Nicotine enhances store-operated calcium entry by upregulating HIF-1α and SOCC components in non-small cell lung cancer cells

YAN WANG[*], JIANXING HE[*], HUA JIANG[*], QI ZHANG[*], HAIHONG YANG, XIAOMING XU, CHENTING ZHANG, CHUYI XU, JIAN WANG and WENJU LU

State Key Laboratory of Respiratory Diseases, Guangzhou Institute of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, Guangdong 510120, P.R. China

Abstract. Store-operated calcium entry (SOCE) is critical for regulating the proliferation and metastasis of various cancer types. The present study aimed to investigate the role of SOCE on nicotine-promoted proliferation of non-small cell lung cancer (NSCLC) A549 cells. Cell proliferation was evaluated by BrdU incorporation assay. The SOCE and basal $[Ca^{2+}]_i$ in NSCLC A549 cells were determined using Fura-2 fluorescence microscopy. The mRNA and protein expression levels were determined by real-time quantitative PCR and western blotting, respectively. The results demonstrated that, in A549 cells, the detectable store-operated calcium channel (SOCC) components were TRPC proteins 1, 3, 4 and 6 and Orai1, among which TRPC1, TRPC6 and Orai1 are expressed at relatively high levels with TRPC3 and TRPC4 at relatively low levels. Nicotine upregulated the mRNA and protein expression of TRPC1, TRPC6 and Orai1, increased basal $[Ca^{2+}]_i$ and enhanced SOCE. Promotion of cell proliferation but not migration was observed in the nicotine-treated cells, which was inhibited by SOCE inhibitor SKF-96365. Furthermore, nicotine upregulated HIF-1α expression in the A549 and NCI-H292 cells. Silencing of HIF-1α abrogated the increases in TRPCs and Orai1 and reversed the increases in basal $[Ca^{2+}]_i$ and SOCE. Meanwhile, suppression of proliferation was observed in cells following HIF-1α silencing. In conclusion, the results indicate that nicotine promotes lung cancer cell proliferation likely by upregulating HIF-1α and SOCC components and therefore enhancing SOCE and increasing basal $[Ca^{2+}]_i$.

Introduction

Lung cancer is the most commonly diagnosed cancer and the most common cause of cancer-related death worldwide (1). Approximately 85% of lung cancer cases are attributed to smoking, not including those occurring in nonsmokers exposed to second-hand smoke (2). As a major component in cigarette smoke, nicotine contributes to tumor progression by activating angiogenesis, promoting cell proliferation and invasion, and inhibiting apoptosis, although it does not provoke tumorigenesis (3,4). Calcium-mediated signal transduction is suggested to be one of the underlying mechanisms involved in the tumor-promoting effects induced by nicotine/nicotinic receptors (5,6). In tumor progression, the diversity of calcium channels, mostly non-voltage gated calcium channels, on the plasma membrane is relevant to the differential behaviors in proliferation and migration of cancer cells (7). Store-operated Ca^{2+} entry (SOCE), activated by intracellular Ca^{2+} store depletion, is the primary mechanism for Ca^{2+} influx in non-exitable cells (8). It has been found that SOCE remodeling is associated with tumor progression in various human cancers (9,10).

The Orai channels and Ca^{2+}-permeable transient receptor potential canonical (TRPC) channels are Ca^{2+}-permeable channels involved in SOCE upon the binding of the stromal interaction molecule (STIM) proteins as Ca^{2+} sensors (11). There are three mammalian Orai homologues (Orai1-3) showing differences in response to the process of Ca^{2+} depotentiation (12) and six TRPC isoforms (TRPC1-6) serving as non-selective Ca^{2+}-permeable cation channels, through which the Ca^{2+} current in SOCE is generated by the formations of Orai1-STIM1 or TRPCs-STIM1 components (13,14).

Hypoxia is a common feature of solid tumor masses and is essential for the formation of the cellular and physiologic characteristics of cancer (15). The association between hypoxia and intracellular $[Ca^{2+}]_i$ regulation has been identified in hepatoma cells, in which HIF-1α, a key regulator for the adaption of cancer cells to a low-oxygen microenvironment, enhanced STIM1 transcription and contributed to SOCE

Correspondence to: Professor Wenju Lu, State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, 151 Yanjiang Road, Guangzhou, Guangdong 510120, P.R. China
E-mail: wlu92@yahoo.com

[*]Contributed equally

and tumorigenesis (16,17). Furthermore, both inhibition of TRPC6-mediated calcium signaling and attenuation of HIF-1α signaling elevated the sensitivity of tumor cells to drugs (18).

In human non-small cell lung cancer cells, it has been found that nicotine induced HIF-1α overexpression (19). Excessive HIF-1α expression was associated with the growth of lung cancer A549 cells *in vitro* and *in vivo* (20). Nevertheless, the association between nicotine-induced HIF-1α change and SOCE in lung tumor cell growth remains unclear. In this present study, we evaluated the effects of nicotine on changes in the expression of store-operated calcium channel (SOCC) components and SOCE in A549 cells. We demonstrated overexpression of HIF-1α-mediated SOCC components enhancement of SOCE in the presence of nicotine.

Materials and methods

Reagents. (-)-Nicotine ditartrate was purchased from Calbiochem (San Diego, CA, USA; cat. no. 481975). Nifedipine, cyclopiazonic acid (CPA) and SKF-96365 were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Fluorescent dye fura-2 AM was from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture and treatment. The non-small cell lung cancer cell line A549 was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For nicotine treatment, the cells were seeded into 6-well plates at a density of $1x10^5$ cells per well, grown until 70% confluence and exchanged with serum-free medium for a 12-h culture to reach growth arrest. Then, the cells were treated with nicotine in medium containing 0.1% FBS for the indicated time before being collected for further analyses.

RNA extraction and quantitative real-time PCR. Total RNA in cultured cells was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific) according to the manu-facturer's instructions. Reverse transcription into cDNA was performed from 1 μg total RNA and quantitative real-time PCR was carried out by using Scofast™ EvaGreen superMix (Bio-Rad Laboratories, Hercules, CA, USA) and primers listed in Table I. The relative concentration of each transcript was calculated using the Pfaffl method (21) and normalized to 18S as an internal control for each sample.

RNA interference. For the transient silencing of HIF-1α gene expression, small interfering RNA (siRNA) targeting to HIF-1α (siHIF-1α, 5'-CCACCACUGAUGAAUUAAATT-3') and negative control small interfering RNA (siNT, 5'-UUCUCC GAACGUGUCACGUTT-3') were transfected into A549 cells using HiPerFect Transfection Reagent (Qiagen, Duesseldorf, Germany) at a final concentration of 5 nM for 24 h. Then the cells were subjected to nicotine treatment for 24 or 48 h.

Western blotting. Cells were homogenized in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1%

Table I. Primers used for quantitative RT-PCR.

TRPC1	Forward:	5' TTGTGGAGGTGGAATTCAGG 3'
	Reverse:	5' CGTTTGTCA AGAGGCTCGTC 3'
TRPC2	Forward:	5' TCATGGTCATTGTGCTGCTC 3'
	Reverse:	5' ACTCCACGTCAGCATCATCC 3'
TRPC3	Forward:	5' CAGCCAACACGTTATCAGCA 3'
	Reverse:	5' CCTCAGTTGCTTGGCTCTTG 3'
TRPC4	Forward:	5' CGAAAGGGTTAACCTGCAAA 3'
	Reverse:	5' CAGGGACTGCAGTGTCTCAA 3'
TRPC5	Forward:	5' GTGCTGCTGAACATGCTGAT 3'
	Reverse:	5' GCTTCGTCCTTGCAAACTTC 3'
TRPC6	Forward:	5' CAGACAATGGCGGTCAAGTT 3'
	Reverse:	5' TGGTCCACGCATTATCTTCC 3'
TRPC7	Forward:	5' GTTAAAACCCTGCCAAACGA 3'
	Reverse:	5' TCCCAGATTTCCTTGCATTC 3'
HIF-1α	Forward:	5' TGCTTGGTGCTGATTTGTGAACC 3'
	Reverse:	5' CTGTCCTGTGGTGACTTGTCC 3'
Orai1	Forward:	5' ACGTGCACAATCTCAACTCG 3'
	Reverse:	5' AGCACCACCTCAGCTAGGAA 3'
18S	Forward:	5' GCAATTATTCCCCATGAACG 3'
	Reverse:	5' GGCCTCACTAAACCATCCAA 3'

sodium deoxycholate, 2 mM EGTA, 2 mM EDTA, 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich Inc.). Equal amount of total proteins for each sample was separated on SDS-PAGE gel, blotted with primary anti-bodies against human TRPC1 (1:1,000; rabbit polyclonal; cat. no. ACC-010; Alomone Labs, Jerusalem, Israel), TRPC6 (1:1,000; rabbit polyclonal; cat. no. ACC-017; Alomone Labs), Orai1 (1:1,000; rabbit polyclonal; cat. no. 4281; ProSci, Inc., San Diego, CA, USA), HIF-1α (1:1,000; mouse monoclone; cat. no. ab113642; Abcam, Cambridge, UK) or β-actin (1:1,000; mouse monoclone; cat. no. ab8226; Abcam) and then with the corresponding HRP-conjugated secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Finally, the signals were visualized with enhanced chemiluminescence reagents (ECL; Bio-Rad Laboratories).

Proliferation analysis. Cell proliferation was evaluated by using a colorimetric BrdU cell proliferation assay kit (Roche, South San Francisco, CA, USA) according to the manufac-turer's instructions. Cell proliferation was quantified by BrdU incorporation and expressed as a multiple of the value of the control cells.

Scrape-wound migration assay. A scrape-wound migration assay was used to assess the effects of nicotine on cell mobility. A wound was produced in a confluent monolayer of A549 cells by scraping the cells with a pipette tip. Then, the cells were replenished with DMEM containing 0.1% FBS with nicotine (1 μM) and/or SKF-96365 (1 μM) to drive cell migration. Bright-field images of the wound area were captured at 0 and

24 h post-wounding with a Leica (DMI3000B) microscope (Leica Microsystems, Frankfurt, Germany) and the total number of pixels in the empty spaces inside the wound were counted using Adobe Photoshop CS5. At least three photographs were taken per group at each time-point. The migration capacity was calculated as the empty space at 0 h minus the empty space at 24 h and was represented as a percentage relative to the control.

Measurement of intracellular $[Ca^{2+}]_i$ and SOCE by fura-2 fluorescence. Intracellular $[Ca^{2+}]_i$ and SOCE were measured according to methods described previously (22). A549 cells seeded on coverslips were incubated with 5 μM fura-2 AM for 1 h in the dark at room temperature. Then, the coverslips were perfused with physiological salt solution (PSS, 130 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 10 mM HEPES and 10 mM glucose) for 10 min to remove the extracellular fura-2 AM. Basal $[Ca^{2+}]_i$ was determined at 12-sec intervals from the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F340/F380) measured using a xenon lamp (Lambda DG4; Sutter Instrument Company, Novato, CA, USA) in 20 to 30 cells.

After basal $[Ca^{2+}]_i$ measurement, the cells were perfused with $[Ca^{2+}]$-free PSS containing 0.5 mM EGTA for 5 min to chelate residual Ca^{2+} and then with PSS containing 5 μM nifedipine and 10 μM CPA to prevent calcium entry through L-type VOCC and to deplete SR Ca^{2+} stores. To assess SOCE, the peak increase in $[Ca^{2+}]_i$ (ratio F340/F380) caused by restoration of extracellular Ca^{2+} (2.5 mM Ca^{2+} in PSS perfusate containing nifedipine and CPA) was determined.

Statistical analysis. All experiments were repeated three times. Data were statistically analyzed using the two-tailed Student's t-test and are represented as means ± SEM. *P<0.05 and **P<0.01 indicate a significant and extremely significant difference, respectively.

Results

Nicotine upregulates the expression of SOCC components in A549 cells. The calcium channel SOCCs in mammalian cells are thought to be composed of TRPCs, Orai1 and STIM1 (23). In NSCLC A549 cells, among the seven TRPC members, TRPC1 and TRPC6 were expressed at relatively high levels with relatively low levels of TRPC3 and TRPC4. The expression levels of the other three TRPCs members, TRPC2, TRPC5 and TRPC7, were not detected in our study (Fig. 1A). The levels of TRPC6 and Orai1 in A549 cells were upregulated following exposure to nicotine at a dose as low as 0.01 μM, and were further upregulated by higher levels of nicotine in a dose-dependent manner (1-100 μM). The protein TRPC1 was not increased by nicotine at a dose lower than 1 μM, although a trend of upregulation was observed at the concentration 1 μM. Further increased nicotine dosages of 10 or 100 μM did not induce further upregulation of the proteins TRPC6 and Orai1 when compared with the dose at 1 μM (Fig. 1B and C), but did induce obvious cytotoxicity-like cell death. In human smokers, the average peak plasma nicotine level in smoking is around 10-50 mg/ml (~60-310 nM) (24). Therefore, we chose to expose cells to 1 μM nicotine in the present study.
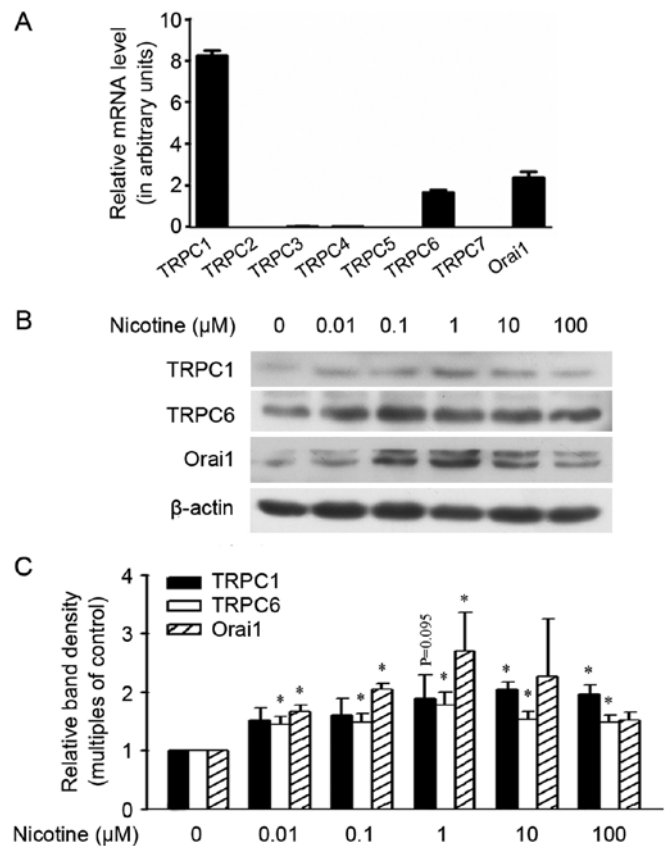
Figure 1. Nicotine induces changes in the expression of SOCC components. (A) Basal mRNA levels of store-operated calcium channel (SOCC) components, TRPC family members 1-7 and Orai1, were determined by quantitative RT-PCR in A549 cells. (B) A549 cells were exposed to nicotine (0-100 μM) for 48 h. The protein levels of TRPC1, TRPC6 and Orai1 were determined using western blotting. The results are from one representative experiment out of three similar experiments. (C) The intensity of each band was quantified, normalized to internal control β-actin and expressed as the multiple of the samples without nicotine exposure. The results are from experiments conducted in triplicate and data are presented as mean ± SEM. *P<0.05, cells exposed to nicotine vs. cells without nicotine exposure.

Nicotine increases SOCE and basal intracellular $[Ca^{2+}]_i$ level. To examine the effects of nicotine on calcium influx, we measured the SOCE and basal $[Ca^{2+}]_i$ in A549 cells. A 48-h exposure to nicotine increased basal $[Ca^{2+}]_i$ (Fig. 2A and B). After washing the cells with Ca^{2+}-free PSS containing 10 μM CPA and 5 μM nifedipine, a low pulse of $[Ca^{2+}]_i$ increase was detected at 10 min which indicated store calcium release and the peak $[Ca^{2+}]_i$ at 20 min reflected SOCE resulting from the restoration of extracellular Ca^{2+} at 2.5 mM. Basically, nicotine exposure enhanced basal $[Ca^{2+}]_i$ and SOCE of A549 cells (Fig. 2A and C).

Blockage of SOCE prevents cell proliferation upon nicotine exposure. Nicotine has been reported to promote the proliferation and migration of A549 cells (25). In our study, a scrape-wound migration assay was conducted to evaluate cell migration capacity. As shown in Fig. 3A and B, nicotine (1 μM in 0.1% FBS) did not induce obvious accelerated cell migration when compared with those cells without nicotine exposure at 24 h. However, TRPC inhibitor SKF-96365 effectively abrogated cell migration, no matter whether nicotine
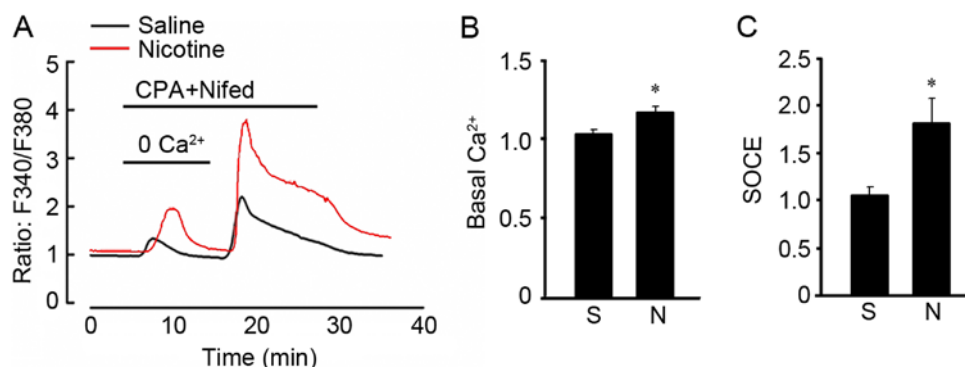
Figure 2. Effects of nicotine on basal $[Ca^{2+}]_i$ and SOCE in A549 cells. A549 cells were exposed to saline (vehicle) or nicotine (1 μM) for 48 h. After the cells were incubated with 5 μM fura-2 AM, the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F340/F380) was determined. (A) Time course of $[Ca^{2+}]_i$ before and after restoration of extracellular Ca^{2+} in cells perfused with Ca^{2+}-free physiological salt solution (PSS) containing 5 μM nifedipine and 10 μM cyclopiazonic acid (CPA) (n=4 experiments in 119 cells). (B) Average basal intracellular $[Ca^{2+}]_i$ and (C) store-operated calcium entry (SOCE) (average peak $\Delta[Ca^{2+}]_i$ shown in A, obtained from cell restored extracellular Ca^{2+}). S, saline; N, nicotine. Data are presented as mean ± SEM. *P<0.05, cells treated with saline vs. cells exposed to nicotine.
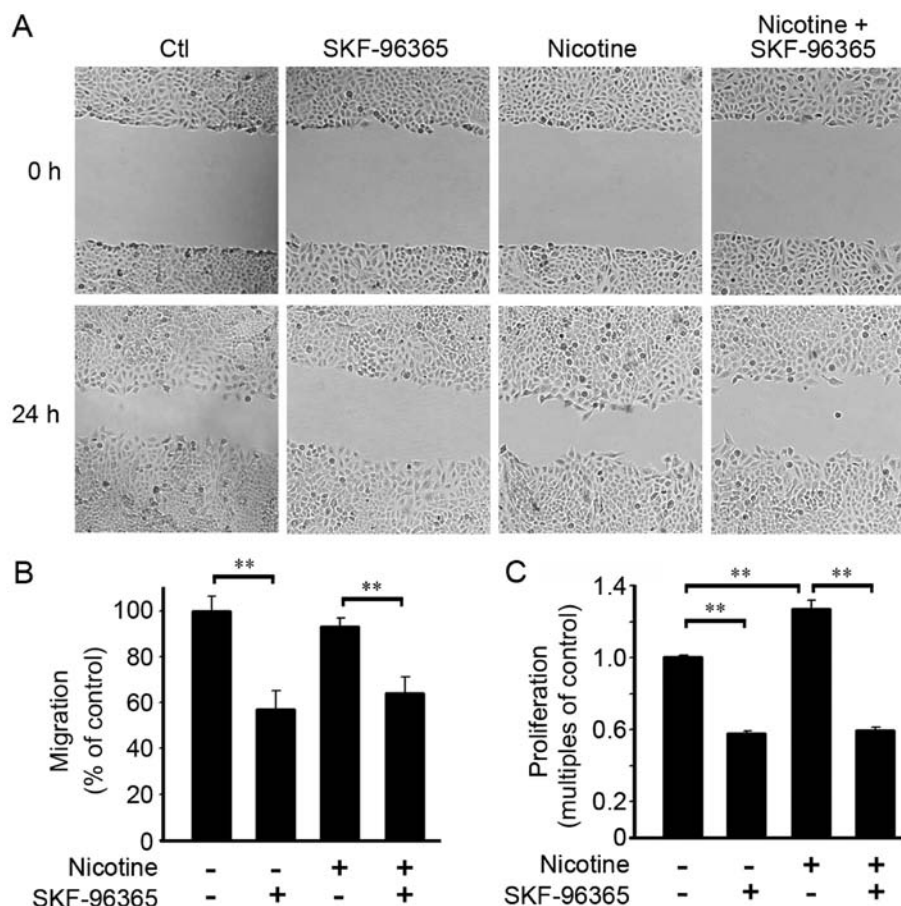

Figure 3. TRPC inhibitor SKF-96365 attenutates cell mobility and proliferation upon nicotine exposure. (A and B) A549 cells were seeded on a 6-well plate and scraped to produce a wound. Then the cells were incubated with 1 μM nicotine in the presence of TRPC inhibitor SKF-96365 (1 μM). Images of the wound area were taken at 0 and 24 h post-wounding. The cell migration is expressed as a percentage of the cells without nicotine and SKF-96365 treatments. At least three images were taken per group at each time point and the results are from one representative group of images. Results are presented as mean ± SEM. **P<0.01. (C) A549 cells were seeded on a 96-well plate and exposed to 1 μM nicotine or 1 μM SKF-96365. The cell proliferation was evaluated by BrdU incorporation assay at 48 h and is expressed as a multiple of the value of the control cells. The results are from three experiments and are presented as mean ± SEM. **P<0.01.

was present. The BrdU incorporation assay showed that nicotine enhanced cell proliferation at 48 h. The TRPC inhibitor SKF-96365 inhibited both basal and nicotine triggered cell proliferation (Fig. 3C).

HIF-1α is required for upregulation of SOCC components induced by nicotine. Since nicotine has been reported to increase HIF-1α in NSCLC cells and HIF-1α modulated expression of SOCC components in pulmonary artery smooth
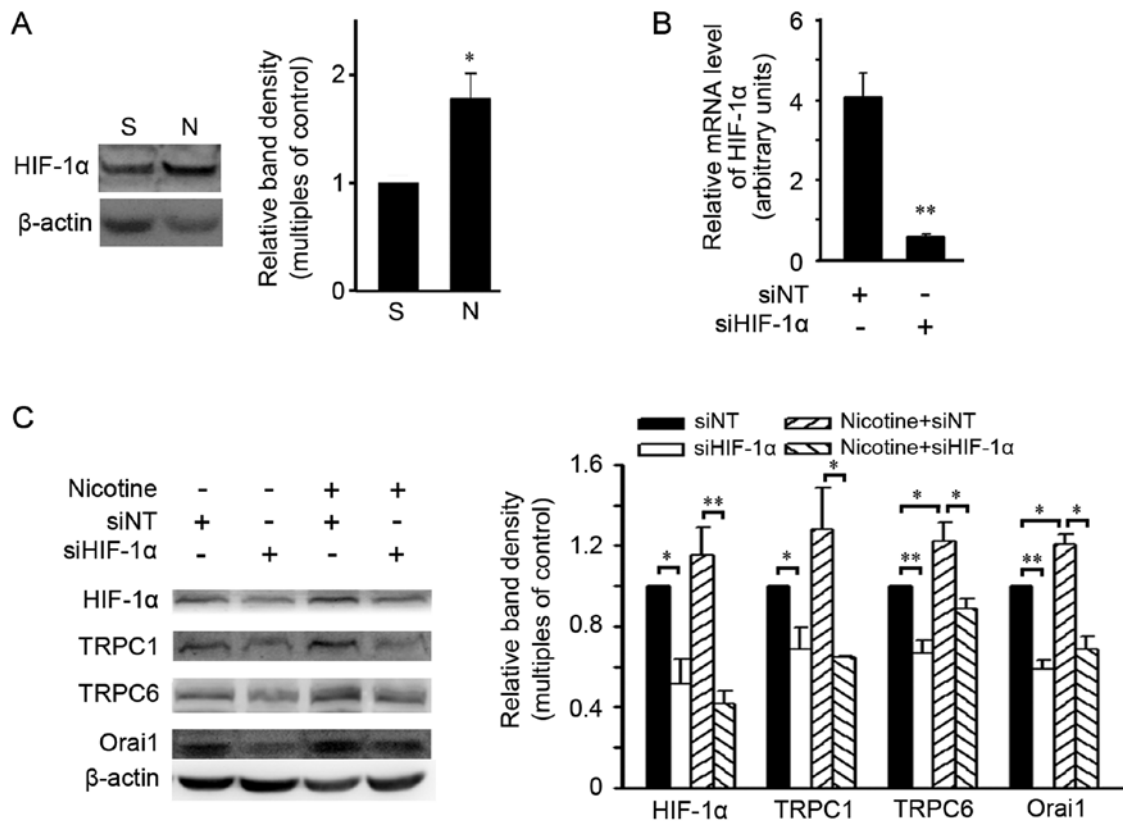
Figure 4. HIF-1α deficiency impacts expression of store-operated calcium channel (SOCC) components in A549 cells. (A) A549 cells were treated with nicotine (1 μM) for 48 h. The protein levels of HIF-1α were determined using western blotting. The relative expression of HIF-1α to β-actin is expressed as the multiple of the control cells after intensity quantification of the bands. S, saline; N, nicotine. (B) A549 cells were transfected with siHIF-1α to silence HIF-1α expression. The mRNA levels of HIF-1α were determined at 24 h after siRNA transfection. (C) Nicotine (1 μM) was used to expose cells at 24 h after siHIF-1α transfection. The protein levels of TRPC1, TRPC6 and Orai1 were determined at 48 h after nicotine exposure. The results are from one representative experiment out of three similar experiments. The intensity of the band was quantified, normalized to internal control β-actin and is expressed as the multiple of the samples treated with only siNT. Data are presented as mean ± SEM. *P<0.05 and **P<0.01.
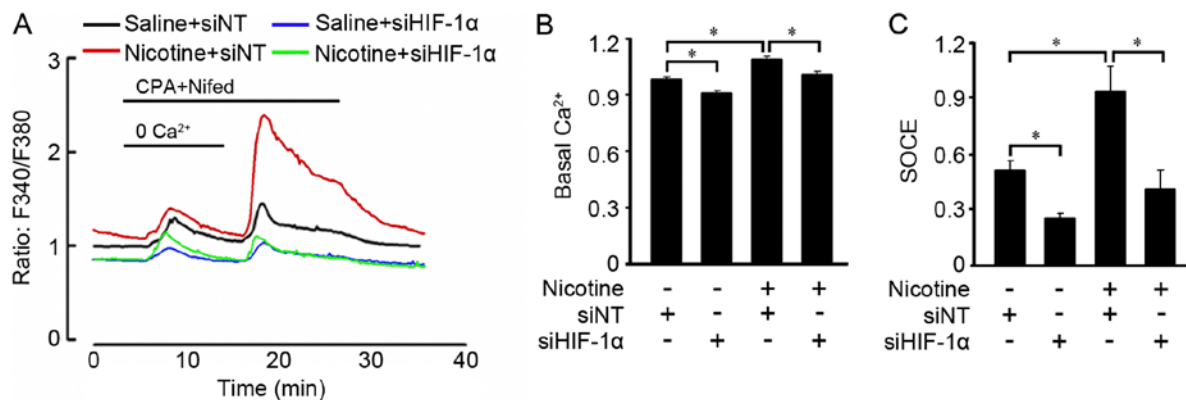

Figure 5. Basal [Ca^{2+}]$_i$ and SOCE are decreased in cells lacking HIF-1α. A549 cells underwent siHIF-1α or siNT transfection and subsequent nicotine (1 μM) exposure. After 48 h, the cells were subjected for assessment of the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F340/F380) under incubation with 5 μM fura-2 AM. (A) Time course of [Ca^{2+}]$_i$ before and after restoration of extracellular Ca^{2+} in cells perfused with Ca^{2+}-free physiological salt solution (PSS) containing 5 μM nifedipine and 10 μM cyclopiazonic acid (CPA) (n=4 experiments in 115 cells). (B) Average basal intracellular [Ca^{2+}]$_i$ and (C) store-operated calcium entry (SOCE) (average peak Δ[Ca^{2+}]$_i$ shown in A, obtained from cells with restored extracellular Ca^{2+}). Data are presented as mean ± SEM. *P<0.05 and **P<0.01.

muscle cells (26,27), the effects of HIF-1α on nicotine-triggered expression of SOCC components were evaluated in A549 cells. As shown in Fig. 4A, nicotine induced an increase in the HIF-1α level in A549 cells at 48 h. A small interfering RNA against HIF-1α (siHIF-1α) was used to silence HIF-1α

expression (Fig. 4B). After 48-h nicotine exposure, siHIF-1α reduced the basal protein levels of HIF-1α and SOCC components TRPC1, TRPC6 and Orai1, and abolished the upregulation of these proteins caused by nicotine exposure (Fig. 4C). Similar upregulatory effects of HIF-1α on SOCC components
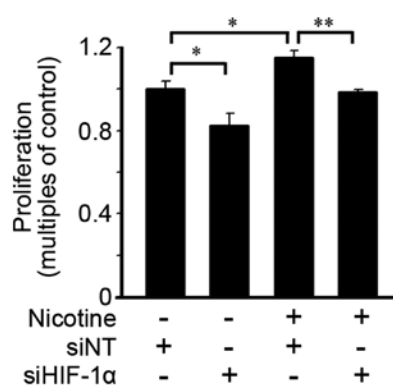
Figure 6. Cell proliferations upon siHIF-1α transfection and nicotine exposure. Cells transfected with siHIF-1α or siNT were seeded on a 96-well plate and exposed to 1 μM nicotine. The cell proliferation was evaluated by BrdU incorporation at 48 h and is expressed as the multiple of the cells transfected only with siNT. The results are from three experiments and are presented as mean ± SEM. *P<0.05 and **P<0.01.

were also found in another NSCLC cell line, NCI-H292, in which the upregulation of SOCC components upon nicotine exposure were attenuated by siHIF-1α (data not shown).

HIF-1α deficiency abolishes the increases in basal [Ca^{2+}]$_i$ and SOCE induced by nicotine. Since HIF-1α deficiency reduced expression of SOCC components in A549 cells, we therefore measured the basal [Ca^{2+}]$_i$ and SOCE when HIF-1α expression was abolished. Downregulation of HIF-1α expression with siHIF-1α decreased basal [Ca^{2+}]$_i$ in the A549 cells and abolished the increase in basal [Ca^{2+}]$_i$ induced by nicotine (Fig. 5A and B). Furthermore, HIF-1α deficiency not only reduced SOCE in the A549 cells, but prevented SOCE increase induced by nicotine (Fig. 5A and C).

Loss of HIF-1α eliminates nicotine-induced cell proliferation. The effects of HIF-1α on cell proliferation were evaluated. As shown in Fig. 6, HIF-1α deficiency induced by siHIF-1α transfection suppressed cell proliferation in cells with or without nicotine exposure at 48 h.

Discussion

Lung cancer is a serious life-threatening disease and cigarette smoking is the primary risk factor. In the present study, we demonstrated that nicotine, the major component in cigarette smoke, upregulated the expression of HIF-1α and SOCC components, and promoted cell proliferation in A549 cells. Enhanced SOCE and elevated intracellular [Ca^{2+}]$_i$ were associated with the upregulation of HIF-1α. Silencing of HIF-1α or blocking SOCE abolished the nicotine-induced cell proliferation. Therefore, HIF-1α mediated the promotive effects of nicotine on SOCC component expression, SOCE and cell proliferation in lung cancer cells.

Intracellular [Ca^{2+}]$_i$ regulated by SOCE is essential for modulating cell migration and proliferation in normal and cancer cells. Suppression of SOCE by abolishing SOCC component expression was found to prevent the proliferation and invasion of lung cancer cells (28-30). In the present study, we determined the expression of SOCC components in

A549 cells. The expression of TRPC isoforms in A549 cells is consistent with the expressional profile of TRPCs in NSCLC tissue, in which the mRNA levels of TRPC1, 3, 4 and 6 are detectable and the levels of TRPC2, 5 and 7 are below the detection limit (31).

Nicotine participates in the progression of lung cancer through the non-neuronal cholinergic system mediated by the nicotine acetylcholine receptor (32,33). Normal bronchial epithelial cells express α3-, α4-, α5- and α7-nAChR subunits which modulate Ca^{2+} metabolism (34). The receptors nAChRs are functionally conserved in mediating nicotine responses on TRPCs in neurons from worms to mammals (35). However, to date it is not known which nAChR subunit mediates the functions of nicotine in regulating expression of SOCC components in epithelial cells. The candidates could be α5- and α7-nAChR, as it has been suggested that the dysregulations of α5- and α7-nAChR in lung cancer tissues are associated with different influences of nicotine on the tumorigenesis of different lung cancer types (36).

The present study demonstrated that HIF-1α mediated the upregulatory effects of nicotine on TRPCs and Orai1. It has been confirmed that nicotine upregulates HIF-1α expression through binding α5-nAChR and activating the downstream Erk1/2 or PI3K/Akt signaling in NSCLC (26,37). Actually, the genes encoding TRPCs could be target genes of HIF-1α in modulating the growth of various types of cells, since, except for nicotine, HIF-1α is believed to be a common mediator of various factors in enhancing the expression of TRPCs, SOCC and intracellular [Ca^{2+}] in pulmonary arterial smooth muscle cells and cardiomyocytes suffering hypoxia, which result in cell proliferation and migration or tissue hypertrophy (27,38,39).

Although the association between Orai1 dysexpression and lung cancer progression remains to be evaluated, Orai1 elevation is related to enhanced tumor cell proliferation and invasion (40). Our results not only demonstrated the involvement of Orai1 in nicotine-triggered lung cancer cell proliferation, but showed that *Orai1* may be a target gene of HIF-1α. Furthermore, like in other cell types, it is reasonable to suppose that the functions of Orai1 on SOCE in lung tumor cells might not be limited as a channel forming protein, but as a regulator for SOCC complex formation, including regulating the recruitment of TRPC1 (41) or the formation of ternary complex of TRPC-Orai1-STIM1 (42).

Based on our results, it is comprehensible to suggest that HIF-1α upregulation upon nicotine stimulation eventually promotes lung tumor cell proliferation by increasing expression of SOCC components and intracellular [Ca^{2+}]. Actually, nicotine-activated signaling mediated by PKC, NF-κB, Srk, PI3K/Akt, Raf-1, ERK1/2 and p90RSK are related with increases in cell proliferation, migration, invasion or inhibition of cell apoptosis (43-46). However, we did not observe an obvious change in migration within 24 to 48 h upon nicotine exposure, although inhibition of SOCE abolished cell migration (Fig. 3B and data not shown). Probably this was due to the lower concentration of nicotine used in this study when compared with other studies, which may need a longer time to trigger a change in migration capacity (25,47).

In summary, the present study demonstrated that nicotine upregulated the expression of SOCC components TRPC6 and Orai1 by increasing HIF-1α expression in NSCLC cells,

which eventually led to enhanced SOCE, elevated intracellular $[Ca^{2+}]_i$ and promotion of cell proliferation. These findings suggest that HIF-1α-SOCE signaling plays a pivotal role in pro-tumor functions of nicotine in NSCLC.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
2. Warren GW and Cummings KM: Tobacco and lung cancer: Risks, trends, and outcomes in patients with cancer. Am Soc Clin Oncol Educ Book: 359-364, 2013.
3. Grozio A, Catassi A, Cavalieri Z, Paleari L, Cesario A and Russo P: Nicotine, lung and cancer. Anticancer Agents Med Chem 7: 461-466, 2007.
4. Cardinale A, Nastrucci C, Cesario A and Russo P: Nicotine: Specific role in angiogenesis, proliferation and apoptosis. Crit Rev Toxicol 42: 68-89, 2012.
5. Improgo MR, Tapper AR and Gardner PD: Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer. Biochem Pharmacol 82: 1015-1021, 2011.
6. Carlisle DL, Liu X, Hopkins TM, Swick MC, Dhir R and Siegfried JM: Nicotine activates cell-signaling pathways through muscle-type and neuronal nicotinic acetylcholine receptors in non-small cell lung cancer cells. Pulm Pharmacol Ther 20: 629-641, 2007.
7. Déliot N and Constantin B: Plasma membrane calcium channels in cancer: Alterations and consequences for cell proliferation and migration. Biochim Biophys Acta 1848: 2512-2522, 2015.
8. Parekh AB and Putney JW Jr: Store-operated calcium channels. Physiol Rev 85: 757-810, 2005.
9. Chen YF, Chen YT, Chiu WT and Shen MR: Remodeling of calcium signaling in tumor progression. J Biomed Sci 20: 23, 2013.
10. Villalobos C, Sobradillo D, Hernández-Morales M and Nuñez L: Remodeling of calcium entry pathways in cancer. Adv Exp Med Biol 898: 449-466, 2016.
11. Smyth JT, Hwang SY, Tomita T, DeHaven WI, Mercer JC and Putney JW: Activation and regulation of store-operated calcium entry. J Cell Mol Med 14: 2337-2349, 2010.
12. DeHaven WI, Smyth JT, Boyles RR and Putney JW Jr: Calcium inhibition and calcium potentiation of Orai1, Orai2, and Orai3 calcium release-activated calcium channels. J Biol Chem 282: 17548-17556, 2007.
13. Ambudkar IS, de Souza LB and Ong HL: TRPC1, Orai1, and STIM1 in SOCE: Friends in tight spaces. Cell Calcium 63: 33-39, 2017.
14. Yazbeck P, Tauseef M, Kruse K, Amin MR, Sheikh R, Feske S, Komarova Y and Mehta D: STIM1 phosphorylation at Y361 recruits Orai1 to STIM1 puncta and induces Ca^{2+} entry. Sci Rep 7: 42758, 2017.
15. Ruan K, Song G and Ouyang G: Role of hypoxia in the hallmarks of human cancer. J Cell Biochem 107: 1053-1062, 2009.
16. Li Y, Guo B, Xie Q, Ye D, Zhang D, Zhu Y, Chen H and Zhu B: STIM1 mediates hypoxia-driven hepatocarcinogenesis via interaction with HIF-1. Cell Rep 12: 388-395, 2015.
17. Semenza GL, Agani F, Feldser D, Iyer N, Kotch L, Laughner E and Yu A: Hypoxia, HIF-1, and the pathophysiology of common human diseases. Adv Exp Med Biol 475: 123-130, 2000.
18. Wen L, Liang C, Chen E, Chen W, Liang F, Zhi X, Wei T, Xue F, Li G, Yang Q, et al: Regulation of multi-drug resistance in hepatocellular carcinoma cells is TRPC6/calcium dependent. Sci Rep 6: 23269, 2016.
19. Guo L, Li L, Wang W, Pan Z, Zhou Q and Wu Z: Mitochondrial reactive oxygen species mediates nicotine-induced hypoxia-inducible factor-1α expression in human non-small cell lung cancer cells. Biochim Biophys Acta 1822: 852-861, 2012.
20. Li W, Chen YQ, Shen YB, Shu HM, Wang XJ, Zhao CL and Chen CJ: HIF-1α knockdown by miRNA decreases survivin expression and inhibits A549 cell growth in vitro and in vivo. Int J Mol Med 32: 271-280, 2013.
21. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45, 2001.
22. Wang J, Shimoda LA and Sylvester JT: Capacitative calcium entry and TRPC channel proteins are expressed in rat distal pulmonary arterial smooth muscle. Am J Physiol Lung Cell Mol Physiol 286: L848-L858, 2004.
23. Smyth JT, Dehaven WI, Jones BF, Mercer JC, Trebak M, Vazquez G and Putney JW Jr: Emerging perspectives in store-operated Ca^{2+} entry: Roles of Orai, Stim and TRP. Biochim Biophys Acta 1763: 1147-1160, 2006.
24. Calderon LE, Liu S, Arnold N, Breakall B, Rollins J and Ndinguri M: Bromoenol lactone attenuates nicotine-induced breast cancer cell proliferation and migration. PLoS One 10: e0143277, 2015.
25. Nair S, Bora-Singhal N, Perumal D and Chellappan S: Nicotine-mediated invasion and migration of non-small cell lung carcinoma cells by modulating STMN3 and GSPT1 genes in an ID1-dependent manner. Mol Cancer 13: 173, 2014.
26. Zhang Q, Tang X, Zhang ZF, Velikina R, Shi S and Le AD: Nicotine induces hypoxia-inducible factor-1alpha expression in human lung cancer cells via nicotinic acetylcholine receptor-mediated signaling pathways. Clin Cancer Res 13: 4686-4694, 2007.
27. Wang Y, Lu W, Yang K, Wang Y, Zhang J, Jia J, Yun X, Tian L, Chen Y, Jiang Q, et al: Peroxisome proliferator-activated receptor γ inhibits pulmonary hypertension targeting store-operated calcium entry. J Mol Med 93: 327-342, 2015.
28. Sweeney M, Yu Y, Platoshyn O, Zhang S, McDaniel SS and Yuan JX: Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. Am J Physiol Lung Cell Mol Physiol 283: L144-L155, 2002.
29. Choi DL, Jang SJ, Cho S, Choi HE, Rim HK, Lee KT and Lee JY: Inhibition of cellular proliferation and induction of apoptosis in human lung adenocarcinoma A549 cells by T-type calcium channel antagonist. Bioorg Med Chem Lett 24: 1565-1570, 2014.
30. Yang LL, Liu BC, Lu XY, Yan Y, Zhai YJ, Bao Q, Doetsch PW, Deng X, Thai TL, Alli AA, et al: Inhibition of TRPC6 reduces non-small cell lung cancer cell proliferation and invasion. Oncotarget 8: 5123-5134, 2017.
31. Zhang Q, He J, Lu W, Yin W, Yang H, Xu X and Wang D: Expression of transient receptor potential canonical channel proteins in human non-small cell lung cancer. Zhongguo Fei Ai Za Zhi 13: 612-616, 2010 (In Chinese).
32. Shiraishi K, Kohno T, Kunitoh H, Watanabe S, Goto K, Nishiwaki Y, Shimada Y, Hirose H, Saito I, Kuchiba A, et al: Contribution of nicotine acetylcholine receptor polymorphisms to lung cancer risk in a smoking-independent manner in the Japanese. Carcinogenesis 30: 65-70, 2009.
33. Millar NS and Gotti C: Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology 56: 237-246, 2009.
34. Zia S, Ndoye A, Nguyen VT and Grando SA: Nicotine enhances expression of the alpha 3, alpha 4, alpha 5, and alpha 7 nicotinic receptors modulating calcium metabolism and regulating adhesion and motility of respiratory epithelial cells. Res Commun Mol Pathol Pharmacol 97: 243-262, 1997.
35. Feng Z, Li W, Ward A, Piggott BJ, Larkspur ER, Sternberg PW and Xu XZ: A C. elegans model of nicotine-dependent behavior: Regulation by TRP-family channels. Cell 127: 621-633, 2006.

36. Bordas A, Cedillo JL, Arnalich F, Esteban-Rodriguez I, Guerra-Pastrián L, de Castro J, Martín-Sánchez C, Atienza G, Fernández-Capitan C, Rios JJ and Montiel C: Expression patterns for nicotinic acetylcholine receptor subunit genes in smoking-related lung cancers. Oncotarget 8: 67878-67890, 2017.
37. Ma X, Jia Y, Zu S, Li R, Jia Y, Zhao Y, Xiao D, Dang N and Wang Y: α5 Nicotinic acetylcholine receptor mediates nicotine-induced HIF-1α and VEGF expression in non-small cell lung cancer. Toxicol Appl Pharmacol 278: 172-179, 2014.
38. Wang J, Fu X, Yang K, Jiang Q, Chen Y, Jia J, Duan X, Wang EW, He J, Ran P, *et al*: Hypoxia inducible factor-1-dependent up-regulation of BMP4 mediates hypoxia-induced increase of TRPC expression in PASMCs. Cardiovasc Res 107: 108-118, 2015.
39. Chu W, Wan L, Zhao D, Qu X, Cai F, Huo R, Wang N, Zhu J, Zhang C, Zheng F, *et al*: Mild hypoxia-induced cardiomyocyte hypertrophy via up-regulation of HIF-1α-mediated TRPC signalling. J Cell Mol Med 16: 2022-2034, 2012.
40. Xia J, Wang H, Huang H, Sun L, Dong S, Huang N, Shi M, Bin J, Liao Y and Liao W: Elevated Orai1 and STIM1 expressions upregulate MACC1 expression to promote tumor cell proliferation, metabolism, migration, and invasion in human gastric cancer. Cancer Lett 381: 31-40, 2016.
41. Cheng KT, Liu X, Ong HL, Swaim W and Ambudkar IS: Local Ca²⁺ entry via Orai1 regulates plasma membrane recruitment of TRPC1 and controls cytosolic Ca²⁺ signals required for specific cell functions. PLoS Biol 9: e1001025, 2011.
42. Berna-Erro A, Redondo PC and Rosado JA: Store-operated Ca²⁺ entry. Adv Exp Med Biol 740: 349-382, 2012.
43. Catassi A, Servent D, Paleari L, Cesario A and Russo P: Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: Implications on lung carcinogenesis. Mutat Res 659: 221-231, 2008.
44. Paleari L, Catassi A, Ciarlo M, Cavalieri Z, Bruzzo C, Servent D, Cesario A, Chessa L, Cilli M, Piccardi F, *et al*: Role of alpha7-nicotinic acetylcholine receptor in human non-small cell lung cancer proliferation. Cell Prolif 41: 936-959, 2008.
45. Dasgupta P, Rizwani W, Pillai S, Kinkade R, Kovacs M, Rastogi S, Banerjee S, Carless M, Kim E, Coppola D, *et al*: Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines. Int J Cancer 124: 36-45, 2009.
46. Egleton RD, Brown KC and Dasgupta P: Nicotinic acetylcholine receptors in cancer: Multiple roles in proliferation and inhibition of apoptosis. Trends Pharmacol Sci 29: 151-158, 2008.
47. Shi J, Liu F, Zhang W, Liu X, Lin B and Tang X: Epigallocatechin-3-gallate inhibits nicotine-induced migration and invasion by the suppression of angiogenesis and epithelial-mesenchymal transition in non-small cell lung cancer cells. Oncol Rep 33: 2972-2980, 2015.