-1-induced invasive potential may be regulated through the N-cadherin-RhoA axis and Rac1 activation. J. Cell. Biochem. 112: 157–168, 2011.

KEY WORDS: ID-1; EMT; ESOPHAGEAL; INVASIVENESS; N-CADHERIN; RHO GTPASES

The Id-1 (inhibitor of differentiation-1) protein belongs to the family of Id transcriptional regulators, which can inhibit the DNA-binding of basic helix-loop-helix transcription factors involved in cellular differentiation [Benezra et al., 1990]. Over-expression of Id-1 is commonly found in a variety of human tumors, including esophageal squamous cell carcinoma (ESCC) [Hu et al., 2001; Yuen et al., 2007]. Its expression has also been shown to correlate with histological grade and aggressiveness in some cancer types, such as prostate cancer [Ouyang et al., 2002] and breast cancer [Schoppmann et al., 2003], thus implicating its role in promoting cancer progression. One of the most threatening features of cancer cells is their ability to invade and metastasize. Epithelial-mesenchymal transition (EMT) is a transcriptional reprogramming process that enables epithelial cells to overcome physical constraints imposed by the junctional complexes and adopt a motile phenotype. During cancer development, EMT represents a fundamentally important process conducive to tumor dissemination and metastatic spread. As an integral component of EMT, cadherin switching elicits a profound effect on cell motility and invasive behavior. It usually refers to a switch from expression of E-cadherin to expression of N-cadherin (or less commonly R-cadherin, T-cadherin, or P-cadherin), but also includes situations in which E-cadherin expression levels do not change significantly but the expression of N-cadherin is increased [Wheelock et al., 2008].

Immortalization is generally regarded as the initial stage in the malignant transformation of normal cells. However, recent studies have shown that EMT can be induced in immortalized cells [Barbieri et al., 2006; Wang et al., 2007; Mani et al., 2008], suggesting that an

Abbreviations: DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ESCC, esophageal squamous cell carcinoma; GST, glutathione S-transferase; GTPases, guanosine triphosphatases; hTERT, human telomerase reverse transcriptase; Id-1, inhibitor of DNA-binding/differentiation-1; MMP, matrix metalloproteinase; ROCK, Rho-activated kinase; TGF-β, transforming growth factor.

P.Y. Cheung and Y.L. Yip contributed equally to this work.

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invasive phenotype may be acquired at an earlier stage of carcinogenesis. A number of studies have also indicated that Id-1 functions as a mediator in transforming growth factor-β (TGF-β)-induced phenotypic switching of smooth muscle cells [Chambers et al., 2003], trans-differentiation of rat hepatic stellate cells [Wiercinska et al., 2006], and dedifferentiation of renal tubular epithelial cells [Li et al., 2007b]. Furthermore, Id-1 disrupts the adherens junction complex in TGF-β-treated immortalized prostate epithelial through cadherin switching and redistribution of β-catenin [Di et al., 2007]. Although TGF-β usually inhibits cell proliferation in normal cells, these findings suggest that cellular Id-1 level may determine the switching of TGF-β function from tumor suppressing (growth inhibiting) to tumor promoting (enhancing cell motility by EMT). In addition, Id-1 expression was found to be elevated in Madin–Darby canine kidney (MDCK) epithelial cells in response to EMT induced by Snail and E47 transcription factors [Jorda et al., 2007]. All these studies suggest that Id-1 can mediate the effects of various EMT inducers, but whether Id-1 on its own can induce or initiate EMT in non-malignant cells in the absence of other EMT stimuli is still unclear.

Cadherin-mediated phenotypic changes can activate a family of small (~21 kDa) signaling G proteins called Rho GTPases, which regulate cell motility and invasion [Wheelock et al., 2008]. The three members of the family, Cdc42, Rac1, and RhoA, operate as binary switches, cycling between a GTP-bound active state and a GDP-bound inactive state. Activated Rac1 induces membrane ruffles and rearrangements of actin cytoskeleton which leads to the formation of lamellipodia and focal contacts at the leading edge that drive cell motility [Ridley et al., 1992]. On the other hand, activated RhoA regulates assembly of stress fibers and focal adhesions through its downstream effectors including the Rho-activated kinase (ROCK) that phosphorylates cytoskeletal proteins, resulting in actomyosin contractility [Ridley and Hall, 1992]. Dynamic activation and inactivation of Rho GTPases are tightly coordinated. Changes in the level and balance of the activities among these Rho GTPases essentially affect cell migratory and invasive potentials [Sahai et al., 2001; Sahai and Marshall, 2003].

In this study, we examined the effects of Id-1 expression on cadherin switching, motility and invasiveness in non-transformed immortalized esophageal epithelial cells. We also explored the roles of Rho GTPases and cadherins in mediating these Id-1-induced EMT phenotypes.

METHODS AND MATERIALS

CELL LINES AND CULTURE CONDITIONS

Primary and immortalized human esophageal epithelial cells were maintained in a 1:1 mixture of defined keratinocyte serum free medium (dKSFM; GIBCO, Invitrogen, Carlsbad, CA) and EpiLife (Cascade Biologics, Portland, Oregon) with the provided supplements. The immortalized cell lines NE2-hTERT [Cheung et al., 2010] and NE083-hTERT [Zhang et al., 2007a] were established through ectopic expression of human telomerase reverse transcriptase (hTERT), whereas NE1-E6E7 and NE6-E6E7 were immortalized with human papillomavirus E6/E7 [Deng et al., 2004]. HeLa cells obtained from American Type Culture Collection (Manassas, VA), KYSE cells obtained from DSMZ [Shimada et al., 1992] and HKESC-1 cells [Hu et al., 2000] were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

TREATMENTS WITH TGF-β AND CHEMICAL INHIBITORS

Cells were treated with recombinant TGF-β1 (1 ng/ml; Calbiochem, La Jolla, CA) diluted in 4 mM HCl containing 0.1% bovine serum albumin for different durations. For experiments involving the use of metalloproteinase inhibitor GM6001 (Calbiochem), Rac1 inhibitor NSC23766 (Calbiochem), Rho inhibitor ADP-ribosyltransferase C3 from *Clostridium botulinum* (Sigma, St. Louis, MO) and the Rho kinase inhibitor Y27632 (Calbiochem), cells were treated with the inhibitors for 24 h before assay. The Rac1 and Rho inhibitors were dissolved in water. Other chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO).

WESTERN BLOT ANALYSIS

Extraction of total cell lysate and Western blotting were performed as described previously [Hui et al., 2006]. Primary antibodies included: Id-1 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), involucrin (Ab-1, NeoMarkers, Fremont, CA), ΔNp63 (63P03, NeoMarkers), N-cadherin (H-63, Santa Cruz) and E-cadherin (BD Transduction Laboratories, San Jose, CA). Actin (I19, Santa Cruz) was included as an internal loading control. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). Chemiluminescent signals were developed by ECL Plus Western blotting detection system (Amersham, Piscataway, NJ) and visualized by exposing the membrane to BioMax Light Film (Kodak, Rochester, NY).

MANIPULATION OF ID-1 AND N-CADHERIN EXPRESSION

The retroviral vectors pBabe-puro and the pBabe-puro-Id-1 (kindly provided by Dr. Joan Massagué, Howard Hughes Medical Institute), together with the VSVG plasmid, were used for transfection into phoenix 293 packaging cell line using Fugene 6 transfection reagent (Roche Diagnostics, Germany). The medium was replaced by fresh medium after 24 h. After 2 days, the supernatant was collected and mixed with fresh medium and polybrene (4 μg/ml) (Sigma). The target cells were incubated in the mixture for 8 h at 37°C, and the infected cells were subsequently selected in medium containing 1 mg/ml of puromycin for 2 weeks to obtain two stable cell lines NE2-hTERT-pBabe and NE2-hTERT-Id-1. Id-1 knockdown in another immortalized esophageal epithelial cell line NE083-hTERT was performed as described above using retroviral vectors pSuper and pSuper-sh-Id-1 (kindly provided by Dr. Joan Massagué, Howard Hughes Medical Institute) [Minn et al., 2005]. Stable transfectants were selected in 1 mg/ml of puromycin for 2 weeks. The two stable cell lines transduced by pSuper and pSuper-sh-Id-1 were designated NE083-hTERT-pSuper and NE083-hTERT-sh-Id-1, respectively. Likewise, N-cadherin knockdown in NE2-hTERT-Id-1 cells was performed using retroviral vectors pSuper and pSuper-sh-N-cadherin (kindly provided by Dr. Margaret J. Wheelock, Eppley Institute) [Maeda et al., 2005] to produce two stable cell lines NE2-hTERT-Id-1-pSuper and NE2-hTERT-Id-1-sh-N-cadherin, respectively. Over-expression of N-cadherin in HeLa cells was

performed by transfecting pcDNA-N-cadherin plasmid (kindly provided by Dr. Laurent Brossay, Brown University) [Tessmer et al., 2007] into HeLa cells for 48 h using Fugene 6 transfection reagent.

TRANSWELL INVASION ASSAY

The invasiveness of cells was assayed in a 24-transwell BD BioCoat Matrigel invasion chamber with an 8-μm-pore-size (BD Biosciences, San Jose, CA). Fifty-thousand cells were layered in the upper compartment of a transwell. The lower chamber was filled with culture medium. After 18 h of incubation at 37°C and 5% CO_2, cells adherent to the upper surface of the membrane were mechanically removed using a cotton swab. The cells that migrated to the lower surface of the membrane were stained with 10% Giemsa solution for 10 min, then counted under a light microscope at 400× magnification in 10 randomly selected fields per well. Data are from at least three independent experiments (mean ± SEM).

WOUND-HEALING ASSAY

Cells were grown to confluence on 6-well plates. A ''wound'' was created in the monolayer using the tip of a P100 micropipette, and was followed by substantial washing with PBS. Photos were taken at different time points to monitor the wound closure.

TIME-LAPSE LIVE CELL IMAGING

For time-lapse observation of cell movement, cells on six-well plates were maintained in a 1:1 mixture of dKSFM and EpiLife. Cells were placed in an incubator with temperature maintained at 37°C and CO_2 at 5%. The cell movement observed under ZEISS Axiovert 200 M microscope (20× objective) and live images were captured at 5-min intervals using ZEISS Axiocam MRm camera and Axio-Vision Release 4.5 software. The cell displacement was determined by tracking the single cells at different time points using Imaris imaging software (Bitplane Scientific Software, Switzerland).

ADHESION ASSAY

Adhesion assays were modified from Neumeister et al. [2003]. In brief, 96-well plates were coated with collagen (100 μg/ml, BD Biosciences), laminin (5 μg/ml, Sigma) or fibronectin (10 μg/ml, Sigma) and the wells were blocked with 10 mg/ml heat-denatured bovine serum albumin for 30 min to avoid non-specific adhesion. Therefore only the particular extracellular matrix (ECM) protein, but not the well surface, would contribute to the readout. Twenty-thousand cells were seeded and allowed to adhere for 4 h and 24 h. Then the wells were washed with PBS, and the cells were fixed in 4% formalin and stained with crystal violet. Assays were performed in triplicate. The absorbance was read at 550 nm and the values at 4 h were normalized to the values at 24 h. Adherent cells were represented as A_{550} (mean ± SEM) of three independent experiments.

DETECTION OF GTP-BOUND RHO FAMILY SMALL GTPASES BY GST PULL-DOWN ASSAY

The pGEX empty vector and pGEX vectors encoding the Cdc42/Rac1 interactive binding region (CRIB/PBD) of p21-activated kinase [Ching et al., 2003] and the Rho binding domain (RBD) of the human Rhotekin protein [Schwartz et al., 1996] were used to express the proteins required for the pull-down assays. One milliliter of BL-21 bacterial culture was incubated in 100 ml Luria–Bertani ampicillin-selection medium (LBA, BD Biosciences) for 2–3 h with shaking at 37°C. To induce protein expression, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) was added to the 100 ml-culture followed by shaking at 28°C for 3 h. Cells were then pelleted by centrifugation at 3,000 *g* for 5 min. Bacterial cells were lysed in bacterial lysis buffer (50 mM Tris pH7.4, 50 mM NaCl, 5 mM MgCl, 10 μg/ml PMSF, 20 μg/ml aprotinin, 10 μg/ml leupeptin) followed by sonication at 5-s intervals for 1–2 min on ice. The bacterial lysates were then centrifuged at 18,000 *g* at 4°C for 20 min. Induced glutathione S-transferase (GST)-tagged proteins in the supernatant were immobilized on glutathione-Sepharose™ beads (GE Healthcare, Freiburg, Germany) by incubating the supernatant with the beads slurry at 4°C for 1 h. Cells were washed twice in TBS and lysed in Buffer B (50 mM Tris pH7.6, 150 mM NaCl, 0.5 mM MgCl, 1% Triton X-100, 10 μg/ml PMSF, 20 μg/ml aprotinin, 10 μg/ml leupeptin). The lysates were then centrifuged at 14,000 rpm for 4 min at 4°C. Three-hundred micrograms protein was incubated with purified GST-, GST-PBD- or GST-RBD-immobilized beads at 4°C for 45 min. The GST-immobilized beads were included to ensure that the target proteins in the cell lysate pulled out were not from non-specific binding to GST protein. The beads were washed four times in an excess of Buffer B. Bound proteins were eluted in 2× sample buffer. The eluates were subjected to SDS–PAGE, followed by immunoblotting with antibodies against Rac1/2/3 (L129, Cell Signaling) and RhoA (67B9, Cell Signaling).

STATISTICAL ANALYSIS

Statistical significance was assessed using two-tailed Student's *t*-test. A *P*-value of <0.05 was considered as significant.

RESULTS

ECTOPIC EXPRESSION OF ID-1 INHIBITS DIFFERENTIATION AND INDUCES CADHERIN-SWITCHING IN IMMORTALIZED ESOPHAGEAL EPITHELIAL CELLS

To investigate the functional role of Id-1 in early carcinogenesis, the expression level of Id-1 was first screened in primary, immortalized esophageal epithelial cells and ESCC cell lines. Compared with primary cells, the immortalized cell lines had variable expression levels of Id-1, but the expression levels were all higher than in primary cells, which lacked Id-1 expression. The esophageal squamous carcinoma cell lines examined, also showed higher endogenous expression of Id-1 compared with primary cells (Fig. 1a). These data suggest that Id-1 may have a functional role in immortalization and malignant transformation. Gain- and loss-of-function studies were then carried out to determine the functional role of Id-1 in pre-neoplastic esophageal epithelial cells. Since a recent study showed that human papillomavirus type 16 E6/E7 oncoproteins can induce cell invasive and metastatic abilities in breast cancer cells through up-regulation of Id-1 [Yasmeen et al., 2007], we prevented this confounding effect by using hTERT-immortalized cell lines. We over-expressed Id-1 in NE2-hTERT which had a low endogenous Id-1 level (Fig. 1b). The effect of Id-1

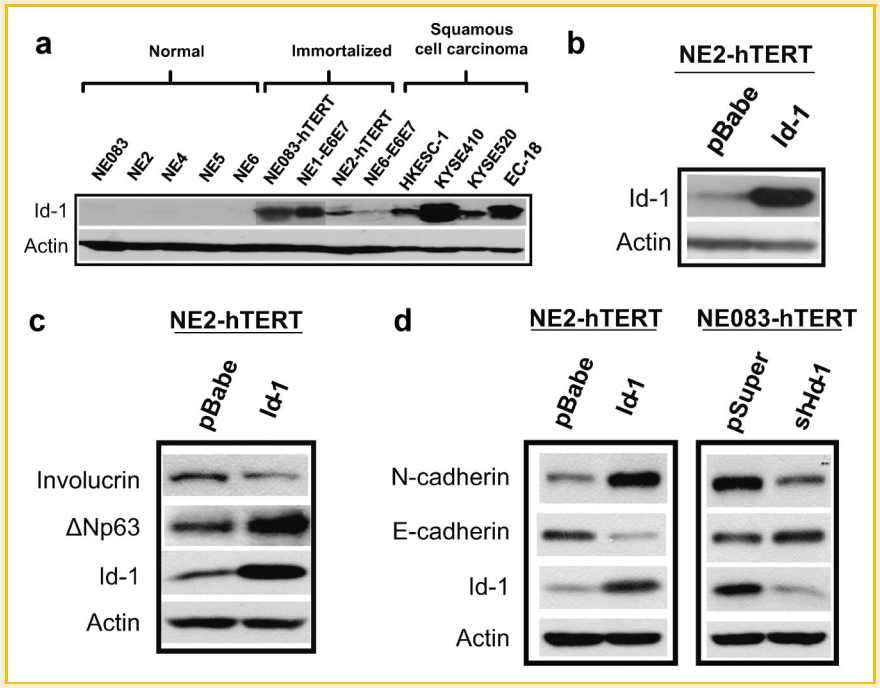

Fig. 1. Manipulation of Id-1 expression level in immortalized esophageal epithelial cells and its effects on cadherin switching. a: Expression of Id-1 in normal primary and immortalized esophageal epithelial cell lines, and in ESCC cell lines. b: Ectopic expression of Id-1 in NE2-hTERT detected by Western blotting. c: Expression of differentiation markers, involucrin and ΔNp63, in NE2-hTERT-pBabe and NE2-hTERT-Id-1 cells. d: E-cadherin and N-cadherin expressions after manipulation of Id-1 expression in NE2-hTERT and NE083-hTERT.

on cell differentiation was determined using differentiation markers, involucrin and ΔNp63 (a squamous differentiation marker, which normally has a higher expression level in the proliferative basal cells than in the uppermost differentiated cells). Cells over-expressing Id-1 showed lower level of involucrin and a higher level of ΔNp63, compared with NE2-hTERT-pBabe cells (Fig. 1c), suggesting that Id-1 inhibits differentiation of NE2-hTERT cells. Ectopic Id-1 expression also led to increase in N-cadherin expression and decrease in E-cadherin expression (Fig. 1d), which represents a major characteristic of cadherin switching during EMT. The effect of Id-1 on cadherin switching was further confirmed by stable knockdown of Id-1 in NE083-hTERT cells, which had high endogenous Id-1 expression, using short-hairpin RNA (shRNA) targeting Id-1. Marked down-regulation of N-cadherin expression was detected although the effect on E-cadherin was less apparent (Fig. 1d).

ID-1 MODULATES TGF-β-INDUCED EMT IN IMMORTALIZED ESOPHAGEAL EPITHELIAL CELLS

Having established that Id-1 could induce cadherin switching in immortalized cells, we proceeded to further explore this function in the context of EMT by treating NE2-hTERT and NE083-hTERT cells with TGF-β, which is a major inducer of EMT. Both cell lines showed induction of Id-1 after exposure to TGF-β for 2 h (Fig. 2a). The effect was particularly marked in NE2-hTERT, which had low endogenous Id-1; and the Id-1 level returned to basal level after 24 h of treatment (Fig. 2b). Since prolonged TGF-β treatment (24 h) induced cadherin switching in both cell lines, and the response was reinforced by ectopic Id-1 expression in NE2-hTERT cells but attenuated by Id-1-knockdown in NE083-hTERT cells (Fig. 2c), our data suggest that Id-1 modulates TGF-β-induced cadherin switching.

ECTOPIC EXPRESSION OF ID-1 ENHANCES ADHESION OF IMMORTALIZED ESOPHAGEAL EPITHELIAL CELLS TO EXTRACELLULAR MATRIX

The effect of Id-1 on matrix adhesion was examined by adhesion assays performed in culture wells coated with ECM proteins, collagen, laminin, and fibronectin. NE2-hTERT cells over-expressing Id-1 were significantly more adherent on laminin and collagen compared with the pBabe control cells (Fig. 3a), whereas knockdown of Id-1 in NE083-hTERT cells significantly reduced adhesion to all three ECM proteins (Fig. 3b), suggesting that Id-1 may have a role in regulating cell–matrix adhesion. Moreover, we detected an increase in integrin β4 level in the NE2-hTERT-Id-1 cells (Supplementary Fig. 1), suggesting that the Id-1-induced increase in cell–matrix adhesion might be integrin-dependent. To exclude the possibility that any increase in absorbance could be due to increased cell proliferation, MTT assay was performed to monitor cell proliferation rate for up to 48 h, and we found no significant difference in cell growth between the Id-1-over-expressing cells and control cells (Supplementary Fig. 2).

ECTOPIC EXPRESSION OF ID-1 PROMOTES MIGRATORY AND MMP-DEPENDENT INVASIVE POTENTIALS OF IMMORTALIZED ESOPHAGEAL EPITHELIAL CELLS

The effect of Id-1 on migratory ability was examined by wound healing assay and live cell imaging. As shown in Figure 4a, NE2-

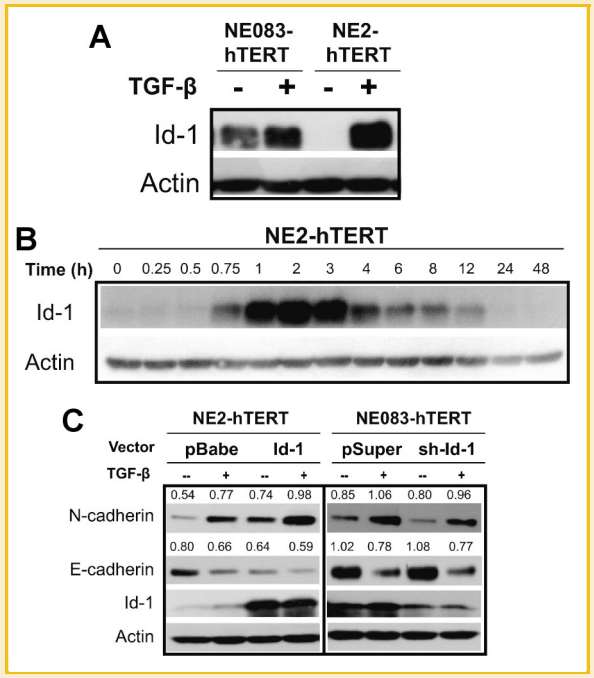

Fig. 2. Effects of TGF-β treatment on Id-1, E-cadherin and N-cadherin. a: Expression of Id-1 in NE083-hTERT and NE2-hTERT cells treated with TGF-β (1 ng/ml, 2 h). b: Induction of Id-1 expression by TGF-β (1 ng/ml). c: Expression of E-cadherin and N-cadherin after TGF-β treatment for 24 h, and response of cadherins to TGF-β after Id-1 manipulation in immortalized esophageal epithelial cells. The signal intensities were quantified using Image J 1.43 V image analysis software (National Institute of Health, MD, USA; http://rsb.info.nih.gov/ij) and were normalized to the corresponding actin signals.

hTERT cells over-expressing Id-1 migrated and closed the wound faster than the cells transduced with the pBabe control vector. To rule out that this effect was due to increased cell proliferation, the movements of Id-1-over-expressing cells and the pBabe control cells were monitored for 6 h using time-lapse microscopy. The arrows in Figure 4b represented the vectorial displacement of the cells with the longest duration time in the captured field. Quantitatively, NE2-hTERT cells ectopically expressing Id-1 had a significantly larger displacement compared with NE2-hTERT-pBabe cells (Fig. 4c), suggesting that Id-1 may enhance the migratory potential of immortalized esophageal epithelial cells. The NE2-hTERT-Id-1 cells were also assessed for their invasive properties. Significantly more NE2-hTERT-Id-1 cells migrated through the Matrigel insert of the transwell invasion chamber, when compared with the pBabe control (Fig. 4d). The Id-1-over-expressing cells were then cultured in medium containing the matrix metalloproteinase (MMP) inhibitor GM6001, and tested for their invasive potential in transwell invasion chamber. Treatment with 20 μM GM6001 significantly inhibited the invasive potential of Id-1-over-expressing cells (Fig. 4e), suggesting that Id-1 may promote the MMP-dependent invasiveness of immortalized esophageal epithelial cells. Since our previous study showed that Id-1 over-expression can induce MMP2 and MMP9 in esophageal cancer cells [Li et al., 2009], gelatin zymography was performed as previously described [Li et al., 2009], to determine if the increased invasiveness of NE2-hTERT-Id-1 cells was attributed to these MMPs. Our results showed that although there was no detectable MMP9 activity, there was a decrease in MMP2 activity after treatment with 20 μM GM6001 (Supplementary Fig. 3), which correlated with the effects of GM6001 on cell invasion.

RHO GTPASES MEDIATE ID-1-INDUCED INVASIVE PHENOTYPE

The Rho family of small GTPases function to regulate cell motility [Hall, 1998], as well as TGF-β-induced EMT [Bhowmick et al., 2001]. Using GST pull-down assay, we found significant increase of GTP-bound Rac1 in the Id-1 over-expressing cells. The regulation of the activity of Rac1 by Id-1 was further confirmed by the significant down-regulation of GTP-bound Rac1 in NE083-hTERT cells transduced with sh-Id-1 (Fig. 5a). Similarly, the effect of Id-1 on RhoA activation was assessed by pull-down of GTP-bound RhoA. The results showed that over-expression of Id-1 had no significant effect on RhoA activity. However, GTP-bound RhoA was significantly reduced in NE083-hTERT cells transduced with sh-Id-1 (Fig. 5a). To determine if Rho GTPases mediate the Id-1-induced invasive potential, NE2-hTERT-Id-1 cells were treated with inhibitors targeting Rac1 and RhoA, and assessed for invasive ability. Cells treated with either Rac1 inhibitor or RhoA inhibitor showed a significant decrease in the number of cells migrating through the transwell invasion chamber, when compared with cells treated with PBS, suggesting that both Rho/Rac GTPases may have a role in mediating Id-1-induced invasive property (Fig. 5b,c). Next, a chemically synthesized Rho kinase inhibitor, Y27632, was used to treat the Id-1-expressing cells. The treatment also significantly reduced the invasive potential of NE2-hTERT-Id1 cells (Fig. 5d). Re-transducing the Y27632-treated NE2-hTERT-Id-1 cells with Id-1 plasmids rescued the invasive phenotype (Fig. 5e).

KNOCKDOWN OF N-CADHERIN SUPPRESSES ID-1-INDUCED INVASIVE PHENOTYPE

Since ectopic expression of Id-1 led to increase in N-cadherin, and N-cadherin has been reported to associate with Rac1 and Cdc42 activity [Kim et al., 2005], we next investigated whether increased N-cadherin mediates Id-1-induced invasive properties and whether it affects Rho GTPases activity. N-cadherin was transiently expressed in HeLa cells, which has low endogenous N-cadherin expression, to detect changes in the activities of RhoGTPases. We found that ectopic expression of N-cadherin in HeLa cells up-regulated both RhoA and Rac1 activities (Fig. 6a). Next, N-cadherin was stably knocked-down by shRNA targeting N-cadherin in NE2-hTERT-Id-1 cells (Fig. 6b) and the cells assessed for invasiveness. The results showed a significant reduction in the number of cells migrated through the transwell invasion chamber, compared with the pSuper control (Fig. 6c), indicating that N-cadherin may mediate Id-1-induced invasive potential. To determine whether the changes in the activity of Rho GTPases were attributed to the increase in N-cadherin expression in Id-1 expressing cells, we compared the Rac1 and RhoA activities in NE2-hTERT-Id-1 cells stably transduced with pSuper and sh-N-cadherin. Knockdown of N-cadherin had no apparent effect on Rac1 activity, whereas RhoA activity was significantly suppressed (Fig. 6d), suggesting that N-cadherin may

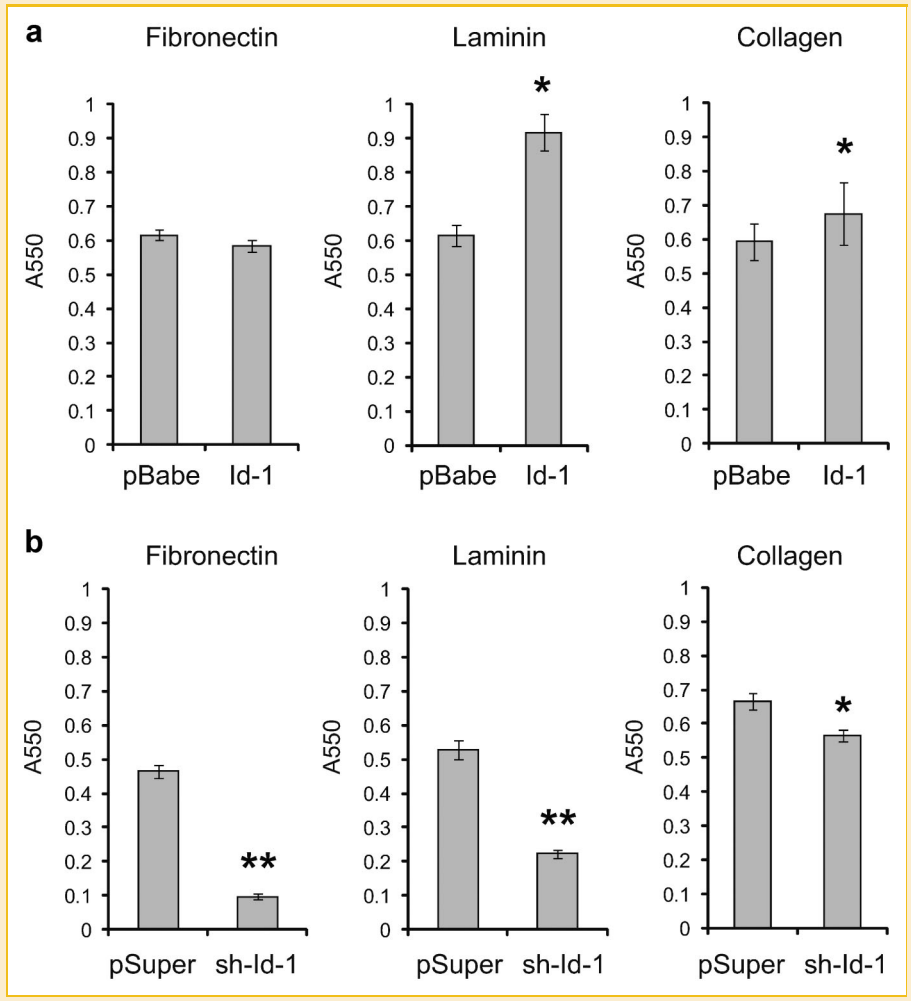

Fig. 3. Effects of Id-1 expression level on the adhesion of esophageal epithelial cells to extracellular matrix proteins. a: Ectopic Id-1 expression significantly increased the adhesion of NE2-hTERT cells to laminin and collagen. b: Knockdown of Id-1 in NE083-hTERT cells significantly reduced the adhesion of cells to fibronectin, laminin, and collagen. $^{*}P < 0.05$, $^{**}P < 0.01$.

be partially responsible for the changes in Rho GTPases activity, particularly RhoA activity, associated with Id-1.

DISCUSSION

A recent report has shown that the phenotype or differentiation state of normal cells can exert lasting influences on the behavior of their transformed, tumorigenic derivatives [Ince et al., 2007]. Therefore, induction of EMT in immortalized cells may predispose these cells to a more aggressive and invasive phenotype when they undergo malignant transformation. Here, we have demonstrated that immortalized esophageal epithelial cells have higher endogenous level of Id-1 protein than normal primary cells, and that Id-1 over-expression can promote EMT and enhance the invasiveness and motility of pre-/non-malignant cells in the absence of other EMT inducers. In this study, we also report for the first time that RhoA and Rac1 mediate the Id-1-induced invasive phenotype.

The relatively high endogenous expression of Id-1 in immortalized esophageal epithelial cells lines and cancer cell lines suggests that Id-1 has a functional role in esophageal carcinogenesis. The down-regulation of involucrin and up-regulation of ΔNp63 in Id-1-over-expressing NE2-hTERT cells supported the classical function of Id-1 as an inhibitor of differentiation [Benezra et al., 1990]. Since cancer cells are often poorly differentiated, and poorly differentiated cancers are usually associated with a more aggressive and metastatic phenotype, the function of Id-1 in regulating the differentiation program of immortalized cells may further facilitate malignant transformation of pre-cancerous cells. We previously reported that Id-1 promotes proliferation and survival of ESCC cells in vitro [Hui et al., 2006; Li et al., 2007a], as well as tumorigenicity and metastasis of ESCC cells in vivo [Li et al., 2009]. In this study, we found that Id-1 also promoted migration and MMP-dependent invasion of immortalized esophageal epithelial cells. Although the function of Id-1 in cell invasion has been described in mammary epithelial cells [Desprez et al., 1998], breast cancer cells [Lin et al.,

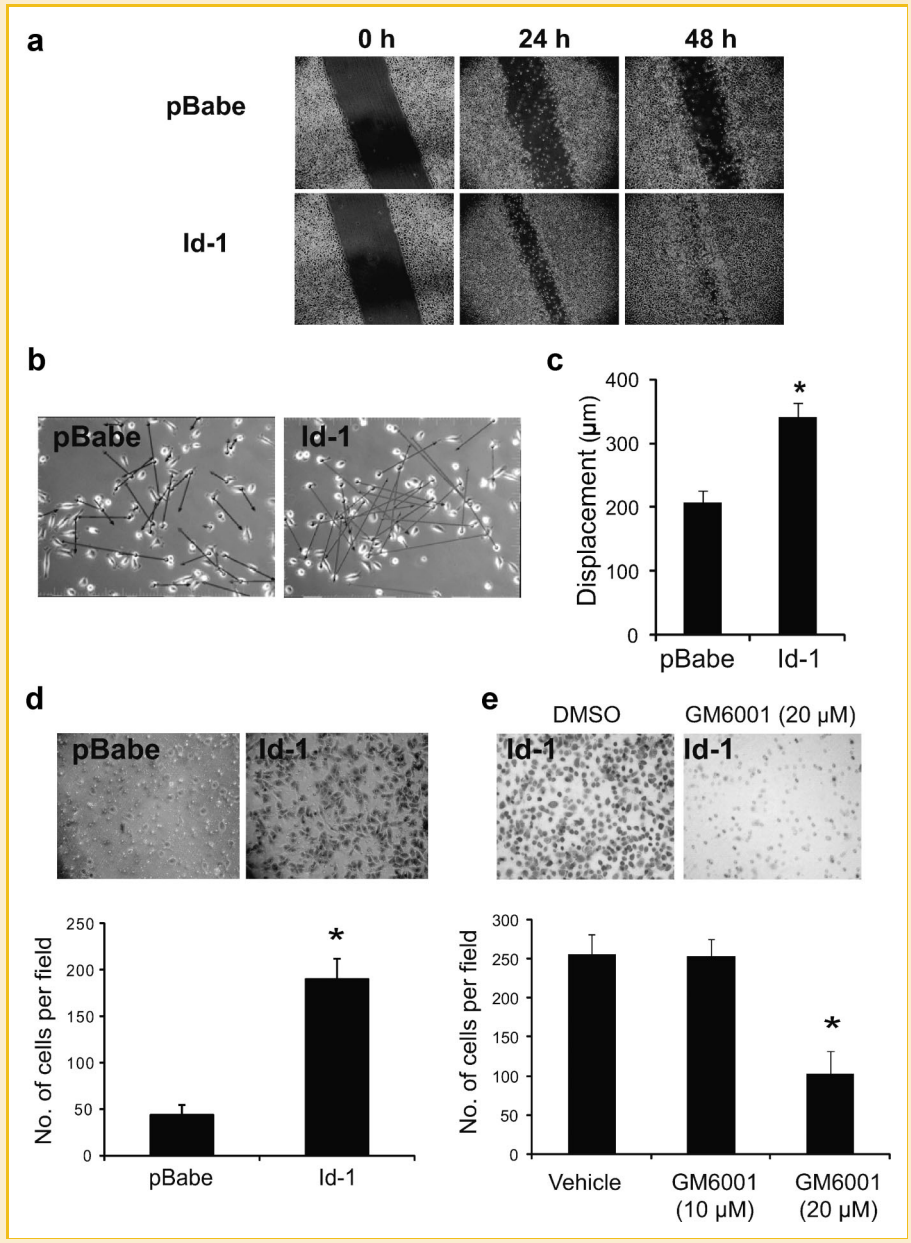

Fig. 4. Effects of ectopic Id-1 expression on the migratory and MMP-dependent invasive potentials of esophageal epithelial cells. a: Ectopic Id-1 expression promoted cell migration on 2-D surface in wound healing assay. Microscopic images were taken under 16× magnification. b: Live cell imaging demonstrated the effect of ectopic expression of Id-1 on vectorial migration. Cells grown in 6-well plate were observed under ZEISS Axiovert 200 M, and images were taken at 5-min intervals for 6 h using ZEISS Axiocam MRm (20× objective). Arrows indicated the direction and magnitude of displacement. c: Graph comparing the displacement of pBabe and Id-1 expressing cells. d: The invasive activity of NE2-hTERT-Id-1 cells was compared with the vector control cells using Matrigel invasion assay. e: Id-1-over-expressing cells were cultured in medium containing MMP inhibitor, GM6001 (10 μM and 20 μM), and tested for invasive potential in Matrigel invasion chamber. $^{*}P < 0.05$.

2000], and prostate cancer cells [Darby et al., 2008], this is the first report showing that Id-1 promotes cell migration and invasion in early esophageal carcinogenesis. We also showed that ectopic Id-1 expression was associated with a gain of cell–matrix interaction involving integrin β4, which is a major epithelial integrin mediating the adhesion of keratinocytes to the basement membrane [De et al., 1990]. In invasion and metastasis, cells must separate from the primary site and interact with the ECM, which requires the acquisition of cell–matrix adhesion properties. A number of studies have shown that increased cell adhesion to ECM is associated with effective cell invasion [Stetler-Stevenson et al., 1993; Rubenstein and Kaufman, 2008; Hu et al., 2009]. Here, we report for the first time the function of Id-1 in integrating cell–matrix adhesion with cell invasion in immortalized cells.

Over-expression of Id-1 was associated with a switch from E-cadherin expression to N-cadherin expression in NE2-hTERT cells.

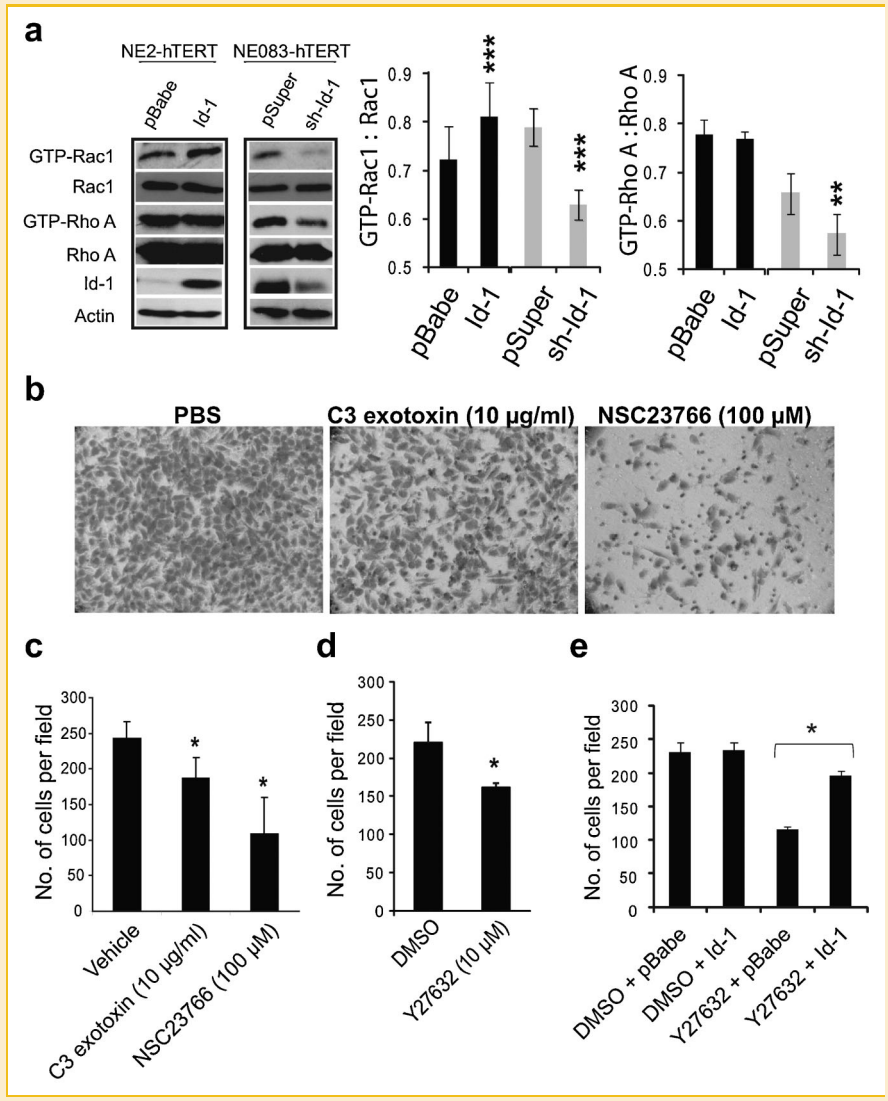

Fig. 5. Rho GTPases and Rho kinase signaling in Id-1-induced invasive phenotype. a: Effects of Id-1 manipulation on Rac1 and RhoA activity as determined by Western blot. Bar charts show the ratios of GTP-bound Rac1/RhoA to total Rac1/RhoA, and RhoA to total RhoA, quantified by densitometry. b–d: Effects of inhibitors targeting Rac1, RhoA, and Rho kinase, on the invasive potential of Id-1 over-expressing cells as detected by Matrigel invasion assay. e: Id-1 expression rescued the invasive phenotype of Y27632-treated NE2-hTERT-Id-1 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Id-1 itself could be induced by TGF-β in our immortalized esophageal epithelial cell lines. A similar phenomenon was reported in human renal tubular epithelial cells, human fetal lung fibroblasts, and rat hepatic stellate cells [Chambers et al., 2003; Wiercinska et al., 2006; Li et al., 2007b]. In addition, Id-1 reinforced the TGF-β-induced switching of EMT markers to the extent that knockdown of Id-1 suppressed the effect of TGF-β, suggesting that Id-1 not only participates in regulating TGF-β signal, but also exerts additive effects on cadherin switching. In prostate epithelial cells, the E-cadherin is suppressed by Id-1 at the transcriptional level with the responsive element(s) located between (−108) and (+1) in the E-cadherin promoter region [Di et al., 2007]. The possible mechanism by which Id-1 regulates E-cadherin was further demonstrated in a renal tubular epithelial system in which the suppression is operated by the formation of a heterodimeric HEB/Id-1 complex to prevent HEB from binding to the E-box and sequestering its ability to transactivate E-cadherin gene transcription [Li et al., 2007b]. These findings on the regulation of E-cadherin by Id-1 extensively highlighted the role of Id-1 in mediating epithelial dedifferentiation. Interestingly, we found that the effect of Id-1 was more pronounced on N-cadherin than E-cadherin, as clearly demonstrated by both the gain-of-function and loss-of-function studies in immortalized esophageal epithelial cells. Knockdown of Id-1 in NE083-hTERT cells, for example, did not lead to apparent changes in E-cadherin expression, suggesting that the regulation of cadherin-switching by Id-1 may be dependent on cellular context.

The strong association between Id-1 and N-cadherin suggests that the invasive phenotype induced by Id-1 may be mediated by

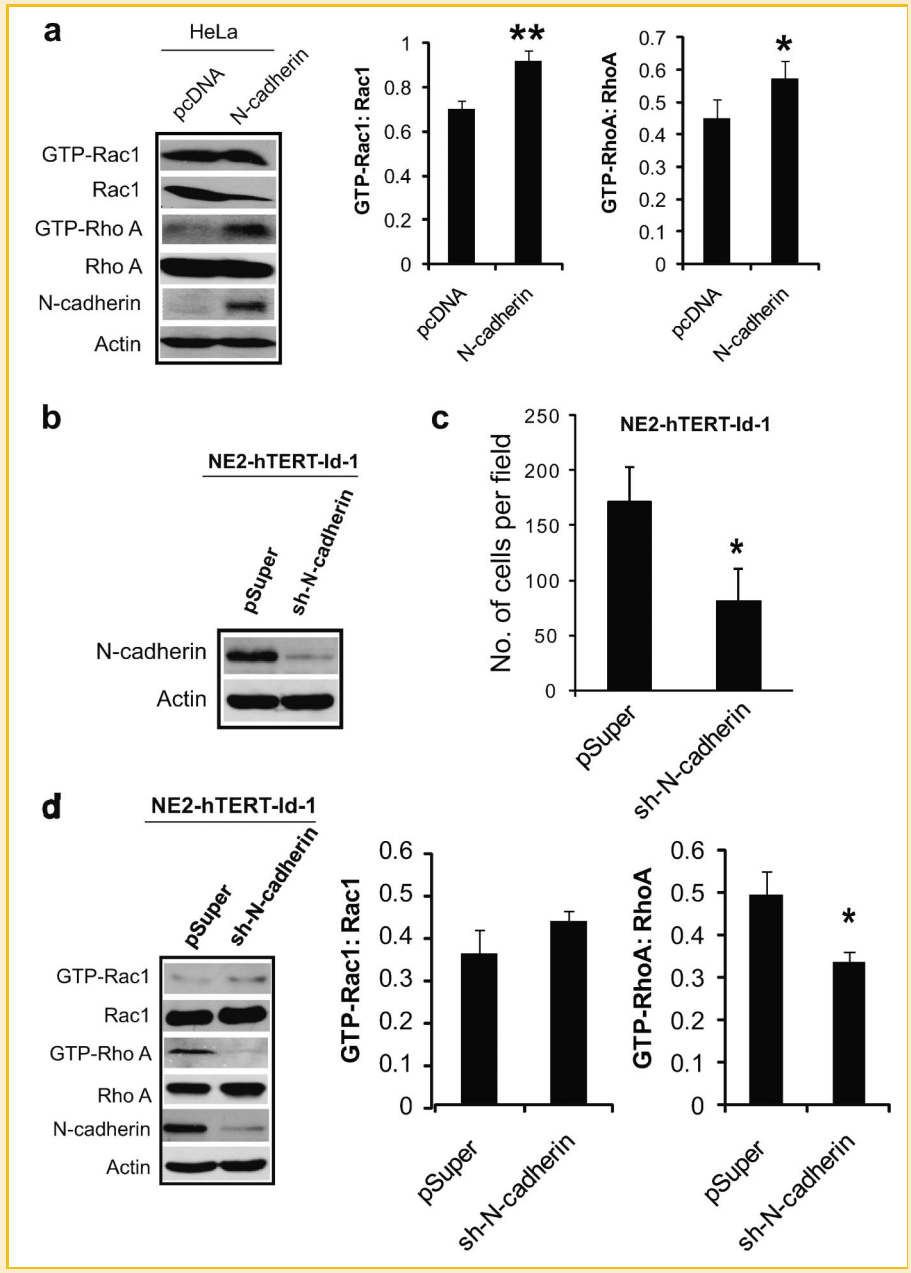

Fig. 6. Involvement of N-cadherin in Id-1-induced invasive phenotype and its relationship with Rho GTPases. a: Effects of transient expression of N-cadherin on the activity of RhoGTPases in HeLa cells. b: Knockdown of N-cadherin in NE2-hTERT-Id-1 cells by shRNA targeting N-cadherin. c: Knockdown of N-cadherin in NE2-hTERT-Id-1 cells significantly decreased the invasive potential of cells in Matrigel invasion assay. d: Western blot analysis of the effect of N-cadherin knockdown on Rac1 and Rho A activity. Bar charts show the ratios of GTP-bound Rho GTPases to total Rho GTPases. $^{*}P < 0.05$. $^{**}P < 0.01$.

N-cadherin. This was confirmed by the reduced invasive potential after the knockdown of N-cadherin. It has long been suggested that tumor cells must lose E-cadherin function for malignant cells to invade or metastasize. Nevertheless, some studies argue that N-cadherin may dominate over E-cadherin in metastatic progression. For example, ectopic expression of N-cadherin in E-cadherin-positive breast cancer cell lines promotes cell motility and metastatic potential in nude mice without reducing E-cadherin expression; conversely, introduction of E-cadherin into N-cadherin expressing breast cancer cell line does not reduce invasive potential [Nieman et al., 1999; Hazan et al., 2000]. There are also many reports suggesting that N-cadherin promotes the migration and invasion of cancer cells independent of E-cadherin expression level [Nieman et al., 1999; Tran et al., 1999; Li et al., 2001; Darby et al., 2008]. Our results support that N-cadherin, rather than E-cadherin, is the critical determinant of cell invasiveness or cancer aggressiveness and, more importantly, that the up-regulation of N-cadherin by Id-1 may be sufficient to promote cell invasion. So far, there are only a

few reports on the clinicopathologic significance of N-cadherin expression in esophageal carcinoma, but there is evidence that the expression of N-cadherin mRNA is associated with the depth of wall invasion, and that patients with high N-cadherin expression have poorer prognosis [Li et al., 2001; Yoshinaga et al., 2004], which supports the role of N-cadherin in the aggressiveness of esophageal cancer. Although the action of Id-1 on N-cadherin had been reported previously, the effect was observed under the stimulation of TGF-β [Di et al., 2007; Zhang et al., 2007b]. Thus, this is the first study showing that Id-1 alone can up-regulate N-cadherin expression, and that N-cadherin mediates the Id-1-induced invasive potential of immortalized esophageal epithelial cells.

In addition to N-cadherin, Id-1 was shown to regulate Rac1 activity and the inhibition of Rac1 reduced the invasive potential of Id-1 over-expressing cells. Since Rac1 is also known to facilitate cell–matrix attachment [Fukata et al., 2003], the increase in Id-1 induced invasiveness and cell–matrix adhesion could be mediated by Rac1 GTPase. The involvement of Rho, on the other hand, was less conclusive since we failed to establish a statistically significant increase in Rho activity with ectopic Id-1 expression although Id-1 knockdown did lead to reduced Rho activity. However, our results showing that Id-1 expression could rescue the invasive phenotype in Y27632-treated NE2-hTERT-Id-1 cells suggest a role for Rho in Id-1-induced invasive phenotype. Aberrant expression of N-cadherin by a variety of cell types has been shown to result in increased steady-state levels of active RhoA, Rac1, and Cdc42, and that the activation of these GTPases is associated with increased cell motility [Kim et al., 2005; Taulet et al., 2009]. We manipulated the expression of N-cadherin in different cell systems to study the relationship between Id-1, N-cadherin, and Rho GTPases. In HeLa cells, transient expression of N-cadherin led to increase in both active Rac1 and RhoA, whereas N-cadherin knockdown reduced RhoA activity but not Rac1 activity in NE2-hTERT-Id-1 cells. As such, our data support that N-cadherin mediates Id-1-induced Rho A activity, but whether it also relays the induction of Rac1 by Id-1 remains inconclusive in this study. We therefore propose N-cadherin, RhoA, and Rac1 as downstream effectors of Id-1 in the regulation of cell adhesion, migration, and invasion. While N-cadherin and RhoA may be mechanistically linked in this function, Rac1 probably acts independently through another signaling axis (Fig. 7).

Taken together, we have demonstrated that cadherin switching, adhesion to ECM, as well as the migratory and invasive properties can be induced by Id-1 in immortalized epithelial cells although these phenotypes are commonly thought to be late events in cancer progression. The significance of Id-1 in imparting these properties in pre-neoplastic cells further substantiates the functional role of Id-1 as an oncogenic protein.

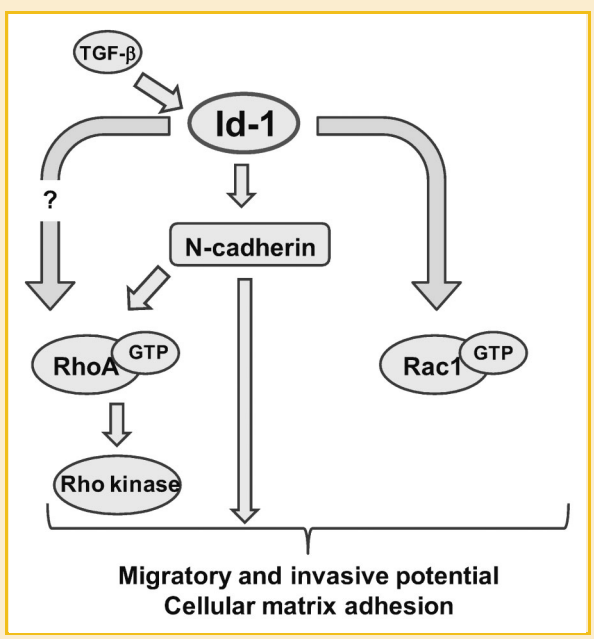

Fig. 7. Schematic diagram showing the possible signaling events involved in Id-1-induced matrix adhesion and cell invasion. Based on the findings in this study, we propose that Id-1, which could be induced by TGF-β, may act separately through N-cadherin, Rho GTPases, or the N-cadherin–RhoA axis to promote matrix adhesion, cell migration and invasion.

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