

Capsaicin-Mediated tNOX (ENOX2) Up-regulation Enhances Cell Proliferation and Migration in Vitro and in Vivo

Nei-Chi Liu,^{†,||} Pei-Fang Hsieh,^{†,‡,||} Ming-Kun Hsieh,[‡] Zih-Ming Zeng,[†] Hsiao-Ling Cheng,[†] Jiunn-Wang Liao,[§] and Pin Ju Chueh^{*,†}

[†]Graduate Institute of Biomedical Sciences, National Chung Hsing University, Taichung, 40227, Taiwan, Republic of China

[‡]Graduate Institute of Microbiology and Public Health, National Chung Hsing University, Taichung, 40227, Taiwan, Republic of China

[§]Graduate Institute of Veterinary Pathobiology, National Chung Hsing University, Taichung, 40227, Taiwan, Republic of China

ABSTRACT: Cancer chemoprevention is employed to block or reverse the progression of malignancies. To date, several thousands of agents have been found to possess chemopreventative activity, one of which is capsaicin, a component of chili peppers that exhibits antigrowth activity against various cancer cell lines. However, the role of capsaicin in tumorigenesis remains controversial because both cancer prevention and promotion have been proposed. Here, we made the unexpected discovery that treatment with low concentrations of capsaicin up-regulates tNOX (tumor-associated NADH oxidase) expression in HCT116 human colon carcinoma cells in association with enhanced cell proliferation and migration, as evidenced by down-regulation of epithelial markers and up-regulation of mesenchymal markers. Importantly, tNOX-knockdown in HCT116 cells by RNA interference reversed capsaicin-induced cell proliferation and migration in vitro and decreased tumor growth in vivo. Collectively, these findings provide a basis for explaining the tumor-promoting effect of capsaicin and might imply that caution should be taken when using capsaicin as a chemopreventive agent.

KEYWORDS: capsaicin, chemoprevention, migration, proliferation, tumor-associated NADH oxidase (tNOX)

■ INTRODUCTION

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), a pungent component of chili peppers, has long been consumed by humans all over the world.¹ Capsaicin also is used as a chemopreventive agent, reflecting its antigrowth activity in various cancer cell systems including human leukemia,^{2–4} prostate,^{5–7} colon,⁸ hepatoma,^{9,10} breast,^{11,12} and gastric cancer.^{13,14} Capsaicin exerts its cytotoxic action by activating an array of signaling mechanisms, including generation of reactive oxygen species (ROS),^{2,15} up-regulation of p53,⁵ and suppression of STAT3 activation.¹⁶ Although capsaicin is commonly considered a chemopreventive agent, conflicting data from animal models and basic research suggest that it may exert either anti- or pro-tumorigenesis effects.^{17–21}

Previous studies have identified tumor-associated NADH oxidase (tNOX; ENOX2) as a member of a family of growth-related NADH (or hydroquinone) oxidases. Unlike the NADH oxidase activity identified in normal rat liver plasma membranes (CNOX; ENOX1), which is responsive to growth factors and hormones, tNOX isolated from rat hepatoma cells is constitutively activated.^{22,23} Further studies have revealed that tNOX is present in numerous cancer cell lines, including those derived from solid cancers, as well as leukemias;^{12,24–26} it is also detected in the sera of cancer patients.^{27–29} Capsaicin and (–)-epigallocatechin-3-gallate (EGCg), another chemopreventive agent, preferentially inhibit tNOX activity and concurrently inhibit cancer cell growth while having little effect on noncancerous cells.^{12,30} However, our recent study reveals a paradoxical role of capsaicin in apoptosis induction, possibly as a result of contrary effect of capsaicin on tNOX expression;

moreover, forced tNOX down-regulation restored capsaicin-induced apoptosis in TMC-1 cells, supporting an essential role for tNOX in cancer cell growth.²⁴ Using small hairpin RNA (shRNA) to knockdown tNOX expression, we showed that reduced tNOX expression attenuates HeLa cell migration via membrane association of Rac protein.²⁶ In contrast, tNOX overexpression in noncancerous MCF-10A cells results in the acquisition of invasivity, an aggressive characteristic of cancer cells, further highlighting a key role for tNOX in cell migration.³¹

We previously demonstrated that capsaicin-repressed cancer cell growth correlates with the inhibition of tNOX activity or tNOX down-regulation, supporting the notion that capsaicin is cytotoxic against cancer cells.^{12,25} We and others have shown that capsaicin is cytotoxic at concentrations of approximately 10 μ M and higher, depending on the cell lines.^{14,15,24,32} In this study, we discovered that tNOX expression in HCT116 human colon carcinoma cells is up-regulated in response to low concentrations (≤ 10 μ M) of capsaicin, leading to enhanced cell proliferation and migration. Importantly, data obtained using a loss-of-function approach demonstrate that tNOX plays a key role in cell proliferation and tumor progression.

Received: November 28, 2011

Revised: February 17, 2012

Accepted: February 17, 2012

Published: February 18, 2012

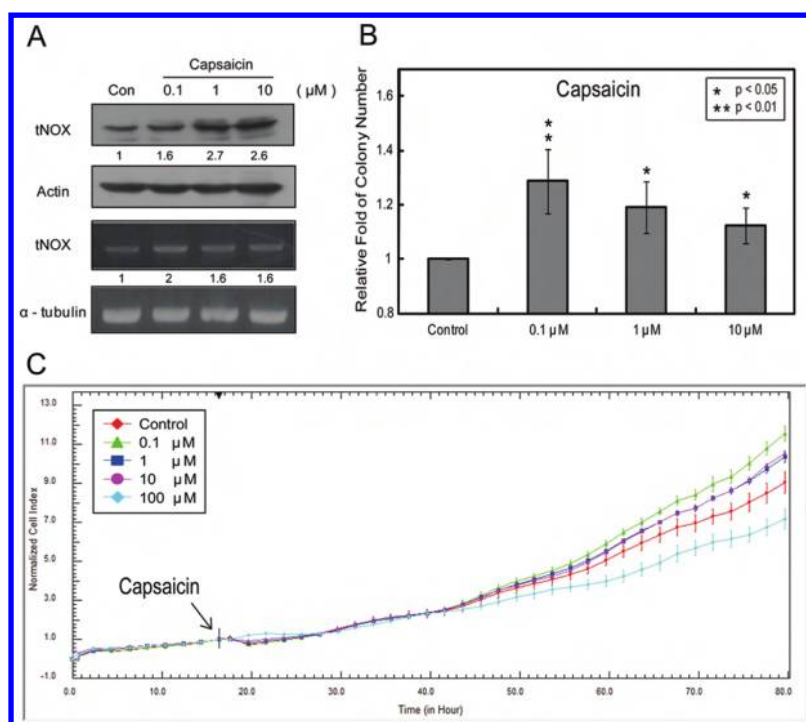


Figure 1. Capsaicin stimulates tNOX protein expression and cell proliferation in HCT116 cells. (A, B) Cells were treated with different concentrations of capsaicin for 24 h. (A) Aliquots of cell lysates were separated by SDS-PAGE and analyzed for tNOX by Western blotting. β -Actin was used as an internal control to monitor for equal loading. tNOX mRNA levels were determined by RT-PCR using α -tubulin as an internal control. Representative images are shown. (B) Cells were seeded at 200 cells/dish, incubated with/without capsaicin, and allowed to form colonies. Colony numbers were counted and recorded. Values (means \pm SEs) are from three independent experiments. Colony numbers were significantly decreased in cells treated with capsaicin as compared with controls ($*p < 0.05$, and $**p < 0.01$). (C) Dynamic monitoring of cell proliferation following incubation with or without capsaicin using impedance technology, as described in the Materials and Methods. Shown are normalized cell index values measured over 80 h.

MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). The antivimentin and phosphorylated ERK1/2 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-E-cadherin and anti-N-cadherin antibodies were from BD Biosciences (San Jose, CA). The anti-ZO-1 antibody was from Invitrogen Corp. (Carlsbad, CA). The ERK2 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti- β -actin antibody was from Millipore Corp. (Temecula, CA). The antitubulin antibody was from Abcam Inc. (Cambridge, MA). The antisera to tNOX used in Western blot analyses were generated as described previously;³³ the tNOX protein band recognized by these antisera was also identified using a commercially available anti-tNOX polyclonal antibody (Protein Tech Group, Inc., Chicago, IL).²⁶ The antimouse IgG antibody, capsaicin, and other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO), unless specified otherwise.

Cell Culture and Transfection. HCT116 human colon cancer cells were grown in McCoy's 5A medium. All media were supplemented with 10% FBS, 100 units/mL penicillin, and 50 μ g/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air, and the media were replaced every 2–3 days. Cells were treated with different concentrations of capsaicin (dissolved in ethanol), as described in the text, or with the same volume of ethanol (vehicle control).

HCT116 cells were transiently transfected with short hairpin RNA (shRNA) against tNOX or scrambled shRNA (control) using the jetPEI transfection reagent according to the manufacturer's protocol (Polyplus-transfection SA, Illkirch Cedex, France), as described previously.²⁶ In another experiment, cells were transiently transfected with siRNA control and siRNA against ERK2 from Santa Cruz

Biotechnology, Inc. using the Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen Corp.).

Continuous Cell Monitoring with the xCELLigence System.

For continuous monitoring of changes in cell growth, cells (1×10^4 cells/well) were seeded onto E-plates and incubated for 30 min at room temperature, after which the E-plates were placed onto the Real-Time Cell Analyzer (RTCA) station (xCELLigence System, Roche, Mannheim, Germany). Cells were grown overnight, and impedance was measured every hour, prior to treatments, as previously described.³⁴ The principle of the xCELLigence system is that cells grown on the surface of electrodes produce an increase in electrode impedance whose magnitude is determined by the number of cells and the degree of cell adhesion.

For continuous monitoring of cell invasion and migration (CIM), cells (1×10^4 cells/well) were seeded onto the top chamber of a CIM plate, which features microelectronic sensors integrated on the underside of the microporous polyethylene terephthalate (PET) membrane of a Boyden-like chamber. After they were incubated for 30 min at room temperature, CIM plates were placed onto the RTCA station (xCELLigence System). Cells migration was continuously monitored throughout the experiments by measuring changes in the electrical impedance at the electrode/cell interface, as a population of cells migrated from the top to the bottom chamber.

Cell impedance is characterized by the cell index (CI) = $(Z_t - Z_0) / [Z_0 - Z_0]$, where Z_0 is background resistance and Z_t is the resistance at an individual time point. A normalized cell index was determined as the cell index at a certain time point (CI_t) divided by the cell index at the normalization time point (CI_{norm_time}).

Colony Formation Assay. Two hundred cells were seeded onto a 6 cm dish and incubated in culture medium with different concentrations of the indicated anticancer agent for 10 days to allow colony formation. After incubation, colonies were fixed in 1.25% glutaraldehyde at room temperature for 30 min, rinsed with distilled

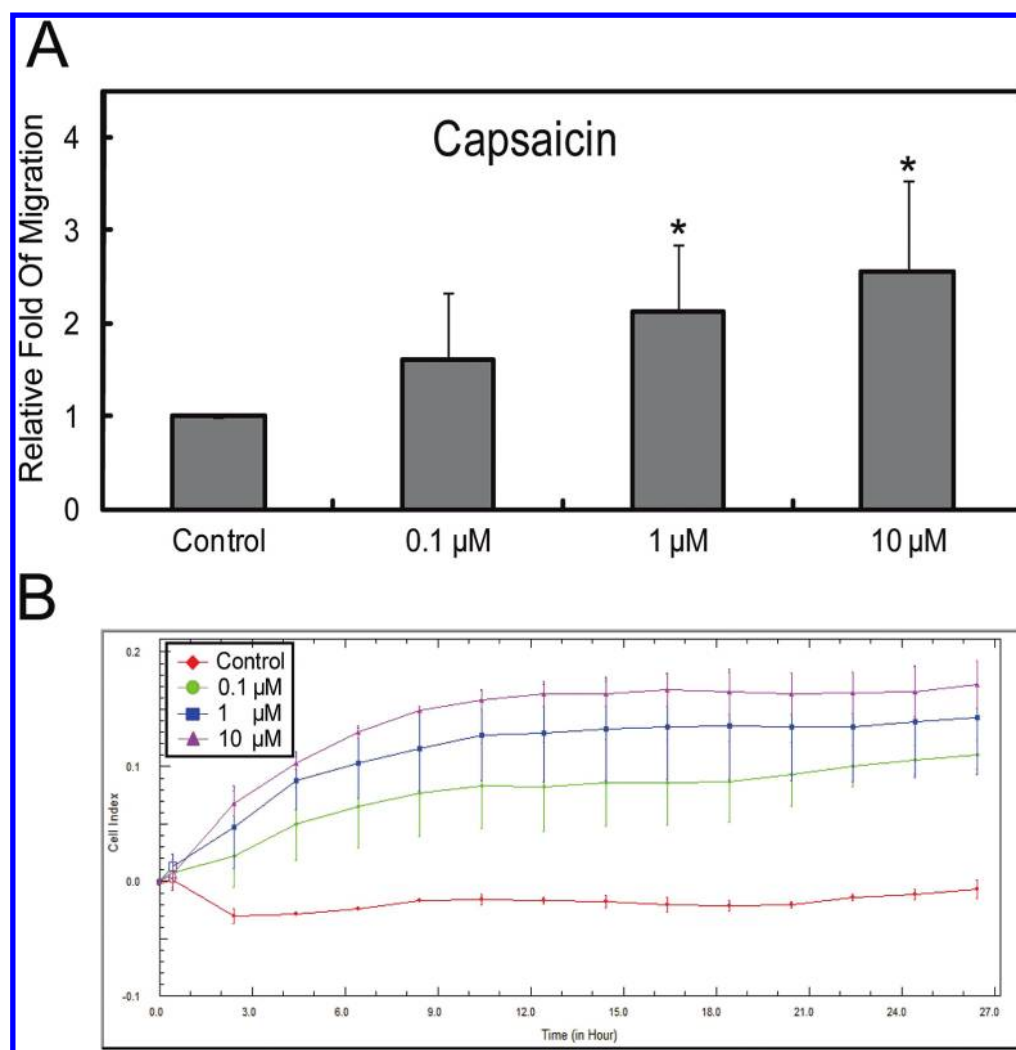


Figure 2. Capsaicin enhances cell migration in HCT116 cells. (A) HCT116 cell migration was measured using a Boyden chamber system. Values (means \pm SEs) are from at least three independent experiments. Cell migration was significantly decreased in cells incubated with capsaicin as compared with controls ($*p < 0.05$). (B) Dynamic monitoring of cell migration following incubation with or without capsaicin using impedance technology, as described in the Materials and Methods. Shown are normalized cell index values measured over 26 h.

water, and stained with a 0.05% methylene blue solution. The number of colonies was counted and recorded.

Boyden Chamber Assay. A Boyden chamber with filter inserts containing 8 μm pores (Neuro Probe, Inc., Gaithersburg, MD) was used to measure cell migration. Cells (2.5×10^3) in DMEM containing 0.5% of FBS were placed in the upper chamber, and the lower chamber was filled with complete DMEM. After 24 h in culture, cells were fixed in methanol for 15 min and then stained with 10% Giemsa in phosphate-buffered saline (PBS) for 30 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. The number of cells on the bottom of filters (migrated cells) was counted and recorded. Data shown are representative of three independent experiments.

Animal Studies. Nine female BALB/c nude mice purchased from National Laboratory Animal Center (Taiwan) were randomly divided into three groups ($n = 3$ per group). Each group of 6 week old mice was injected in the tail vein with 2×10^6 HCT116 cells suspended in 150 μL of PBS. Group 1 animals (wild type) were injected with parental HCT116 cells, groups 2 animals were injected with HCT116 cells transfected with scrambled shRNA (control-shRNA), and group 3 animals (tNOX-shRNA) were injected with HCT116 cells transfected with shRNA against tNOX. Animals were euthanized and sacrificed after 60 days postinjection (DPI = 60). Brain, heart, lung, liver, kidney, spleen, intestine, and stomach were quickly removed and formalin-fixed, and 2 μm sections were subjected to

H&E staining. Tumor masses were counted for each tissue, and degree of lesions was graded for each H&E staining section from one to five depending on the severity [1 = minimal (<1%); 2 = slight (1–25%); 3 = moderate (26–50%); 4 = moderate/severe (51–75%); and 5 = severe/high (76–100%)].

Western Blot Analysis. Cell extracts were prepared in lysis buffer [20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 ng/mL leupeptin, and 10 $\mu\text{g}/\text{mL}$ aprotinin]. Volumes of extract containing equal amounts of proteins (40 μg) were applied to SDS-PAGE gels, and resolved proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were blocked, washed, and probed with primary antibody. After they were washed to remove unbound primary antibody, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The blots were washed again and developed using enhanced chemiluminescence (ECL) reagents, according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA from gastric cancer cells was isolated using the TRIzol reagent (GIBCO, Carlsbad, CA), and first strand cDNA was synthesized from 1 μg of total RNA using Superscript II (Life Technologies, Rockville, MD). The following primers sets were used for PCR amplifications: tNOX, 5'-TGG CTG TGG GTC TAC GAA A-3' (sense) and 5'-GGA GTC TGC CTG TGT CCT TC-3'

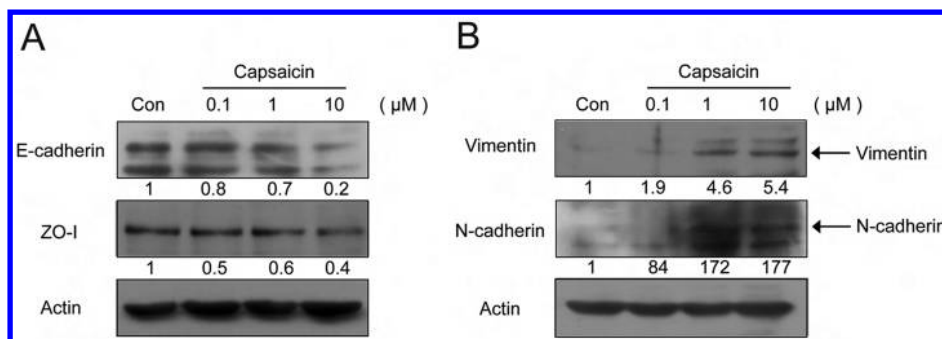


Figure 3. Capsaicin induces changes in EMT protein markers. (A, B) HCT116 cells were incubated with or without capsaicin for 24 h, and aliquots of cell lysates were separated by SDS-PAGE and analyzed for expression of epithelial markers (A) or mesenchymal markers (B). β -Actin was used as an internal control to monitor for equal loading. Representative images are shown.

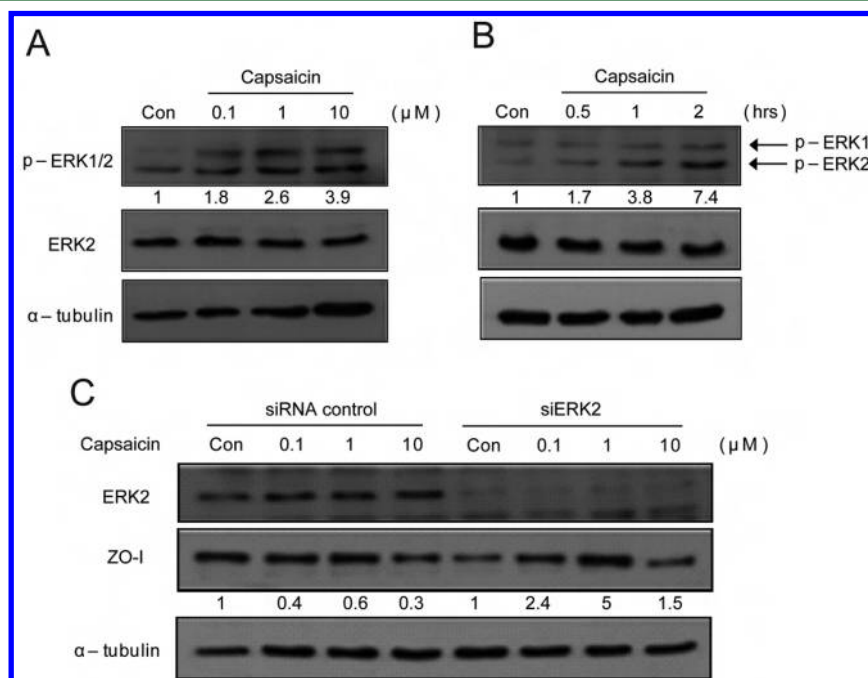


Figure 4. Capsaicin induces activation of ERK and ERK2 knockdown reverses the capsaicin-induced ZO-1 downregulation. (A, B) HCT116 cells were incubated with or without capsaicin, and aliquots of cell lysates were separated by SDS-PAGE and analyzed by Western blotting for ERK2 and phosphorylated ERK1/2. (A) Cells were treated with different concentrations of capsaicin for 24 h. (B) Cells were treated with 10 μ M capsaicin for 0.5, 1, or 2 h. (C) HCT116 cells were transfected with either siRNA control or siERK2 as described in the Materials and Methods and then treated with different concentrations of capsaicin for 24 h. Aliquots of cell lysates were separated by SDS-PAGE and analyzed for ERK2 and ZO-1 by Western blotting. α -Tubulin was used as an internal control to monitor for equal loading. Representative images are shown.

(antisense); α -tubulin, 5'-GGA GGA TGC TGC CAA TAA CT-3' (sense) and 5'-GGT GGT GAG GAT GGA ATT GT-3' (antisense). Reaction conditions consisted of 30 cycles of 95 $^{\circ}$ C for 1 min, 58 $^{\circ}$ C for 1 min, and 72 $^{\circ}$ C for 1 min, followed by a final extension of 7 min at 72 $^{\circ}$ C. PCR products were resolved by electrophoresis on 0.8% agarose gels and visualized by ethidium bromide staining.

Statistics. All data are expressed as the means \pm SEs of three independent experiments. The differences between control and treatment groups were calculated using a one-way analysis of variance followed by Dunnett's test to evaluate significance levels.

RESULTS

Capsaicin-Induced tNOX Expression Is Associated with Enhanced Cell Proliferation. Given that capsaicin is proposed to possess paradoxical effects in tumorigenesis, we decided to study the effect of capsaicin at concentrations below 10 μ M. Unexpectedly, we found that exposure of HCT116 human colon cancer cells to capsaicin (0.1–10 μ M) for 24 h

up-regulated tNOX expression at both RNA and protein levels (Figure 1A). Next, because tNOX is associated with cancer cell growth, we investigated whether this capsaicin-mediated tNOX up-regulation altered the growth of HCT116 cells. Capsaicin (0.1–10 μ M) significantly induced the proliferation of HCT116 cells, as determined by colony-forming assay (Figure 1B). To continuously monitor the effects of capsaicin, we measured cell proliferation using xCELLigence, a real-time cell monitoring system. Consistent with the data from colony-forming assays, we found that capsaicin significantly enhanced cell growth at 0.1–10 μ M, whereas cell growth was inhibited by 100 μ M capsaicin (Figure 1C), indicating concentration-dependent, differential effects of capsaicin on cell growth.

Low Concentrations of Capsaicin-Induced tNOX Expression Is Associated with Cell Proliferation Enhancement. We previously showed that tNOX knockdown attenuates cell migration in HeLa cells, whereas tNOX

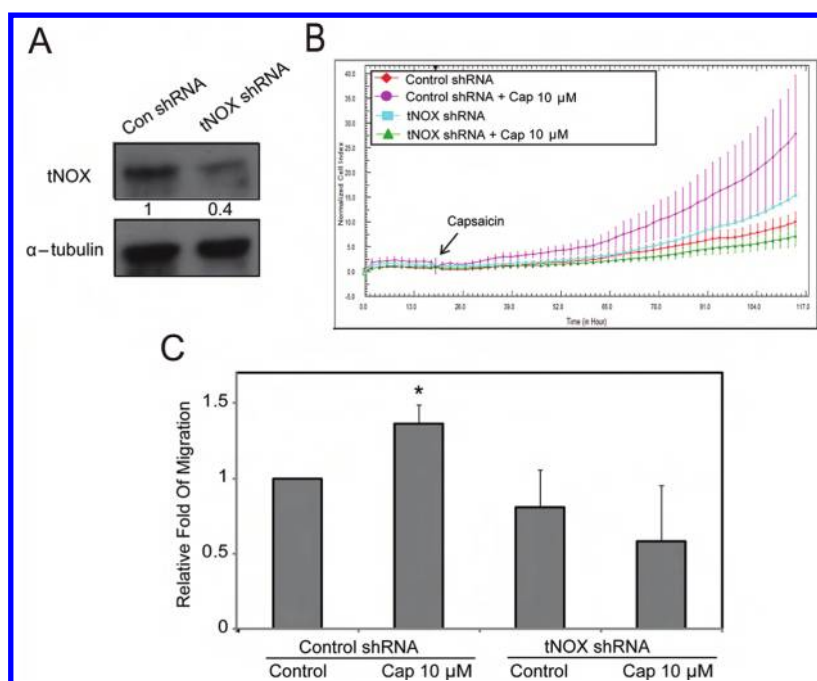


Figure 5. tNOX knockdown reverses capsaicin-induced cell migration and growth. HCT116 cells were transfected with control shRNA or tNOX shRNA as described in the Materials and Methods. (A) tNOX protein expression was analyzed in cells expressing control shRNA or tNOX shRNA. Aliquots of cell lysates were separated by SDS-PAGE and analyzed by Western blotting. β -Actin was used as an internal control to monitor for equal loading. Representative images are shown. (B) Dynamic monitoring of proliferation of cells expressing control shRNA or tNOX shRNA following incubation with or without capsaicin using impedance technology, as described in the Materials and Methods. Shown are normalized cell index values measured over 116 h. (C) Cell migration was measured using a Boyden chamber system. Values (means \pm SEs) are from at least three independent experiments. Migration of control shRNA was significantly increased by treatment with 10 μ M capsaicin as compared with untreated controls ($*p < 0.05$). There was no statistical difference between the tNOX-shRNA cells and the capsaicin-treated tNOX-shRNA cells.

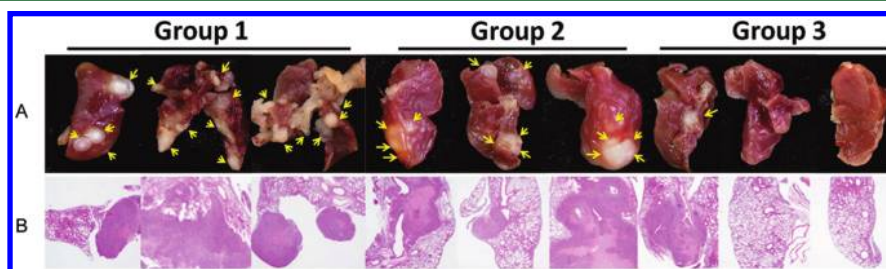


Figure 6. tNOX knockdown suppresses the growth of HCT116 xenografts in vivo. HCT116 xenografts were established in nude mice as described in the Materials and Methods. (A) The lung tissues of mice injected with wild type (group 1), shRNA control-expressing cells (group 2), or shRNA tNOX-expressing cells (group 3) HCT116 cells were examined. The tumor masses are indicated with arrowheads and are from in all mice in groups 1 and 2 and one mouse in group 3. (B) Representative H&E-stained lung sections showed the histopathological tumor lesions in mice of groups 1 and 2 but not the two mice of group 3.

overexpression promotes acquisition of invasiveness in non-cancerous MCF-10A cells. It is of interest to know whether capsaicin-enhanced tNOX expression also affects cell-migration ability. Boyden chamber assays revealed that cell migration was significantly enhanced by 1 and 10 μ M capsaicin as compared to controls (Figure 2A). Assays employing the xCELLigence System confirmed these results (Figure 2B).

To determine whether capsaicin-enhanced cell migration is associated with epithelial-to-mesenchymal transition (EMT), we used Western blot analyses to evaluate the expression levels of individual protein markers. E-cadherin and ZO-1 expression were attenuated by capsaicin treatment as compared to the control (Figure 3A). In contrast, expression of mesenchymal markers, vimentin and N-cadherin, was increased in HCT116 cells treated with capsaicin (Figure 3B). We also investigated the involvement of Ras-ERK1/2 (extracellular signal-regulated

kinases) signaling in capsaicin-mediated EMT and found that phosphorylated ERK1/2 levels were increased by treatment with low concentrations of capsaicin for 24 h (Figure 4A). At 10 μ M capsaicin, enhanced ERK2 phosphorylation was observed after treatment of cells for 1 h (Figure 4B). Next, we examined the involvement of ERK2 in capsaicin-induced EMT using siRNA knockdown. These experiments showed that capsaicin-induced ZO-1 down-regulation was reversed in ERK2 knockdown cells (Figure 4C).

tNOX Knockdown Attenuates Capsaicin-Enhanced Cell Proliferation. To show that the tNOX protein level is important for cell proliferation and migration, we employed a loss-of-function approach, using shRNA-tNOX knockdown in HCT116 cells. Our data demonstrated that shRNA knockdown significantly repressed tNOX expression as compared to controls (Figure 5A) and reversed the stimulatory effect of

10 μM capsaicin on cell proliferation observed in control HCT116 cells (Figure 5B). Similarly, Boyden chamber assays showed that capsaicin-enhanced cell migration was attenuated in tNOX-knockdown cells (Figure 5C).

HCT116 colon cancer cells have been previously used to study tumorigenesis and metastatic potential following tail-vein injections in mice.³⁵ To investigate whether tNOX reduction in HCT116 cells is sufficient to repress tumor growth in mice, we injected mice via the tail vein with parental HCT116 cells, or HCT116 cells transfected with control- or tNOX-shRNA, and assessed subsequent tumor formation. Sixty days postinjection, multiple tumor masses were found in only lung tissues of mice injected with wild-type HCT116 or cells expressing control-RNAi, whereas only a single tumor mass was observed in lung of one mouse injected with cells expressing tNOX-shRNA (Figure 6A), indicating that tNOX depletion significantly repressed the growth of HCT116 xenograft tumors. Moreover, tNOX depletion did not significantly affect body weight (control-shRNA = 21.6 ± 0.9 g; tNOX-shRNA = 21.7 ± 1.0 g). Furthermore, hematoxylin and eosin (H&E) staining of tumor masses in lung sections showed that tumor cells grew from arteries as single oval round cells with highly mitotic figures in predominant nucleoli, and necrosis was seen in the central area. The averages of degree of lesions were 3.67 for groups 1 and 2 and 1 for group 3 (Figure 6B). These data suggest that tNOX down-regulation in HCT116 cells decreases the capacity of these cells to induce tumors *in vivo*.

DISCUSSION

The goal of this study was to investigate the effects of low concentrations ($\leq 10 \mu\text{M}$) of capsaicin on tNOX expression. Surprisingly, we found that at concentrations $\leq 10 \mu\text{M}$, capsaicin up-regulated tNOX in association with enhanced cell proliferation and migration. Importantly, *in vitro* studies demonstrated that tNOX knockdown in HCT116 cells decreased cell proliferation and migration. In addition, tNOX depletion in HCT116 cells attenuated the growth of HCT116 xenografts in nude mice, further confirming a vital role for tNOX in cancer progression.

Capsaicin is one of the most commonly used anticancer drugs because of its inhibitory effects on cell proliferation. However, data from *in vivo* and *in vitro* studies suggest that capsaicin may exert dual effects—antitumor or tumor-promoting—depending on the cellular targets of capsaicin and the molecular mechanisms initiated by capsaicin. For instance, capsaicin has been shown to enhance p53 gene expression to induce apoptotic cell death in SNU-1 stomach cancer cells.¹³ Capsaicin is also shown to augment the protein stability of I κ B, an inhibitor of NF- κ B, thereby inhibiting NF- κ B activation.³⁶ On the contrary, capsaicin acts via epidermal growth factor receptor (EGFR) signaling to function as a cocarcinogen in the TPA (tetradecanoylphorbol-13-acetate)-induced skin cancer model.³⁷ In addition, Erin et al. reported that capsaicin promotes a more aggressive gene expression phenotype and represses expression of pro-apoptotic proteins in breast cancer cells.³⁸ A recent study also demonstrated that capsaicin triggers genotoxicity-induced autophagy through ATM-mediated DNA repair, showed that the resulting autophagy led to chemoresistance, and sustained survival of breast cancer cells.³⁹ These evidence all support the idea that capsaicin acts on numerous cellular targets to initiate unanticipated pathways, subsequently leading to tumorigenesis. In this study, we demonstrated that low concentrations of

capsaicin induce tNOX expression in association with enhanced cell proliferation and migration, suggesting that tNOX is a potential cellular target of capsaicin and the resulting mechanisms are to account for the tumor-promoting effects of capsaicin.

Of the diverse array of signaling pathways involved in capsaicin-induced responses, one that is often mentioned is oxidative stress, which can result in modulation of protein expression and function, and eventually, apoptosis.^{2,7,15} Capsaicin considerably induces ROS production and tNOX down-regulation concurrently with mitochondria-dependent apoptosis, resulting in decreased growth of SNU-1 cells, derived from a poorly differentiated human gastric carcinoma. On the contrary, capsaicin is largely ineffective in provoking ROS generating and tNOX down-regulation in TMC-1 cells, a metastatic gastric carcinoma line; accordingly, apoptosis induction is absent, and cell viability is increased.²⁴ These divergent actions of capsaicin on the growth of gastric cancer cells are reflected in its effects on ROS generation and tNOX protein expression. Combined with these findings, as well as results from this present study, we propose that tNOX may be involved in the complicated system of cellular redox homeostasis modulated by capsaicin, and the tNOX/ROS subsequently initiates signaling pathways that lead to important cellular responses to capsaicin.

Migration of tumor cells is a requirement for cancer metastasis and invasion. Capsaicin has been shown to significantly inhibit cell migration in B16-F10 melanoma cells via targeting the phosphatidylinositol 3-K (PI3-K)/Akt/Rac1-mediated pathways.⁴⁰ In addition, Hwang et al. reported that capsaicin inhibits the EGF-induced invasion and migration via EGFR-dependent signaling in human fibrosarcoma cells.⁴¹ On the contrary, the work of Waning et al. provides a precedent for the reinforcing effect of capsaicin on cell migration thru TRPV1 channels in hepatoma cells.⁴² Capsaicin has also been shown to stimulate calcium entry via TRPV4 channels, leading to a migratory phenotype.⁴³ Seemingly, capsaicin acts on TRPV family, and the resulting mechanisms affect cellular outcomes. In this study, we provide evidence indicating that capsaicin acts on tNOX protein and initiates molecular mechanisms that mediate cell migration.

Migration of cancer cells is often associated with EMT, a trans-differentiation process in which epithelial cells lose their characteristics morphology and adhesive properties and acquire a mesenchymal phenotype.⁴⁴ Recently, Shin et al. reported that ERK2, but not ERK1, induces EMT, an effect that is correlated with increased migration, invasion, and survival.⁴⁵ In this study, we provided evidence suggesting that low concentrations of capsaicin induce tNOX up-regulation, which in turn, enhances cell migration in association with EMT. We also demonstrated that ERK2 plays a key role in capsaicin-mediated EMT using siRNA knockdown. Capsaicin ($< 20 \mu\text{M}$) has been demonstrated to induce LNCaP cell proliferation through phosphoinositol-3-kinase (PI3K) and ERK pathways,²⁰ supporting the findings of the present study.

Together, our results show that capsaicin induces tNOX protein up-regulation, leading to enhanced cell proliferation and migration in HCT116 cells, and that tNOX knockdown attenuates cell proliferation and cell migratory ability in these cells. These results thus offer a basis for explaining the tumor-promoting effects of capsaicin. Moreover, tNOX deficiency in HCT116 cells effectively represses tumor capacity *in vivo*, further confirming an essential role for tNOX in cancer

progression and suggest that caution should be taken when using capsaicin as a chemopreventive agent.

AUTHOR INFORMATION

Corresponding Author

*Tel: +886 4 22840896. Fax: +886 4 22853469. E-mail: pjchueh@dragon.nchu.edu.tw.

Author Contributions

[†]These authors contributed equally.

Funding

This work was supported by grants from the National Science Council (NSC 100-2320-B-005-005) and the Ministry of Education, Taiwan, Republic of China, under the ATU plan.

Notes

The authors declare no competing financial interest.

REFERENCES

- (1) Dorai, T.; Aggarwal, B. B. Role of chemopreventive agents in cancer therapy. *Cancer Lett.* **2004**, *215* (2), 129–140.
- (2) Ito, K.; Nakazato, T.; Yamato, K.; Miyakawa, Y.; Yamada, T.; Hozumi, N.; Segawa, K.; Ikeda, Y.; Kizaki, M. Induction of apoptosis in leukemic cells by homovanillic acid derivative, capsaicin, through oxidative stress: implication of phosphorylation of p53 at Ser-15 residue by reactive oxygen species. *Cancer Res.* **2004**, *64* (3), 1071–1078.
- (3) Lawen, A.; Martinus, R. D.; McMullen, G. L.; Nagley, P.; Vaillant, F.; Wolvetang, E. J.; Linnane, A. W. The universality of bioenergetic disease: The role of mitochondrial mutation and the putative interrelationship between mitochondria and plasma membrane NADH oxidoreductase. *Mol. Aspects Med.* **1994**, *15* (Suppl.), s13–s27.
- (4) Wolvetang, E. J.; Larm, J. A.; Moutsoulas, P.; Lawen, A. Apoptosis induced by inhibitors of the plasma membrane NADH-oxidase involves Bcl-2 and calcineurin. *Cell Growth Differ.* **1996**, *7* (10), 1315–1325.
- (5) Mori, A.; Lehmann, S.; O'Kelly, J.; Kumagai, T.; Desmond, J. C.; Pervan, M.; McBride, W. H.; Kizaki, M.; Koeffler, H. P. Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res.* **2006**, *66* (6), 3222–3229.
- (6) Sanchez, A. M.; Sanchez, M. G.; Malagarie-Cazenave, S.; Olea, N.; Diaz-Laviada, I. Induction of apoptosis in prostate tumor PC-3 cells and inhibition of xenograft prostate tumor growth by the vanilloid capsaicin. *Apoptosis* **2006**, *11* (1), 89–99.
- (7) Sanchez, A. M.; Malagarie-Cazenave, S.; Olea, N.; Vara, D.; Chiloeches, A.; Diaz-Laviada, I. Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation. *Apoptosis* **2007**, *12* (11), 2013–2024.
- (8) Kim, Y. M.; Hwang, J. T.; Kwak, D. W.; Lee, Y. K.; Park, O. J. Involvement of AMPK signaling cascade in capsaicin-induced apoptosis of HT-29 colon cancer cells. *Ann. N.Y. Acad. Sci.* **2007**, *1095*, 496–503.
- (9) Baek, Y. M.; Hwang, H. J.; Kim, S. W.; Hwang, H. S.; Lee, S. H.; Kim, J. A.; Yun, J. W. A comparative proteomic analysis for capsaicin-induced apoptosis between human hepatocarcinoma (HepG2) and human neuroblastoma (SK-N-SH) cells. *Proteomics* **2008**, *8* (22), 4748–4767.
- (10) Lee, Y. S.; Kang, Y. S.; Lee, J. S.; Nicolova, S.; Kim, J. A. Involvement of NADPH oxidase-mediated generation of reactive oxygen species in the apoptotic cell death by capsaicin in HepG2 human hepatoma cells. *Free Radical Res.* **2004**, *38* (4), 405–412.
- (11) Kang, H. J.; Soh, Y.; Kim, M. S.; Lee, E. J.; Surh, Y. J.; Kim, H. R.; Kim, S. H.; Moon, A. Roles of JNK-1 and p38 in selective induction of apoptosis by capsaicin in ras-transformed human breast epithelial cells. *Int. J. Cancer* **2003**, *103* (4), 475–482.
- (12) Morre, D. J.; Chueh, P. J.; Morre, D. M. Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (6), 1831–1835.
- (13) Kim, J. D.; Kim, J. M.; Pyo, J. O.; Kim, S. Y.; Kim, B. S.; Yu, R.; Han, I. S. Capsaicin can alter the expression of tumor forming-related genes which might be followed by induction of apoptosis of a Korean stomach cancer cell line, SNU-1. *Cancer Lett.* **1997**, *120* (2), 235–241.
- (14) Wang, H. M.; Chueh, P. J.; Chang, S. P.; Yang, C. L.; Shao, K. N. Effect of Capsaicin on tNOX (ENOX2) protein expression in stomach cancer cells. *Biofactors* **2009**, *34* (3), 209–217.
- (15) Zhang, R.; Humphreys, I.; Sahu, R. P.; Shi, Y.; Srivastava, S. K. In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway. *Apoptosis* **2008**, *13* (12), 1465–1478.
- (16) Bhutani, M.; Pathak, A. K.; Nair, A. S.; Kunnumakkara, A. B.; Guha, S.; Sethi, G.; Aggarwal, B. B. Capsaicin is a novel blocker of constitutive and interleukin-6-inducible STAT3 activation. *Clin. Cancer Res.* **2007**, *13* (10), 3024–3032.
- (17) Agrawal, R. C.; Wiessler, M.; Hecker, E.; Bhide, S. V. Tumour-promoting effect of chilli extract in BALB/c mice. *Int. J. Cancer* **1986**, *38* (5), 689–695.
- (18) Toth, B.; Gannett, P. Carcinogenicity of lifelong administration of capsaicin of hot pepper in mice. *In Vivo* **1992**, *6* (1), 59–63.
- (19) Bode, A. M.; Dong, Z. The two faces of capsaicin. *Cancer Res.* **2011**, *71* (8), 2809–2814.
- (20) Malagarie-Cazenave, S.; Olea-Herrero, N.; Vara, D.; Diaz-Laviada, I. Capsaicin, a component of red peppers, induces expression of androgen receptor via PI3K and MAPK pathways in prostate LNCaP cells. *FEBS Lett.* **2009**, *583* (1), 141–147.
- (21) Surh, Y. J.; Lee, S. S. Capsaicin in hot chili pepper: Carcinogen, co-carcinogen or anticarcinogen? *Food Chem. Toxicol.* **1996**, *34* (3), 313–316.
- (22) Bruno, M.; Brightman, A. O.; Lawrence, J.; Werderitsh, D.; Morre, D. M.; Morre, D. J. Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes. *Biochem. J.* **1992**, *284* (Part 3), 625–628.
- (23) Chueh, P. J. Cell membrane redox systems and transformation. *Antioxid. Redox Signaling* **2000**, *2* (2), 177–187.
- (24) Wang, H. M.; Chuang, S. M.; Su, Y. C.; Li, Y. H.; Chueh, P. J. Down-regulation of tumor-associated NADH oxidase, tNOX (ENOX2), enhances capsaicin-induced inhibition of gastric cancer cell growth. *Cell Biochem. Biophys.* **2011**, *61* (2), 355–366.
- (25) Mao, L. C.; Wang, H. M.; Lin, Y. Y.; Chang, T. K.; Hsin, Y. H.; Chueh, P. J. Stress-induced down-regulation of tumor-associated NADH oxidase during apoptosis in transformed cells. *FEBS Lett.* **2008**, *582* (23–24), 3445–3450.
- (26) Liu, S. C.; Yang, J. J.; Shao, K. N.; Chueh, P. J. RNA interference targeting tNOX attenuates cell migration via a mechanism that involves membrane association of Rac. *Biochem. Biophys. Res. Commun.* **2008**, *365* (4), 672–677.
- (27) Chueh, P. J.; Morre, D. J.; Wilkinson, F. E.; Gibson, J.; Morre, D. M. A 33.5-kDa heat- and protease-resistant NADH oxidase inhibited by capsaicin from sera of cancer patients. *Arch. Biochem. Biophys.* **1997**, *342* (1), 38–47.
- (28) Morre, D. J.; Reust, T. A circulating form of NADH oxidase activity responsive to the antitumor sulfonyleurea N-4-(methylphenyl-sulfonyl)-N'-(4-chlorophenyl)urea (LY181984) specific to sera from cancer patients. *J. Bioenerg. Biomembr.* **1997**, *29* (3), 281–289.
- (29) Morre, D. J.; Caldwell, S.; Mayorga, A.; Wu, L. Y.; Morre, D. M. NADH oxidase activity from sera altered by capsaicin is widely distributed among cancer patients. *Arch. Biochem. Biophys.* **1997**, *342* (2), 224–230.
- (30) Morre, D. J.; Bridge, A.; Wu, L. Y.; Morre, D. M. Preferential inhibition by (–)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture. *Biochem. Pharmacol.* **2000**, *60* (7), 937–946.
- (31) Chueh, P. J.; Wu, L. Y.; Morre, D. M.; Morre, D. J. tNOX is both necessary and sufficient as a cellular target for the anticancer

actions of capsaicin and the green tea catechin (–)-epigallocatechin-3-gallate. *Biofactors* **2004**, *20* (4), 235–249.

(32) Lee, S. H.; Richardson, R. L.; Dashwood, R. H.; Baek, S. J. Capsaicin represses transcriptional activity of beta-catenin in human colorectal cancer cells. *J. Nutr. Biochem.* **2011**, DOI: 10.1016/j.jnutbio.2011.03.009.

(33) Chen, C. F.; Huang, S.; Liu, S. C.; Chueh, P. J. Effect of polyclonal antisera to recombinant tNOX protein on the growth of transformed cells. *Biofactors* **2006**, *28* (2), 119–133.

(34) Kuo, Y. F.; Su, Y. Z.; Tseng, Y. H.; Wang, S. Y.; Wang, H. M.; Chueh, P. J.; Flavokawain, B a novel chalcone from *Alpinia pricei* Hayata with potent apoptotic activity: Involvement of ROS and GADD153 upstream of mitochondria-dependent apoptosis in HCT116 cells. *Free Radical Biol. Med.* **2010**, *49* (2), 214–226.

(35) Langlois, M. J.; Bergeron, S.; Bernatchez, G.; Boudreau, F.; Saucier, C.; Perreault, N.; Carrier, J. C.; Rivard, N. The PTEN phosphatase controls intestinal epithelial cell polarity and barrier function: Role in colorectal cancer progression. *PLoS One* **2010**, *5* (12), e15742.

(36) Singh, S.; Natarajan, K.; Aggarwal, B. B. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a potent inhibitor of nuclear transcription factor-kappa B activation by diverse agents. *J. Immunol.* **1996**, *157* (10), 4412–4420.

(37) Hwang, M. K.; Bode, A. M.; Byun, S.; Song, N. R.; Lee, H. J.; Lee, K. W.; Dong, Z. Cocarcinogenic effect of capsaicin involves activation of EGFR signaling but not TRPV1. *Cancer Res.* **2010**, *70* (17), 6859–6869.

(38) Erin, N.; Zhao, W.; Bylander, J.; Chase, G.; Clawson, G. Capsaicin-induced inactivation of sensory neurons promotes a more aggressive gene expression phenotype in breast cancer cells. *Breast Cancer Res. Treat.* **2006**, *99* (3), 351–364.

(39) Yoon, J. H.; Ahn, S. G.; Lee, B. H.; Jung, S. H.; Oh, S. H. Role of autophagy in chemoresistance: Regulation of the ATM-mediated DNA-damage signaling pathway through activation of DNA-PKcs and PARP-1. *Biochem. Pharmacol.* **2011**, *83*, 747–757.

(40) Shin, D. H.; Kim, O. H.; Jun, H. S.; Kang, M. K. Inhibitory effect of capsaicin on B16-F10 melanoma cell migration via the phosphatidylinositol 3-kinase/Akt/Rac1 signal pathway. *Exp. Mol. Med.* **2008**, *40* (5), 486–494.

(41) Hwang, Y. P.; Yun, H. J.; Choi, J. H.; Han, E. H.; Kim, H. G.; Song, G. Y.; Kwon, K. I.; Jeong, T. C.; Jeong, H. G. Suppression of EGF-induced tumor cell migration and matrix metalloproteinase-9 expression by capsaicin via the inhibition of EGFR-mediated FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 signaling. *Mol. Nutr. Food Res.* **2011**, *55* (4), 594–605.

(42) Waning, J.; Vriens, J.; Owsianik, G.; Stuwe, L.; Mally, S.; Fabian, A.; Frippiat, C.; Nilius, B.; Schwab, A. A novel function of capsaicin-sensitive TRPV1 channels: involvement in cell migration. *Cell Calcium* **2007**, *42* (1), 17–25.

(43) Vriens, J.; Janssens, A.; Prenen, J.; Nilius, B.; Wondergem, R. TRPV channels and modulation by hepatocyte growth factor/scatter factor in human hepatoblastoma (HepG2) cells. *Cell Calcium* **2004**, *36* (1), 19–28.

(44) Cannito, S.; Novo, E.; di Bonzo, L. V.; Busletta, C.; Colombatto, S.; Parola, M. Epithelial-mesenchymal transition: from molecular mechanisms, redox regulation to implications in human health and disease. *Antioxid. Redox Signaling* **2010**, *12* (12), 1383–1430.

(45) Shin, S.; Dimitri, C. A.; Yoon, S. O.; Dowdle, W.; Blenis, J. ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events. *Mol. Cell* **2010**, *38* (1), 114–127.