ABSTRACT

Title of Thesis: **ANTI-CANCER MECHANISM OF ARCTIGENIN (ARC) IN HUMAN LUNG CANCER CELLS**

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Arctigenin (ARC) is a lignan and is abundant in *Asteraceae* plant which possesses anti-inflammatory and anti-cancer activities. The current study was performed to investigate if ARC affects cancer progression and metastasis focusing on epithelial–mesenchymal transition (EMT) using invasive human lung cancer cell line, A549. No toxicity was observed in the cells treated with different doses of ARC (12-100 μM). The treatment of ARC repressed TGF-β-stimulated changes of metastatic morphology and cell invasion and migration. ARC inhibited TGF-β-induced phosphorylation and transcriptional activity of SMAD2/3 and expression of snail in dose-dependent and time-dependent manners. ARC also decreased expression of N-cadherin and increased expression of E-cadherin in dose-dependent and time-dependent manners. These changes were accompanied with decreased amount of nuclear phospho-SMAD2 and SMAD3, and nuclear translocation of SMAD2 and SMAD3. Moreover, ARC repressed TGF-β-induced phosphorylation of ERK. Our data demonstrate anti-metastatic activity of ARC in lung cancer model.

Key words: ARC, TGF-β, EMT, Lung cancer

ANTI-CANCER MECHANISM OF ARCTIGENIN (ARC)

IN HUMAN LUNG CANCER CELLS

By

Yanrui Xu

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Chapter 1: Background

1.1 Lung cancer overview

Lung cancer, also known as lung carcinoma, is a malignant lung tumor characterized by uncontrolled cell growth in tissues of lungs. This growth can spread beyond the lung by the process of metastasis into nearby tissue or other parts of the body [1]. Most cancers that start in the lung, known as primary lung cancers, are carcinomas. The two broad histological subtypes of lung cancer are small-cell lung cancer (SCLC), which is the cause of 15% of cases, and non-small-cell lung cancer (NSCLC), which accounts for 85% of cases and includes adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma [2]. The most common symptoms of lung cancer are coughing (including coughing up blood), weight loss, shortness of breath, and chest pains [3]. Worldwide in 2012, lung cancer occurred in 1.8 million people and resulted in 1.6 million deaths [4]. This makes it the most common cause of cancer-related death in men and second most common in women after breast cancer [4].

Treatment option for lung cancer depends on the cancer's specific cell type, how far it has spread, and the person's performance status. Common treatments include palliative care, surgery, chemotherapy, and radiation therapy. However, in many cases that the cancer cells have widely spread, surgery is no longer available. Furthermore, during the process of chemotherapy and radiotherapy, there exists severe side effects including fatigue, hair loss, easy bleeding, infection, anemia, nausea and vomiting [5, 6]. Therefore, new therapy against human lung cancer with less side effects and toxicities is required for a better prognosis of lung cancer patients.

1.2 Metastasis

Metastasis is one of the hallmarks of cancer, distinguishing it from benign tumors [7], and plays an important role in the malignancy and poor prognosis of cancer. It is a complex process whereby cancer cells spread from a primary organ site and form tumors at distant organ sites. During this process, some cancer cells known as circulating tumor cells acquire the ability to penetrate the walls of lymphatic or blood vessels by loss of cell-cell and cell-matrix adhesion, increased cellular motility and invasion [8], after which they are able to circulate through the bloodstream to other sites and tissues in the body. This process is known as lymphatic or hematogenous spread. After the tumor cells come to rest at another site, they re-penetrate the vessel or walls and continue to multiply, eventually forming another clinically detectable tumor. This new tumor is known as a metastatic tumor. Most cancers can metastasize, although in varying degrees.

Metastasis is a key element in cancer staging systems such as the TNM, where it represents the "M". In overall stage grouping, metastasis places a cancer in Stage IV. The possibilities of curative treatment are greatly reduced, or often entirely removed, when a cancer has metastasized. Therefore, around 90% of all cancer deaths are the result of metastases, rather than of the primary tumors [9].

Lung cancer, which is one of the most common and malignant cancers worldwide, is most often diagnosed at late stages, when it has already presented local invasion and distal metastases. The cascade of events during lung cancer metastasis include: (1) Detachment of tumor cells from the extracellular matrix (ECM) and degradation of the ECM by several proteolytic enzymes. (2) Invasion of neighboring tissues and basement membrane. (3) Intravasation into the blood stream or lymphatic vessels, by attachment

on the endothelial cells with adhesion molecules, infiltration of the vessels, survival and transport through the blood stream. (4) Arrest and extravasation at a distal site and formation of a metastatic lesion. Research in past decades highlighted the steps of metastasis at a microscopic level. However, this had little or no effects on improving treatment outcome, since still almost 90% of lung cancer patient deaths are metastasis-related. Therefore, further research of novel and more effective strategies for preventing and treating lung cancer is still needed [10].

1.3 Epithelial–Mesenchymal Transition (EMT)

The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells [8]. It is an important cellular process during embryonic morphogenesis, organogenesis and tissue repair and remodeling [11], and it also plays an important role in the initiation of metastasis in cancer progression [12].

EMT was first recognized as a feature of embryogenesis by Betty Hay in the 1980s [13, 14]. EMT, and its reverse process, MET (mesenchymal-epithelial transition) are critical for development of many tissues and organs in the developing embryo, and numerous embryonic events such as gastrulation, neural crest formation, heart valve formation, palatogenesis and myogenesis [15]. Epithelial and mesenchymal cells differ in phenotype as well as function, though both share inherent plasticity [14]. Epithelial cells are closely connected to each other by tight junctions, gap junctions and adherens junctions, have an apico-basal polarity, polarization of the actin cytoskeleton and are bound by a basal lamina at their basal surface. On the other hand, mesenchymal cells lack this polarization, have a spindle-shaped

morphology and interact with each other only through focal points [16]. Epithelial cells express high levels of E-cadherin, whereas mesenchymal cells express those of N-cadherin, fibronectin and vimentin. Thus, EMT entails profound morphological and phenotypic changes to the cells.

TGF-β is a multifunctional cytokine and most well-known EMT stimulant [17]. TGF-β-treated cells *in vitro* undergo mesenchymal-like spindle-shaped morphology with a decrease in cell-cell and cell-matrix adhesion and loss of polarity [18]. Regarding to cellular and molecular event, TGF-β first binds to type II receptor which leads to recruitment and phosphorylation of the type I receptor. The activated type I receptor subsequently induces phosphorylation of SMAD2 and SMAD3 (R-SMADs) and form heterodimeric complexes with a SMAD4 (co-SMAD). The complex of SMAD2/3/4 trans-locates into the nucleus and binds to SMAD response element (SRE) on the promoter of their specific target genes, and mediates EMT [19]. Additionally, TGF-β activates non-SMAD signaling pathways such as extracellular-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK) during signal transduction of TGF-β-induced cell migration and invasion [20].

1.4 E-cadherin

E-cadherin is one of the most important molecules in cell-cell adhesion in epithelial tissues. It is localized on the surfaces of epithelial cells in regions of cell-cell contact known as adherens junctions [21]. E-cadherin molecules are critical for the formation and maintenance of adherent junctions in areas of epithelial cell-cell contact, and it also plays a major role in malignant cell transformation, and especially in tumor development and progression. The suppression of E-cadherin expression is regarded as one of the main molecular events responsible for dysfunction in cell-cell adhesion. Most

tumors have abnormal cellular architecture, and loss of tissue integrity can lead to local invasion. Thus, loss of function of E-cadherin tumor suppressor protein correlates with EMT and increases invasiveness and metastasis of tumors [22].

1.5 N-cadherin

N-cadherin is encoded by the CDH2 gene [23] and expressed in multiple tissues and functions to mediate cell–cell adhesion. N-cadherin is associated with a lot of molecules that regulate its function. It is involved in a lot of processes like cell-cell adhesion, differentiation, embryogenesis, migration, invasion and signal transduction. During EMT, the aberrant expression of N-cadherin attributes a more fibroblastic phenotype to the cancer cells, and as a result, the cells become more motile and invasive [24].

1.6 Snail

Snail is a protein that is encoded by the SNAI1gene [25]. It is a family of transcription factor that promotes the repression of the adhesion molecule E-cadherin to regulate EMT during embryonic development. As a critical regulator of multiple signaling pathways leading to EMT, the expression of Snail is closely associated with cancer metastasis. It has been demonstrated that Snail is required for lymph node metastasis of human breast carcinoma MDA-MB-231 cells [26]. A set of genes of the "lung metastasis signature" are direct or indirect targets of Snail. They include ID1, SPARC or MMP2 [27]. It has also been observed that the level of expression of Snail is elevated in metastatic lesions in ovarian cancer [28]. A recent study demonstrated that Snail-induced EMT accelerates metastasis through induction of immune-suppression [29]. Knockdown of Snail significantly inhibits tumor growth and

metastasis by increasing tumor-infiltrating lymphocytes and systemic immune responses [29]. Therefore, Snail is an effective target for preventing metastasis.

1.7 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF), originally known as vascular permeability factor (VPF), is a signal protein produced by cells that stimulates the formation of blood vessels. To be specific, VEGF is a sub-family of growth factors, the platelet-derived growth factor family of cystine-knot growth factors. They are important signaling proteins involved in both vasculogenesis (the de novo formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature) [30].

VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (the VEGFRs) on the cell surface, causing them to dimerize and become activated through transphosphorylation, although to different sites, times, and extents. The VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region, and an intracellular portion containing a split tyrosine-kinase domain [31].

VEGF's normal function is to create new blood vessels during embryonic development, new blood vessels after injury, muscle following exercise, and new vessels (collateral circulation) to bypass blocked vessels. When VEGF is overexpressed, it can contribute to diseases such as cancer. Solid cancers cannot grow beyond a limited size without an adequate blood supply, however, cancers that can express VEGF are able to grow and metastasize. It is reported that VEGF family members play an important role in the development of pancreatic cancer [32], and EMT confers efficient tumorigenicity to murine breast cancer cells by the upregulated expression of the

proangiogenic factor VEGF-A and by increased tumor angiogenesis [33]. At the same time, VEGF mediates promotion of human ovarian cancer cell invasion [34]. Some other researcher also pointed out that high expression of VEGF is associate with poor prognosis of gastric cancer [35]. Meanwhile, VEGF is also involved in the treatment of certain cancers. It has been reported that the anti-tumor effect is enhanced by targeting VEGF in colorectal cancer [36].

1.8 Cyclin D1

Cyclin-D1 is a protein that in humans is encoded by the CCND1 gene [37, 38], it is a protein required for progression through the G1 phase of the cell cycle. During the G1 phase, it is synthesized rapidly and accumulates in the nucleus, and is degraded as the cell enters the S phase. Cyclin D1 is a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6. The protein dimerizes with CDK4/6 to regulate the G1/S phase transition and entry into the S-phase.

Cyclin D1 overexpression has been shown to correlate with early cancer onset and tumor progression [39] and it can lead to oncogenesis by increasing anchorage-independent growth and angiogenesis via VEGF production [40]. Cyclin D1 overexpression can also down-regulate Fas expression, leading to increased chemotherapeutic resistance and protection from apoptosis [40], which plays an important role in the formation of tumor.

1.9 Plant polyphenols

Plant polyphenols is one of the important groups of phytochemicals, it is a structural class of mainly natural, but also synthetic or semisynthetic, organic chemicals characterized by the presence of large multiples of phenol structural units [41].

It has been reported that polyphenols exert anticancer effects within human bodies. Accumulating evidence suggests that pomegranate polyphenols targets a broad spectrum of genes and proteins to suppress cancer growth and progression. The anticancer activity of pomegranate can be seen in a variety of cancers include breast cancer, colon cancer, liver cancer, skin cancer [42] and lung cancer [43]. Meanwhile, Green tea, which has higher concentrations of polyphenols than other teas, has been correlated with reduced risk of various malignancies with most data supporting a potential protective role in prostate neoplasia [44].

1.10 Lignan

The lignans are a large group of polyphenols found in plants, which also show anticancer effects. According to the previous research, lignans can have a protective effect on bladder cancer [45], and it can also inhibit breast cancer-mediated bone destruction by blocking the vicious cycle between cancer cells, osteoblasts and osteoclasts [46]. At the same time, it is reported that a dietary pattern rich in lignans decreases the risk of oesophageal cancer [47]. Furthermore, researchers also found that higher lignan intakes were associated with lower risks of breast cancer with more favorable prognostic characteristics [48].

1.11 Arctigenin (ARC) and cancer

Arctium lappa L. (Asteraceae/Compositae), a biennial plant known as burdock, is regarded as an effective Chinese medicine for alleviation of rheumatic pain and fever [49]. ARC ($C_{21}H_{24}O_6$; molecular weight: 372.41) is a phenylpropanoid dibenzylbutyrolactone lignan which extracted from the Arctium lappa [50].

ARC has various biological activities including anti-inflammatory, antioxidative,

anticancer, and antiviral activities [50]. As a new anticancer agent, ARC exerts anti-cancer effects in various cancers. In human breast cancer, ARC induces apoptosis of estrogen receptor-negative breast cancer cells through the reactive oxygen species (ROS)/p38 mitogen-activated protein kinases (MAPKs) pathway and epigenetic regulation [51], inhibits the activation of the mTOR pathway, resulting in autophagic cell death and decreased ER expression in ER-positive human breast cancer cells [52], inhibits metastasis of human breast cancer cells through the downregulation of MMP-2/-9 and heparanase in MDA-MB-231 cells [53], inhibits STAT3 and exhibits anticancer potential in human triple-negative breast cancer therapy [54]. In bladder cancer cells, ARC induces cell-cycle arrest and apoptosis [55]. In lung cancer cells, ARC enhances chemo-sensitivity of human non-small lung cancer H460 cells to cisplatin through down-regulation of survivin expression and inhibition of the signal transducer and activator of transcription 3 (STAT3) signaling pathway [56]. ARC also shows pro-apoptotic activity in ovarian cancer cells, which is associated largely with modulation of the iNOS/NO/STAT3 axis [57]. The combination of ARC and quercetin enhances the anti-proliferative effect in prostate cancer cells [58]. In human colorectal cancer, ARC inhibited angiogenesis and migration and invasion of HCT-116 adenocarcinoma cells through regulating the H1F4A and Wnt/β-catenin pathway [59]. ARC also induces apoptosis of HT-29 colon cancer cells by regulating ROS and p38 MAPK pathways [60]. ARC inhibits lung metastasis of colorectal cancer, in which ARC induced cell cycle arrest and apoptosis in CT26 cells through the intrinsic apoptotic pathway via MAPKs signaling, controlled EMT through increasing the expression of epithelial marker E-cadherin and decreasing the expressions of mesenchymal markers; N-cadherin, vimentin, β-catenin, and Snail, as well as inhibited migration and invasion through reducing of matrix metalloproteinase-2 (MMP-2) and

MMP-9 expressions [61]. In gastric cancer, the proliferation of SNU-1 and AGS cells was significantly inhibited by ARC with cell cycle arrest from G(1) to S phase, which was accompanied with changes of cell cycle regulatory proteins such as Rb, cyclin D1, cyclin E, CDK4, CDK2, p21Waf1/Cip1 and p15 INK4b [62]. In gallbladder cancer cells, ARC decreased the expression of EGFR and led to the senescence of cancer cells [60]. ARC can also dose-dependently inhibit the growth of oral squamous cell carcinomas, and this effect may be related to down regulation of VEGF expression [63]. However, the effect of ARC on metastasis of lung cancer was not determined so far. In the present study, we examined whether treatment of ARC could repress TGF-β-induced EMT in human lung cancer cells. We found that TGF-β-induced mesenchymal phenotype was noticeably suppressed by pretreatment with ARC. We further found that TGF-β-induced migration and invasion of human lung cancer cells were also suppressed by ARC, and we also found that the mechanism of above effects of ARC is mainly through suppressing TGF-β-induced phosphorylation of SMAD2 and ERK. Thus, ARC could be applied in the new method of treatment toward human lung cancer.

Chapter 2: Materials and Methods

2.1 Materials

ARC was purchased from Tocris Bioscience (Bristol, UK) (Fig. 1A). Dulbecco's modified Eagle medium (DMEM) and trypsin were purchased from GE Healthcare (Logan, UT, USA). Antibodies for phospho-SMAD2 (CST#3101S), phospho-SMAD3 (CST#9520S), SMAD2 (CST#5339S), SMAD3 (CST#9513S), E-cadherin (3195P), N-cadherin (4061S), snail (CST#3879S), active β-catenin (CST#19807), β-catenin (CST#9582S), phospho-β-catenin (CST#9561L), phosphorylated ERK (CST#4370P) and ERK (CST#9102S) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for β-actin (SC#1616), SMAD4 (SC#H2216), Tubulin (SC#L1813) and U1SnRNP (SC#K2613) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). TGF-β (#240-B) was purchased from RD system (Minneapolis, MN, 55413, USA). ERK inhibitor PD98059 (#513000) was purchased from Calbiochem (Billerica, MA, USA) All other chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA), unless otherwise specified.

2.2 Cell culture and treatment

Human NSCLC cells (A549, H1299, H1703, H358) were maintained at 37°C with 5% CO2 in media supplemented with 10% fetal bovine serum. The cells were plated onto 96-well (for MTT assay), 24-well (for luciferase activity) and 6-well plates (for western blot). The cells were treated with TGF-β and different concentrations of ARC for indicated times as described in the figure legends in detail.

2.3 Cell Proliferation

Briefly, the cells were plated onto a 96-well culture plate (1×10^4 cells/well) with media containing different concentrations of ARC for different time periods (0-2 days). After removing the media at different time points, 100 ml mixture of serum free media and 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5:1) was added into each well then incubated for 3 hours at 37°C in a CO_2 incubator. Then, MTT dye was extracted using 200 µL of dimethylsulfoxide (DMSO), and the absorbance was measured at 540 nm using the microplate reader (Add model).

2.4 Migration and invasion assay

For migration assay, the cells were plated onto a 12-well plate and maintained until they reach 100% confluence. In a sterile environment (in a biosafety hood), a 200 µl pipette tip was used to press firmly against the top of the plate and swiftly make a vertical and a horizontal wound through the cell monolayer. A different sized pipette tip was used to make the wound size that is desired. Excessive force against the plate was avoided with the pipette tip as it may damage the surface. The media and cell debris were carefully removed and enough culture media was added slowly with different concentrations of ARC against the well wall to cover the bottom of the well and avoid detaching additional cells, initial pictures for each well were taken, the culture plate was placed in an incubator with 37°C and 5% CO_2. After 24 hours, TGF-β was added into certain wells, the culture plate was placed in an incubator with 37°C and 5% CO_2. After 24 hours, pictures for each well were taken. To analyze the results, the distance of one side of the wound to the other was measured.

For invasion assay, A549 cells were treated with or without 50 µM ARC and 5

ng/ml TGF-β on top of the filter membrane in a transwell insert and incubated for 10 minutes at 37°C and 5% CO_2 to allow the cells to settle down. 600 µl of complete media which works as chemo-attractant was added very carefully with a pipette into the bottom of the lower chamber in a 24-well plate, then complete media was added without moving the transwell insert and avoid generating bubbles. Make sure the complete media liquid in the bottom well made contact with the membrane in the upper well. The system was incubated at 37°C and 5% CO_2 for 48 hours. After the incubation, the transwell insert was removed from the plate, a cotton-tipped applicator was used as many times as needed to carefully remove the media and remaining cells that have not migrated from the top of the membrane without damage. 1 ml of 70% ethanol was added into a well of a 24-well plate and the transwell insert was placed into the 70% ethanol for 10 minutes to allow cell fixation. After that, the transwell insert was removed from the 24-well plate and a cotton-tipped applicator was used to remove the remaining ethanol from the top of the membrane. The transwell membrane was allowed to dry (typically 10-15 minutes). 1 ml of 0.2% crystal violet was added into a well of a 24-well plate and position the membrane into it for staining at room temperature for 5-10 minutes. The crystal violet was gently removed from the top of the membrane with a pipette tip or cotton tipped applicator, then the membrane was dipped into distilled water as many times as needed to remove the excess crystal violet. The transwell membrane was allowed to dry for 10-15 minutes. Downside of the transwell membrane was observed with microscope and the number of cells in different fields of view was counted to get an average sum of cells that have migrated through the membrane toward the chemo-attractant and attached on the underside of the membrane.

2.5 SDS-PAGE and Western Blot

Cells were washed with 1×phosphate-buffered saline (PBS), sat on ice for 15 minutes in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA) supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma Aldrich) and then harvested. The cell lysate was centrifuged at 12,000×g for 15 minutes at 4°C. Protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN) and blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 hour at room temperature. Membranes were probed with specific primary antibodies in 3% Bovine Serum Albumin (Santa Cruz) at 4°C overnight and then with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 hour at room temperature. Chemiluminescence was detected with Pierce ECL Western blotting substrate (Thermo Scientific) and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

2.6 Transient transfection and reporter gene assay

Transient transfection was performed using PolyJet reagent (SignaGen Laboratories, Ijamsville, MD, USA). The cells (1×10^5 cells/well) were plated in a 12-well plate. Media was changed on the next day, then master mixtures of reporter plasmid were prepared and dispensed to each well and incubated with cells for 24 hours. After that, the media was removed, and the wells were rinsed with PBS, then serum free

media was added, the transfected cells were then treated with or without different concentrations of ARC for 24 hours, then the cells were co-treated with TGF-β for 24 hours. After that, the media was removed, the wells were rinsed with PBS, and 250 µl PLB was added into each well, the plate was shaken for 15 minutes at room temperature. 100 µl LAR was pre-dispensed into luminometer tube. 20 µl PLB lysate mixture was transferred into the tube, firefly luciferase activity was measured. 100 µl stop and Glo reagent was dispensed and renilla luciferase activity was measured. (Promega, Madison, WI, USA).

2.7 Separation of nuclear and cytoplasmic fraction from cells

The nuclear and cytoplasmic fractions were isolated separately using an Active Motif Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). After the cell pellet was acquired, the cells were gently resuspended in 500 µl hypotonic buffer by pipetting up and down several times and transferred to a pre-chilled microcentrifuge tube. The cells were allowed to swell by incubating for 15 minutes on ice. 25 µl detergent was added and vortexed for 10 seconds at the highest setting. The suspension was centrifuged for 30 seconds at 14000 g in a microcentrifuge tube. The supernatant was collected (cytoplasmic fraction) and the pellet was used for nuclear fraction collection. The nuclear pellet was resuspended in 50 µl complete lysis buffer by pipetting up and down and the suspension was incubated for 30 minutes on ice on a rocking platform set at 150 rpm. After that, the suspension was vortexed for 30 seconds at the highest setting and centrifuged for 10 minutes at 14000 g in a microcentrifuge pre-cooled at 4°C, then the supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube.

2.8 Statistical Analysis

All experiments were conducted in triplicates, and the results were presented as mean values ± standard deviations (SD). Significant differences ($p<0.05$) were analyzed using a Student t test.

Chapter 3: Arctigenin (ARC) represses TGF-β-induced epithelial mesenchymal transition (EMT) in human lung cancer cells

3.1 Effect of ARC on viability of A549

In order to test the viability of the experimental system, we explored if treatment of different doses of ARC (0, 12, 25, 50 and 100 μM) leads to toxicity in A549 cells. The cells were plated onto 96-well culture plates with media containing different concentrations of ARC for different time periods (0-2 days). The media was removed at indicated time points, and 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) was added, and the cells were incubated for 3 hours at 37°C in a CO_2 incubator. Then, MTT dye was extracted using 200 μL of dimethylsulfoxide (DMSO), and the absorbance was measured at 540 nm using the microplate reader. The result indicated that ARC with concentration below 100 μM had no cytotoxicity in A549 cells within 48 hours compared to control group treated with vehicle (Fig.1), and the experimental system is viable.

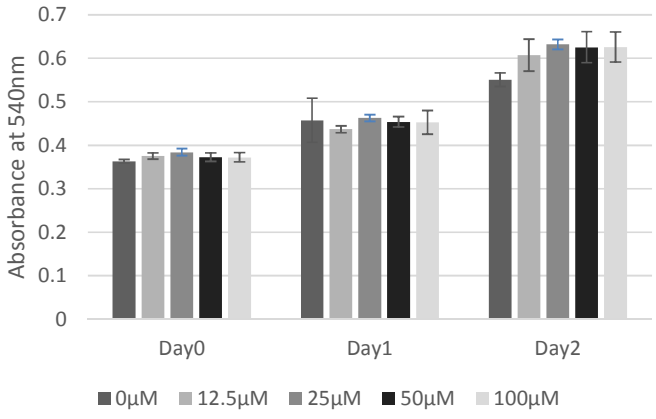


Figure 1. Effect of ARC on viability of A549 cells. Effect of different concentrations of ARC on A549 cells proliferation was determined using MTT assay. A459 cells were treated with ARC at concentrations of 0 (DMSO was used as a control), 12, 25, 50 and

100 μM for 24 hours and 48 hours, respectively. Values were represented as means ± SD for triplicates.

3.2. Effect of ARC on morphological changes of A549 cells during TGF-β-induced EMT

During the EMT, epithelial cells that are closely connected by tight junction turn into mesenchymal cells that have a spindle-shaped morphology and interact one another through focal adhesion [16]. Therefore, we examined the effect of ARC on TGF-β-induced EMT in A549 cells through observing the morphological changes of the cells after co-treatment with ARC and TGF-β.

In this experiment, A549 cells were pre-incubated with ARC (12, 25 and 50 μM) in serum-free media. After 24 hours, cells were co-incubated with 5 ng/mL TGF-β for additional 24 hours. Then the morphology of cells was observed through microscope. The result showed that treatment with TGF-β markedly induced the mesenchymal phenotype of A549 cells, including elongated and spindle-like shapes. However, this morphological change was noticeably suppressed by ARC (Fig.2).

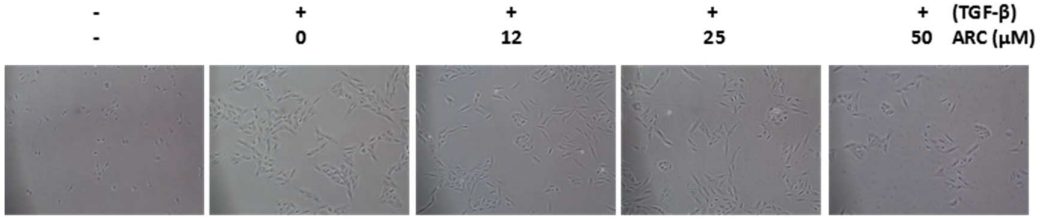


Figure 2. ARC inhibits TGF-β-induced morphological change in A549 cells. A549 cells were pretreated with ARC for 24 hours and then co-treated with 5 ng/mL TGF-β for 24 hours. The cell morphology was observed under microscope.

3.3. Effects of ARC on motility (migration and invasion) of A549 cells

It is generally accepted that EMT led to increased metastatic progression through

increased migration and invasiveness [64]. Therefore, we examined if treatment of ARC affects TGF-β-induced migration and invasion of A549 cells.

First, to determine whether ARC prevents TGF-β-induced migration of A549 cells, a wound healing assay was performed by scratching the cell layer prior to TGF-β treatment with or without ARC and imaging subsequent cell migration after 48 hours. As shown in Fig. 2B, TGF-β-treated cells enhanced cell migration activity compared with that of control cells, whereas migration ability of TGF-β-treated cells was significantly inhibited by ARC in a dose-dependent manner (Fig.3A). Second, we examined whether ARC prevents TGF-β-induced invasion of A549 cells. The cells were cultured in an invasion chamber in Transwells (inner chamber) and treated with TGF-β and different doses of ARC for 24 hours in the Transwells. The invasive cells, presented on the lower surface of the chamber, were then stained and imaged, and invasion ability was measured through counting the number of invasive cells. The result showed that TGF-β treatment promoted cell invasion ability by approximately 3.5-fold compared with that of the control cells, and this effect was significantly suppressed by ARC (Fig.3B and 3C). All taken together, these results demonstrated that ARC prevents TGF-β-induced migration and invasion in A549 cells.

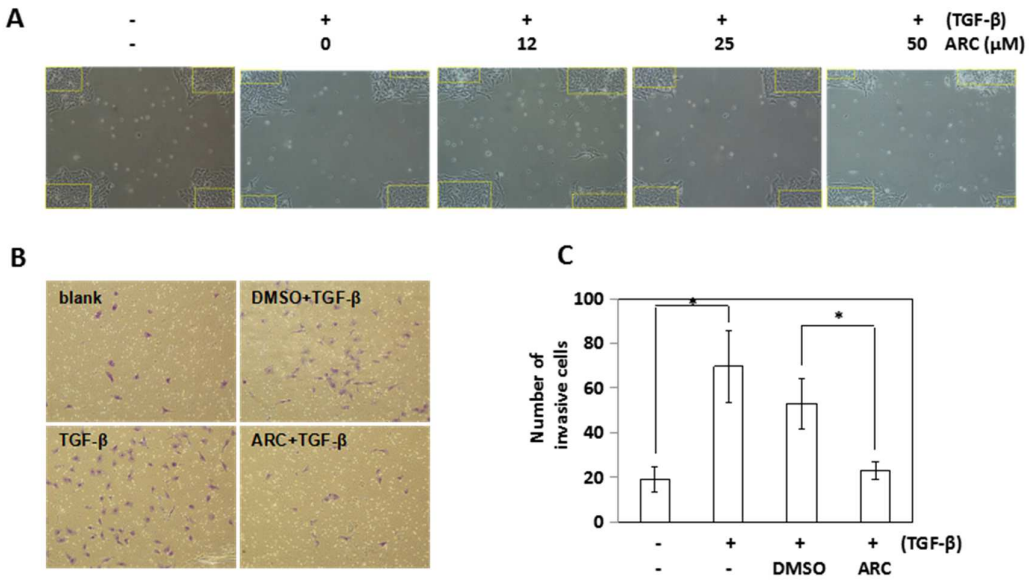


Figure 3. ARC inhibits TGF-β-induced migration and invasion in A549 cells.

(A) A549 cells were pretreated with ARC for 24 hours and then co-treated with 5 ng/mL TGF-β for 24 hours. The cells were photographed under microscope 48 hours after scratching. (B) A549 cells were treated with 50 μM ARC and 5 ng/ml TGF-β for 48 hours. The invasion was measured using Matrigel invasion assay. In vitro invasiveness of A549 cells were imaged and measured by counting cells that migrated through the extracellular matrix layer of invasion chambers. (C) The graph represents mean ± SD for five replicates. *$p<0.05$ versus control.

3.4 The effect of ARC and TGF-β on expression of VEGF and cyclin D1 in A549 cells

VEGF and Cyclin D1 are closely involved in tumorigenesis of lung cancer (References). Therefore, in order to investigate potential mechanism, we examined the effect of ARC TGF-β on expression of VEGF and Cyclin D1 in A549 cells. The result showed that neither TGF-β nor ARC affect the expression of VEGF and Cyclin D1 in A549 cells.

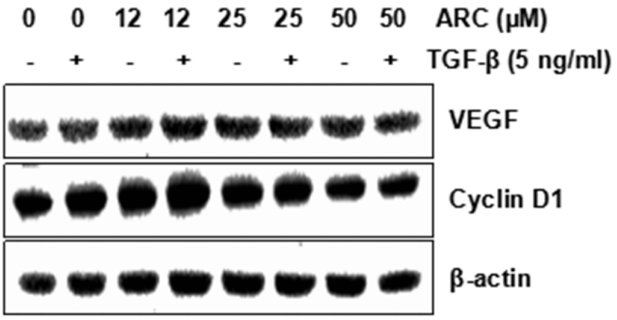


Figure 4. ARC and TGF-β have no effect on the expression of VEGF and Cyclin D1 in A549 cells. A549 cells were pretreated with the indicated concentration of ARC for 24 hours and then stimulated with TGF-β (5 ng/mL) for 24 hours. Western Blot was performed to measure the expression of VEGF and Cyclin D1.

3.5 ARC's effect on TGF-β-induced phosphorylation of SMAD2/3

TGF-β-induced EMT is mediated through SMAD signaling pathways. After TGF-β stimulation, SMAD2 and SMAD3 is phosphorylated, translocated into nucleus, formed SMAD2/3 complex, and then induce the EMT [65]. Therefore, we explored the mechanism of ARC's effect on TGF-β-induced EMT through examining its effect on TGF-β-induced phosphorylation of SMAD2/3.

The result showed that TGF-β treatment promoted the phosphorylation of SMAD2, which was significantly decreased in the presence of ARC (Fig.5A). This result indicated that ARC prevented the TGF-β-induced phosphorylation of SMAD2, by which to inhibit TGF-β-induced EMT in A549 cells. However, although TGF-β treatment also promoted the phosphorylation of SMAD3, it was not suppressed by ARC.

In addition, we found that ARC also inhibited TGF-β-induced phosphorylation of SMAD2 in another three lung cancer cell lines H1299, H1703 and H358 (Fig.5B), which further enhances our conclusion of ARC's inhibitory effects on TGF-β-induced

EMT in lung cancer cells.

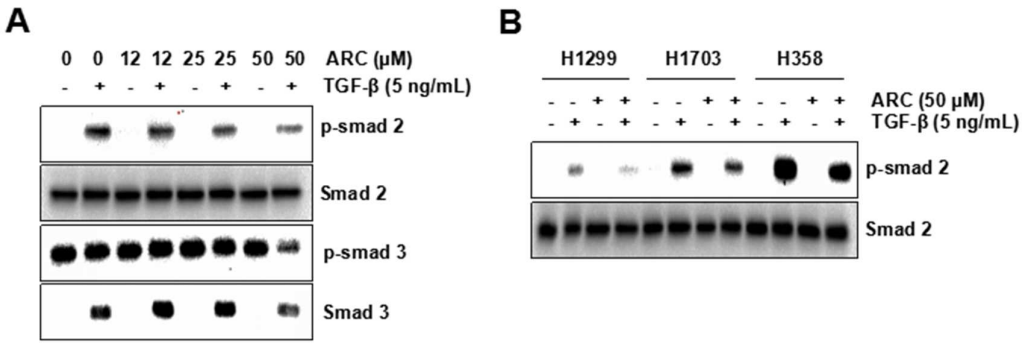


Figure 5. ARC's effects on TGF-β-induced phosphorylation of SMAD2/3.

(A) A549 cells were pretreated with the indicated concentration of ARC for 24 hours and then stimulated with TGF-β (5 ng/mL) for 30 minutes. Western blot was performed to measure phospho- and total SMAD2/3. (B) Three different types of human NSCLC cells (H1299, H1703, H358) were pre-treated with ARC (50 μM) for 24 hours and then co-treated with TGF-β (5 ng/mL) for 30 minutes. Western blot was performed to measure phospho- and total SMAD2.

3.6 ARC inhibits expression of N-cadherin and snail, rescues the decrease of E-cadherin expression during TGF-β-induced EMT.

During the EMT, epithelial cells lose E-cadherin expression and turn into mesenchymal cells that overexpress N-cadherin. Therefore, by examining ARC's effect on TGF-β-induced change of expression of E-cadherin and N-cadherin, we can further verify ARC's effect on TGF-β-induced EMT in A549 cells.

The results showed that E-cadherin expression was decreased significantly by TGF-β treatment, whereas N-cadherin expression increased in TGF-β-treated A549 cells compared with that in control cells. In contrast, ARC treatment remarkably reversed the decrease of E-cadherin expression and increase of N-cadherin expression (Fig.6).

Furthermore, downregulation of E-cadherin in the TGF-β-induced EMT process can be induced by transcriptional repressor, snail [66]. Thus, we also investigated whether ARC represses snail expression stimulated by TGF-β. As a result, expression of snail increased prominently in TGF-β-treated A549 cells compared with that in control cells. However, snail expression decreased following ARC treatment in a dose-dependent manner (Fig.6). Taken together, these results strongly suggested that ARC plays an important role in suppressing TGF-β-induced EMT in A549 cells by rescuing TGF-β-induced suppression of E-cadherin expression and suppressing TGF-β-induced expression of N-catenin and snail.

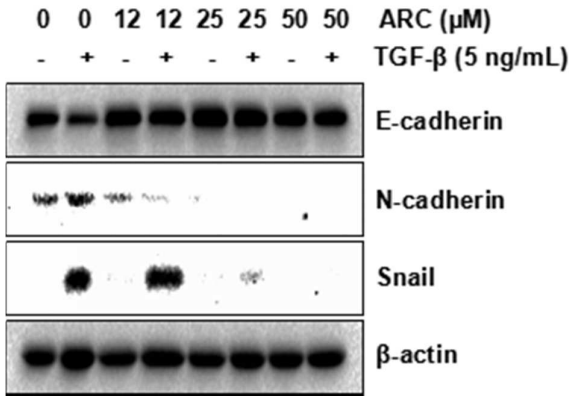


Figure 6. ARC inhibits expression of N-cadherin and snail, rescues the decrease of E-cadherin expression during TGF-β-induced EMT. A549 cells were pretreated with the indicated concentration of ARC for 24 hours and then stimulated with TGF-β (5 ng/mL) for 24 hours. Western Blot was performed to measure expression of the E-cadherin, N-cadherin, snail and β-actin.

3.7 ARC inhibits TGF-β-induced phosphorylation of SMAD2, expression of N-cadherin and snail, and rescues E-cadherin expression in time-dependent manner.

To investigate the time-sequential changes of EMT-related genes, A549 cells were pre-treated with ARC for 24 hours, and then co-treated with TGF-β for 15 minutes, 30 minutes, 1 hour, 8 hours and 24 hours respectively. Western Blot was performed to measure phosphorylation of SMAD2 and expression of E-cadherin, N-cadherin and snail.

The result showed that ARC exerts its inhibitory effect on TGF-β-induced phosphorylation of SMAD2 from 15 minutes to 1 hour. For ARC's rescuing the expression of E-cadherin, the time point is 24 hours, and ARC can inhibit TGF-β-induced expression of N-cadherin and snail from 15 minutes to 8 hours and 15 minutes to 24 hours respectively (Fig.7).

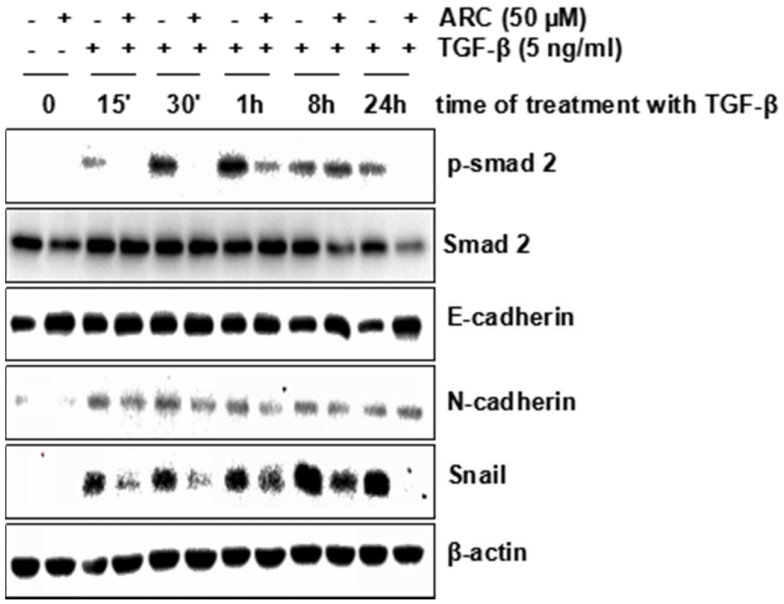


Figure 7. ARC inhibits TGF-β-induced phosphorylation of SMAD2, expression of N-cadherin and snail, and rescues E-cadherin expression in time-dependent manner. A549 cells were pretreated with the indicated concentration of ARC for 24

hours and then stimulated with TGF-β (5 ng/mL) for a series of time periods (15 minutes, 30 minutes, 1 hour, 8 hours and 24 hours). Western Blot was performed to measure expression of phospho-SMAD2/3, E-cadherin, N-cadherin, snail and β-actin.

3.8 ARC's effect on TGF-β signaling pathway measured by transfecting the reporter p3TP-Lux.

To test transcriptional activity of TGF-β signaling, we did report gene analysis using p3TP-Lux reporter gene containing SMAD-binding sites at 5'-flaking region of luciferase genes. The result of reporter gene p3TP-Lux transfection and subsequent luciferase assay showed that ARC does inhibit TGF-β signaling pathway (Fig.8), which is consistent to the above results.

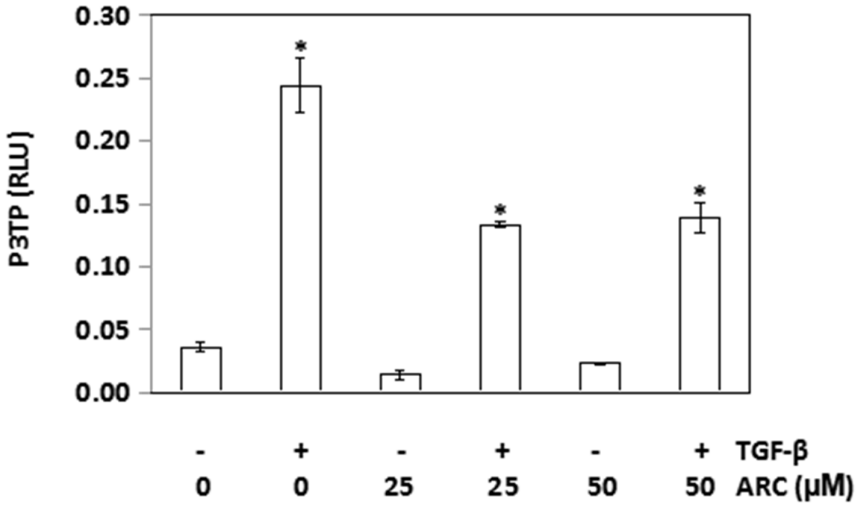


Figure 8. ARC's effect on TGF-β signaling pathway measured by transfecting the reporter p3TP-Lux. A549 cells were transfected with the p3TP-Lux reporter, then pretreated with the indicated concentration of ARC for 24 hours and then stimulated with TGF-β (5 ng/mL) for 24 hours. Luciferase activity was measuring as indicated in Materials and Methods. The data are expressed as mean ± SD for triplicates. *$p<0.05$ versus control.

3.9 ARC inhibits translocation of phospho-SMAD2/3 during TGF-β-induced EMT.

During TGF-β-induced EMT, the activated TGF-β receptor phosphorylates SMAD2 and SMAD3, which then complex with SMAD4 and translocate to the nucleus [67]. Therefore, we examined the ARC's effect on translocation of phospho-SMAD2/3 during TGF-β-induced EMT, by which to further verify ARC's effect on TGF-β-induced EMT.

In this experiment, A549 cells were exposed to ARC for 24 hours and then co-treated with 5 ng/mL TGF-β for 6 hours. Cells were harvested, cytoplasm protein and nuclear protein were extracted separately and subjected to Western Blot analysis. The expression of tubulin and U1snRNP were used as markers for cytoplasmic and nuclear protein, respectively.

The result showed that ARC effectively inhibits the TGF-β-induced translocation of phosphorylated SMAD2 and SMAD3 from cytoplasm to nucleus (Fig.9), which indicates that ARC does inhibit TGF-β-induced EMT in A549 cells.

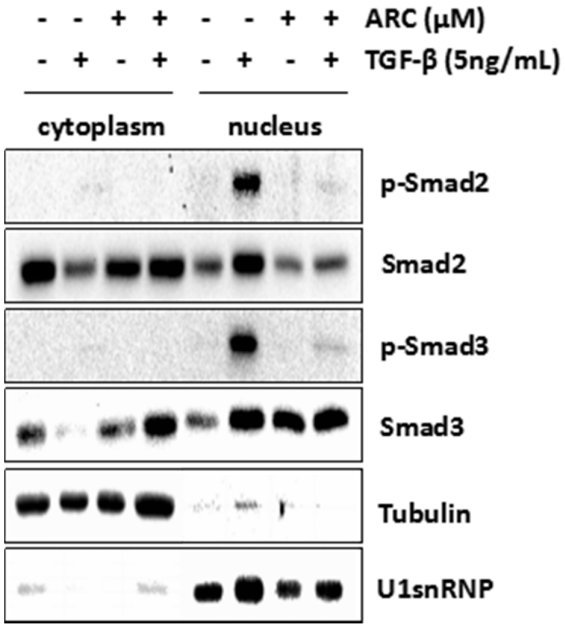


Figure 9. ARC inhibits translocation of phospho-SMAD2/3 during TGF-β-induced EMT. A549 cells were exposed to ARC for 24 hours and then co-treated with 5 ng/mL TGF-β for 6 hours. Cells were harvested, cytoplasm and nuclear protein were extracted separately and subjected to Western Blot analysis for phosphorylated and total SMAD2/3, tubulin (cytosolic marker) and U1snRNP (nuclear marker).

3.10 ARC inhibits TGF-β-induced phosphorylation of ERK

Since it is reported that ERK is responsible for activation of SMAD2 after TGF-β treatment [68], we proposed that ARC may exert the effect of suppressing TGF-β-induced EMT through inhibiting TGF-β-induced phosphorylation of ERK other than inhibiting TGF-β-induced phosphorylation of SMAD2. In order to verify this proposal, we examined the effect of ARC on TGF-β-induced phosphorylation of ERK in A549 cells.

The result showed that ARC does prevent the TGF-β-induced phosphorylation of ERK (Fig.10), indicating that ARC suppress TGF-β-induced EMT through inhibiting TGF-β-induced activation of ERK.

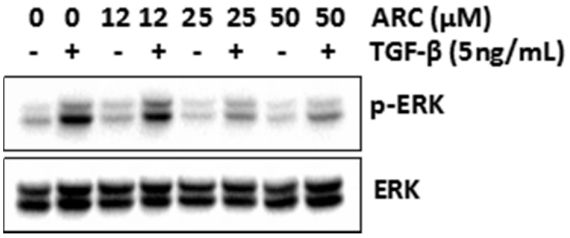


Figure 10. ARC inhibits TGF-β-induced phosphorylation of ERK. A549 cells were exposed to ARC for 24 hours and then co-treated with 5 ng/mL TGF-β for 30 minutes. Western Blot was performed to measure phosphorylated and total ERK.

3.11 ARC inhibits transcriptional activity of β-catenin.

It is reported that β-catenin, which is a multifunctional protein involved in two independent processes: cell to cell adhesion and signal transduction, is released from the E-cadherin complexes into the cytoplasm during EMT where it interacts with other proteins, raising the possibility that β-catenin signaling contributes to EMT [69]. In addition, β-catenin functions as a critical downstream transcriptional activator in the Wnt signaling pathway, and it may translocate into the nucleus where it functions as a cofactor of the T-cell factor family of transcription factors to drive the expression of important cell cycle proteins, oncogenes, and proteases including c-myc, cyclin-D1 [70], and MMP-7 [71]. In summary, β-catenin plays an important role in EMT and its related cell signaling pathway. Therefore, we suppose that ARC may inhibit transcriptional activity of β-catenin, by which to suppress TGF-β-induced EMT in A549 cells.

In this experiment, we transfected TOP/FOP flash luciferase reporter plasmid into A549 cells and then co-treated with TGF-β in the absence or presence of 50 μM of ARC. The result of the subsequent luciferase assay showed that TGF-β increased transcriptional activity of β-catenin by 2.5-fold and 1.8-fold in the absence and presence of ARC, respectively (Fig.11), which indicates that ARC suppresses TGF-β-induced increasing of transcriptional activity of β-catenin, which is also one of the mechanisms of ARC's inhibitory effect on TGF-β-induced EMT.

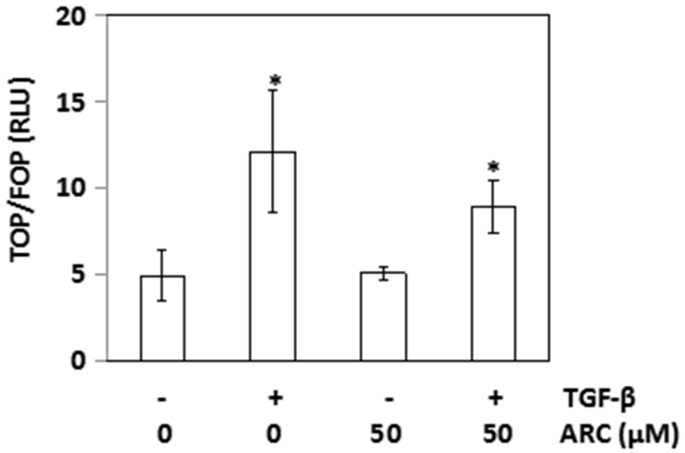


Figure 11. ARC inhibits transcriptional activity of β-catenin. A549 cells were transfected with TOP/flash containing β-catenin/TCF binding site or FOP/flash (negative control) and then co-treated with TGF-β in the presence or absence of ARC for 24 hours. The luciferase activity was measured and expressed as mean ± SD for triplicates. *$p<0.05$ versus control.

Chapter 4: Discussion, propose mechanism and conclusion

4.1 Discussion

Lung cancer is one of the most common cancers in the world, it is a leading cause of cancer death in men and women in the United States. Overall, 17.4% of people in the United States diagnosed with lung cancer survive five years after the diagnosis while outcomes on average are worse in the developing countries due to less access to diagnosis and medical treatment [4].

One of the most important reasons of lung cancer's malignancy, poor prognosis and high death rate is metastasis. Metastasis is the most fatal characteristics of malignancy tumor, which accounted for more than 90% of tumor-related mortality. Distant organ or tissue metastasis is a sign of poor prognosis in patients with lung cancer. Tumor cells metastasis is a very complex process including tumor cell transformation, growth, angiogenesis, invasion, dissemination and survival in the circulation, and subsequent adhesion and colonization the secondary organ or tissue [7].

During metastasis, TGF-β-induced EMT of cancer cells plays an important role. EMT is the process that epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells [8]. During this process, cancer cells gain increased invasive property, by which to break through the basement membrane and enter the bloodstream through intravasation. Later, when these circulating tumor cells (CTCs) exit the bloodstream to form micro-metastases, they undergo MET for clonal outgrowth at these metastatic sites. Thus, EMT and MET form the initiation and completion of the invasion-metastasis cascade [72]. At this new metastatic site, the tumor may undergo other processes to optimize growth. For example, EMT has been associated with PD-L1 expression, particularly in

lung cancer. Increased levels of PD-L1 suppresses the immune system which allows the cancer to spread more easily [73].

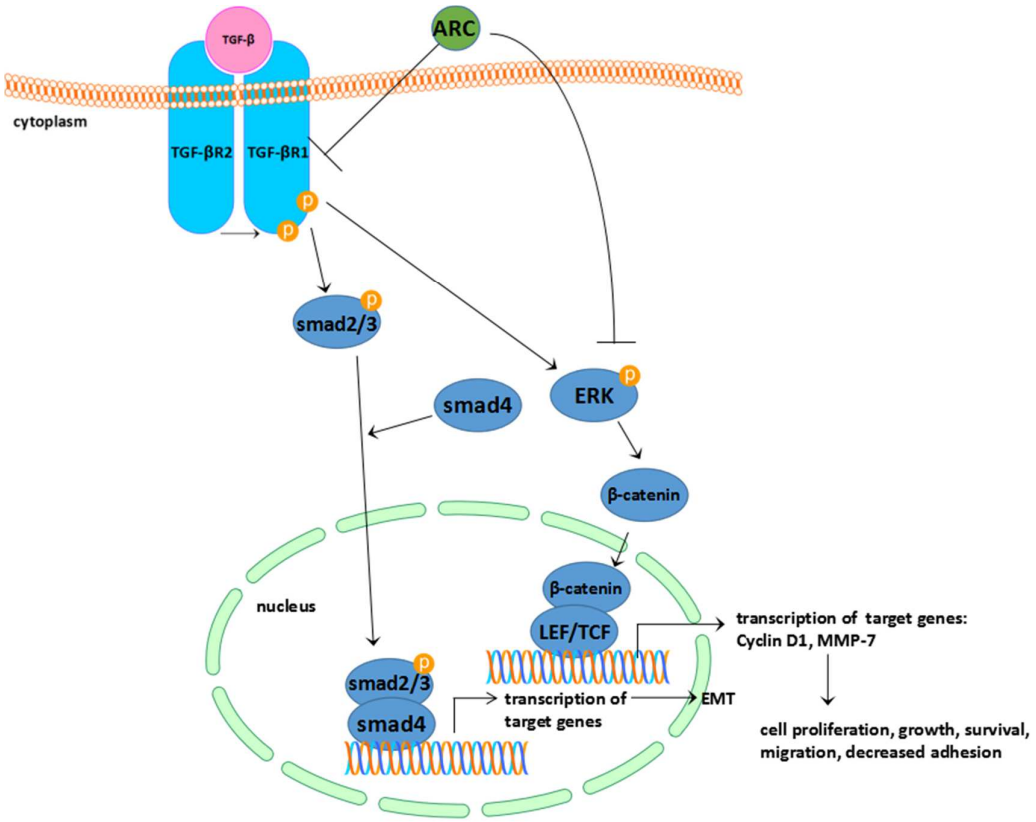
As mentioned above, TGF-β is one of the most important stimulants of EMT. When TGF-β ligands bind to their type II and type III receptors (TGF-β RII and TGF-β RIII), which causes recruitment and phosphorylation of the type I receptor (TGF-β RI). This activates various signaling pathways, including those mediated by SMAD2/3, Ras, and PI3K (simplified here), which activate transcription factors that induce the expression of genes encoding EMT-inducing transcription factors. At the surface of the cell, TACE cleaves the intracellular domain of TGF-β RI, which can then act as a transcriptional regulator to mediate EMT. Additionally, the Par3–Par6–aPKC (atypical PKC) complex also associates with TGF-β Rs at the cell membrane and is involved in cytoskeletal remodeling to promote the mesenchymal phenotype. SMAD-independent pathways (such as through PI3K and ILK) can activate Akt, which in turn can inhibit the function of GSK-3β, a kinase that inhibits nuclear translocation of Snail and β-catenin. Inhibition of SMAD signaling is mediated by SMAD6/7, which prevent the binding and phosphorylation of SMAD2/3 at TGF-β Rs, and by Smurf2, which is known to degrade the activated complex of SMAD2/3/4 [74].

Arctigenin (ARC) is an active ingredient of Arctium lappa and exhibits anti-inflammatory [75], anti-viral [76] and anti-cancer [55] activity in various types of cancer cells. For example, ARC inhibits the growth of colon cancer [77], ovarian cancer [57], hepatocellular carcinoma [78], gastric cancer [62], breast cancer [51], and bladder cancer [55]. The reliable mechanisms include induction of apoptosis [79] or cell cycle arrest [80] through downregulating STAT3 (signal transducer and activator of transcription-3)/survivin signaling [57], phosphatidylinositol-3-kinase (PI3K)/AKT signaling [78], reactive oxygen species (ROS)/p38 MAPK (mitogen-activated protein

kinase) pathway [51], and phosphorylation of retinoblastoma (Rb) [62]. Recently a few studies reported anti-metastatic activity of ARC in breast cancer cells [53, 81] and colon cancer [61].

Here, for the first time, we revealed that ARC significantly suppresses the TGF-β-induced migration, invasion and EMT of NSCLC cells. Our results showed that ARC inhibited TGF-β-induced EMT development in A549 lung cancer cells at concentrations of 25 μM and 50 μM, while no toxic effects toward the cells was observed in these concentrations. Moreover, we found that ARC inhibited TGF-β-induced phosphorylation of SMAD2 and translocation of SMAD2/3 in the TGF-β-induced EMT, as well as TGF-β-induced phosphorylation of ERK, suggesting that ARC prevents EMT and metastasis in the TGF-β-induced signaling pathway by inhibiting SMAD2 phosphorylation as well as ERK phosphorylation. We also found that the increase in snail expression and transactivation of β-catenin via the TGF-β-induced EMT was also inhibited by ARC. These results are consistent with the inverse relationship between E-cadherin and snail/β-catenin in human cancer cells.

4.2. The proposed anti-metastatic mechanism by ARC in lung cancer



As shown above, regarding to cellular and molecular event, TGF-β first binds to type II receptor which leads to recruitment and phosphorylation of the type I receptor. The activated type I receptor subsequently induces phosphorylation of SMAD2 and SMAD3 (R-SMADs) and form heterodimeric complexes with a SMAD4 (co-SMAD). The complex of SMAD2/3/4 translocate into the nucleus and binds to SMAD response element (SRE) on the promoter of their specific target genes, and mediates EMT [19]. According to our results, ARC can suppress TGF-β-induced phosphorylation of SMAD2 by which to prevent the down-stream signaling pathway and the triggering of EMT.

On the other hand, the activated type I receptor subsequently induces phosphorylation of ERK [82], which is required for accumulation of β-catenin in the nucleus [83], where it binds to LEF/TCF transcription factors, displacing co-repressors and recruiting additional co-activators to Wnt target genes such as Cyclin D1 and MMP-7 [84], by which to alter the cells' biological features. For example, increased ability of proliferation, migration, survival, growth, and decreased adhesion property, which are all key factors in triggering and development of cancer. Additionally, β-catenin cooperates with several other transcription factors to regulate specific targets. Moreover, Wnt signaling has also been shown to promote nuclear accumulation of other transcriptional regulator implicated in cancer, such as TAZ and Snail1.

Based on our results, ARC inhibits TGF-β-induced phosphorylation of ERK and SMAD2, by which to exert its anti-cancer effects against human lung cancer cells A549.

4.3 Conclusion

We demonstrated that ARC suppresses progression and metastasis of A549 cells through suppressing TGF-β-induced EMT, with the mechanisms of inhibiting TGF-β-induced phosphorylation of SMAD2 and ERK, suppressing TGF-β-induced translocation of SMAD2/3, and inhibiting transcriptional activity of β-catenin.

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