

# Mouse Melanoma Cell Migration is Dependent on Production of Reactive Oxygen Species under Normoxia Condition

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## Abstract

Cell migration plays a role in many physiological and pathological processes. Reactive oxygen species (ROS) produced in mammalian cells influence intracellular signaling processes which in turn regulate various biological activities. Here, we investigated whether melanoma cell migration could be controlled by ROS production under normoxia condition. Cell migration was measured by wound healing assay after scratching confluent monolayer of B16F10 mouse melanoma cells. Cell migration was enhanced over 12 h after scratching cells. In addition, we found that ROS production was increased by scratching cells. ERK phosphorylation was also increased by scratching cells but it was decreased by the treatment with ROS scavengers, N-acetylcysteine (NAC). Tumor cell migration was inhibited by the treatment with PD98059, ERK inhibitor, NAC or DPI, well-known ROS scavengers. Tumor cell growth as judged by succinate dehydrogenase activity was inhibited by NAC treatment. When mice were intraperitoneally administered with NAC, the intracellular ROS production was reduced in peripheral blood mononuclear cells. In addition, B16F10 tumor growth was significantly inhibited by *in vivo* treatment with NAC. Collectively, these findings suggest that tumor cell migration and growth could be controlled by ROS production and its downstream signaling pathways, *in vitro* and *in vivo*.

**Key Words:** Reactive oxygen species, N-acetylcysteine, ERK, Cell migration, Tumor growth

## INTRODUCTION

Cell migration plays a role in many physiological and pathological processes, including tumor metastasis (Singer and Kupfer, 1986). Cell migration is a multistep cycle including extension of a protrusion, formation of stable attachments near the leading edge of the protrusion, translocation of the cell body forward, release of adhesions and retraction at the cell rear (Lee *et al.*, 1993; Lauffenburger and Horwitz, 1996). Cell migration requires many cellular factors such as Arp2/3 complex (Welch *et al.*, 1997), profilin, G-actin-binding protein (Borisy and Svitkina, 2000), FAK, encoding focal adhesion kinase (Luedde, 2010) and so on. As a result, cell migration is resulted from the integration and temporal coordination of many different processes that occur in spatially distinct locations in the cell (Webb *et al.*, 2002).

Tumor cell migration through the circulatory system establish metastasis at peripheral sites, which is enhanced by new blood vessel formation (=angiogenesis) (Folkman, 1990). Since oxygen supply in solid tumors is often diminished to the tumor cells (=hypoxia), solid tumor growth is also dependent upon angiogenesis. Angiogenesis could be induced by soluble factors such as vascular endothelial growth factor (VEGF)

released from a tumor under hypoxia. Hypoxic condition enhances prostate and breast cancer cell migration (Jiang *et al.*, 2006; Lin *et al.*, 2009) through the sequential induction of HIF-1 $\alpha$  and VEGF (Park *et al.*, 2007). HIF-1 $\alpha$  is stabilized by reactive oxygen species (ROS) produced in hypoxic condition (Oh *et al.*, 2010).

ROS are produced in mammalian cells in response to the activation of various cell surface receptors and by serum-deprivation, LPS-stimulation and peroxiredoxin II-deficiency (Moon, 2008; Kim and Moon, 2009). ROS cause tissue injury in one hand and promote tissue repair in another hand (Maulik and Das, 2002). ROS contribute to intracellular signaling processes which in turn regulate various biological activities including host defense (Rhee *et al.*, 2000) and the progression of various tumors originated from prostate, lung, colon, breast and so on (Luanpitpong *et al.*, 2010; Tobar *et al.*, 2010; Chetram *et al.*, 2011; Lei *et al.*, 2011). The motility of cancer cells is enhanced by the activation of ERK and p38 MAPK, catalase activity, and by the reduction of ROS levels (Shim *et al.*, 2006). However, little is known about whether the ROS could be involved in migration of melanoma cells under normoxia condition.

In this study, we investigated whether melanoma cell migra-

www.biomolther.org

Open Access <http://dx.doi.org/10.4062/biomolther.2012.20.2.165>

pISSN: 1976-9148 eISSN: 2005-4483

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Received Jan 3, 2012 Revised Feb 6, 2012 Accepted Feb 21, 2012

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tion could be controlled by ROS production under normoxia condition. Cell migration was measured by wound healing assay with scratching confluent monolayer of B16F10 mouse melanoma cells. We found that ROS production was increased by scratching monolayer of cells. B16F10 cell migration *in vitro* and tumor growth *in vivo* were inhibited by the treatment with ROS scavengers, N-acetylcysteine (NAC). Data suggest that tumor cell migration and growth could be controlled by ROS production and its downstream signaling pathways.

**MATERIALS AND METHODS**

**Mice and reagents**

C57BL/6 mice were obtained from Daehan Biolink (Chungju, Korea). Animals were maintained in authorized facility where the temperature at 20-22°C, the humidity at 50-60%, the dark/light cycles with 12 hour. All animal procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee, Sejong University. Antibodies which are reactive with ERK and phospho-ERK were from Cell Signalling (Beverly, MA, USA). PD98059 was obtained from Calbiochem (La Jolla, CA, USA). DCF-DA was purchased from Molecular Probe (Eugene, OR, USA). Except where indicated, all other materials are obtained from the Sigma chemical company (St. Louis, MO, USA).

**Cell purification and culture**

B16F10 mouse melanoma cells were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) cell bank (Taejeon, Korea). Cells were maintained and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Kansas City, MO, USA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Blood was drawn by eye puncture in EDTA tubes from control and NAC-treated mice. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation over Histopaque 1077 (Sigma Chemical Co., St. Louis, MO, USA). Then, mouse PBMCs were washed and re-suspended in DMEM supplemented with 10% fetal bovine serum.

**Measurement of cell migration**

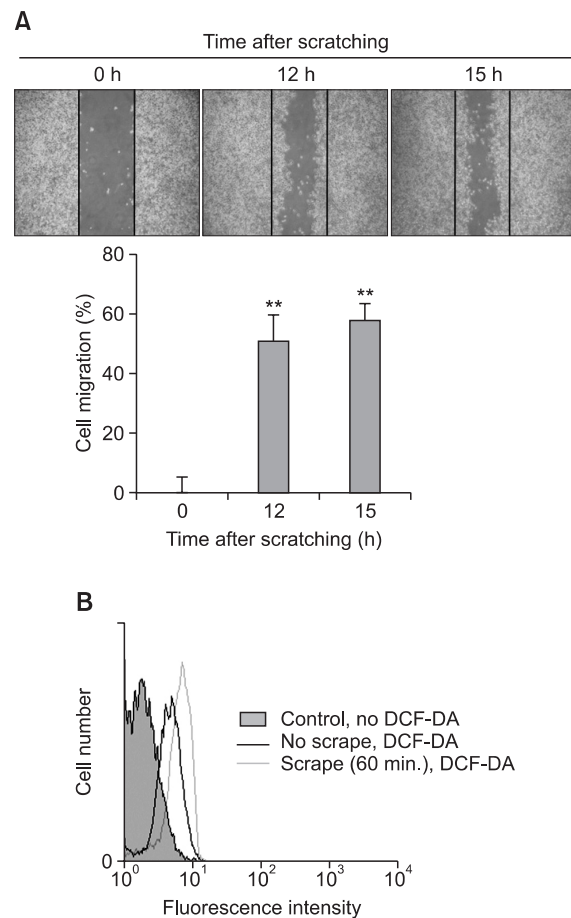
When B16F10 cell density was confluent in 35 mm culture dish (Corning, NY, USA), three wound lines in the form of a cross were made by scratching cellular monolayer with a plastic pipette tip. Then, floating cells were washed out and fresh medium was added (Moon *et al.*, 2010; Ryu *et al.*, 2010). As the incubation time passed under normoxia condition, the width of scratch was narrowed and it was recorded by taking photographs under phase contrast microscope from 6 h after the scratch.

**Flow cytometric analyses**

To analyze intracellular amount of ROS, B16F10 cells (1-10×10<sup>5</sup>) and mouse PBMCs were stained with fluorescence dye, DCF-DA (Molecular Probe, Eugene, OR) for 30 minute (Oh and Moon, 2010). Then, 10,000 cells were acquired to analyze intracellular amount of ROS by CELLQuest™ software in FACScalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA).

**Western blot analysis**

Cells were lysed in ice-cold lysis buffer, containing 0.5% Nonidet P-40 (vol./vol.) in 20 mM Tris-HCl, at a pH of 8.3; 150 mM NaCl; protease inhibitors (2 µg/ml aprotinin, pepstatin, and chymostatin; 1 µg/ml leupeptin and pepstatin; 1 mM phenylmethyl sulfonyl fluoride (PMSF); and 1 mM Na<sub>4</sub>VO<sub>3</sub>. Lysates were incubated for 30 minutes on ice prior to centrifugation at 14,000 rpm for 5 minute at 4°C. Proteins in the supernatant were denatured by boiling for 5 minute in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. Following this



**Fig. 1.** Cell migration was induced by scratching monolayer of cells. (A) When B16F10 cell density was confluent in 35 mm culture dish, wound lines were made by scratching cellular monolayer with a plastic pipette tip. Cells were incubated for an appropriate time under normoxia condition. The width of scratch was recorded by taking photographs under phase contrast microscope. Data were representative of three experiments (top). Empty area in each time point was quantified with NIH image analysis software (version 1.62) and compared with that in the initiation of cell migration. Percentage of cell migration was represented with bar graph. Data in bar graph represent mean ± SED. \*\*p<0.01, Significantly different from the initiation of cell migration (bottom). (B) B16F10 cells were incubated under normoxia condition for 1 h after scratching cellular monolayer. Cells were stained with fluorescence dye, 5 µM DCF-DA for 30 minute. Then, 10,000 cells were analyzed by flow cytometer.

transfer, equal loading of protein was verified by Ponceau staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5% Tween 20), then incubated with the indicated antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA).

**MTT assay**

B16F10 cells were incubated in 96 well plates and treated with PD98059 or NAC for 48 h. Then, we quantified cell survival using colorimetric assay described for measuring intracellular succinate dehydrogenase content with MTT [3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Denizot and Lang, 1986). Cells were then incubated with 50 µg/ml of MTT at 37°C for 2 h. Formazan crystals were dissolved in dimethyl-sulfoxide (DMSO). Optical density (OD) was read at 540 nm.

**In vivo tumor growth**

C57BL/6 mice were subcutaneously implanted with 2×10<sup>5</sup> B16F10 mouse melanoma cells. N-acetylcysteine (NAC) was intraperitoneally administered at dose of 32 mg/kg into mice everyday (Tomomura *et al.*, 2003). Tumor growth was monitored by the measurement of short axis and long axis of tumor mass with vernier calipers (Mizuyo, Japan). Tumor volume was calculated by the multiplication of (long axis)/2 and (short

axis)<sup>2</sup>.

**Statistical analyses**

Experimental differences were tested for statistical significance using ANOVA and Student's *t*-test. *p* value of <0.05 was considered to be significant.

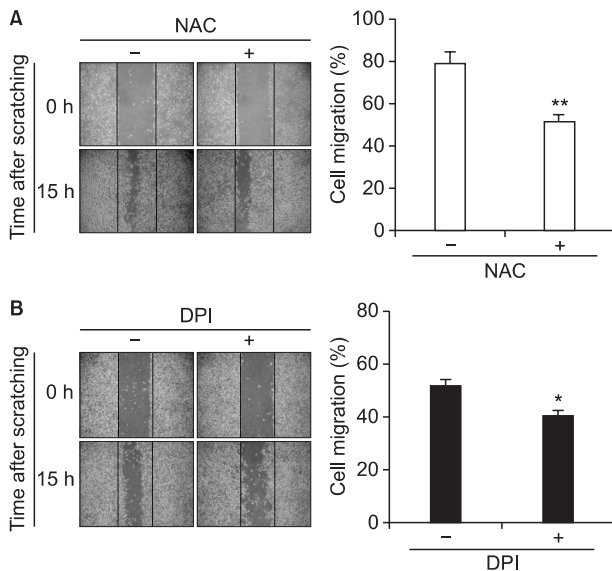
**RESULTS**

**Reactive oxygen species (ROS) production was increased during tumor cell migration**

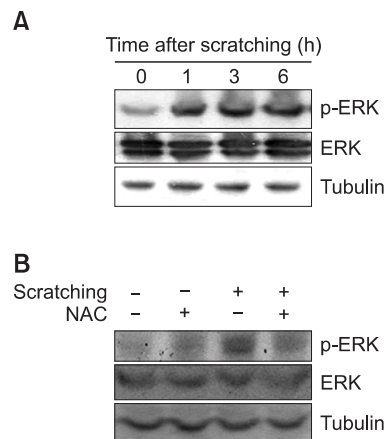
We investigated whether reactive oxygen species (ROS) are involved in tumor cell migration. We used wound migration assay by scratching a monolayer of mouse B16F10 melanoma cells. When a monolayer of B16F10 cells was scratched, wound migration was enhanced as compared to the initiation of assay (Fig. 1A). ROS production was also increased by scratching a monolayer of B16F10 cells (Fig. 1B). To confirm the effect of ROS on wound migration, B16F10 cells were incubated in the presence or absence of N-acetylcysteine (NAC) or diphenylene iodonium (DPI), well-known ROS scavengers after scratching tumor cells. As shown in Fig. 2A and B, wound migration was reduced by the incubation with NAC or DPI. Data suggest that tumor cell migration could be induced by ROS produced by scratching a monolayer of B16F10 cells.

**ERK phosphorylation was dependent on ROS production**

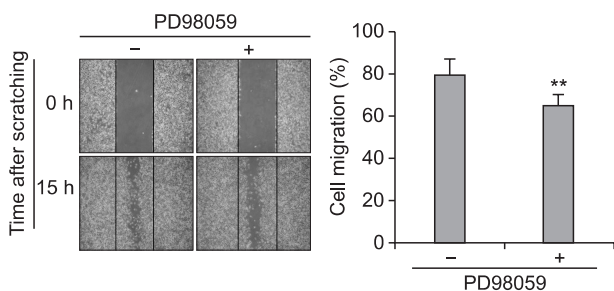
Previous report showed that extracellular signal-regulated kinase (ERK) was stimulated by ROS which was constitutively increased by the deletion of antioxidant enzyme, peroxiredoxin II (Prx II) (Han *et al.*, 2006). Therefore, we examined whether ERK phosphorylation could be changed by wounding a monolayer of tumor cells and during migration. As shown in Fig. 3A, ERK phosphorylation was increased by wounding B16F10 cells. In addition, ERK phosphorylation was inhibited by the in-



**Fig. 2.** Cell migration was reduced by the treatment with ROS scavengers. (A, B) When B16F10 cell density was confluent in 35 mm culture dish, wound lines were made by scratching cellular monolayer with a plastic pipette tip. Cells were incubated in the presence or absence of ROS scavengers, 20 mM NAC (A) or 5 µM DPI (B) for an appropriate time under normoxia condition. The width of scratch was recorded by taking photographs under phase contrast microscope. Data were representative of three experiments (top). Empty area in each time point was quantified with NIH image analysis software (version 1.62) and compared with that in the initiation of cell migration. Percentage of cell migration was represented with bar graph. Data in bar graph represent mean ± SED. \**p*<0.05; \*\**p*<0.01. Significantly different from the initiation of cell migration (bottom).



**Fig. 3.** ERK phosphorylation was dependent on ROS production. (A, B) When B16F10 cell density was confluent in 35 mm culture dish, wound lines were made by scratching cellular monolayer with a plastic pipette tip. B16F10 cells were incubated for an appropriate time under normoxia condition (A). B16F10 cells were also incubated in the presence or absence of 20 mM NAC for 1 h under normoxia condition (B). Cell lysates were prepared and phospho-ERK was detected by western blot analysis. Data were representative of three experiments (A, B).



**Fig. 4.** Cell migration was inhibited by the treatment with PD98059, ERK inhibitor. When B16F10 cell density was confluent in 35 mm culture dish, wound lines were made by scratching cellular monolayer with a plastic pipette tip. B16F10 cells were incubated in the presence or absence of 20  $\mu$ M PD98059 for 1 h under normoxia condition. The width of scratch was recorded by taking photographs under phase contrast microscope. Data were representative of three experiments (top). Empty area in each time point was quantified with NIH image analysis software (version 1.62) and compared with that in the initiation of cell migration. Percentage of cell migration was represented with bar graph. Data in bar graph represent mean  $\pm$  SED. \*\* $p < 0.01$ , Significantly different from the initiation of cell migration (bottom).

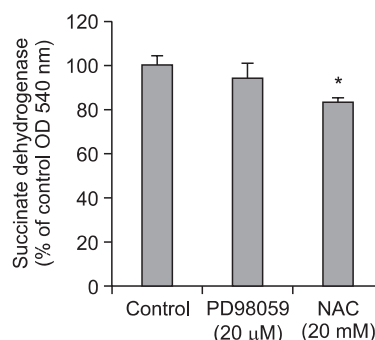
cubation in the presence of NAC (Fig. 3B). These demonstrate that tumor cell wound induced ERK phosphorylation through ROS production. To confirm the involvement of ERK phosphorylation in tumor cell migration, we treated B16F10 cells with PD98059, ERK inhibitor. Wound migration of B16F10 cells was decreased by the treatment with PD98059 (Fig. 4). It suggests that tumor cell migration could be induced by ERK phosphorylation through ROS production.

#### Tumor growth was inhibited by the administration of NAC into mice

As wound migration of B16F10 cells was decreased by the treatment with NAC Fig. 2A or PD98059 (Fig. 4), we also tested their effect on tumor cell growth. While B16F10 cell growth was retarded by the incubation with NAC, no significant changes were found in the group incubated with 20  $\mu$ M PD98059 (Fig. 5). It suggests that NAC might be effective anti-tumor agent. Then, we examined whether NAC administration led to the inhibition of tumor growth. When mice were administered with NAC, B16F10 melanoma growth was significantly reduced by the administration with NAC to the mice (Fig. 6A). In addition, ROS production in mouse PBMCs was reduced by NAC administration (Fig. 6B). Data suggest that tumor cell growth could be effectively interfered by scavenging ROS or by blocking their production *in vivo*.

## DISCUSSION

Cell migration is an important multistep cycle in many physiological and pathological processes, including tumor metastasis (Singer and Kupfer, 1986; Lee *et al.*, 1993; Lauffenburger and Horwitz, 1996). Many cellular factors including Arp2/3 complex, profilin, and FAK, are required in cell migration (Welch *et al.*, 1997; Borisy and Svitkina, 2000; Luedde, 2010). Tumor cell migration is enhanced by angiogenesis, which is induced by soluble factors such as VEGF under hypoxia (Folkman, 1990). VEGF expression is stimulated by transcrip-



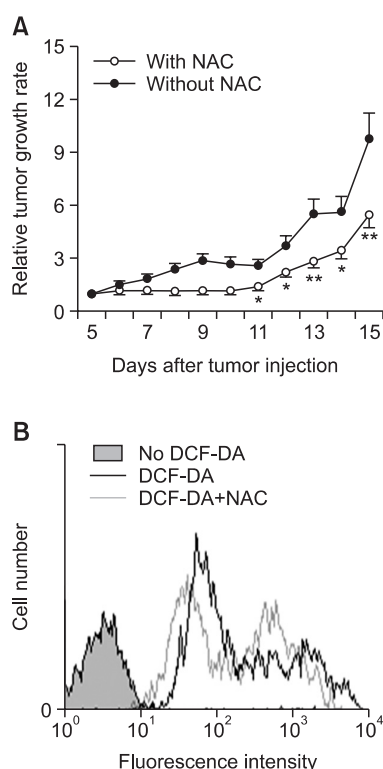
**Fig. 5.** Cell growth was reduced by the treatment with NAC but no significant changes were detected by PD98059. B16F10 cells were incubated with 20  $\mu$ M PD98059 or 20 mM NAC for 48 h under normoxia condition. Cell density was measured by MTT assay. Data represent mean  $\pm$  SED. \* $p < 0.05$ , cell density in NAC-treated group was significantly different from control.

tion factor, HIF-1 $\alpha$  that is stabilized by hypoxia-induced ROS production (Park *et al.*, 2007; Oh *et al.*, 2010). ROS influence intracellular signaling processes which in turn regulate various biological activities such as the motility of cancer cells (Shim *et al.*, 2006; Luanpitpong *et al.*, 2010; Tobar *et al.*, 2010; Chetram *et al.*, 2011; Lei *et al.*, 2011). However, little is known about whether the ROS could be involved in migration of melanoma cells under normoxia condition. Here, we investigated whether melanoma cell migration could be controlled by ROS production under normoxia condition. We found that ROS production was increased during scratch-induced B16F10 cell migration (Fig. 1), which is attenuated by the treatment with ROS scavengers or ERK inhibitor (Fig. 2 and 4). In addition, *in vivo* tumor growth was retarded by NAC administration (Fig. 6A). Data suggest that tumor cell activities such as migration and growth could be controlled by ROS production.

ROS-mediated signaling pathways are generally related to various cell functions such as proliferation, development, cellular senescence, death, etc. (Finkel, 2003). Mild effect of ROS may indirectly increase cell proliferation whereas high amount of ROS show the acute direct effect on cell survival which are sensitive to apoptotic death (Moon *et al.*, 2004). ROS augment the activity of various kinases including ERK, p38 (Han *et al.*, 2006), PKA and Rap1 (Moon *et al.*, 2011). ROS-dependent ERK activation might be mediated by an increase in ras gene expression in peroxiredoxin II-deficient mouse embryonic fibroblast (MEF) that produced higher amount of ROS than wild type MEF (Han *et al.*, 2006). In addition redox-based mechanism in cells affects cytoskeletal dynamics and the GTPase RhoA (Aghajanian *et al.*, 2009). ROS formed by NADPH oxidases regulate cytoskeletal organization and cell migration through a Slingshot-1L (SSH-1L), a selective cofilin regulatory phosphatase-cofilin pathway (Kim *et al.*, 2009). Melanoma cell intravasation is in part mediated by ROS-sensitive cellular signaling cascades (Cheng *et al.*, 2004). It is consistent with our data that melanoma cell migration is mediated by ROS produced by scratch-induced stimulation under normoxia condition. In addition, it remains to be cleared the effect of other signaling pathways on tumor cell migration through ROS-mediated regulation in various cancer cell types.

In conclusion, even though we could not explain all of the mechanism on the regulatory effect of ROS on melanoma





**Fig. 6.** Tumor growth was increased by the administration of NAC. (A, B) Mice were subcutaneously implanted with B16F10 mouse melanoma cells. Then, mice were intraperitoneally injected with 32 mg NAC/kg once a day for 14 d. Tumor size was measured daily with Vernier calipers. Tumor volume was calculated by the equation of the  $[(\text{long axis})/2] \times (\text{short axis})^2$ . Data shown are in terms of mean  $\pm$  SE. Value significantly ( $*p < 0.05$ ,  $**p < 0.01$ ) different from that of the control group (A). Blood was drawn by eye puncture in EDTA tubes from control and NAC-treated mice. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation over Histopaque 1077. PBMCs were stained with fluorescence dye, 5  $\mu$ M DCF-DA for 30 minute. Then, 10,000 cells were analyzed by flowcytometer (B).

cell migration, data demonstrate that tumor cell migration and growth could be increased through ROS production and downstream signaling pathways under normoxia condition. It suggests that ROS could be a modulator to control tumor cell migration and growth through the regulation of tumor cell microenvironment and these findings might be useful to develop anti-metastatic therapeutics.

## ACKNOWLEDGMENTS

This work was supported by grants (#0920270) from National Cancer Control Program, Ministry of Health and Welfare and National Nuclear R&D Program (#2010-0018545) through National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST), Korea.

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