ARTICLE
Journal of Cellular Biochemistry 112:3459–3468 (2011)

Journal of Cellular Biochemistry

Downregulation of $p57^{kip2}$ Promotes Cell Invasion via LIMK/Cofilin Pathway in Human Nasopharyngeal Carcinoma Cells

Shu-Er Chow,[1,2*] Jong-Shyan Wang,[2,3] Ming-Rung Lin,[1] and Chien Lin Lee[4]

[1] *Center for General Studies, Chang Gung University, Taoyuan, Taiwan*

[2] *Healthy Aging Research Center, Chang Gung University, Taoyuan, Taiwan*

[3] *Graduate Institute of Rehablishment Science and Healthy Aging Research Center, Chang Gung University, Taoyuan, Taiwan*

[4] *Department of Medicine, Chang Gung University, Taoyuan, Taiwan*

ABSTRACT

The members of Rho family are well known for their regulation of actin cytoskeleton to control cell migration. The Cip/kip members of cyclin-dependent (CDK) inhibitors have shown to implicate in cell migration and cytoskeletal dynamics. $p57^{kip2}$, a CDK inhibitor, is frequently downregulated in several malignancy tumors. However, its biological roles in human nasopharyngeal carcinoma (NPC) cells remained to be investigated. Here, we found $p57^{kip2}$ has nuclear and cytoplasm distributions and depletion of endogenous $p57^{kip2}$ did not change the cell-cycle progression. Inhibition of cell proliferation by mitomycin C promoted FBS-mediated cell migration and accompanied with the downregulation of ΔNp63α and $p57^{kip2}$, but did not change the level of $p27^{kip1}$, another CDK inhibitor. By using siRNA transfection and cell migration/invasion assays, we found that knockdown of $p57^{kip2}$, but not ΔNp63α, involved in promotion of NPC cell migration and invasion via decrease of phospho-cofilin (p-cofilin). Treatment with Y-27632, a specific ROCK inhibitor, we found that dysregulation of ROCK/cofilin pathway decreased p-cofilin expression and induced cell migration. This change of p-cofilin induced actin remodeling and pronounced increase of membrane protrusions. Further, silence of $p57^{kip2}$ not only decreased the interaction between $p57^{kip2}$ and LIMK-1 assayed by immunoprecipitation but also reduced the level of phospho-LIMK1/2. Therefore, this study indicated that dysregulation of $p57^{kip2}$ promoted cell migration and invasion through modulation of LIMK/cofilin signaling and suggested this induction of inappropriate cell motility might contribute to promoting tumor cell for metastasis. J. Cell. Biochem. 112: 3459–3468, 2011.

KEY WORDS: $p57^{kip2}$; LIMK; COFILIN; CELL MIGRATION; NPC

Nasopharyngeal carcinoma (NPC) is a malignant tumor that is distinctive in head and neck carcinomas for its close association with Epstein-Barr virus and its highly metastatic nature [Lo et al., 2004]. ΔNp63α is highly expressed in NPC and interacts with the Epstein-Barr virus nuclear antigen 5 (EBNA-5) associated with the differential stage in the pathogenesis of NPC [Lo et al., 2004; Guo et al., 2006; Chou et al., 2008]. The function of ΔNp63α is in cellular survival and antiapoptosis, whereas downregulation of ΔNp63α induces cellular apoptosis [Yip and Tsao, 2008; Chow et al., 2010]. However, downregulation of ΔNp63α is involved in cell migration of primary keratinocytes and squamous cancer cell lines [Barbieri et al., 2006; Horikawa et al., 2007; Ichikawa et al., 2008]. However, its role in migration is not explored in NPC cells.

Tumor invasiveness and metastsis both depend on altered regulation of cell migration. The actin cytoskeleton and its regulatory proteins are dynamic remodeled and force for cell migration [Yamazaki et al., 2005]. $p57^{kip2}$ has a direct functional link between cyclin-dependent kinase (CDK) inhibitor and regulators of cytoskeletal organization [Besson et al., 2004; Lee and Helfman, 2004; Besson et al., 2008; Pateras et al., 2009]. Ectopic expression of $p57^{kip2}$ interacts with the actin cytoskeleton modifying enzyme, LIMK-1 to enhance the activity of LIMK-1 and increase the level of phosphorylation of cofilin [Pateras et al., 2009]. Cofilin is one of the key proteins that regulating actin remodeling [DesMarais et al., 2005]. It is postulated to drive protrusion and migration by depolymerizing actin filaments and by

Grant sponsor: Chang Gung Memorial Hospital; Grant number: CMRPD 180332; Grant sponsor: Healthy Aging Research Program, Chang Gung University; Grant number: EMRPD 1A0841.

*Correspondence to: Dr. Shu-Er Chow, 259, Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan, Tao-Yuan, 333. E-mail: chowse@mail.cgu.edu.tw

Received 7 July 2011; Accepted 8 July 2011 • DOI 10.1002/jcb.23277 • © 2011 Wiley Periodicals, Inc.
Published online 18 July 2011 in Wiley Online Library (wileyonlinelibrary.com).

serving actin filaments to provide new barbed ends for filament elongation [Ghosh et al., 2004]. LIM kinases (LIMK-1 and LIMK-2) are serine/threonine kinases that involved in actin cytoskeletal regulation downstream of Rho-family GTPases, PAKs and ROCK [Bernard, 2007]. PAK1 and ROCK phosphorylate LIMK-1 or LIMK-2 at the conserved Thr508 or Thr505 residues in the activation loop, increasing the LIMK activity [Besson et al., 2004]. Activated LIMKs inhibit the actin depolymerization activity of cofilin by phosphorylation of cofilin at ser3 [Yang et al., 1998].

$p57^{kip2}$, a CDK inhibitor of Cip/kip family, together with $p21^{cip2}$ and $p27^{kip1}$, plays a critical role in regulating cell proliferation. In particular, only $p57^{kip2}$ is the CDK inhibitor has a specific role for mouse embryogenesis[11,19]. In addition, $p57^{kip2}$ play important roles in cell differentiation and apoptosis [Vlachos et al., 2007]. Emerging evidences indicate the CDK inhibitors of the Cip/kip family included $p57^{kip2}$, are involved in the regulation of Rho family signaling that controlled cytoskeleton dynamics and cell migration [Yokoo et al., 2003; Besson et al., 2004; Besson et al., 2008; Vlachos and Joseph, 2009]. $p57^{kip2}$ is frequently down-regulated in many cancers, and its decreased expression is correlated with aggressiveness in several malignancies [Nakai et al., 2002; Nan et al., 2005; Pateras et al., 2009]. Inducible expression of $p57^{kip2}$ in cancer cells significantly reduced invasive ability [Sakai et al., 2004; Jin et al., 2008] and overexpression of $p57^{kip2}$ in LNCaP cells inhibits tumor formation in nude mice[25]. Thus, $p57^{kip2}$ plays an important role in cancer cell metastasis. Diminished expression of $p57^{kip2}$ predicts poor prognosis in carcinoma cells [Pateras et al., 2009] and its up-regulation is strongly the prolongation of time and dosage after irradiation in patients with NPC [Sun et al., 1992]. So far it is not known whether the endogenous $p57^{kip2}$ is involved in the regulation of cell migration. In this study, we examine the impact of expression of ΔNp63α and $p57^{kip2}$ on cell migration. Our study indicates that inactivation of ROCK/LIMK pathway induced cell migration and silence of endogenous $p57^{kip2}$, but not ΔNp63α, induced an enhancement of cell migration. Further study indicate $p57^{kip2}$ interacts with LIMKs to regulate the phosphorylated status of cofilin to exert its function in migration/invasion in NPC cells.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human NPC cell lines NPC-TW076 and NPC-TW039 were isolated from keratinized nasopharyngeal squamous cell carcinoma [Lin et al., 1990]. The cells were maintained in basal medium (DMEM/F-12 at 1:1 v/v; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum in a humidified incubator at 37°C under 5% CO_2/95% air. Most chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Antibodies to $p57^{kip2}$ and $p27^{kip1}$ were purchased from Santa Cruz Biotechnology (Delaware, DE). Antibodies to p-cofilin, cofilin, LIMK1, LIMK2, and phospho-LIMK-1/2 were purchased from Cell Signaling (Beverly, MA). Antibody to ΔNp63α was purchased from Biolegend (San Diego, CA).

CELL CYCLE-DISTRIBUTION ANALYSIS

Propidium iodide (PI) staining was used to analyze the DNA content. Cells were plated in 35-mm dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were then treated with $p57^{kip2}$ siRNA for 48–72 h. Cells were harvested and resuspended in PBS, fixed with 70% ethanol, labeled with 0.05 mg/ml PI, and incubated at room temperature in the dark for 30 min. DNA content was then analyzed using a FACScan instrument equipped with FACStation running cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

siRNA TRANSFECTION

To explore the function of ΔNp63α or $p57^{kip2}$ in NPC cells, small interfering RNAs (siRNA) were used to silence their expressions. The siRNA targeting ΔNp63α mRNA was designed and synthesized by Dharmacon Research Inc. (Lafayette, CO, USA) [Chow et al., 2010]. The siRNA sequence for ΔNp63α targeting was 5′ACAAUG-CCCAGACUCAAUU3′. Commercially available siRNA-$p57^{kip2}$ (sc-35751) duplexes and control siRNA-A (sc-37007) were from Santa Cruz Biotechnology. $p57^{kip2}$ siRNA is a pool of three target-specific 20- to 25-nucleotide siRNAs designed to knock down $p57^{kip2}$ gene expression. Negative control siRNA (Ngi) consisted of a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA. The transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The effectiveness of siRNA silencing was assayed by western blot analysis using anti-ΔNp63α or anti-$p57^{kip2}$ antibody. After 24–72 h, the transfected cells were reseeded per well of 6-well plate (3×10^5) for 24 h and cell migration, the distribution of cell cycle or protein expression analysis were measured.

SCRATCH WOUND MIGRATION ASSAYS

For scratch wound migration assays, NPC cells were seeded on 6-well plates (5×10^5 cells per well; Falcon, Becton Dickinson) in DMEM containing 5% FBS. The cells were grown to confluence after which the cultures were scratch wounded with a 200-μl pipette tip. Two perpendicular wounds were created. Where indicated, NPC cells were exposed to mitomycin C (10 μg/ml) for 2 h before wound healing. Loose cells were removed by washing with serum free medium. Wound edges were photographed 0 and 24 h after FBS addition.

CELL MIGRATION AND INVASION ASSAYS

Boyden chamber cell migration assay was performed using transwell chambers with 8-μm pore size membranes and 6.5-mm diameter (Becton Dickinson). NPC cells were transfected with the indicated siRNAs. Twenty-four hours after transfection, medium was changed to fresh medium and subsequently incubated for 24 h. Cells were resuspended in medium containing 2% FBS and added to upper chamber at 1×10^5 cells/well. The lower chamber was filled with DMEM containing 10% FBS. After 24 h, the cells on the upper side of the filters were mechanically removed and the cells that had migrated to the lower side were fixed with 4% paraformaldehyde, stained with 0.25% crystal violet, and counted under a light microscope in five randomed fields (200X). All assays were independently repeated at least three experiments. For the cell invasion assay, the procedure was similar to the cell migration assay, except that the transwell membranes were precoated with 10 μg/ml Matrigel (R&D Systems, Bedford, MA) and the cells were incubated

for 16 h at 37°C in a 5% CO_2 atmosphere. Cells adhering to the lower surface were counted the same way as for the cell migration assay.

PREPARATION OF CELL LYSATES AND WESTERN BLOT ANALYSIS

The cells were seeded at a 3×10^5 per 60-mm culture dish. The cells were incubated for 24 h and were transfected with $p57^{kip2}$ siRNA with at the desired concentrations. Twenty-four hours after treatment, the cells were washed with ice-cold phosphate-buffered saline and lysed in Mammalian Protein Extraction Reagent (M-PER; Pierce Chemical Co., Rockford, IL). Cytoplasmic and nuclear protein extracts were prepared according to the manufacturer's protocol described by Perkiman. Protein samples (20 μg per lane) were separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene difluoride membranes (Immobilon(TM)-P, Millipore, Bedford, MA), blocked in 5% nonfat milk in PBS and probed with primary antibodies overnight at 4°C. The membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2,000). The immunoreactive protein bands were developed by Enhanced Chemiluminescence (ECL) (Amersham Pharmacia Biotech, Freiburg, Germany).

IMMUNOPRECIPITATION ASSAY

For immunoprecipitation, the cell lysates were precleared with protein-A/G Sepharose and incubated with the indicated antibody overnight at 4°C. Immunocomplexes bound to protein-A/G Sepharose were collected by centrifugation and washed in radioimmunoprecipitation (RIPA) buffer. Immunoprecipitated proteins were analyzed by SDS-PAGE and detected with anti-$p57^{kip2}$ or anti-LIMK1 antibody.

IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked in 1% BSA for 1 h at room temperature. After blocking, cells were incubated with rabbit anti-$p57^{kip2}$ polyclonal antibody for 1 h, then incubated with anti-rabbit IgG-conjugated FITC for 45 min at room temperature. To visualize F-actin, cells were then incubated with Alex *Fluor® 555 phalloidin* and labeled with 0.2 μg/ml DAPI in PBS for 1 min and photographed with *Olympus* BH2 *fluorescence microscope* or a laser scanning confocal microscope (Leica, TCSNT, Bensheim, Germany).

STATISTICAL ANALYSES

Data are presented as means ± SEM. The statistical differences were determined using Student-Newman-Keuls Test and Dunn's Test (Sigma Stat Software Program, Jandel Scientific, San Rafael, CA). A *P*-value of 0.05 or less was considered as significant.

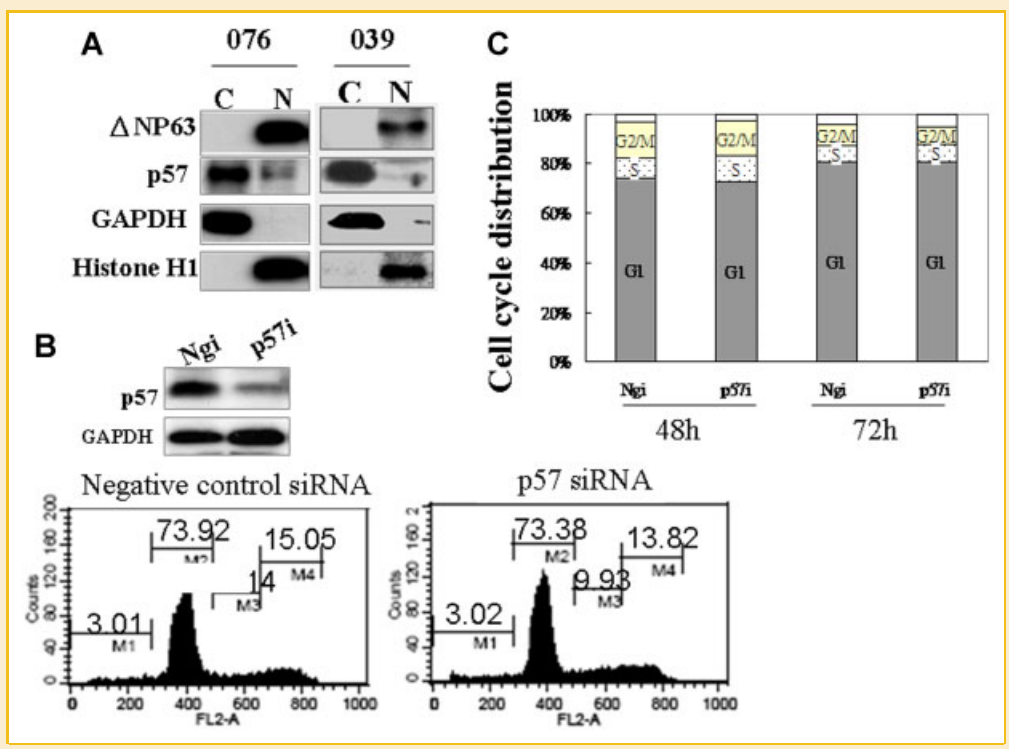

Fig. 1. $p57^{kip2}$ downregulation on cell cycle progression. A: The subcellular distribution of $p57^{kip2}$ and ΔNp63α in NPC-TW076 and NPC-TW039 cells. Cytoplasmic and nuclear extracts (17 μg/lane) were subjected to immunoblotting with anti-$p57^{kip2}$ and ΔNp63α antibodies. GAPDH as an internal control of cytoplasmic fraction. Anti-histone H1 as an internal control of nuclear fraction. B: Representative plots of cell cycle distribution after siRNA transfection for 48 h. The effective of $p57^{kip2}$ siRNA was shown by Western blotting. Cell cycle was determined using PI staining and detected by flow cytometry. C: Statistical plots of cell cycle distribution were shown after transfection with negative control siRNA (Ngi) or $p57^{kip2}$ siRNA (p57i) for 48 or 72 h in NPC-TW076 cells. The data from three independent experiments are graphed as the mean ± SD. $^*P < 0.05$.

RESULTS

SILENCE OF $p57^{kip2}$ DOES NOT AFFECT THE CELL CYCLE PROGRESSION

To characterize the function of $p57^{kip2}$ and ΔNp63α, the distributions of the subcellular compartments were investigated in NPC-TW076 and NPC-TW039 cells. Figure 1A indicates that ΔNp63α, an oncogenic protein only presented in the nucleus fraction, whereas $p57^{kip2}$ existed in the nucleus and abundantly in the cytosol fraction, indicating their different functions in the subcellular compartment. Next, cell-cycle distribution were analyzed by flow cytometry to determine whether p57kip2 was associated with a disturbance in cell-cycle regulation. NPC-TW076 cells were analyzed after $p57^{kip2}$ siRNA or negative control siRNA transfection for 48–72 h. Figure 1B indicates $p57^{kip2}$ siRNA transfection for 48h did not significantly affect the distribution of the G1, S, and G2/M phases. Figure 1C also indicates there was not significantly statistical difference in the cell-cycle distribution after $p57^{kip2}$ siRNA transfection for 48 or 72 h. These data indicate that $p57^{kip2}$ downregulation was not involved in the regulation of cell cycle progression.

MITOMYCIN C INDUCES FBS-MEDIATED CELL MIGRATION

The effect of proliferation on NPC cell migration was further investigated by pretreatment with 10 μg/ml of mitomycin C for 2 h before FBS deprivation for 24 h. A cross of mechanical scratch was introduced and the cells were further incubated with or without FBS (1 and 5%) for 24 h. As shown in Figure 2A, NPC cells migrated into wound in the presence of FBS in a dose-dependent manner. Importantly, NPC cells had a remarked increase of migration after mitomycin C plus FBS treatment. The quantitative assay indicated the percentage of wound healing markedly increased after mitomycin C plus 5% FBS treatment (Fig. 2B). These results suggested FBS-mediated cell migration is cell-division independent.

To investigate whether the promotion of FBS-mediated migration in NPC cells by mitomycin C would lead to changes in the levels of $p27^{kip1}$, $p57^{kip2}$ and ΔNp63α. NPC cells were preteatmented with

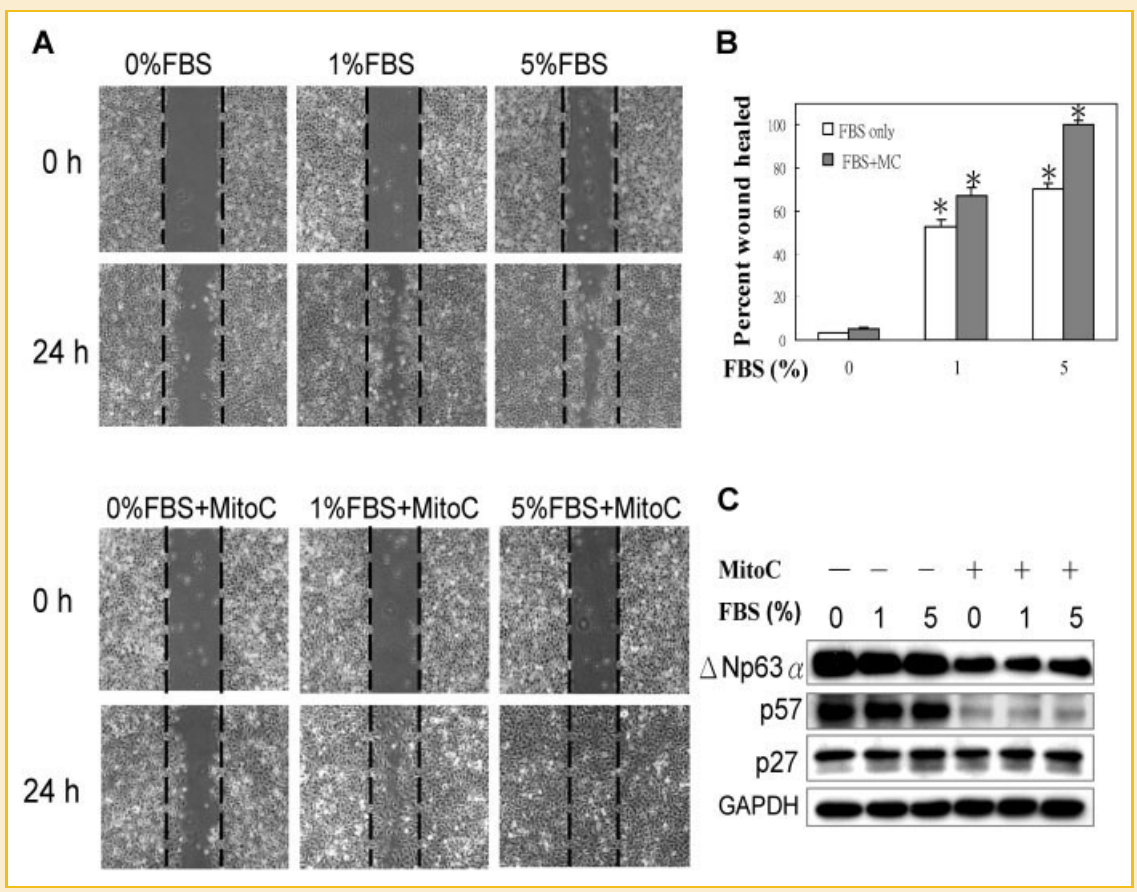

Fig. 2. Mitomycin C pretreatment enhances FBS-induced cell migration. A–C: Confluent NPC cells were incubated with or without 10 μg/ml of mitomycin C for 2 h before FBS deprivation for 24 h. After FBS deprivation, wounds were introduced and cells were cultured in fresh medium containing 0, 1, and 5% FBS for 24 h. A: Wound healing assay. Wound edges were photographed 0 and 24 h after FBS addition. Dotted lines indicate positions of wound edge 0 h after FBS addition. B: Statistical plots of migration were shown from three independent experiments. C: Mitomycin C pretreatment inhibits the expression level of $p57^{kip2}$, $p27^{kip1}$, and ΔNp63α proteins. The lysates were subjected to SDS PAGE to assay the expression of $p57^{kip2}$, $p27^{kip1}$, and ΔNp63α proteins. The data from three independent experiments are graphed as the mean ± SD. $^{*}P < 0.05$. GAPDH as an internal control.

mitomycin C for 2 h and incubated with or without FBS for 24 h and the lysates were assessed by Western blotting. As shown in Figure 2C, mitomycin C pretreatment remarkably inhibited the expression level of $p57^{kip2}$ and ΔNp63α, but did not change the level of $p27^{kip1}$. These data indicated the downregulations of $p57^{kip2}$ and ΔNp63α in response to mitomycin C might mediate the promotion of FBS-mediated cell migration.

$p57^{kip2}$siRNA TRANSFECTION PROMOTES CELL MIGRATION AND INVASION

To investigate whether the downregulation of $p57^{kip2}$ or/and ΔNp63α might promote the FBS-mediated cell migration, NPC cells were transfected with the indicated $p57^{kip2}$ siRNA, ΔNp63α siRNA, or negative control siRNA for 24 h and the wound healing assay was applied. As shown in Figure 3A, $p57^{kip2}$ or ΔNp63α siRNAs transfection effectively inhibited their protein expressions. Figure 3B shows the downregulation of $p57^{kip2}$ enhanced cell migration into wound. However, the reduction in ΔNp63α expression did not change significantly to promote the NPC cell migration (Fig. 3B).

To confirm the migration effect of $p57^{kip2}$ downregulation, Boyden chamber assay was applied to measure (Fig. 4). The siRNAs-transfected cells were plated on the upper chamber and migrated toward the lower chamber (10% FBS) for 24 h. Figure 4A shows that there was detectable increase of cell numbers on the lower side of membrane in $p57^{kip2}$ siRNA-transfected cells as compared to the negative control siRNA-transfected cells. ΔNp63α siRNA-transfected cells did not change significantly the migration status. Further, the ability of $p57^{kip2}$ involved in cell invasion was assayed by matrigel assay. As shown in Figure 4B, reduction of $p57^{kip2}$ also enhanced the cell numbers on lower side of membrane. These data

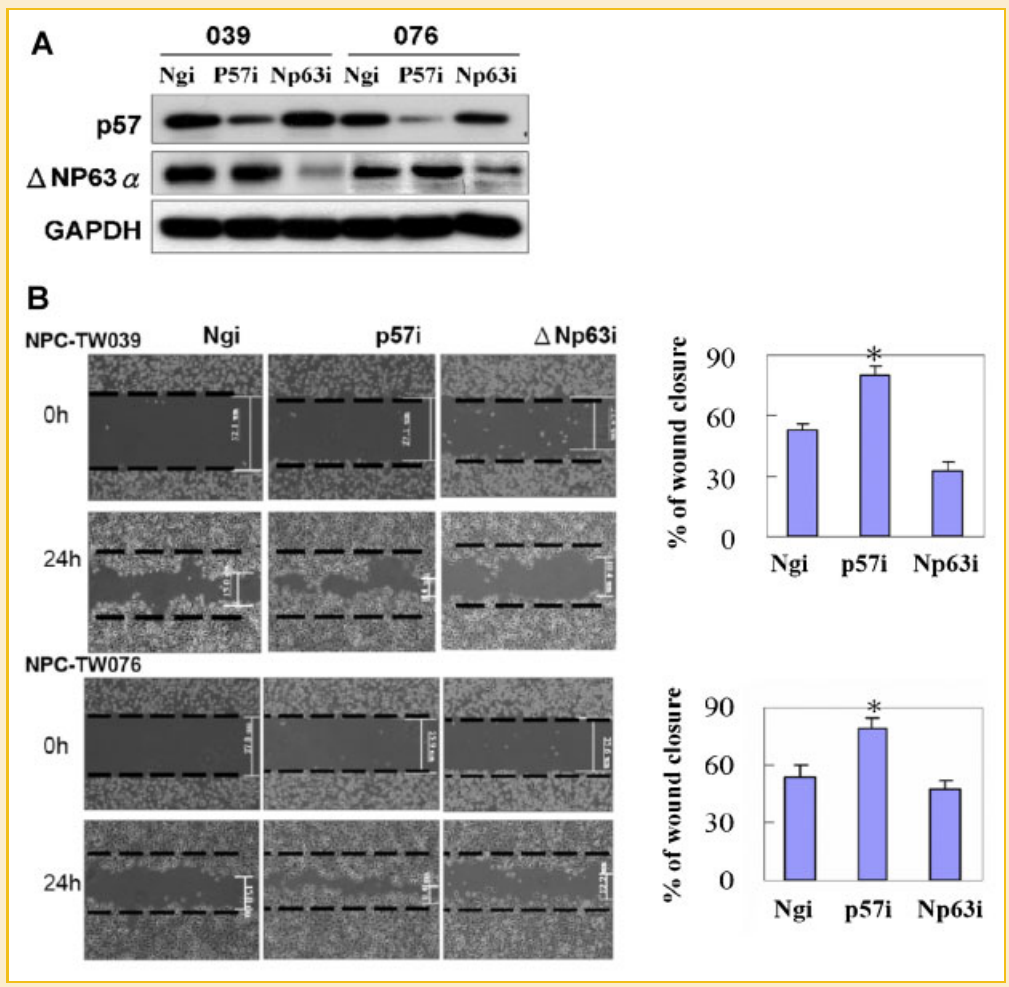

Fig. 3. Downregulation of $p57^{kip2}$ enhances cell migration. NPC cells were transfected with the indicated $p57^{kip2}$ and ΔNp63α siRNAs for 24 h. A: The cell extracts were prepared from treated cells and subjected to immunoblotting with anti-$p57^{kip2}$ and anti-ΔNp63α antibodies. Ngi: negative control siRNA. B: Wound healing assay. Confluent NPC cells transfected with the indicated siRNAs were incubated with FBS-free medium for 24 h. FBS-deprived cells were scratched and medium was changed into fresh medium containing 5% of FBS. Wound edges were photographed 24 h after FBS addition. Dotted lines indicate positions of wound edge 0 h after FBS addition. Statistical plots of migration were from three independent experiments shown in right panel. The data from three independent experiments are graphed as the mean ± SD. $^{*}P < 0.05$.(p57i vs. Ngi).

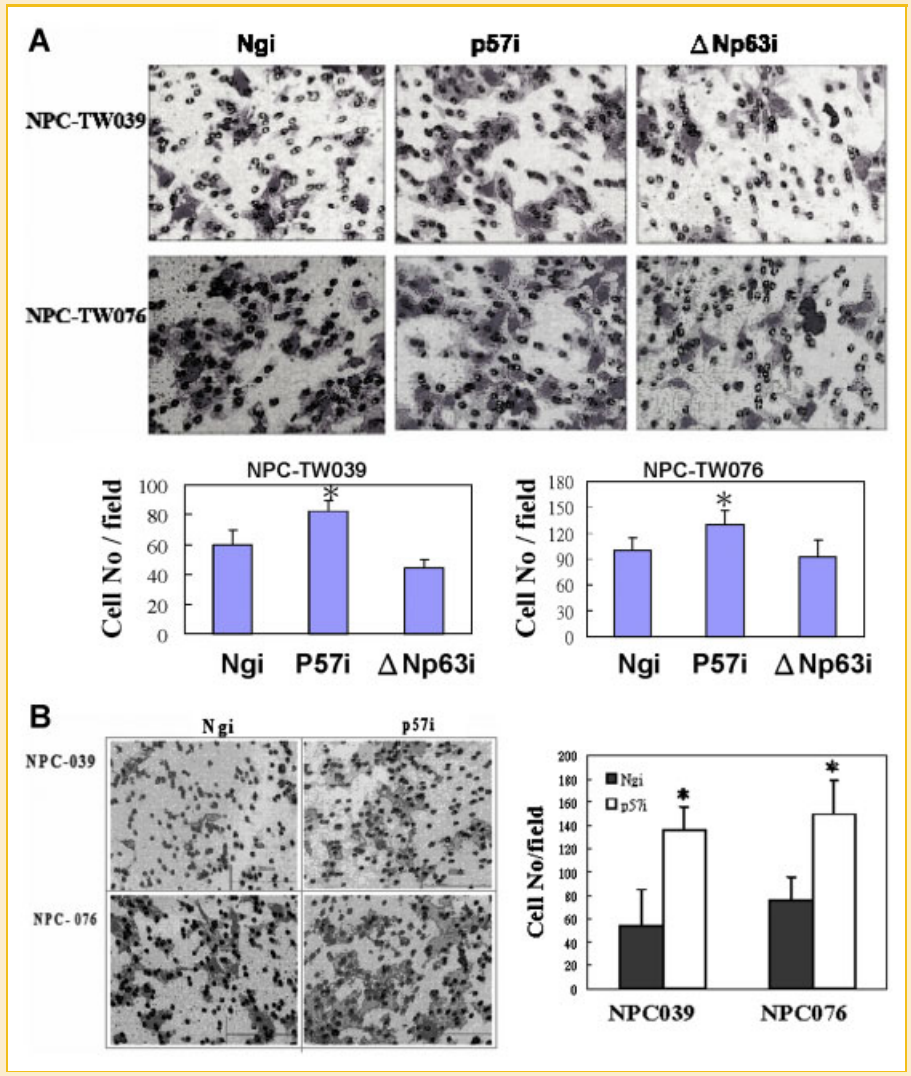

Fig. 4. Promotion of cell migration and invasion by reduction of $p57^{kip2}$ with siRNA. A: $p57^{kip2}$ siRNA treatment enhanced cell migration. NPC cells were transfected with the indicated $p57^{kip2}$ or ΔNp63α siRNAs for 24 h. 1×10^5 cells were seeded into Boyden chamber and incubated for 18 h. Cells in the upper side of the membrane was mechanical removed, and the cells that migrated to the lower side of membrane were fixed with 4% paraformaldehyde, followed by 0.25% crystal violet staining. The lower side of membrane was counted and photographed under a light microscope. The mean of the number were used to quantify the migration. B: $p57^{kip2}$ siRNA transfection enhanced cell invasion. The $p57^{kip2}$ siRNA-transfected cells (1×10^5) were seeded into Boyden chamber precoating with matrigel for 18 h. The cells were counted as shown in (A). Represented and statistical plots of matrigel invasion assay after $p57^{kip2}$ siRNA and Ngi transfection. The data from three independent experiments are graphed as the mean ± SD. $^*P < 0.05$ (p57i or ΔNp63α siRNA vs. Ngi). Ngi: negative control siRNA.

indicated $p57^{kip2}$, but not ΔNp63α, was involved in the NPC cell migration and invasion.

$p57^{kip2}$ AND Y-27632 CHANGE THE PHOSPHORYLATION STATUS OF COFILIN

Cofilin has emerged as one of the protein families playing an essential role in actin dynamics at the plasma membrane during cell protrusion [DesMarais et al., 2005]. To examine the migration effect of $p57^{kip2}$ on the phosphorylation status of cofilin (p-cofilin), NPC cells were transfected with $p57^{kip2}$ siRNA for 24 h and the lysates were investigated by Western blotting. Figure 5 shows $p57^{kip2}$ siRNA transfection (80 nM) induced a decrease of p-cofilin, but did not affect the total cofilin protein. This data suggested a change of p-cofilin might be involved in the regulation of actin remodeling and cell migration. To confirm the effect of p-cofilin in NPC cell migration, Y-27632, a ROCK specific inhibitor, was used to detect the level of p-cofilin expression. Figure 6A,B show that ROCK inhibition effectively reduced p-cofilin expression and induced a significantly enhanced cell migration. Y-27632 also induced a remarked increase of membrane protrusion and had a dendritic-like morphology (Fig. 6C). These data indicated inhibition of ROCK/LIMK/cofilin pathway induced cell motility.

$p57^{kip2}$ DOWNREGULATION AFFECTS THE ACTIN REMODELING

Since cell migration is dependent on actin reorganization, we employed the immunofluorescence staining and fluorescence

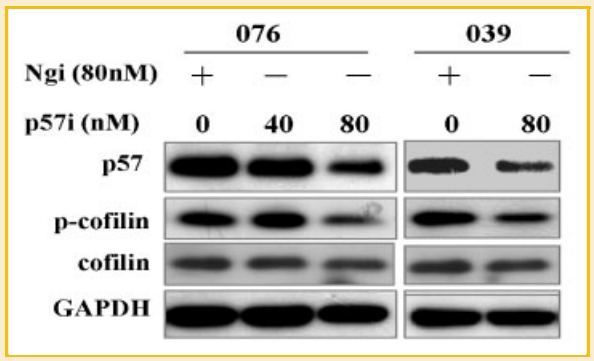

Fig. 5. Silence of p57^{kip2} induces decrease of p-cofilin expression. NPC cells were transfected with the indicated p57^{kip2} siRNAs for 24 h. Total cell extracts were prepared and subjected to immunoblotting with anti-p57^{kip2}, anti-p-cofilin and cofilin antibodies. GAPDH as a loading control.

microscope or confocal laser microscope to observe the changes of cytoskeletal morphology. As shown in Figure 7A, p57^{kip2} siRNA transfection induced a dramatically different morphology as compared to control siRNA transfection. In contrast to control siRNA-transfected cells with broader morphology, the p57^{kip2} siRNA-transfected cell had a dendritic-like morphology with remarked membrane protrusions. Further investigation by confocal laser microscope indicated these radial dentritic-like cells had a relatively lower level of p57^{kip2} expression (green) in their cytosol fraction (Fig. 7B,C). However, in contrast, the staining with rabbit polyclonal p57^{kip2} antibody and secondary antibody conjugated FITC showed a high background in the nucleus of NPC cells. To clearly show the merge image of Figure 7B, the magnification of merged images were presented in Figure 7C. These data indicated loss of p57^{kip2} expression had a significant effect on the cytoskeletal remodeling in NPC cells.

LIMK-1 AS A TARGET OF p57^{kip2} AFFECTS THE CYTOSKELETON REMODELING

To explore the effect mechanisms of p57^{kip2} on actin remodeling, the interaction of p57^{kip2} with LIMK-1 was assayed by immunoprecipitation and Western blotting. As shown in Figure 8A, there was remarked decrease in coprecipitated binding of the endogenous p57^{kip2} with the anti-LIMK1 antibody in p57^{kip2} siRNA-transfected cells as compared to that of control siRNA-transfected cells. Furthermore, this physical interaction was shown to inhibit the phosphorylation level of LIMKs (Figure 8B). These data suggested the expression level of p57^{kip2} protein held the actin cytoskeleton remodeling properties through modulation the activation of LIMKs/cofilin pathway.

DISCUSSION

p57^{kip2} is frequently down-regulated in many common human malignancies and its decreased expression is correlated with aggressiveness in oral squamous cell carcinoma [Fan et al., 2006a], laryngeal squamous cell carcinoma [Fan et al., 2006b],

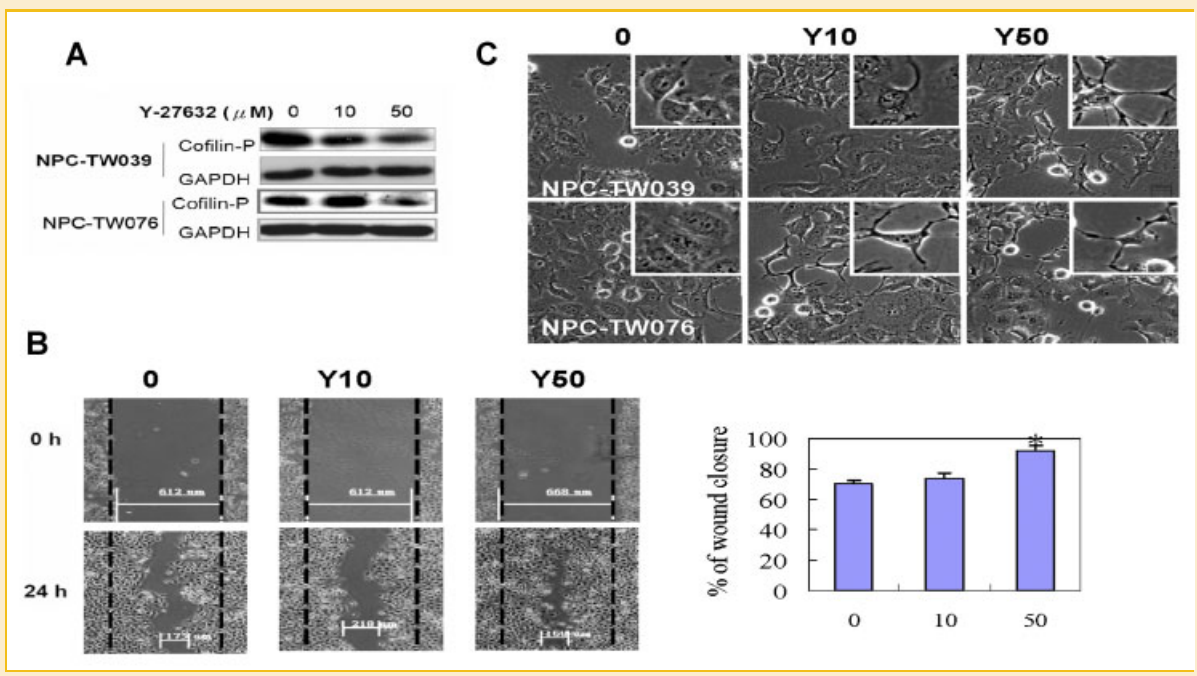

Fig. 6. Inhibition of ROCK induces cell migration. A: NPC cells treated with Y-27632 (10 and 50 μM; Y10 and Y50) for 24 h, total cell extracts were prepared and subjected to immunoblotting with anti-p-cofilin antibodies. GAPDH antibody as a loading control. B: Wound healing assay. After FBS deprivation, wounds were introduced in confluent cells and incubation with or without Y-27632 in fresh medium containing 5% FBS. Wound edges were photographed 0 and 24 h after FBS addition in NPC-TW076 cells. Represented plot shown in right panel. Statistical plot was shown in left panel. C: The active membrane protrusions of Y-27632-treated cells were shown in higher magnification (10 × 40) and amplification figure was shown in the right upper panel.

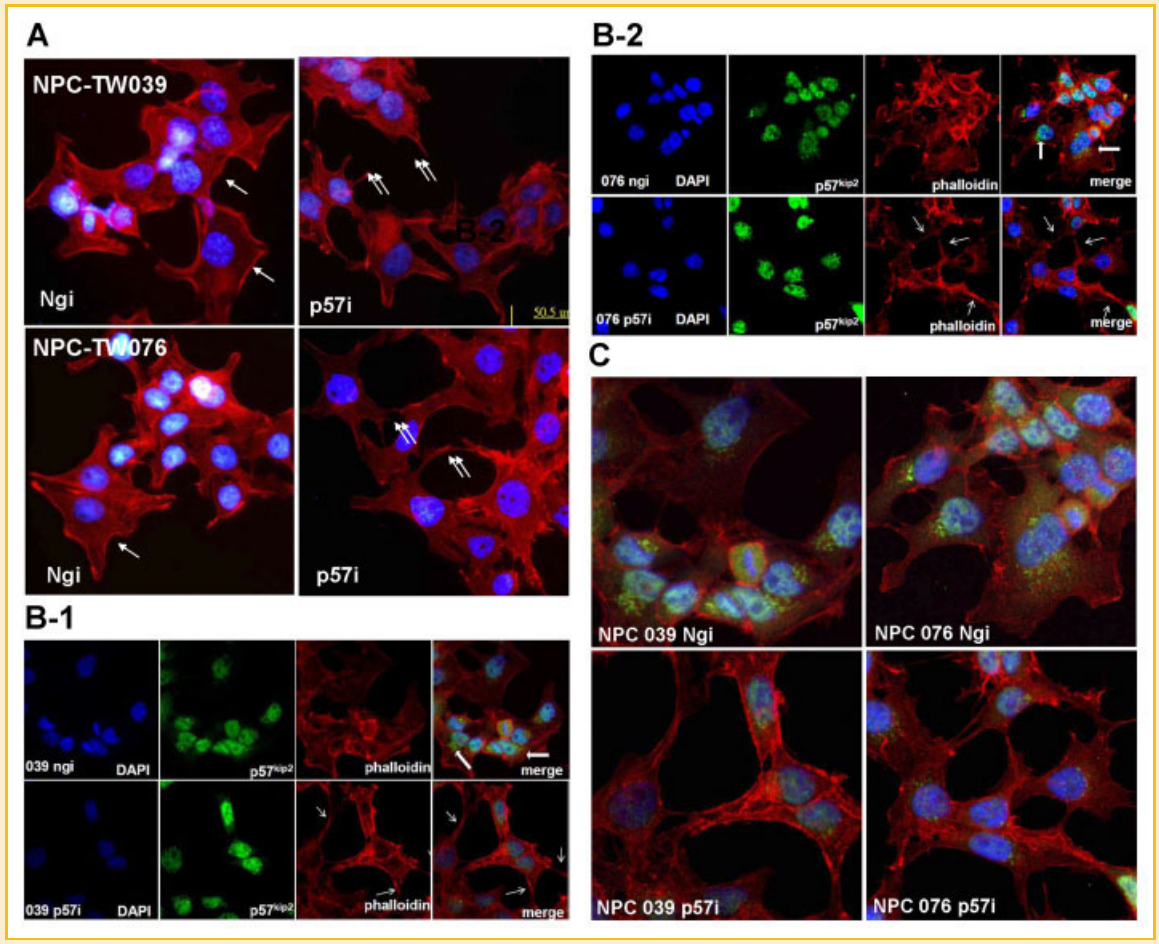

Fig. 7. Immunofluoresence staining indicates the membrane protrusion after $p57^{kip2}$ siRNA transfection. A–C: NPC cells were transfected with $p57^{kip2}$ siRNAs or negative control siRNA (Ngi) for 24 h. NPC cells (5×10^4) were seeded into 4-well chamber slide and incubated for 24 h in 5% FBS. A: Fluorescence microscopy images. Cells were fixed and stained with Alexa Fluor 555 phalloidin. Nuclei were counterstained by DAPI staining. Representative images of F-actin (Alexa Fluor 555, red) and nuclear DAPI staining (blue) of NPC cells were presented. Single arrow indicated the position of cells with cortical filament. Double arrows indicated the cells with long membrane protrusions. B: Confocal images. Cells were fixed and detected with anti-$p57^{kip2}$ antibody and FITC-conjugated secondary antibody. Samples were also stained with Alexa Fluor 555 phalloidin and nuclei were counterstained by DAPI staining. Representative confocal images of immunostaining with $p57^{kip2}$ (FITC, green), F-actin (Alexa Fluor 555, red) and nuclear DAPI staining (blue) of NPC cells were presented. Dendritic-like membrane protrusions shown in "arrow," $p57^{kip2}$ in cytoplasm fraction (green) shown in " bold arrow." Similar results were obtained from $\geq$3 independent experiments. C: The magnification of "merged images" of (B).

hepatocellular carcinoma [Ito et al., 2001], and malignant ovarian tumors [Sui et al., 2002], denoting its anti-oncogenic function. Decreased $p57^{kip2}$ expression is related with poor outcome in breast cancer [Yang et al., 2009]. In the present study, we found $p57^{kip2}$ protein existed abundantly in the cytosol in NPC cell lines and denoted the impact on NPC cell migration. We found that suppression of LIMK/cofilin signaling by ROCK inhibition induced the cytoskeletal remodeling that remarkably increased the membrane protrusions and led to more pronounced cell motility. Importantly, knockdown of endogenous $p57^{kip2}$ induced the reduction of p-cofilin via inactivation of LIMK and thereby induced increases of membrane protrusions and enhanced cell migration. In addition, this study also indicated that the function of ΔNP63α that presented in the nucleus of NPC cells is unrelated with the cell migration. These results indicated dysregulation of $p57^{kip2}$ might contribute to cell mobility through regulation of LIMK/cofilin pathway. Thus, targeting of $p57^{kip2}$/LIMK/cofilin signaling molecules might provide the opportunities in metastasis of cancer cells.

Directed cell movement is a multi-step process involving changes in cytoskeleton, cell substrate adhesion, and the extracellular matrix. It is initially in response to extracellular cues, which can be diffusible factors, signals on neighboring cells, and/or signals from the extracellular matrix [Ridley, 2001]. In the present study, initiation of NPC cell migration depended on the signal molecules presented in FBS to stimulate intracellular signaling pathway to induce cell migration. Mitomycin C pretreatment enhanced the FBS-induced cell migration. This effect suggests NPC cell migration is independent of cell proliferation and may be activated by the same upstream signaling pathways of cell division [Besson et al., 2004]. Importantly, mitomycin C inhibited cell proliferation and determined a cell chooses to migrate that accompanied with the downregulation of $p57^{kip2}$ and ΔNp63α, but not $p27^{kip1}$. Employing siRNA technology, this study clearly ruled out the possible role of

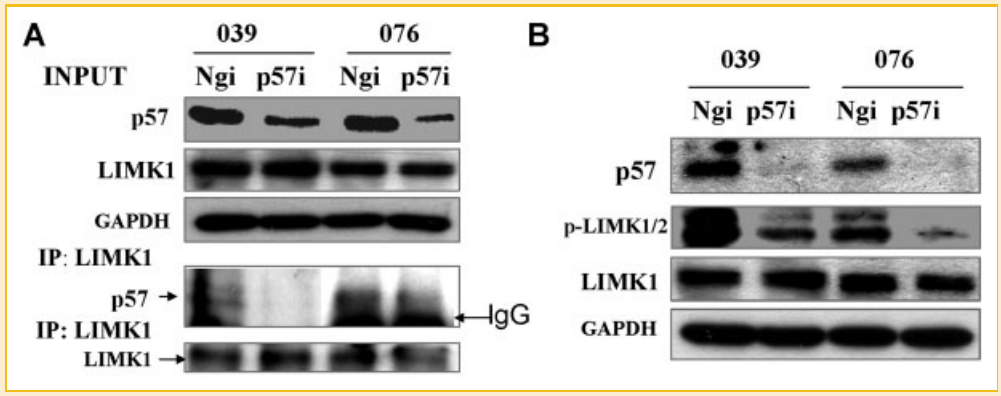

Fig. 8. Interaction of $p57^{kip2}$ and LIMK-1. A–B: NPC cells were transfected with $p57^{kip2}$ siRNA and negative control siRNA (Ngi) for 24 h A: Immunoprecipitation assay. For immunoblot analysis of cell extracts before immunoprecipitation (INPUT), the cell extracts (20 μg/lane) were subjected to immunoblotting using anti-$p57^{kip2}$ and LIMK1 antibodies. For immunoprecipitation, the cell lysates (1 mg/ml) were immunoprecipitated with anti-LIMK1 antibody and the immune complexes were subjected to immunoblotting using anti-$p57^{kip2}$ and LIMK1 antibodies (as an IP control). B: Silence of $p57^{kip2}$ reduced the phosphorylation status of LIMK-1. The cell extracts (20 μg/lane) were subjected to immunoblotting using anti-p-LIMK1/2 and LIMK1 antibodies. GAPDH as a loading control.

ΔNp63α implicated in NPC cell migration. This result may be contrast with the data of some recent findings [Yip and Tsao, 2008; Kommagani et al., 2009]. They show that loss of the endogenous ΔNp63α expression results in up-regulation of genes associated with invasion and metastasis in squamous carcinoma cell lines. Interestingly, our previous studies and other reports indicate ΔNp63α is involved in cell division and has antiapoptotic effect in NPC cells [Muller et al., 2006; Chiang et al., 2009; Chow et al., 2010; Chow et al., 2011]. Downregulation of ΔNp63α induces cell apoptosis [Yip and Tsao, 2008]. Thus, ΔNp63α downregulation might be involved in the anti-growth effect of mitomycin C.

$p57^{kip2}$, a member of the Cip/kip family, shares a conserved N-terminal domain with $p21^{cip1}$ and $p27^{kip1}$ that involved in the binding to cyclins-CDK complexes [Pateras et al., 2009]. CDK inhibitors directly modulate the processes of cell movement and cell proliferation [Lee and Helfman, 2004]. $p57^{kip2}$ presented in nuclear distribution was supposed to involve in its CDK inhibitor capacity, however, silence of $p57^{kip2}$ did not affect the cell-cycle progression. Overexpression of $p57^{kip2}$ in prostate cancer and glioma significantly suppress cell proliferation and reduce invasive ability [Sakai et al., 2004; Jin et al., 2008]. In contrast, ectoptic expression of $p57^{kip2}$ reduces the actin protein mobile fraction and affects the cytoskeleton dynamic in HeLa or HEK293 cells [Vlachos and Joseph, 2009]. These studies might be consistent with our finding that silence of endogenous $p57^{kip2}$ induced the enhanced cell migration. Thus, $p57^{kip2}$ existed abundantly in the cytosol fraction might raise the possible link with cell migration. Thus, modulation of $p57^{kip2}$ expression might be involved in the regulation of NPC metastasis. Interestingly, mitomycin C treatment did not affect the $p27^{kip1}$ levels. In addition, other significant cell cycle regulators such as dephosphorylation of retinoblastoma tumor suppressor protein (RB) or $p21^{cip1}$ should be considered [Muller et al., 2006].

The motility of a cancer cell is governed by the regulators of cytoskeletal dynamics, in particular the Rho family of GTPase [Ridley, 2001]. Upon stimulation, GTP-bound Rho A activates its effectors, Rho-associated kinase (ROCK) and/or PAK4, and phosphorylates its substrate LIMKs [Ridley, 2001]. Activation of LIMK enhances its ability to phosphorylate cofilin, an actin-depolymerizing protein, resulting in a reduction of cell motility [Bernard, 2007]. Cofilin is a key regulator of actin filament dynamics and reorganization by stimulating depolymerization and severance of actin filament [DesMarais et al., 2005]. This cofilin effect is inhibited by phosphorylation on Ser3 that resulted in abolishing its actin-binding activity [Ghosh et al., 2004]. The $p57^{kip2}$ siRNA transfection significantly induces an increase of dendritic-like protrusions through reduction of the p-cofilin expression that is determined with the help of ROCK inhibitor (Y-27632). Reduction of p-cofilin might be one of major mechanisms for serving free barbed ends on pre-existing actin filaments for generation protrusions [DesMarais et al., 2005; Yamazaki et al., 2005]. Members of the CDK inhibitors of the Cip/kip family have a distinct function in the cytoplasm, different from their cyclin-CDK inhibitory role [Besson et al., 2008; Pateras et al., 2009]. They have the ability to regulate the Rho signaling pathway, therefore interfering with the cytoskeleton dynamic [Besson et al., 2008; Vlachos and Joseph, 2009]. $p27^{kip1}$ is shown to bind to Rho A resulted in an increase of migration. Cytosolic $p21^{cip1}$ is shown to bind ROCK that inhibits its kinase activity, resulting in decreased stress fiber formation [Besson et al., 2008]. $p57^{kip2}$ is tightly coupled to LIMK1 to regulate the cytoskeletal dynamics and the locoalization of $p57^{kip2}$ prevents cell motility in a RhoA/ROCK independent manner in ectoptotic $p57^{kip2}$-expressed cancer cell or normal cells [Besson et al., 2008]. In this study, we clearly indicated there is a functional linkage between endogenous $p57^{kip2}$and LIMK/cofilin signaling pathway in NPC cells. However, the possible roles of $p57^{kip2}$ might be interacted with the upstream molecules of LIMK signal pathway that regulated cell migration need to be explored in NPC.

$p57^{kip2}$ is increased in the initial stages of cancer followed by a gradual decrease as malignancy progresses, implying that a selective pressure leading to its loss takes place [Pateras et al., 2009]. This study indicated that suppression of ROCK/LIMK/cofilin pathway induced cell migration. Silence of endogenous $p57^{kip2}$ or dysregula-

tion the LIMK/cofilin pathway, leading to cell migration through actin remodeling. Thus, dysregulation of $p57^{kip2}$/LIMK/cofilin signaling network may be inappropriate to stimulate cell motility to undergo metastasis in NPC cancer cells.

REFERENCES

Barbieri CE, Tang LJ, Brown KA, Pietenpol JA. 2006. Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. Cancer Res 66:7589–7597.

Bernard O. 2007. Lim kinases, regulators of actin dynamics. Int J Biochem Cell Biol 39:1071–1076.

Besson A, Assoian RK, Roberts JM. 2004. Regulation of the cytoskeleton: An oncogenic function for cdk inhibitors? Nat Rev Cancer 4:948–955.

Besson A, Dowdy SF, Roberts JM. 2008. CDK inhibitors: Cell cycle regulators and beyond. Dev Cell 14:159–169.

Chiang CT, Chu WK, Chow SE, Chen JK. 2009. Overexpression of delta Np63 in a human nasopharyngeal carcinoma cell line downregulates CKIs and enhances cell proliferation. J Cell Physiol 219:117–122.

Chou J, Lin YC, Kim J, You L, Xu Z, He B, Jablons DM. 2008. Nasopharyngeal carcinoma—Review of the molecular mechanisms of tumorigenesis. Head Neck 30:946–963.

Chow SE, Wang JS, Chuang SF, Chang YL, Chu WK, Chen WS, Chen YW. 2010. Resveratrol-induced p53-independent apoptosis of human nasopharyngeal carcinoma cells is correlated with the downregulation of [Delta]Np63. Cancer Gene Ther 17:872–882.

Chow SE, Chang YL, Chuang SF, Wang JS. 2011. Wogonin induced apoptosis in human nasopharyngeal carcinoma cells by targeting GSK-3β and ΔNp63. Cancer Chemother Pharmacol [Epub ahead of reprint]. DOI: 10.1007/s00280-010-1552-1.

DesMarais V, Ghosh M, Eddy R, Condeelis J. 2005. Cofilin takes the lead. J Cell Sci 118:19–26.

Fan GK, Chen J, Ping F, Geng Y. 2006a. Immunohistochemical analysis of P57(kip2), p53 and hsp60 expressions in premalignant and malignant oral tissues. Oral Oncol 42:147–153.

Fan GK, Xu F, Yang B, Fujieda S. 2006b. p57kip2 expression is related to carcinogenesis and tumor progression in laryngeal tissues. Acta Otolaryngol 126:301–305.

Ghosh M, Song X, Mouneimne G, Sidani M, Lawrence DS, Condeelis JS. 2004. Cofilin promotes actin polymerization and defines the direction of cell motility. Science 304:743–746.

Guo C, Pan Z-G, Li D-J, Yun J-P, Zheng M-Z, Hu Z-Y, Cheng L-Z, Zeng Y-X. 2006. The expression of p63 is associated with the differential stage in nasopharyngeal carcinoma and EBV infection. J Transl Med 4:23.

Horikawa T, Yang J, Kondo S, Yoshizaki T, Joab I, Furukawa M, Pagano JS. 2007. Twist and epithelial-mesenchymal transition are induced by the EBV oncoprotein latent membrane protein 1 and are associated with metastatic nasopharyngeal carcinoma. Cancer Res 67:1970–1978.

Ichikawa T, Suenaga Y, Koda T, Ozaki T, Nakagawara A. 2008. [Delta]Np63/BMP-7-dependent expression of matrilin-2 is involved in keratinocyte migration in response to wounding. Biochem Biophysl Res Commun 369:994–1000.

Ito Y, Takeda T, Sakon M, Tsujimoto M, Monden M, Matsuura N. 2001. Expression of p57/Kip2 protein in hepatocellular carcinoma. Oncology 61: 221–225.

Jin RJ, Lho Y, Wang Y, Ao M, Revelo MP, Hayward SW, Wills ML, Logan SK, Zhang P, Matusik RJ. 2008. Down-regulation of p57Kip2 induces prostate cancer in the mouse. Cancer Res 68:3601–3608.

Kommagani R, Leonard MK, Lewis S, Romano RA, Sinha S, Kadakia MP. 2009. Regulation of VDR by {Delta}Np63{alpha} is associated with inhibition of cell invasion. J Cell Sci 122:2828–2835.

Lee S, Helfman DM. 2004. Cytoplasmic p21Cip1 is involved in Ras-induced inhibition of the ROCK/LIMK/Cofilin pathway. J Biol Chem 279(3):1885–1891.

Lin CT, Wong CI, Chan WY, Tzung KW, Ho JK, Hsu MM, Chuang SM. 1990. Establishment and characterization of two nasopharyngeal carcinoma cell lines. Lab Invest 62:713–724.

Lo KW, To KF, Huang DP. 2004. Focus on nasopharyngeal carcinoma. Cancer Cell 5:423–428.

Muller M, Schleithoff ES, Stremmel W, Melino G, Krammer PH, Schilling T. 2006. One, two, three–p53, p63, p73 and chemosensitivity. Drug Resist Updat 9:288–306.

Nakai S, Masaki T, Shiratori Y, Ohgi T, Morishita A, Kurokohchi K, Watanabe S, Kuriyama S. 2002. Expression of p57KIP2 in hepatocellular carcinoma: Relationship between tumor differentiation and patient survival. Int J Oncol 20:769–775.

Nan K, Guo H, Ruan ZP, Jing Z, Liu SX. 2005. Expression of p57kip2 and its relationship with clinicopathology, PCNA and p53 in primary hepatocellular carcinoma. World J Gastroenterol 11:237–1240.

Pateras IS, Apostolopoulou K, Niforou K, Kotsinas A, Gorgoulis VG. 2009. p57KIP2: ''Kip''ing the Cell under Control. Mol Cancer Res 7:1902–1919.

Ridley AJ. 2001. Rho GTPases and cell migration. J Cell Sci 114:2713–2722.

Sakai K, Peraud A, Mainprize T, Nakayama J, Tsugu A, Hongo K, Kobayashi S, Rutka J. 2004. Inducible expression of p57KIP2 inhibits glioma cell motility and invasion. J Neuro-Oncol 68:217–223.

Sui L, Dong Y, Ohno M, Atanabe Y, Sugimoto K, Tokuda M. 2002. Expression of p57kip2 and its clinical relevance in epithelial ovarian tumors. Anticancer Res 22:3191–3196.

Sun Y, Hegamyer G, Cheng Y, Hildesheim A, Chen J, Chen I, Cao Y, Yao K, Colburn N. 1992. An infrequent point mutation of the p53 gene in human nasopharyngeal carcinoma. Proc Natl Acad Sci USA 89:6516–6520.

Vlachos P, Joseph B. 2009. The Cdk inhibitor p57Kip2 controls LIM-kinase 1 activity and regulates actin cytoskeleton dynamics. Oncogene 28:4175–4188.

Vlachos P, Nyman U, Hajji N, Joseph B. 2007. The cell cycle inhibitor p57Kip2 promotes cell death via the mitochondrial apoptotic pathway. Cell Death Differ 14:1497–1507.

Yamazaki D, Kurisu S, Takenawa T. 2005. Regulation of cancer cell motility through actin reorganization. Cancer Sci 96:379–386.

Yang N, Higuchi O, Ohashi K, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K. 1998. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 393:809–812.

Yang X, Karuturi RK, Sun F, Aau M, Yu K, Shao R, Miller LD, Tan PB, Yu Q. 2009. CDKN1C (p57KIP2) is adirect target of EZH2 and suppressed by multiple epigenetic mechanisms in breast cancer cells. PLoS One 4:e5011.

Yip YL, Tsao SW. 2008. Regulation of p63 expression in primary and immortalized nasopharyngeal epithelial cells. Int J Oncol 33:713–724.

Yokoo T, Toyoshima H, Miura M, Wang Y, Iida KT, Suzuki H, Sone H, Shimano H, Gotoda T, Nishimori S, Tanaka K, Yamada N. 2003. p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus. J Biol Chem 278:52919–52923.