

ABSTRACT

Title of Document: THE POTENTIAL DICHOTOMOUS ROLE OF
ACTIVATING TRANSCRIPTION FACTOR 3 (ATF3)
IN COLON CANCER

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Colorectal cancer (CRC) is the third leading cause of cancer-related death in the United States. During the tumorigenesis and metastasis of CRC, cells encounter numerous cellular and molecular events. ATF3, a member of the ATF/CREB transcription factor family, plays an important role on regulation of apoptosis and is regarded as a potential molecular target for chemoprevention and chemotherapy of colon cancer. The current study was performed to investigate cellular and molecular mechanisms by which ATF3 affects colon cancer-related phenotypes including apoptosis and metastasis. Here, we demonstrated that knockdown of ATF3 using small interfering RNA (siRNA) promotes the expression of anti-apoptotic protein, B-cell lymphoma 2 (Bcl-2), in colon cancer cells, while overexpression of ATF3 resulted in a dramatic decrease in Bcl-2 protein. Gain of function of ATF3 in colon cancer cell line HCT116 led to an increase of pro-apoptotic protein Bcl-2 homologous antagonist killer (Bak), followed by the induction of apoptosis. Furthermore, we observed that ATF3 overexpression downregulated expression of epithelial-mesenchymal transition (EMT)-related transcription factors. However, mammosphere forming assay indicated that ATF3 overexpressed colon cancer cells form larger and more budding sites compared to control, which is associated with an increase of cluster of differentiation 44 (CD44) expression and a decrease of retinoblastoma (Rb) and tight junction protein zonula occludens (ZO)-1. This study suggested that ATF3 may play a dichotomous role in regulation of apoptosis and metastasis in colon cancer.

THE POTENTIAL DICHOTOMOUS ROLE OF ACTIVATING TRANSCRIPTION
FACTOR 3 (ATF3) IN COLON CANCER

By

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Table of Contents

Table of Contents	ii
List of Figures	iii
Chapter 1. Introduction	1
1.1 Introduction	1
1.2 Overview of cancer development and tumorigenesis.....	2
1.2.1 Tumor cell apoptosis and Bcl-2 protein family	2
1.2.2 Tumor cell cycle	3
1.2.3 Tumor cell metastasis: Single cell migration (EMT) vs. Collective invasion.....	4
1.2.4 Tumor growth and cancer stem cell	7
1.3 The biological activities of ATF3 in cancer development	9
1.3.1 Characteristics of ATF/CREB family of transcription factors	9
1.3.2 Dichotomous role of ATF3 in cancer progression.....	10
Chapter 2. Materials and Methods.....	13
2.1 Cell culture, reagents, and antibodies	13
2.2 Generation of colon cancer cells with ATF3 knockdown or ectopic expression of ATF3	13
2.3 SDS-PAGE and western blot.....	14
2.4 Isolation of RNA and semi-quantitative RT-PCR.....	14
2.5 Mammosphere assay	15
2.6 Boyden chamber single-cell migration assay.....	15
2.7 Detection of apoptotic cells.	16
2.8 Cell cycle analysis	16
2.9 Statistics	17
Chapter 3: The dual biological function of ATF3 in colon cancer progression	18
3.1 Low expression of ATF3 in colon cancer cell lines	18
3.2 ATF3 induces apoptosis in colon cancer	20
3.3 ATF3 modulates Bcl-2 family members.....	21
3.4 ATF3 inversely regulates Bcl-2 expression.	23
3.5 ATF3 has limited effect on cell cycle regulation.	26
3.6 Regulation of EMT-related gene expression by ATF3 in colon cancer cell lines. ...	29
3.7 ATF3 overexpressed cells do not induce single cell migration	32
3.8 Formation of cancer initiating cell features and collective cell invasion.....	34
Chapter 4. Discussion, Conclusions, and Future Perspective	36
4.1 Discussion	36
4.2 Conclusion and Future Perspectives	40
Reference	42

List of Figures

Figure 3.1. ATF3 expression in colon cancer cell lines

Figure 3.2. ATF3 induces apoptosis in colon cancer

Figure 3.3. ATF3 modulates the protein expression of Bcl-2 family genes

Figure 3.4. ATF3 inversely regulates Bcl-2 protein levels.

Figure 3.5. ATF3 has limited effect on cell cycle regulation.

Figure 3.6. Differential regulation of EMT-related gene expression by ATF3 in different colon cancer cell lines

Figure 3.7. ATF3 overexpressed cells do not induce single cell migration

Figure 3.8. Formation of cancer initiating cell features and collective cell invasion

Chapter 1. Introduction

1.1 Introduction

Cancer is a major cause of death in the world. The American Cancer Society estimates that 1,658,370 men and women will be diagnosed and 589,430 cancer deaths will occur in the United States in 2015 (1). Colorectal cancer (CRC) is the third leading cause of cancer mortality in the Western world (1). Cost-effective therapies and preventive approaches to treat colorectal cancer are urgently needed. Chemopreventive strategy using dietary compounds which prevents or delays the onset of several types of cancer, has gained a great deal of attention, (2). Various bioactive phytochemicals, such as flavonoids (3-8), proanthocyanidins (9-11), carotenoids (12-16), isothiocyanates (17-24), and sphingolipids (25-29) have been shown to exert anti-cancer activities in CRC (30). The mechanisms of their chemopreventive actions include targeting specific genes and modulating of oncogenic or tumor suppressive signaling pathways that lead to changes of cell proliferation, differentiation, survival, cell cycle progression, and metastasis (30).

During the course of tumorigenesis, cells encounter numerous cellular and physical stresses (31). Cells respond to stress in various ways ranging from promotion of cell survival to elicitation of the programmed cell death and removal of the damaged cells (32). However, the failure to restrain and eliminate stress signals can increase the risk of cancer (33-35). Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family, which shares basic region-leucine zipper (bZip) DNA binding domain (36). Previous research has demonstrated that ATF3 is encoded by an immediate early gene (36) and its baseline mRNA level is low in normal cells. ATF3 can be induced by various stress stimuli and signals that damage the cells or tissues. These stress signals include hypoxia, anoxia, carcinogens, DNA damage, UV exposure and radiation (36, 37).

Comprehensive research found that ATF3 can participate in several cellular processes and adapt to different extra- and intra-cellular stimuli (36, 38).

In previous studies, we and other groups reported that ATF3 is a molecular target of many anti-cancer compounds and mediates the compound-stimulated apoptosis (39-42). However, several current studies demonstrated that ATF3 could either promote or suppress the cell proliferation and apoptosis in cancer cells (43). Besides, xenograft models showed ATF3 possessed either tumor suppressive or oncogenic activity (44-47). To better understand the role of ATF3 in colon cancer progression and increase the efficacy of anti-tumorigenic compounds targeting ATF3, in this project, we examined the changes of cellular gene expression and cell morphology after overexpression or knockdown of ATF3.

1.2 Overview of cancer development and tumorigenesis

1.2.1 Tumor cell apoptosis and Bcl-2 protein family

Apoptosis is a major mode of programmed cell death, which serves as an essential barrier for cancer development in multicellular organisms. By eliminating the damaged or aged cells, cell death balances the cell proliferation and contributes to the tissue homeostasis, development and immunity (48). The cellular cascade of molecular events involved in apoptosis is highly complex, and they can be categorized into two main pathways: the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway, with evidence indicating that the two pathways are interactive (49).

The function and activity of mitochondrial pathway mainly depends on the dynamic interaction between subgroups of Bcl-2 family proteins on the mitochondrial outer membrane. The Bcl-2 family proteins, which control the mitochondrial membrane permeability, can be further categorized into BH3-only proteins (delivering initiation

signals) (e.g. BIM, PUMA, BAD, BIK), anti-apoptotic proteins (e.g. Bcl-2, Bcl-x, Bcl-XL, Bcl-w, BAG), and pro-apoptotic proteins (e.g. Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk) (31, 48). Accumulation of cytotoxic stresses suppresses the activity of anti-apoptosis proteins, whereas it triggers the pro-apoptotic proteins BAX and BAK to form the oligomers and increases the permeability of mitochondrial outer membrane. As a result, cytochrome c is released to cytosol where they bind to apoptotic protease activating factor 1 (APAF1), recruits caspase 9 enzyme and activate caspase 3 (50). Aberrant expression of Bcl-2 family proteins is widely observed in various malignancies, and therapeutic strategy targeting Bcl-2 family proteins is promising. For example, anti-apoptotic protein Bcl-2 is frequently overexpressed in estrogen receptor-positive breast cancer, and the BH3 mimetic ABT-737 targeting Bcl-2 can improve tumor response to antiestrogen tamoxifen (51).

With better understanding of the signaling cascades, many approaches and assays are available for detecting and counting the apoptotic cells. The annexin V, a Ca^{2+} -dependent phospholipid-binding protein, is widely used as a marker of apoptosis. During early apoptosis, annexin V can bind to phosphatidylserine residues that translocate from inner surface of membrane to the outside of the cell due to the increased permeability (52).

1.2.2 Tumor cell cycle

Cell cycle refers to the sequence of activities carried out by a cell leading to self-division and duplication. It proceeds through the activation of cyclins and cyclin-dependent kinases (CDKs) to ensure successive transition from G1 to S and G2 phase and then the initiation of mitosis. Cyclin/CDK complexes are formed and switched on and off during different phases of the cell cycle progression through phosphorylation of target proteins (53). For example, the Cyclin D and CDK4/6 complex phosphorylates and inactivates its target protein, retinoblastoma and subsequently

cause G1/S transition and stimulate cell cycle (55). Whereas, cyclin A/E and CDK2/1 complex regulates progression through S/G2/M phases (53, 54).

The activities of cyclin/CDK complexes are suppressed by CDK inhibitors, depending on the specificity between target CDK and inhibitor (56). The first class of inhibitors includes the INK4 proteins that bind to catalytic subunits of CDK4 and CDK6, but not other CDKs or D-type cyclins. The second family inhibitors of Cip/Kip proteins like p21 and p27 are more broadly acting, which inhibit all types of cyclin and CDK complexes at any phase without dissociating cyclin–CDK complexes (56, 57).

Structural studies show that p21 and p27 share similar primary structure and exert inhibition effect over the target proteins (58). At the beginning of the catalytic reaction, α -helix of Cip/Kip protein first makes contact with cyclin, followed by a deep insertion of second helix inside the catalytic cleft, and blocks the loading of ATP. Further conformational changes of CDK2 are observed to lock the catalytic cleft to an inactive form (57). Some studies have shown that highly expressed p27 can also inhibit CDK4 activity (59).

1.2.3 Tumor cell metastasis: Single cell migration (EMT) vs. Collective invasion

Metastasis is a collective term which describes a process that a tumor cell leaves the primary tumor, travels to a distant site via the circulatory system, and establishes a secondary tumor. Metastasis is initiated by invasion. During the invasion, cancer cells may (or may not, depends on the migration model chosen) lose cell-cell or cell-matrix attachment which triggers matrix degradation followed by migration. Depending on the cell context and tissue environment, cells use two different models for migration: single

cell migration with the absence of cell-cell attachment, and collective cell migration along with the maintenance of the cell-cell junction. Cancer cells move into lumina blood and lymphatic vessels during intravasation and then circulates in the blood vessel. Many cancer cells will be trapped, however, those escape from the arrest can be transported to the new site (60).

Metastasis is responsible for 80% of death in cancer patients. Invasive tumor cells divide rapidly in an aggressive primary mass and variety of stromal cells in the surrounding microenvironment can be recruited to tumors. The hijacked tumor-associated stroma cells can be educated and in turn, support tumor growth by producing growth factors and proteolytic enzymes (61).

Cells migration occurs in two major modes. Single cell migration, when cell-cell adhesions are absent, and multicellular, collective cell migration, when cell-cell junctions are retained (60). The single cell migration is executed in a series of physicochemical steps within the same cell, which allows the cell body to protrude and generate traction force (60).

Epithelial Mesenchymal Transition

Epithelial Mesenchymal Transition (EMT) is an essential embryonic process that causes the epithelial cells to lose their characteristics and switch to motile mesenchymal ones. This transition involves changes in cell morphology, size and behavior that is characterized by loss of cell junctions and apical-basal polarity, followed by the acquisition of a fibroblastic motility. These changes lead to a degradation of extracellular matrix and facilitate the cell migration and invasion (62-64). EMT is initiated by the change in gene expression profile. The main EMT-associated transcription factors include of the Snail, Slug, ZEB-1, SIP-1 and Twist

(64-68). Although a majority of EMT transcription factors are highly up-regulated at early EMT, each type shows distinct expression profiles depending on the cellular context, tissue type and progression stage of EMT. In addition, they control reciprocally and synergistically target a specific gene, thus leading to either repression of the epithelial genes or activation of mesenchymal genes (67, 68).

Activation of EMT transcription factors leads to a downregulation of E-cadherin (epithelial marker) and an upregulation of N-cadherin (mesenchymal marker), thereby decreasing the cell adhesion. For example, SNAIL proteins, such as Snail and Slug, repress transcription of the E-cadherin by directly binding to E-box DNA sequences in the promoter region. This transcriptional suppression is associated with histone modifications of Polycomb repressive complex2 (PRC2), specifically methylation and acetylation at histone H3 Lys4 (H3K4), H3K9 and H3K27 (68). Additionally, SNAIL can also induce the mesenchymal phenotype by activating N-cadherin.

Multiple signaling pathways have been implicated to regulate the initiation and progression of EMT by either activating or suppressing SNAIL expression. Glycogen synthasekinase-3 β (GSK3 β) directly phosphorylates Snail at two Ser-rich consensus motifs and inactivates its transcriptional activity (68). However, Wnt and PIK2-AKT pathway activate transcriptional activity of SNAIL by suppressing GSK3 β activity (69-71). Activation of Wnt and EMT factors lead to an increase of stability of cytoplasmic β -catenin (68). Increased β -catenin translocate to nucleus where it forms β -catenin-TCF-4 complex and promotes the transcription of target gene (72).

Similarly, Twist expression is associated with the E-cadherin and N-cadherin gene expression along with SNAIL. Twist can be up-regulated by transcription factor hypoxia-inducible factor 1 α (HIF1 α) under hypoxia conditions, inducing angiogenesis

(73). Additionally, TWIST can also interact with methyltransferase SET8, thus mediates the H4K20 monomethylation, which is a histone mark linked to repression of E-cadherin and promotion of N-cadherin (74).

Collective invasion

Over the decades, scientists have studied the possible mechanisms underlying single cell migration in both normal and malignant cells, involving the governing of cell adhesion and cytoskeleton dynamics (75-77). However, they failed in defining the rate-limiting mechanisms, such as dominant signaling pathway, receptor-ligand interaction, or protease-substrate interaction, which regulate invasive cancer cell migration. As an alternative, cancer invasion is now considered as a heterogeneous and adaptive process. The “plasticity” of invasion in cell adhesion, cytoskeletal dynamics, and mechanotransduction, together with other hallmarks of cancer, orchestrates morphological, signaling, and genetic alteration, and help the adaptation of cancer cells in even adverse tumor microenvironment (77-85).

Collective invasion requires cell-cell adhesion as well as coordination between various cells, which results in the co-existence of multicellular groups at the interface between tumor and stroma (85-87). Collective invasion exhibit different morphologies depending on the cell types, the number of jointly moving cells, and the tissue structure (85). For example, cells may form small clusters, solid strands or even an inner lumen, if epithelial polarity is retained. In some other ways, collective invasion can also present with a bud-like protruding site consisting of multiple cells with variable positions (60, 85).

1.2.4 Tumor growth and cancer stem cell

Carcinomas are cancers and malignancies that caused by multistep progression from benign low-grade adenomas. Researchers using different models have shown that unrestricted growth occurred in both benign and malignant tumors during complex tumor-progression process can be attributed to cancer stem cells (88). Physiologically, stem cells are undifferentiated cells that are capable of differentiating to all tissues during embryonic development, resulting in both self-renewal and differentiation to second daughter cell. Variety of signaling pathways are involved in the generation of stem cells (89, 90). Mutations in crucial pathways, such as Notch, Wnt and Hedgehog pathways, can cause normal stem cells to function abnormally, and accumulation of these genetic alterations can be responsible for malignancy (91). These malignant stem cells are considered as cancer stem cells (CSCs).

CSCs are considered as the driving forces for tumor growth malignancy, and the seeds for metastatic expansion of the tumor and cancer reoccurrence after surgery or chemotherapy (92). Certain characteristics were associated with CSC-enriched cancer cell populations *in vitro* (93): (i) CSCs in solid tumors can be identified using an extensive list of cell-surface markers (94-99). For example, colon cancer stem cell populations are rich in cell surface proteins CD133, CD24 and CD44 markers, which are usually associated with aggressive cancer types and poor prognosis (100). (ii) CSC-enriched cell populations are prone to form tumor mammospheres or tumorspheres (101), which are spherical colonies in suspension cultures. (iii) CSC-enriched cell populations are hard to eliminate, which are associated with increased resistance to chemotherapeutic agents and radiotherapy (102-107).

CSCs protein marker CD44 is a transmembrane glycoprotein, together with its alternatively spliced variants, functions as receptors of hyaluronan and connects the actin cytoskeleton by the adaptor protein. CD44 expression is essential for

maintaining the cancer stem cell phenotype and promoting primary tumor growth of mammary cells (157). In particular, CD44 expression is essential for collective cell migration and subsequent metastatic progression initiated by loss of Rb function (138). Rb can suppress collective invasion, circulation and metastasis of cancer cells in CD44-dependent manner (138).

1.3 The biological activities of ATF3 in cancer development

1.3.1 Characteristics of ATF/CREB family of transcription factors

Activating transcription factor, or ATF, was first identified in 1987 by Lee and his colleagues as a factor that can activate the transcription of the E1A-inducible adenovirus promoters E2A, E3, and E4 (109). In the same year, Montminy and Bilezikjian characterized a nuclear protein that can bind to the cAMP response element (CRE) in the somatostatin gene, and they named it CRE binding protein or CREB. Later, ATF was found to be identical to the CREB, since ATF directly binds to the consensus sequence 5'-TGACGTCA-3' and transactivates cAMP-inducible somatostatin gene (110). Since then, multiple factors that bind to this consensus sequence have been isolated (38). Those factors constitute ATF/CREB family with nearly 20 members and were classified into subgroups according to their amino acid similarity: the CREB/CREM, CRE-BP1 (commonly known as ATF2), ATF3, ATF4, ATF6 and B-ATF subgroups (38, 43).

All ATF/CREB proteins share similar basic region-leucine zipper (bZip) DNA binding domain, within each subgroups (38). ATF/CREB proteins form homodimers and selective heterodimers with other bZip proteins (for example, the AP-1 and C/EBP families of proteins) (36, 38). Despite their structural similarity, ATF/CREB proteins exhibit various different biological functions because different types of

homo- or hetero-dimers determine DNA binding specificity and transcriptional activities of their genes (38). One common biological response of the ATF/CREB family is induction of a variety of stress signals in the cells (44).

The human ATF3 was first cloned by Dr. Hai and her colleagues in 1989 from cDNA library prepared of HeLa cells (111). The human ATF3 gene is localized in chromosome 1q32.3, and derived from four exons (as exons A, B, C, and E) with over 15 kilobases (112). Exon A contains the 5'-untranslated region with a length of 167bp. Exon B includes the AUG initiation codon and encodes first 80 amino acids of ATF3. Exon C encodes 36 amino acids that made up by basic region. Exon E encodes 65 amino acids, which contains both the leucine zipper (ZIP) domain and the 3'-untranslated region. Each exon B, C, and E each encodes a functional domain.

Like other ATF/CREB family members, ATF3, is a transcriptional factor and can both activate and repress the transcription of the target gene through formation of either hetero- or homo- dimers. ATF3 homodimer auto-represses itself transcriptionally by recruiting inhibitory cofactors to its own promoter whereas ATF3 heterodimer can either activate or repress the transcription of target gene (36, 113-116).

1.3.2 Dichotomous role of ATF3 in cancer progression

ATF3 expression is induced by various cellular stresses such as hypoxia, anoxia, carcinogens, cytokines, genotoxic agents, and cell death-inducing compounds, which suggested that ATF3 expression may associate with and mediates diverse biological consequences. Currently, the debate on the role ATF3 plays in the cancer progression is rather conflicting and confusing. The following part will briefly review the several biological processes associated with ATF3 expression.

ATF3 and apoptosis

Apoptosis, referred as cell programmed death, which is an essential cellular process in the normal cell progression and maintain the hemostasis in the body. The resistance to apoptosis is significant mechanism of malignance and poor prognosis in many types of human cancers. Quite a few research evidences studied the role of ATF3 in apoptosis; nevertheless, a conclusion is still obscure.

ATF3 expression remains low in many cancer cell lines (36, 113). However, stable or transient overexpression of ATF3 increased caspase protease activation and promoted the etoposide or camptothecin induced apoptosis in HeLa cells (117). Furthermore, ATF3 expression can be induced by PI3K inhibitor, LY294002, followed by the induction of apoptosis in several colorectal cancer cell lines (118). ATF3 is molecular target of many anti-cancer compounds such as epicatechin gallate (39), indole-3-carbinol (40), genistein (119), 5'-FU (120), Zerumbone (121), Dexrazoxane (122), sulindac sulfide (123), tolfenamic acid (124), HDAC inhibitor (125), troglitazone (45), metformin (126). Consistently, in *in vivo* condition, expression of ATF3 is linked with functional defects in several target tissues. Transgenic mice expressing *ATF3* showed dysfunction in the liver and defects in endocrine pancreas development (127, 128). Overexpression of ATF3 in the heart displayed atrial enlargement and ventricular hypertrophy (129), which is associated with pro-apoptotic functions of ATF3.

On the other hand, ATF3 was also reported to play a role in protecting the cells from apoptosis. It was reported that overexpression of ATF3 by adenovirus can inhibit the apoptosis through activating cell survival signal such as mitogen-activated kinase kinase 1 (MEKK1) c-Jun N-terminal kinase (JNK)-dependent pathway and enhanced neurite elongation through Akt activation in PC12 cells (130). ATF3 also protected human umbilical vein endothelial cells from tumor necrosis factor (TNF)- α -induced

apoptosis (122, 131). Recently, Yin reported that ATF3 enhances apoptosis in the untransformed MCF10A mammary epithelial cells, whereas protects the aggressive MCF10CA1a cells and enhances its cell motility (132). Taken together, the role of ATF3 in apoptosis depends on the cell type, tissue context.

ATF3 and metastasis

The first paper studying a role of ATF3 in metastasis came out in the late of twenty century. Ishiguro's group found that transfection of ATF3 into the low metastatic clone F1 can switch the parental cells from low- to high- metastatic cells (133). In their follow-up research, ATF3 antisense oligonucleotide changed cell morphology and enhanced migration of HT29 colon cancer cells, and improved mouse survival *in vivo* (46). Another research group also found that ATF3 was highly expressed at higher levels in the cell lines derived from metastatic sites than in those from original tumor sites when they are screening cancer cell lines and surgically excised human colon cancer samples (134). Bottone's group found overexpression of full-length ATF3 protein in colorectal cancer cells was associated with decreased tumor cell invasion, indicating a anti-tumorigenic role for ATF3 (45). However, recently study indicated the involvement of ATF3 in metastasis. A paper published recently found that ATF3 knockout mice have less cancer metastasis, indicating that ATF3 facilitated metastasis (135). Breast cancer model showed that ATF3 protects the high-grade malignant breast cells MCF10CA1a and enhances its cell motility by promoting transcription of the TWIST1, FN-1, Snail and Slug genes (132). ATF3 also promoted *in vitro* motility and invasion in both HT29 and CaCO2 cells (136).

Chapter 2. Materials and Methods

2.1. Cell culture, reagents, and antibodies

Human colorectal adenocarcinoma cells HCT116, LoVo, CaCo2, SW480, HT-29 were purchased from American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS). Normal human colon cells, CCD112CoN was purchased from ATCC and grown in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% FBS. The cells were maintained at 37 °C under a humidified atmosphere of 5% CO₂. Primary and secondary antibodies are obtained from the following source: anti-ATF3 (Santa Cruz, Santa Cruz, CA), anti-Bcl-2 (BD Biosciences, San Jose, CA), anti-Bim, anti-Bcl-xL, anti-Bak, anti-Bad, anti-Bax, anti-Cyclin D1, anti-p21, anti-p27, anti-Gsk3beta, anti-beta-catenin, anti-p-ERK, anti-p-Akt, anti-Rb, anti-CD44, anti-ZO-1, and anti-beta-actin (Cell Signaling, Beverly, MA).

2.2. Generation of colon cancer cells with ATF3 knockdown or ectopic expression of ATF3

Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The pCG-ATF3 expression construct was generously provided by Dr. T Hai (Ohio State University, Columbus, OH, USA). pcDNA3.1 his/v5 empty vector was used as control. HCT116, HCT15, SW480 and HT29 cells were plated in 6-well plates at the concentration of 4×10^5 cells/well. The next day, plasmid mixtures containing 2.5 µg of ATF3 promoter linked to luciferase and 2.5 µg of pcDNA3.1 his/v5 vector were transfected for 48h, respectively.

Endogenous knockdown of ATF3 was performed using TransIT-TKO transfection reagent (Mirus) according to the manufacturer's instruction. 10 µl of TransIT-TKO

reagent was added into 100 μ L of Opti-MEMI Reduced-Serum Medium (Solution A). Then control (cont) small interfering RNA (siRNA; 100 nM) or ATF3 siRNA (siATF3; 100 nM) was mixed with Solution A (Solution B and Solution C). HCT116 cells were transfected with Solution B and Solution C for 24 hours.

2.3. SDS-PAGE and western blot

Cells were washed with ice-cold 1 \times phosphate-buffered saline (PBS), and cell pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO). The cell suspension was centrifuged at 12,000 \times g for 10 min at 4°C. Protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated on 8%, 10%, 12% or 14% SDS-PAGE, transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN, USA) and blocked for non-specific binding with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were probed with specific primary antibodies in 5% nonfat dry milk at 4 °C overnight and then with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h 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, USA).

2.4. Isolation of RNA and semi-quantitative RT-PCR

Total RNA was prepared using a RNeasy Mini kit (Qiagen). Total RNA (1 μ g) was reverse-transcribed with the Verso cDNA kit (Thermo Scientific) according to the

manufacturer's instruction. PCR was carried out using ReadyMix Taq polymerase (Sigma).

Primers used in this study

Gene	Forward (5'-3')	Reverse (5'-3')
ATF3	GTTTGAGGATTTTGCTAACCTGAC	AGCTGCAATCTTATTTCTTTCTCGT
GAPDH	GGGCTGCTTTTAACTCTGGT	TGGCAGGTTTTTCTAGACGG
E-Cadherin	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC
N-Cadherin	ACAGTGGCCACCTACAAAGG	CCGAGATGGGGTTGATAATG
Vimentin	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC
Snail	CCTCCCTGTCAGATGAGGAC	CCAGGCTGAGGTATTCCTTG
Twist	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG
Slug	GGGGAGAAGCCTTTTTCTTG	TCCTCATGTTTGTGCAGGAG

2.5. Tumorsphere assay

Cells were plated at 20,000 cells/mL and grown in 1% methylcellulose on poly-HEMA coated 6-well plates in DMEM supplemented with 10% serum and 20 ng/ml hEGF. Suspension cultures were cultured for 14 days. Then, microscope images were taken by phase-contrast microscope and tumorsphere-forming ability was quantified by ImageJ.

2.6. Boyden chamber single-cell migration assay

Cells (1×10^5) were plated on 8 μ m pore size Transwell filters in either DMEM or RPMI medium and allowed to migrate for 24 hours. The transmigrated cells were detected with Hoechst fluorescent dye and counted.

2.7 Detection of apoptotic cells.

The DNA contents for control and ATF3 overexpressed HCT-116, HCT15, SW480, and HT29 cells were determined by fluorescence-activated cell sorting (FACS) as previously described. For detection of apoptosis by ATF3 overexpression, HCT-116 cells were stained with FITClabeled Annexin V and propidium iodide using Annexin V-FITC apoptosis detection kit (BD Biosciences PharMingen, San Diego, CA) according manufacturer instruction. Briefly, HCT-116 cells were plated at 3×10^5 per well in six-well plates and transfected with pCG-ATF3 by FuGENE 6 (Roche, Indianapolis, IN) for 24 hours. Subsequently, the cells were grown for 24 hours and stained with Annexin V-FITC and propidium iodide. A total of 10,000 cells were examined by flow cytometry using a Beckman Coulter Epixs XL equipped with ADC and ModFit LT software. Cells were gated on side scatter and forward scatter to exclude debris. Doublets were eliminated using peak versus integral analysis. Annexin V-positive/propidium iodide-positive and Annexin V-positive/propidium iodide-negative cell populations were determined as apoptotic cells from the total gated cells.

2.8. Cell cycle analysis

Analysis of cell cycle progression was performed by using flow cytometry. The confluent 2-day HCT116 cells were harvested. Then, harvested cells were fixed with 70% ethanol for 2 h at 4 °C, washed with PBS, and centrifuged. The resulting pellet was stained with 40 µg per mL propidium iodine solution containing 1 mg per mL RNase A at 37 °C for 30 min. The cell cycle progression of samples 10,000 cells was analyzed using flow cytometer according to the manufacturer's instructions.

2.9. Statistics

Statistical analysis was performed with IBM SPSS and the data was analyzed by Student t test. Data was expressed as means \pm SD and differences were considered significant at $p \leq 0.05$.

Chapter 3: The dual biological function of ATF3 in colon cancer progression

3.1 Low expression of ATF3 in colon cancer cell lines

Previous evidences indicated that ATF3 is stress-inducible gene, however its basal mRNA and protein level is nearly undetectable in most colon cancer cell lines. In order to compare basal expression of ATF3 in colorectal cancer cell lines with different genetic backgrounds, we examined the mRNA and protein level of endogenous ATF3 in multiple colon cancer cell lysates including normal human colon CCD-112CoN, human colon adenocarcinoma HCT116, SW480, LoVo, CaCO₂, HCT15 and HT29. Indeed, we found ATF3 mRNA expression was almost undetectable in normal human colon CCD-112CoN, colon cancer cell line HCT116, SW480 at mRNA level by RT-PCR (Figure 3.1A). Protein level of ATF3 was detected by western blot. Similarly, low level of indigenous ATF3 was observed in HCT116, HCT15, HT29 and SW480 (Similarly 3.1.B), however higher expression of ATF3 was observed in LoVo and Caco-2 cells. Based on these results, we choose HCT116, SW480, HT15, and HT29 to further investigate the effect of ATF3 in colon cancer.

Figure 3.1. ATF3 expression in colon cancer cell lines

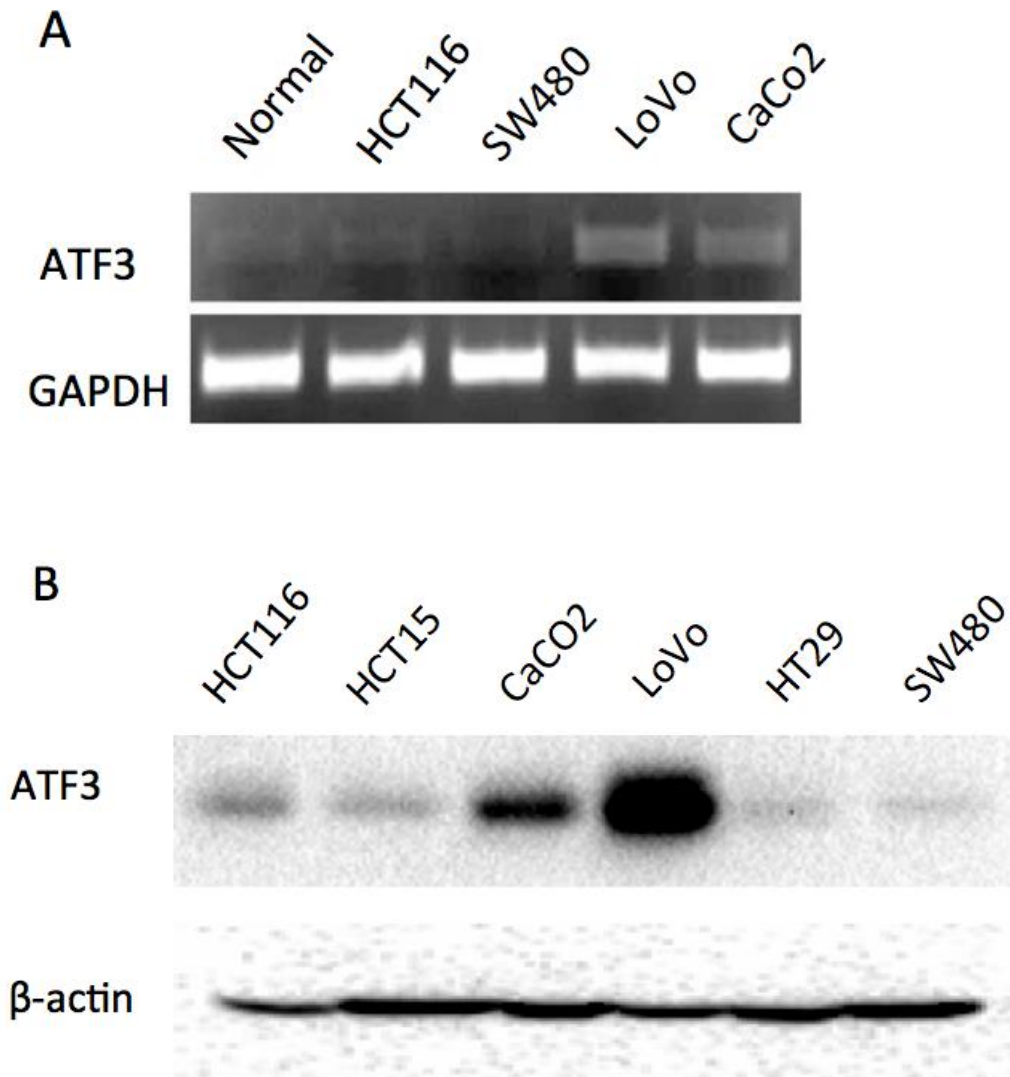


Figure 3. 1. ATF3 expression in colon cancer cell lines. (A) Colon cancer cell lysates were harvested and semiquantitative reverse transcriptase (RT)-PCR was performed for ATF3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Western blot was performed to analyze the basal expression level of ATF3 and β -actin in colon cancer cells.

3.2 ATF3 induces apoptosis in colon cancer

To determine whether gain of function of ATF3 in colon cancer cells can induce the apoptosis, we stained HCT116 cells with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. We observed significance for the apoptotic cell populations after transfection with pCG-ATF3. Result was calculated by Student's t test.

Figure 3.2. ATF3 induces apoptosis in colon cancer

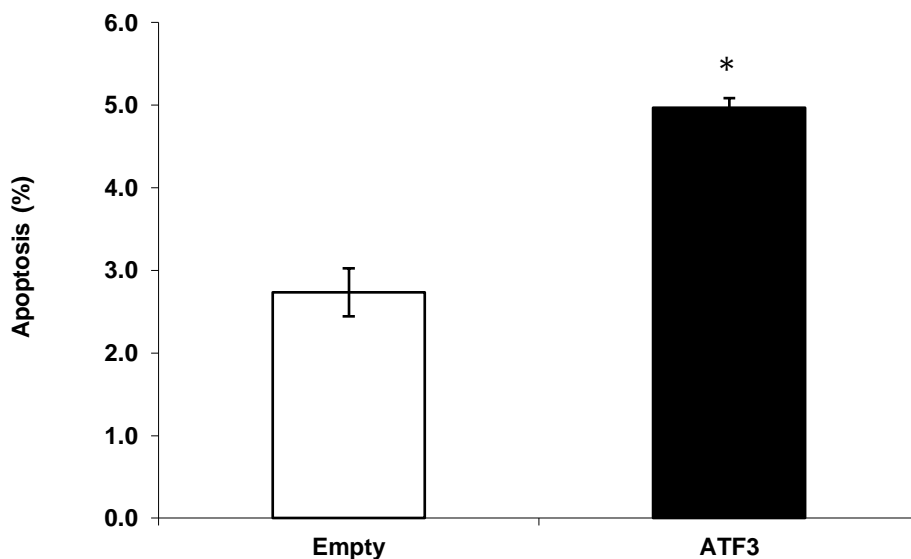


Figure 3.2 Overexpression of ATF3 induces apoptosis in HCT116 cells. HCT-116 cells were transfected with empty and pCG-ATF3 expression vectors. The cells were grown for 24 hours and subsequently stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. Columns, mean from three independent transfections; bars, SD. Apoptotic cell populations after transfection with pCG-ATF3 or empty vector were calculated by Student's t test; *, $P < 0.01$, versus pcDNA3-transfected cells.

3.3 ATF3 modulates Bcl-2 family members

Next we tested if ATF3-mediated apoptosis in human colorectal cancer cells is associated with Bcl-2 family proteins. Then western blot was performed to detect the protein level of Bcl-2 family members. As shown in Fig. 3.3, overexpression of ATF3 decrease the protein level of Bcl-2, whereas increase the expression level of Bak. No change was detected in other Bcl-2 family members. This result provides evidence of potential interaction between ATF3, Bcl-2 and Bak.

Figure 3. 3. ATF3 modulates the protein and mRNA expression of Bcl-2 family genes

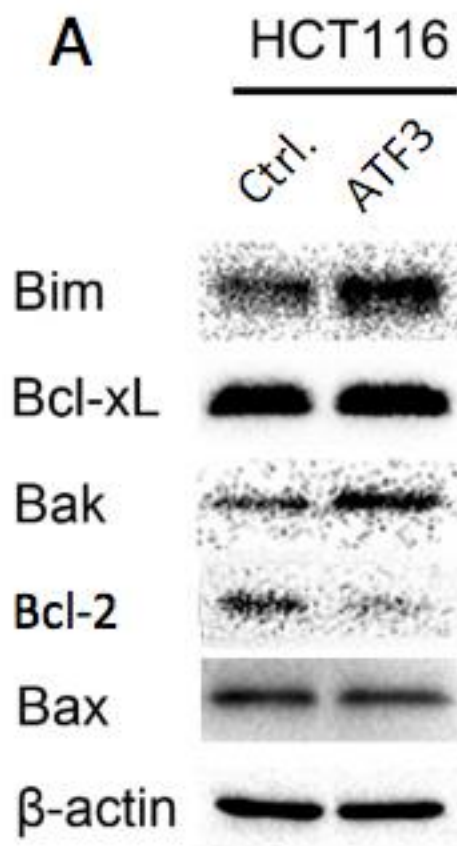
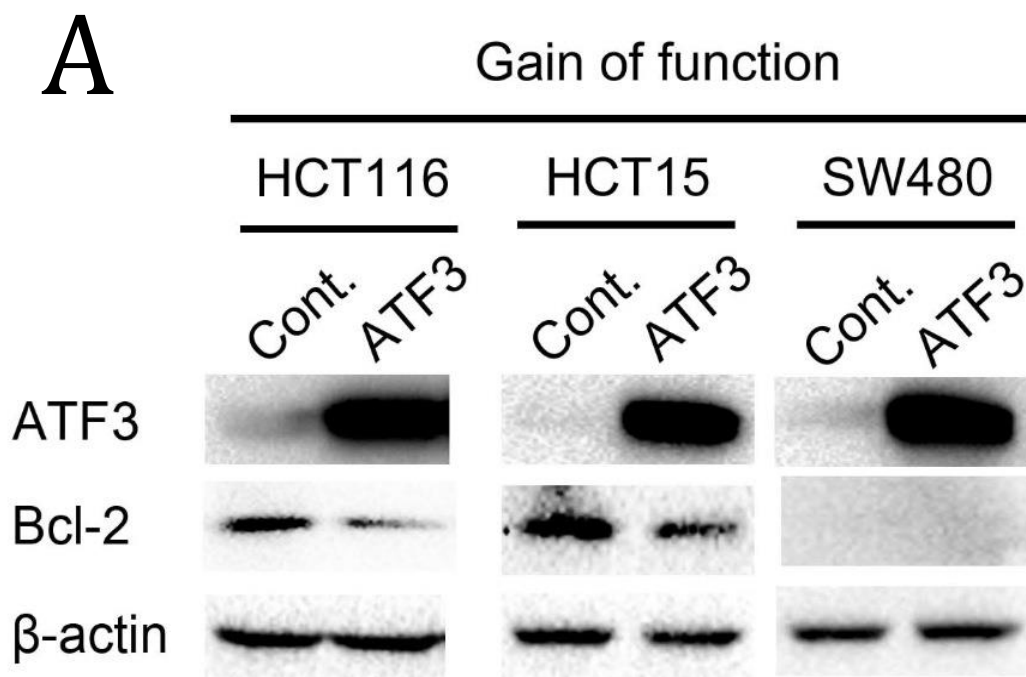


Figure 3. 3. ATF3 modulates the protein and mRNA expression of some Bcl-2 family genes (a) ATF3 expression vector was transfected into colon cancer cells HCT-116 using lipofectamine2000 for 48 hours and then the cells were harvested. Western blot analysis was performed for Bim, Bcl-xl, Bak, Bcl-2, Bax and beta-actin.

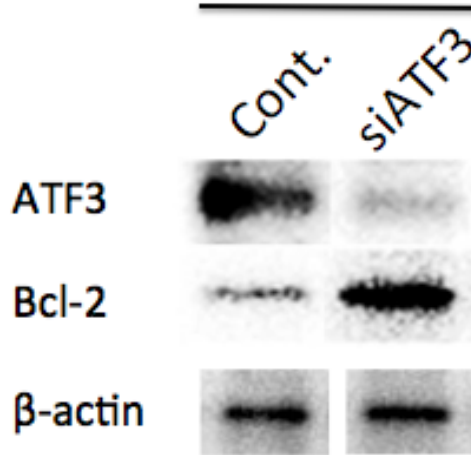
3.4 ATF3 inversely regulates Bcl-2 expression.

To further determine whether ATF3 mediates the expression of Bcl-2, we overexpressed ATF3 in three different types of colorectal cancer cells (HCT116, HCT15 and SW480) and carried out western blot analysis for ATF3, Bcl-2 and β -actin. Decreased of Bcl-2 expression was observed after ATF3 expression in HCT116, HCT15, but SW480 cells did not express Bcl-2 (figure 3.4A). Meanwhile, we knockdown endogenous ATF3 in HCT116 cell line using small interfering RNA (siRNA) targeting ATF3 (siRNA). Inversely, Bcl-2 protein was elevated when ATF3 was suppressed (figure 3.4B). To access whether these changes occurred at a transcriptional level, a semi-quantitative RT-PCR was performed. As shown in Figure 3.4C, overexpressed ATF3 resulted in decrease in the Bcl-2 on mRNA expression level. Whereas, Bak was increased in ATF3 overexpressed HCT116 cells (Figure3.4D) in mRNA level.



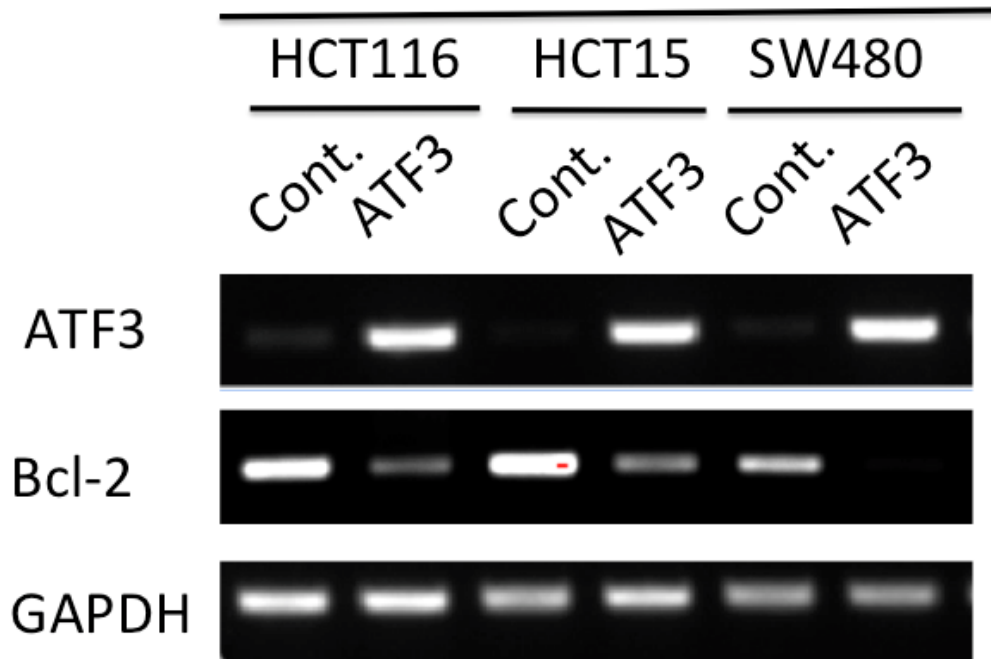
B

Loss of Function



C

Gain of function



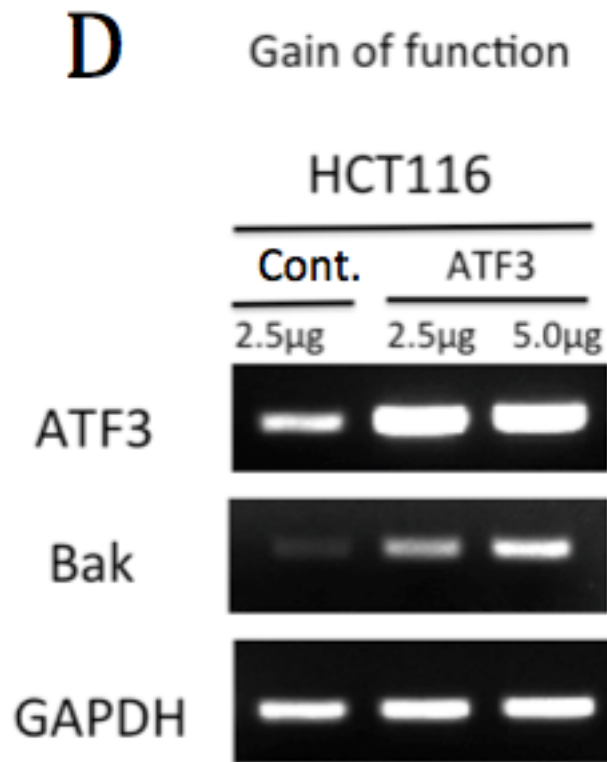


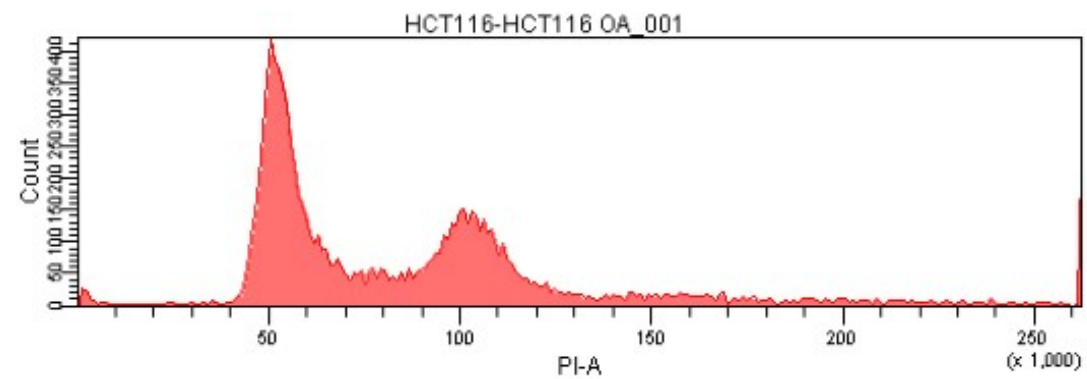
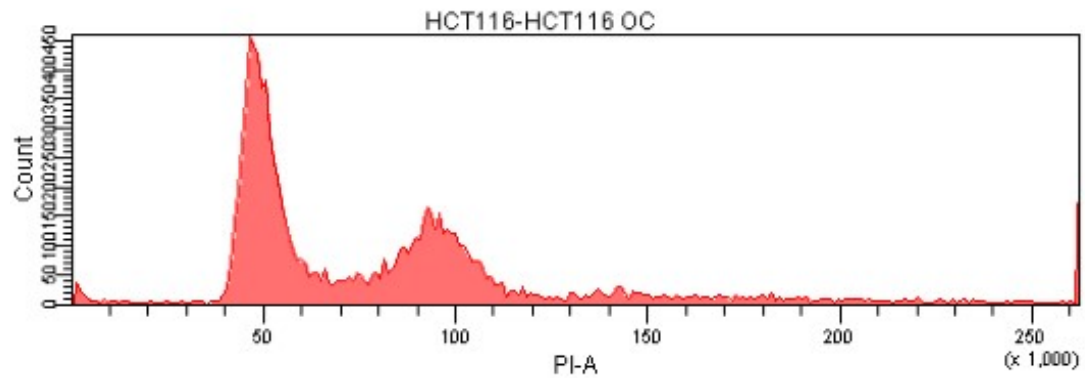
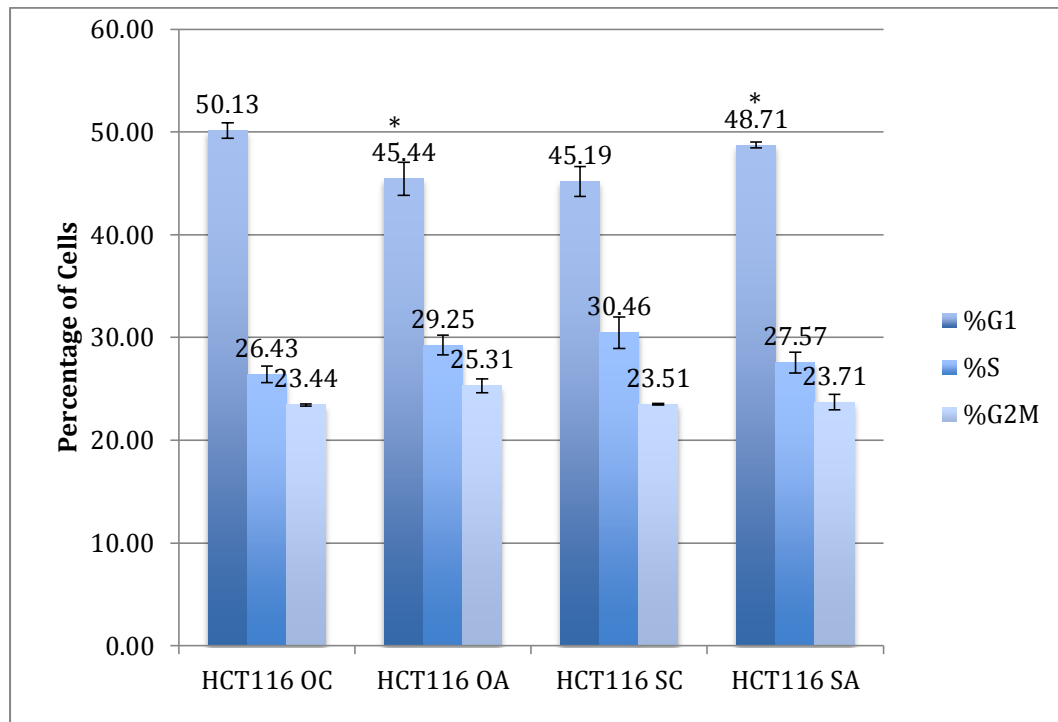
Figure 3.4 Overexpression and knockdown of ATF3 inversely regulate Bcl-2 protein levels. (A) Control or pCG-ATF3 expression vector was transfected into colon cancer cells HCT116, HCT15 and SW480 using lipofectamine2000 for 48 hours and then the cells were harvested. Western blot analysis was performed for ATF3, Bcl-2 and β -actin. (B) HCT-116 cells were transfected with control (cont) small interfering RNA (siRNA; 100nM) or ATF3 siRNA (siATF3; 100nM) for 24 hours using a TransIT-TKO transfection reagent. Western blot analysis was performed for ATF3, Bcl-2 and beta-actin. (C) Control or pCG-ATF3 expression vector was transfected into colon cancer cells HCT116, HCT15 and SW480 using lipofectamine2000 for 48 hours and then RT-PCR was performed for ATF3, Bcl-2 and GAPDH.(D) Control or pCG-ATF3 expression vector was transfected into colon cancer cells HCT-116 using lipofectamine2000 for 48 hours and then RT-PCR was performed for ATF3, Bak and GAPDH.

3.5 ATF3 has limited effect on cell cycle regulation.

According to literature, protective activity of ATF3 in invasive breast cancer cells is mediated by activating cell cycle arrest (Yin, 2008). To determine whether ATF3 affects cell cycle in colon cancer, we analyzed the fraction of each phase in cell cycle after ATF3 expression using FACS analysis and western blot. As shown in Figure 3.5 A, restoration of ATF3 in HCT116 cells results in a slight decrease in G1 phase, but a gain in S and G2-M phase. Inversely, suppression of ATF3 in HCT116 cells resulted in minimal G1 arrest, and decreased S and G2-M phase. However, we did not observe any changes associated with cell cycle regulatory gene such as cyclin D1 and cyclin-dependent kinase (CDK) inhibitor p21, p27 (Figure 3.5 C). These data suggest that ATF3 may have minimal effect on the cell cycle regulation.

Figure 3.5. ATF3 has limited effect on cell cycle regulation.

A



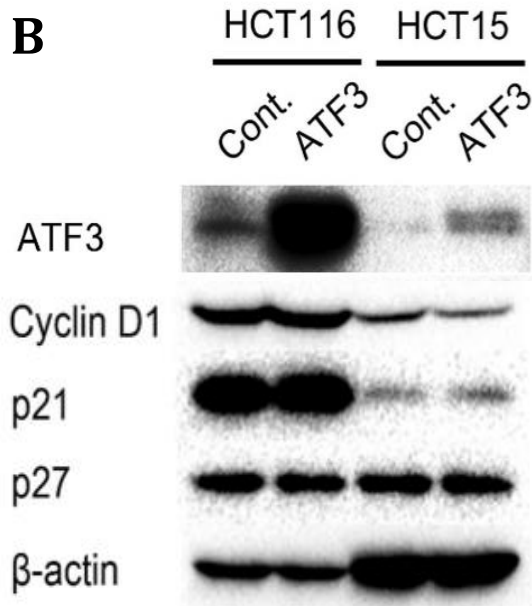
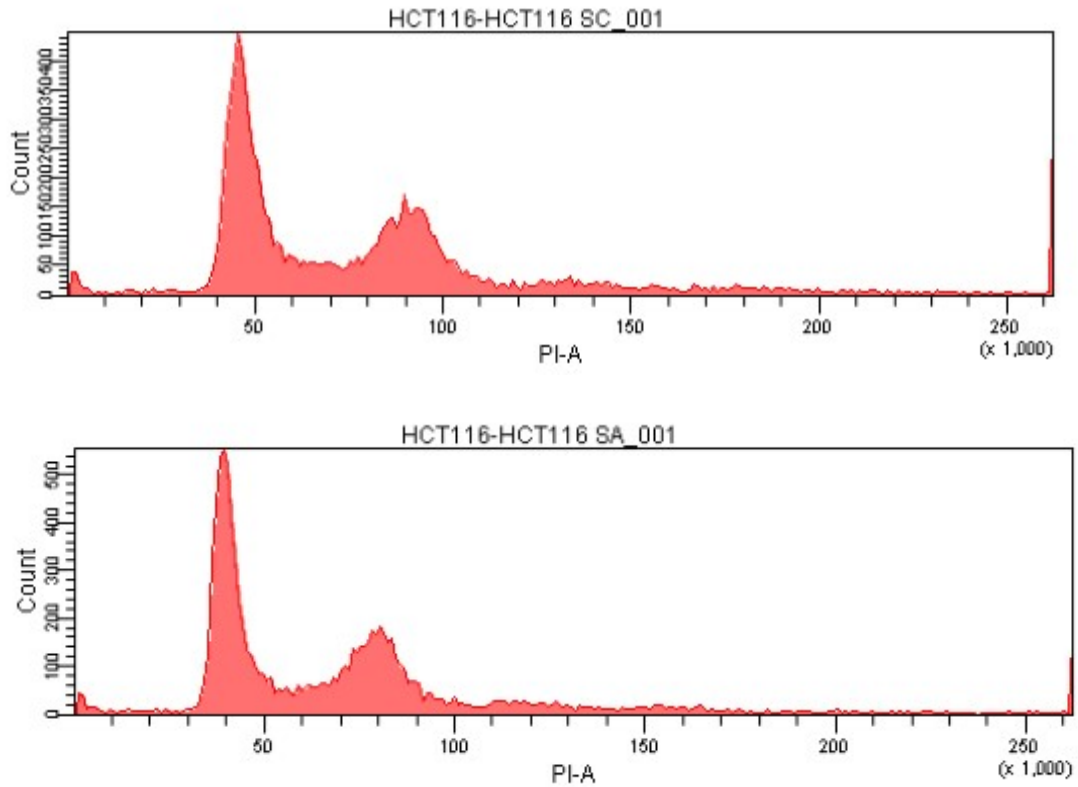


Figure 3.5. ATF3 has limited effect on cell cycle regulation. (a) HCT116 cells were transfected with control vector (OC), ATF3 vector (OA), control knockdown (SC), ATF3 knockdown (SA) for 48h and then cultured and analyzed by FACS. Values are means \pm SD, n=3. Means without a common letter differ, $P \leq 0.05$. (b) ATF3 was transfected into colon cancer cells HCT116 and HCT15 using PolyJet for 48 hours and then the cells were harvested. Western blot analysis was performed for ATF3, Bcl-2, Cyclin D1, p21, p27 and beta-actin.

3.6 Regulation of EMT-related gene expression by ATF3 in colon cancer cell lines.

To test whether ectopic expression of ATF3 affects EMT-related gene expression profile, we performed RT-PCR to assess Slug, Snail, Vimentin, E-cadherin, N-cadherin, and Twist in HCT116, HCT15, SW480 and HT29 cells. Slug was decreased in HCT116, SW480, and HT29; Snail was reduced in HT29 and Vimentin was lessened in SW480. Interestingly, HT15 showed opposite expression of Slug, Snail and Vimentin, all of which were elevated after the introduction of ectopic ATF3.

To further elucidate the mechanism associated with the change of EMT-related gene profile, we checked the protein expression level of Gsk3beta, Beta-catenin, p-ERK, ERK, and p-Akt. Gsk3beta was enforced associated with the diminished expression of beta-catenin in ATF3 overexpressed HCT116 cells. In addition, the expression of p-ERK and p-AKT were also raised after gain function of ATF3 in HCT116 cells.

Figure 3.6 Regulation of EMT-related gene expression by ATF3 in colon cancer cell lines.

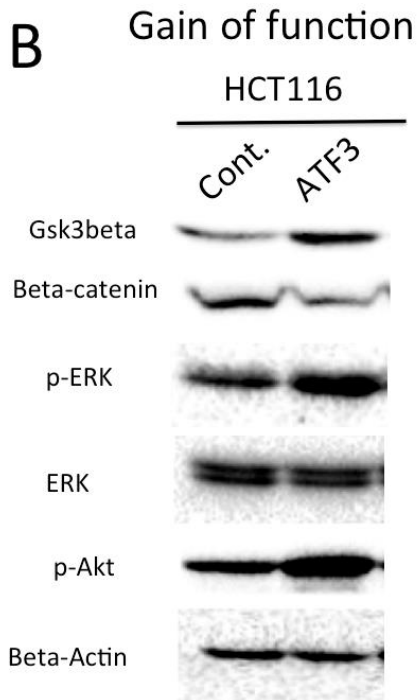
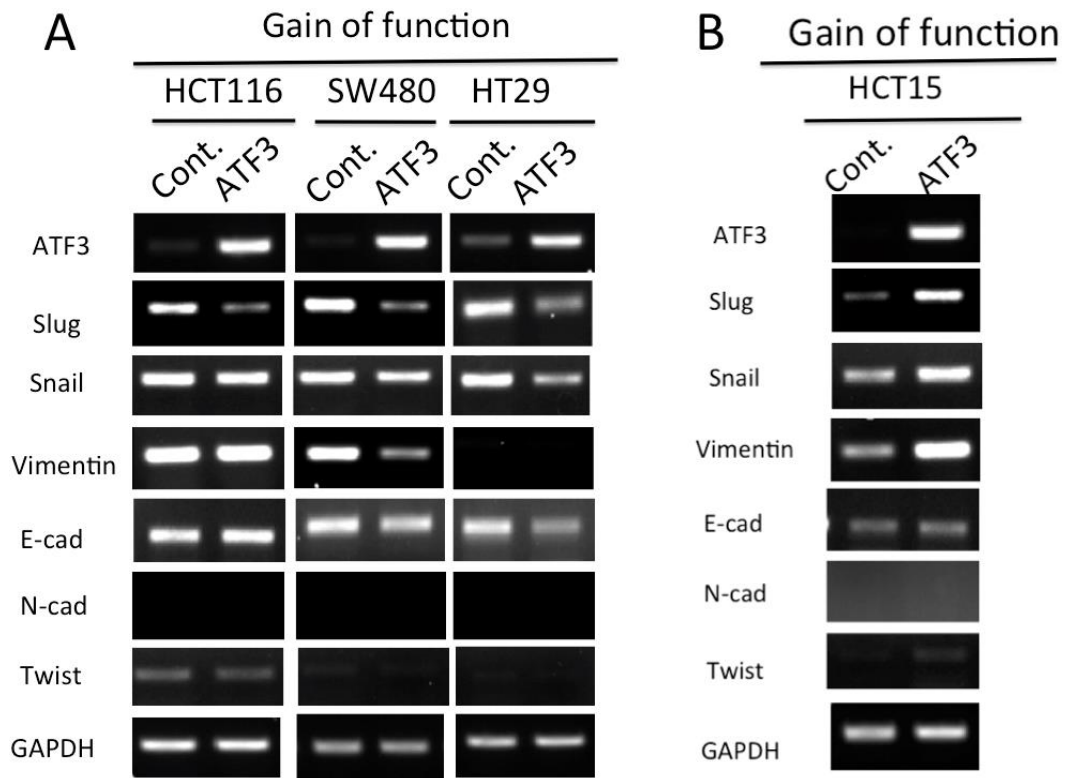
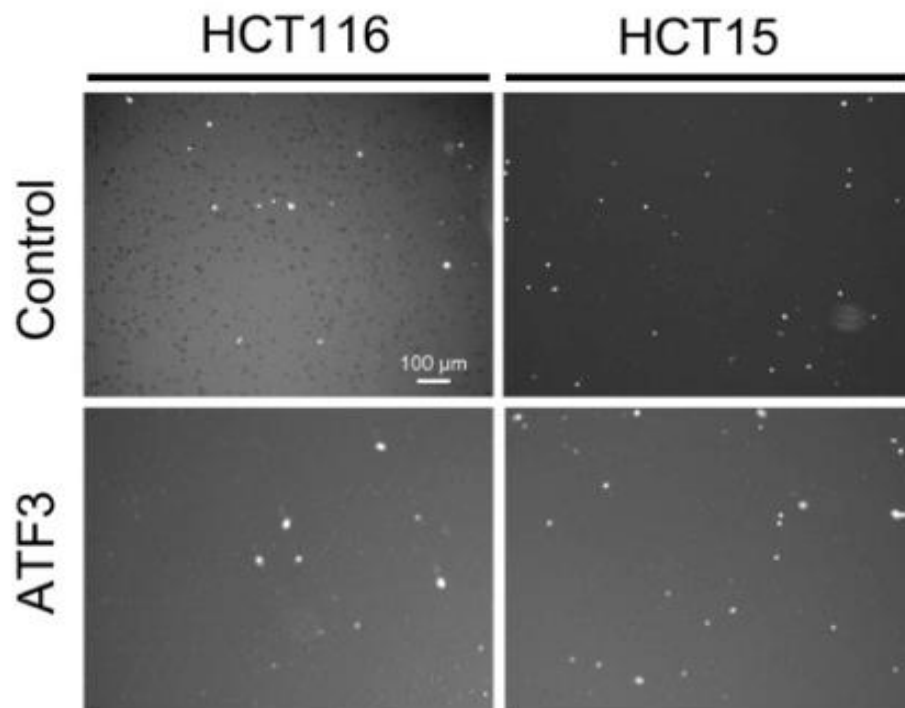


Figure 3.6 Regulation of EMT-related gene expressions by ATF3 in colon cancer cell lines. (A) Colon cancer HCT116, HCT15, SW480, HT29 cell lysates were harvested and RT-PCR was performed for ATF3, GAPDH and EMT-related genes. (B) Western blot was performed to analyze the expression level of Gsk3beat, beta-catenin, p-ERK, ERK, p-Akt, and Beta-Actin after gain function of ATF3 in HCT116 cells.

3.7 *ATF3* overexpressed cells do not induce single cell migration

To address whether *ATF3* promotes colon cancer cell motility, we analyzed the cells by using Boyden chamber. The result indicates that ectopic expression of *ATF3* in HCT116 and HCT15 cells does not affect colon cancer cell motility when compared to the control cells, representative pictures of the motility assay is shown (Figure 3.7A).

A Boyden chamber single cell migration



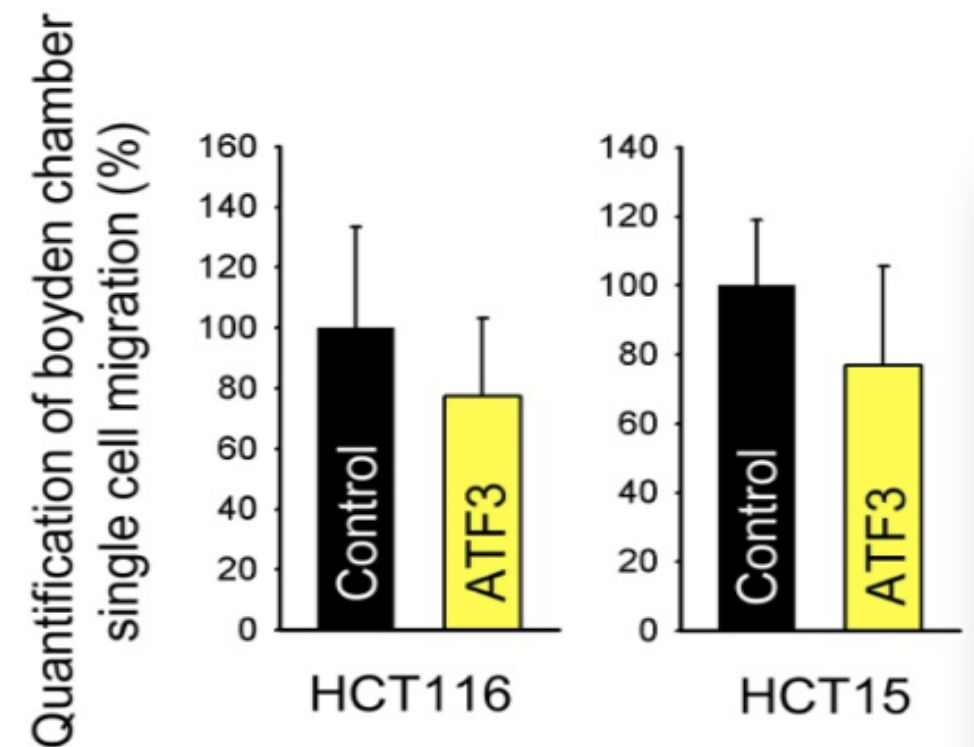


Figure 3.7. ATF3 overexpressed cells do not induce single cell migration. (a) HCT116 and HCT15 cells were analyzed by Boyden chamber assay for single cell migration ability with and without the overexpression of ATF3. Fluorescent images and quantification of migrated cells numbers were initiated one week after the transient transfection with ATF3 expression vector. Numbers of metastases were quantified by ImageJ. Unequal variance Student's t-test, $p \leq 0.05$

3.8 Formation of cancer initiating cell features and collective cell invasion

Rb acts as the key suppressor in metastatic progression and upregulation of CD44 can decrease the expression of Rb, which is crucial for initiating collective cell migration and metastatic growth (138). To test whether ATF3 expression affects collective cell migration, we examined expression level of Rb, CD44, and ZO-1 after overexpression of ATF3. The western blot assay showed that ectopic expression of ATF3 in HCT116 cells can elevate the expression of CD44 alternative splicing v and suppress the protein level of Rb. (Fig. 3.8 left)

The mammosphere forming assay was originally developed by Dontu et al. (101) as a way to propagate mammary epithelial stem cells (MaSC) in *in vitro*. Later, researchers started to use it as a surrogate reporter of stem cell and cancer stem cell activity for tissue samples, tumors and cell lines (92, 139-141). During circulation, undifferentiated stem cells survive, but others die by anoikis in blood. The self-renewal ability of the stem cells permits the formation of mammospheres in serial non-adherent passage. This form of culture system proved to be as a feasible approach for isolating and propagation of tumorigenic breast cancer cells from primary tumors (140) and metastasis (139). Therefore, to test the tumorsphere forming ability of colon cancer cells and their morphology under microscope after overexpression of ATF3, we performed tumorspheres assay under non-adherent conditions. Morphology of the colon cancer cell lines HCT116 with and without ectopic expression of ATF3 included in the study. Compared to the control, ATF3 overexpressing HCT116 cells showed more condensed cell density and formed more protruding and budding sites (Figure 3.8, right).

Figure 3.8. Formation of cancer initiating cell features and collective cell invasion

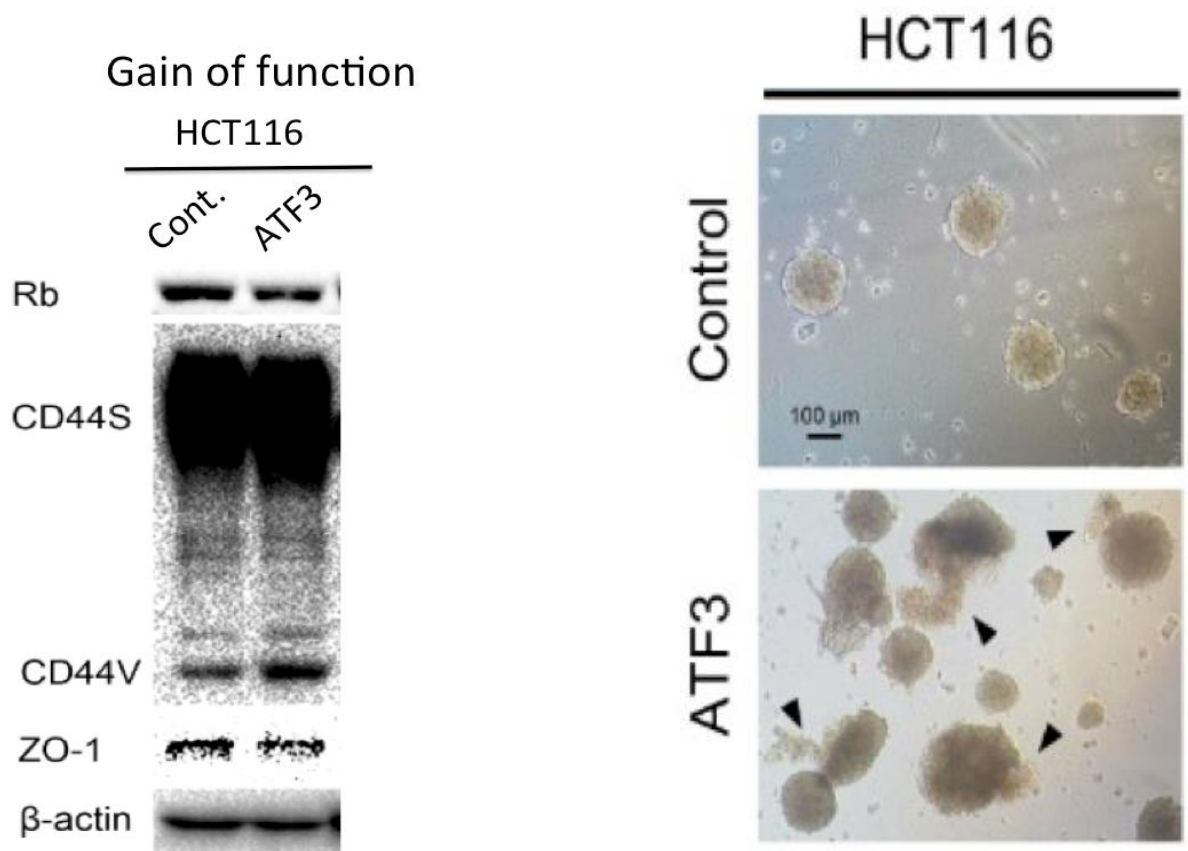


Figure 3.8. Formation of cancer initiating cell features and collective cell invasion (A) HCT-116 cells were transfected with ATF3 expression vector using PolyJet for 48 hours and western blot was performed for ATF3, Rb, CD44, ZO-1 and beta-actin. (B) Phase contrast images and quantification of tumorsphere-forming were initiated to test the collective invasion potency of HCT116 with ATF3 overexpression.

Chapter 4. Discussion, Conclusions, and Future Perspective

4.1 Discussion

ATF3 gene expression can be induced by a variety of stress signals, including those encountered by cancer cells during their development (carcinogens, DNA damage, hypoxia, anoxia etc.) (36, 38), as well as signals commonly present in the tumor microenvironment (142, 143). Therefore, ATF3 is considered as an adaptive-response gene that participates in cellular processes to adapt to extra- and/or intracellular changes and functions as a hub in the biological network that allows cell to respond to signals disrupting homeostasis (144).

The cyclic AMP response element (CRE) is a major positive regulatory site in the bcl-2 promoter, which plays an important functional role in the regulation of endogenous Bcl-2 expression and apoptosis (145). Previous researches revealed that CRE binding protein ATF/CREB can form selective heterodimers with each other or other bZip proteins such as the AP-1 and C/EBP families of proteins. The formation of the heterodimer can alter DNA binding specificity and transcriptional activities (43). Therefore, ATF3 may regulate Bcl-2 by binding to the promoter region of Bcl-2 in colon cancer cell lines including HCT116, HT29, and SW480. Whether Bak, the pro-apoptosis protein from Bcl-2 family, is activated directly by ATF3, or indirectly as a consequence of neutralization of pro-survival targets Bcl-2 is the subject deserved further discussion. A detailed understanding of the interactions between ATF3 and Bcl-2 family members provided by the current research has notable implications for designing anti-cancer drugs to target the Bcl-2 family, thus triggering apoptosis in cancer cells (146).

Interestingly, expression of ATF3 protein is shown to have limited effect on cell cycle regulation. In agreement with this observation, we have also noticed that

cell cycle progression of protein markers, such as cyclins and cyclin-dependent kinases (CDKs), and their inhibitors p21 and p27, were not changed after induction of ATF3 in HCT116 and HCT15 cells. However, previous research found that overexpression of ATF3 using the tetracycline-inducible system can moderately slow down progression of cells from G1 to S phase, indicating that ATF3 protein might be a candidate in the control of cell cycle progression (147). This might be due to the variation of tissue and cellular environment and use of different means of inducing ATF3. The future investigation is required to understand whether ATF3 protein function differently when cellular or tissue environment changed.

In our study, ATF3 potentially suppresses EMT by regulating of EMT-related gene expression in HCT116, HT29 and SW480 cell lines. Here, we propose three mechanisms. Firstly, we observed that the overexpression of ATF3 leads to stimulation of Wnt pathway, which induces the expression of GSK-3 β , consequently, lower the expression level of β -catenin (Figure 3.6B). The glycogen synthase kinase-3 β (GSK3 β) promotes stabilization of cytoplasmic β -catenin, which inhibits the translocation of β -catenin to the nucleus and stops its binding to the transcription factors lymphoid enhancer-binding factor 1 (LEF) and T cell factor (TCF). This action inhibits gene expression program that suppress EMT (68). Secondly, in the present study, we also observed that SNAIL family transcription factors, Slug and Snail, are decreased after induction of ATF3. Snail family transcription factors are well known to stimulate EMT. Previous studies have shown that Snail or Slug can repress the E-cadherin by binding to E-box DNA sequences in the promoter region (68). Additionally, SNAIL can also induce the mesenchymal phenotype by activating N-cadherin. Downregulation of E-cadherin and induction of N-cadherin can result in the alteration of cell adhesion, which is essential of the initiation of EMT. Thirdly,

EMT responses can also be induced by crosstalk and cooperation between distinct pathways. Our result showed that the increase of exogenous ATF3 in colon cancer cell line can activate the phosphorylation of Akt and Eer proteins, which ameliorates the EMT signaling. The integrin-induced Akt activation can cooperating with Wnt signaling by inhibiting GSK3 β , thus inducing SNAIL expression through nuclear factor- κ B (NF- κ B) and stabilizing SNAIL and β -catenin (68).

In addition to promoting EMT through Wnt pathway, transforming growth factor- β (TGF β) can activate the PI3K–Akt, Erk MAPK, p38 MAPK and Jun N-terminal kinase (JNK) pathways. TGF- β 3 signaling induces formation of beta-catenin–LEF-1 complexes that initiate EMT by up-regulating the synthesis of Snail and Slug (72).

Interestingly, HCT15 cell lines showed different expression profile of EMT-related genes. Unlikely HCT116, SW480 and HT29 cells, HCT15 cells have elevated Slug, Snail and vimentin mRNA expression level after ATF3 expression. One of the possible reasons is that HCT15 carry disrupted MSH6. He et al., proposed a correlation between low content of MSH6 and elevated EMT (152). Low level of MSH6 (153, 154) activates alternative lengthening of telomeres (ALT) and promote cancer progression at advanced stages through promoting EMT (152). However, whether the low level of MSH6 can trigger the display of EMT characteristic remains unknown. Further investigation is required to address this issue.

Another interesting finding in current study is that ATF3 suppresses Rb and stimulates the expression of CD44, a marker of colon cancer stem cell. The development of metastasis consists of underlying rate-limiting multistage steps (148). However, metastatic colonization, the successful initiation of metastatic growth, is not frequently seen and inefficient for many cancer types except a minority of cancer cells

that reach distant sites (149, 150). A subpopulation of cancer stem cells is pivotal for metastatic colonization, and the interaction with stromal niche signals are crucial to the expansion process (151).

Previous research indicates that ATF3 enhance cancer-initiating cell features by increasing the population of CD44^{high} cells, the mammosphere-forming ability, and the tumor-initiating frequency in breast cancer (144). We observed that ectopic expression of ATF3 in colon cancer cells processes the ability to increase cancer-initiating cell features and holds the metastasis potential, which paves the way for various stromal signals to exert their impact by inducing ATF3, a hub of the biological network, to promote cancer development.

4.2 Conclusion and Future Perspectives

The present study suggests potential dual function of ATF3 in the development of human colorectal cancer. Data presented in Chapter 3 revealed that ATF3 can modulate the protein and mRNA expression of some Bcl-2 family genes, and enhance apoptosis in different colorectal cancer cells. Though, minimal effect of ATF3 generated on cell cycle regulation. This result indicated a tumor suppressive role of ATF3. Without triggering the single cell migration, however, induction of ATF3 exerts differential regulation effect of EMT-related gene markers in different colon cancer cells. Overexpression of ATF3 can enhance the tumorsphere forming ability and promote primary tumor growth, consequently, contribute to the collective tumor invasion. This finding provides correlative evidence to support an oncogenic role of ATF3.

However, much more work remains to further investigate the mechanisms and functions of ATF3 in human colon cancer. First, in our study, we use overexpression of ectopic ATF3 in colon cancer, but the effects and function of signal-induced or compound-induced expression of ATF3 may be different. Secondly, using the gene overexpression and knockdown strategy, we demonstrated that ATF3 directly regulate the gene expression of Bcl-2. While we do not know whether Bcl-2 can inversely regulate ATF3. Double overexpression or double knockdown strategy is needed to elucidate the relationship between Bcl-2 and ATF3. Thirdly, to further examine the collective cell invasion activity after gain function of ATF3 in colon cancer cells, we can test cell surface proteases, including MT1MMP and MMP2, which are acclaimed to be engaged in degrading the ECM substrate and participate in the ECM remodeling, an early event in collective cancer cell movement (158, 159). Fourthly, given the importance of ATF3 in cellular context, it is also crucial to investigate how ATF3

function *in vivo* situation. Physiological mechanisms that regulate biological events are very complex and the biological system for investigating disease process sometimes can be different from *in vitro* study. To test whether ATF3 can induce the metastasis in colorectal cancer *in vivo*, we can use tail vein injection lung metastasis animal model.

Previous studies have revealed that ATF3 can be induced by a variety of anti-tumorigenic compounds including indole-3-carbinol (40), conjugated linoleic acid (41), tolfenamic acid (124), epicatechin gallate (39), resveratrol (42), paclitaxel (160) and PI3 kinase inhibitor (118). Some of these compounds have entered into clinical trails. However, the possible oncogenic property of ATF3 can bring the side effect to drugs made by these compounds. Thus, it is essential to understand the molecular context which determines the function of ATF3 as a tumor suppressor or an oncogene, providing the rationale for designing anti-cancer treatment and benefiting the clinical practice in the future.

Reference

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA: a cancer journal for clinicians* 2015;65(1):5-29.
2. Wang H, Khor TO, Shu L, et al. *Plants Against Cancer: A Review on Natural Phytochemicals in Preventing and Treating Cancers and Their Druggability*. *Anti-cancer agents in medicinal chemistry* 2012;12(10):1281.
3. Yang CS, Maliakal P, Meng X. Inhibition of Carcinogenesis by Tea*. *Annual Review of Pharmacology and Toxicology* 2002;42(1):25-54.
4. Shimizu M, Shirakami Y, Sakai H, et al. (-)-Epigallocatechin gallate suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-db/db mice. *Cancer Prevention Research* 2008;1(4):298-304.
5. Adachi S, Shimizu M, Shirakami Y, et al. (-)-Epigallocatechin gallate downregulates EGF receptor via phosphorylation at Ser1046/1047 by p38 MAPK in colon cancer cells. *Carcinogenesis* 2009;30(9):1544-52.
6. Larsen CA, Dashwood RH. (-)-Epigallocatechin-3-gallate inhibits Met signaling, proliferation, and invasiveness in human colon cancer cells. *Archives of biochemistry and biophysics* 2010;501(1):52-7.
7. Kim J-M, Kim J-S, Yoo H, Choung M-G, Sung M-K. Effects of black soybean [*Glycine max* (L.) Merr.] seed coats and its anthocyanidins on colonic inflammation and cell proliferation in vitro and in vivo. *J Agric Food Chem* 2008;56(18):8427-33.
8. Yun JM, Afaq F, Khan N, Mukhtar H. Delphinidin, an anthocyanidin in pigmented fruits and vegetables, induces apoptosis and cell cycle arrest in human colon cancer HCT116 cells. *Molecular carcinogenesis* 2009;48(3):260-70.
9. Su C-C, Chen G-w, Lin J-G, WU L-T, CHUNG J-G. Curcumin inhibits cell migration of human colon cancer colo 205 cells through the inhibition of nuclear factor kappa B/p65 and down-regulates cyclooxygenase-2 and matrix metalloproteinase-2 expressions. *Anticancer research* 2006;26(2A):1281-8.
10. Chen A, Xu J, Johnson A. Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. *Oncogene* 2006;25(2):278-87.
11. Johnson SM, Gulhati P, Arrieta I, et al. Curcumin inhibits proliferation of colorectal carcinoma by modulating Akt/mTOR signaling. *Anticancer research* 2009;29(8):3185-90.
12. Choi SY, Park JHY, Kim JS, Kim MK, Aruoma OI, Sung MK. Effects of quercetin and β - carotene supplementation on azoxymethane- induced colon carcinogenesis and inflammatory responses in rats fed with high- fat diet rich in ω - 6 fatty acids. *Biofactors* 2006;27(1- 4):137-46.
13. Tang FY, Shih CJ, Cheng LH, Ho HJ, Chen HJ. Lycopene inhibits growth of human colon cancer cells via suppression of the Akt signaling pathway. *Molecular nutrition & food research* 2008;52(6):646-54.
14. Joo Y-E, Karrasch T, Muhlbauer M, et al. Tomato lycopene extract prevents lipopolysaccharide-induced NF-kappaB signaling but worsens dextran sulfate sodium-induced colitis in NF-kappaBEGFP mice. *PLoS One* 2009;4(2):e4562.
15. Palozza P, Colangelo M, Simone R, et al. Lycopene induces cell growth inhibition by altering mevalonate pathway and Ras signaling in cancer cell lines. *Carcinogenesis* 2010;31(10):1813-21.

16. Nagendraprabhu P, Sudhandiran G. Astaxanthin inhibits tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFkB and COX-2. *Investigational new drugs* 2011;29(2):207-24.
17. Cheung KL, Khor TO, Yu S, Kong A-NT. PEITC induces G1 cell cycle arrest on HT-29 cells through the activation of p38 MAPK signaling pathway. *The AAPS journal* 2008;10(2):277-81.
18. Lai K-C, Huang A-C, Hsu S-C, et al. Benzyl isothiocyanate (BITC) inhibits migration and invasion of human colon cancer HT29 cells by inhibiting matrix metalloproteinase-2/-9 and urokinase plasminogen (uPA) through PKC and MAPK signaling pathway. *Journal of agricultural and food chemistry* 2010;58(5):2935-42.
19. Shen G, Khor TO, Hu R, et al. Chemoprevention of familial adenomatous polyposis by natural dietary compounds sulforaphane and dibenzoylmethane alone and in combination in ApcMin/+ mouse. *Cancer Research* 2007;67(20):9937-44.
20. Rudolf E, Andělová H, Červinka M. Activation of several concurrent proapoptotic pathways by sulforaphane in human colon cancer cells SW620. *Food and Chemical Toxicology* 2009;47(9):2366-73.
21. Choi HJ, Lim DY, Park JH. Induction of G1 and G2/M cell cycle arrests by the dietary compound 3, 3'-diindolylmethane in HT-29 human colon cancer cells. *BMC gastroenterology* 2009;9(1):39.
22. Kim M, Miyamoto S, Yasui Y, Oyama T, Murakami A, Tanaka T. Zerumbone, a tropical ginger sesquiterpene, inhibits colon and lung carcinogenesis in mice. *International Journal of Cancer* 2009;124(2):264-71.
23. Kato T, Kolenic N, Pardini RS. Docosahexaenoic acid (DHA), a primary tumor suppressive omega-3 fatty acid, inhibits growth of colorectal cancer independent of p53 mutational status. *HNUC* 2007;58(2):178-87.
24. Bocca C, Bozzo F, Gabriel L, Miglietta A. Conjugated linoleic acid inhibits Caco-2 cell growth via ERK-MAPK signaling pathway. *The Journal of nutritional biochemistry* 2007;18(5):332-40.
25. Dillehay DL, Webb SK, Schmelz E-M, Merrill Jr AH. Dietary sphingomyelin inhibits 1, 2-dimethylhydrazine-induced colon cancer in CF1 mice. *The Journal of nutrition* 1994;124(5):615.
26. Schmelz EM, Dillehay DL, Webb SK, Reiter A, Adams J, Merrill AH. Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1, 2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. *Cancer Research* 1996;56(21):4936-41.
27. Schmelz EM, Sullards MC, Dillehay DL, Merrill AH. Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glycosphingolipids in 1, 2-dimethylhydrazine-treated CF1 mice. *The Journal of nutrition* 2000;130(3):522-7.
28. Symolon H, Schmelz EM, Dillehay DL, Merrill AH. Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1, 2-dimethylhydrazine-treated CF1 mice and ApcMin/+ mice. *The Journal of nutrition* 2004;134(5):1157-61.
29. Schmelz E-M, Crall KJ, Larocque R, Dillehay DL, Merrill Jr AH. Uptake and metabolism of sphingolipids in isolated intestinal loops of mice. *The Journal of nutrition* 1994;124(5):702.

30. Pan MH, Lai CS, Wu JC, Ho CT. Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. *Molecular nutrition & food research* 2011;55(1):32-45.
31. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *cell* 2011;144(5):646-74.
32. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. *International journal of cell biology* 2010;2010.
33. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 2006;441(7092):437-43.
34. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and cellular biochemistry* 2004;266(1-2):37-56.
35. Valko M, Rhodes C, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-biological interactions* 2006;160(1):1-40.
36. Hai T, Wolfgang CD, Marsee DK, Allen AE, Sivaprasad U. ATF3 and stress responses. *GENE EXPRESSION-CHICAGO THEN ELMSFORD NY--* 1999;7:321-36.
37. Ameri K, Hammond E, Culmsee C, et al. Induction of activating transcription factor 3 by anoxia is independent of p53 and the hypoxic HIF signalling pathway. *Oncogene* 2007;26(2):284-9.
38. Hai T, Hartman MG. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 2001;273(1):1-11.
39. Baek SJ, Kim J-S, Jackson FR, Eling TE, McEntee MF, Lee S-H. Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells. *Carcinogenesis* 2004;25(12):2425-32.
40. Lee S-H, Kim J-S, Yamaguchi K, Eling TE, Baek SJ. Indole-3-carbinol and 3, 3'-diindolylmethane induce expression of NAG-1 in a p53-independent manner. *Biochemical and biophysical research communications* 2005;328(1):63-9.
41. Lee S-H, Yamaguchi K, Kim J-S, et al. Conjugated linoleic acid stimulates an anti-tumorigenic protein NAG-1 in an isomer specific manner. *Carcinogenesis* 2006;27(5):972-81.
42. Whitlock NC, Baek SJ. The anticancer effects of resveratrol: modulation of transcription factors. *Nutrition and cancer* 2012;64(4):493-502.
43. Hai T. The ATF transcription factors in cellular adaptive responses. *Gene Expression and Regulation* 2006:329-40.
44. Lu D, Wolfgang CD, Hai T. Activating transcription factor 3, a stress-inducible gene, suppresses Ras-stimulated tumorigenesis. *Journal of biological chemistry* 2006;281(15):10473-81.
45. Bottone FG, Moon Y, Kim JS, Alston-Mills B, Ishibashi M, Eling TE. The anti-invasive activity of cyclooxygenase inhibitors is regulated by the transcription factor ATF3 (activating transcription factor 3). *Molecular cancer therapeutics* 2005;4(5):693-703.
46. Ishiguro T, Nagawa H, Naito M, Tsuruo T. Inhibitory effect of ATF3 antisense oligonucleotide on ectopic growth of HT29 human colon cancer cells. *Cancer Science* 2000;91(8):833-6.

47. Bandyopadhyay S, Wang Y, Zhan R, et al. The tumor metastasis suppressor gene Drg-1 down-regulates the expression of activating transcription factor 3 in prostate cancer. *Cancer research* 2006;66(24):11983-90.
48. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nature reviews Molecular cell biology* 2014;15(1):49-63.
49. Elmore S. Apoptosis: a review of programmed cell death. *Toxicologic pathology* 2007;35(4):495-516.
50. Jiang X, Wang X. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *Journal of Biological Chemistry* 2000;275(40):31199-203.
51. Vaillant F, Merino D, Lee L, et al. Targeting BCL-2 with the BH3 mimetic ABT-199 in estrogen receptor-positive breast cancer. *Cancer cell* 2013;24(1):120-9.
52. Langdon SP. *Cancer cell culture: methods and protocols*: Springer Science & Business Media, 2004.
53. Giacinti C, Giordano A. RB and cell cycle progression. *Oncogene* 2006;25(38):5220-7.
54. Sherr CJ. Cancer cell cycles. *Science* 1996;274(5293):1672-7.
55. Sherr CJ. D-type cyclins. *Trends in biochemical sciences* 1995;20(5):187-90.
56. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & development* 1999;13(12):1501-12.
57. Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends in cell biology* 2003;13(2):65-70.
58. Russo AA, Jeffrey PD, Patten AK, Massagué J, Pavletich NP. Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. *Nature* 1996;382(6589):325-31.
59. Olashaw N, Pledger W. Paradigms of growth control: relation to Cdk activation. *Science Signaling* 2002;2002(134):re7-re.
60. Ewald AJ, Brenot A, Duong M, Chan BS, Werb Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. *Developmental cell* 2008;14(4):570-81.
61. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nature medicine* 2013;19(11):1423-37.
62. Nawshad A, LaGamba D, Polad A, Hay ED. Transforming growth factor- β signaling during epithelial-mesenchymal transformation: implications for embryogenesis and tumor metastasis. *Cells Tissues Organs* 2005;179(1-2):11-23.
63. Moreno-Bueno G, Portillo F, Cano A. Transcriptional regulation of cell polarity in EMT and cancer. *Oncogene* 2008;27(55):6958-69.
64. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *cell* 2009;139(5):871-90.
65. Peinado H, Portillo F, Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. *International Journal of Developmental Biology* 2004;48(5-6):365-75.
66. Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004;117(7):927-39.

67. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nature Reviews Cancer* 2007;7(6):415-28.
68. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. *Nature reviews Molecular cell biology* 2014;15(3):178-96.
69. Yook JI, Li X-Y, Ota I, Fearon ER, Weiss SJ. Wnt-dependent regulation of the E-cadherin repressor snail. *Journal of Biological Chemistry* 2005;280(12):11740-8.
70. Wang H, Wang H-S, Zhou B-H, et al. Epithelial-mesenchymal transition (EMT) induced by TNF-alpha requires AKT/GSK-3beta-mediated stabilization of snail in colorectal cancer. *PLoS One* 2013;8(2):e56664.
71. Zhou BP, Deng J, Xia W, et al. Dual regulation of Snail by GSK-3β-mediated phosphorylation in control of epithelial–mesenchymal transition. *Nature cell biology* 2004;6(10):931-40.
72. Medici D, Hay ED, Olsen BR. Snail and Slug promote epithelial-mesenchymal transition through β-catenin–T-cell factor-4-dependent expression of transforming growth factor-β3. *Molecular biology of the cell* 2008;19(11):4875-87.
73. Yang M-H, Wu M-Z, Chiou S-H, et al. Direct regulation of TWIST by HIF-1α promotes metastasis. *Nature cell biology* 2008;10(3):295-305.
74. Yang F, Sun L, Li Q, et al. SET8 promotes epithelial–mesenchymal transition and confers TWIST dual transcriptional activities. *The EMBO journal* 2012;31(1):110-23.
75. Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. *Cell* 1996;84(3):359-69.
76. Ridley AJ, Schwartz MA, Burridge K, et al. Cell migration: integrating signals from front to back. *Science* 2003;302(5651):1704-9.
77. Sanz-Moreno V, Marshall CJ. The plasticity of cytoskeletal dynamics underlying neoplastic cell migration. *Current opinion in cell biology* 2010;22(5):690-6.
78. Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. *The Journal of cell biology* 2010;188(1):11-9.
79. Honeth G, Bendahl P-O, Ringnér M, et al. The CD44+/CD24-phenotype is enriched in basal-like breast tumors. *Breast Cancer Res* 2008;10(3):R53.
80. Lopes FF, da Costa Miguel MC, Pereira ALA, et al. Changes in immunoexpression of E-cadherin and β-catenin in oral squamous cell carcinoma with and without nodal metastasis. *Annals of diagnostic pathology* 2009;13(1):22-9.
81. Sahai E. Illuminating the metastatic process. *Nature Reviews Cancer* 2007;7(10):737-49.
82. Shapiro IM, Cheng AW, Flytzanis NC, et al. An EMT-driven alternative splicing program occurs in human breast cancer and modulates cellular phenotype. *PLoS Genet* 2011;7(8):e1002218-e.
83. Stoecklein NH, Hosch SB, Bezler M, et al. Direct genetic analysis of single disseminated cancer cells for prediction of outcome and therapy selection in esophageal cancer. *Cancer cell* 2008;13(5):441-53.
84. Wang X, Zhang J, Fan M, et al. The expression of E-cadherin at the invasive tumor front of oral squamous cell carcinoma: immunohistochemical and RT-PCR analysis with clinicopathological correlation. *Oral Surgery, Oral*

- Medicine, Oral Pathology, Oral Radiology, and Endodontology
2009;107(4):547-54.
85. Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell* 2011;147(5):992-1009.
 86. Friedl P, Noble PB, Walton PA, et al. Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants in vitro. *Cancer research* 1995;55(20):4557-60.
 87. Friedl P, Weigelin B. Interstitial leukocyte migration and immune function. *Nature immunology* 2008;9(9):960-9.
 88. Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. *Nature Reviews Cancer* 2003;3(12):895-902.
 89. Kielman MF, Rindapää M, Gaspar C, et al. Apc modulates embryonic stem-cell differentiation by controlling the dosage of β -catenin signaling. *Nature genetics* 2002;32(4):594-605.
 90. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature medicine* 2004;10(1):55-63.
 91. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Migrating cancer stem cells—an integrated concept of malignant tumour progression. *Nature Reviews Cancer* 2005;5(9):744-9.
 92. Manuel Iglesias J, Beloqui I, Garcia-Garcia F, et al. Mammosphere formation in breast carcinoma cell lines depends upon expression of E-cadherin. *PloS one* 2013;8(10):e77281.
 93. Gupta PB, Onder TT, Jiang G, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* 2009;138(4):645-59.
 94. Medema JP. Cancer stem cells: the challenges ahead. *Nature cell biology* 2013;15(4):338-44.
 95. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences* 2003;100(7):3983-8.
 96. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer research* 2007;67(3):1030-7.
 97. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007;445(7123):111-5.
 98. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer research* 2003;63(18):5821-8.
 99. Zhang M, Behbod F, Atkinson RL, et al. Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. *Cancer research* 2008;68(12):4674-82.
 100. Sahlberg SH, Spiegelberg D, Glimelius B, Stenerlöw B, Nestor M. Evaluation of cancer stem cell markers CD133, CD44, CD24: association with AKT isoforms and radiation resistance in colon cancer cells. *PloS one* 2014;9(4):e94621.
 101. Dontu G, Abdallah WM, Foley JM, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & development* 2003;17(10):1253-70.
 102. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *nature* 2006;444(7120):756-60.

103. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nature Reviews Cancer* 2005;5(4):275-84.
104. Diehn M, Clarke MF. Cancer stem cells and radiotherapy: new insights into tumor radioresistance. *Journal of the National Cancer Institute* 2006;98(24):1755-7.
105. Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *Journal of Clinical Oncology* 2008;26(17):2839-45.
106. Li X, Lewis MT, Huang J, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *Journal of the National Cancer Institute* 2008;100(9):672-9.
107. Woodward WA, Chen MS, Behbod F, Alfaro MP, Buchholz TA, Rosen JM. WNT/ β -catenin mediates radiation resistance of mouse mammary progenitor cells. *Proceedings of the National Academy of Sciences* 2007;104(2):618-23.
108. Jothy S. CD44 and its partners in metastasis. *Clinical & experimental metastasis* 2003;20(3):195-201.
109. Lee K, Hai T-Y, SivaRaman L, et al. A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proceedings of the National Academy of Sciences* 1987;84(23):8355-9.
110. Lin Y-S, Green MR. Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1a-and cyclic AMP-inducible promoters. *Proceedings of the National Academy of Sciences* 1988;85(10):3396-400.
111. Hai T, Liu F, Coukos WJ, Green MR. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes & development* 1989;3(12b):2083-90.
112. Liang G, Wolfgang CD, Chen BP, Chen T-H, Hai T. ATF3 gene genomic organization, promoter, and regulation. *Journal of Biological Chemistry* 1996;271(3):1695-701.
113. Chen B, Liang G, Whelan J, Hai T. ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. *Journal of Biological Chemistry* 1994;269(22):15819-26.
114. Wolfgang CD, Chen B, Martindale JL, Holbrook NJ, Hai T. gadd153/Chop10, a potential target gene of the transcriptional repressor ATF3. *Molecular and cellular biology* 1997;17(11):6700-7.
115. Wolfgang CD, Liang G, Okamoto Y, Allen AE, Hai T. Transcriptional Autorepression of the Stress-inducible Gene ATF3. *Journal of Biological Chemistry* 2000;275(22):16865-70.
116. Chu H-M, Tan Y, Kobierski LA, Balsam LB, Comb MJ. Activating transcription factor-3 stimulates 3', 5'-cyclic adenosine monophosphate-dependent gene expression. *Molecular Endocrinology* 1994;8(1):59-68.
117. Mashima T, Udagawa S, Tsuruo T. Involvement of transcriptional repressor ATF3 in acceleration of caspase protease activation during DNA damaging agent- induced apoptosis. *Journal of cellular physiology* 2001;188(3):352-8.
118. Yamaguchi K, Lee S-H, Kim J-S, Wimalasena J, Kitajima S, Baek SJ. Activating transcription factor 3 and early growth response 1 are the novel targets of LY294002 in a phosphatidylinositol 3-kinase-independent pathway. *Cancer research* 2006;66(4):2376-84.

119. Lee W-Y, Huang S-C, Tzeng C-C, Chang T-L, Hsu K-F. Alterations of metastasis-related genes identified using an oligonucleotide microarray of genistein-treated HCC1395 breast cancer cells. *HNUC* 2007;58(2):239-46.
120. De Angelis PM, Svendsrud DH, Kravik KL, Stokke T. Cellular response to 5-fluorouracil (5-FU) in 5-FU-resistant colon cancer cell lines during treatment and recovery. *Molecular cancer* 2006;5(1):20.
121. Edagawa M, Kawauchi J, Hirata M, et al. Role of activating transcription factor 3 (ATF3) in endoplasmic reticulum (ER) stress-induced sensitization of p53-deficient human colon cancer cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through up-regulation of death receptor 5 (DR5) by zerumbone and celecoxib. *Journal of Biological Chemistry* 2014;289(31):21544-61.
122. Nobori K, Ito H, Tamamori-Adachi M, et al. ATF3 inhibits doxorubicin-induced apoptosis in cardiac myocytes: a novel cardioprotective role of ATF3. *Journal of molecular and cellular cardiology* 2002;34(10):1387-97.
123. Bottone FG, Martinez JM, Collins JB, Afshari CA, Eling TE. Gene Modulation by the Cyclooxygenase Inhibitor, Sulindac Sulfide, in Human Colorectal Carcinoma Cells POSSIBLE LINK TO APOPTOSIS. *Journal of Biological Chemistry* 2003;278(28):25790-801.
124. Lee S-H, Bahn JH, Whitlock NC, Baek SJ. Activating transcription factor 2 (ATF2) controls tolfenamic acid-induced ATF3 expression via MAP kinase pathways. *Oncogene* 2010;29(37):5182-92.
125. Zhong S, Fields C, Su N, Pan Y, Robertson K. Pharmacologic inhibition of epigenetic modifications, coupled with gene expression profiling, reveals novel targets of aberrant DNA methylation and histone deacetylation in lung cancer. *Oncogene* 2007;26(18):2621-34.
126. Kim J, Kwak HJ, Cha J-Y, et al. Metformin suppresses lipopolysaccharide (LPS)-induced inflammatory response in murine macrophages via activating transcription factor-3 (ATF-3) induction. *Journal of Biological Chemistry* 2014;289(33):23246-55.
127. Allen-Jennings AE, Hartman MG, Kociba GJ, Hai T. The roles of ATF3 in glucose homeostasis A transgenic mouse model with liver dysfunction and defects in endocrine pancreas. *Journal of Biological Chemistry* 2001;276(31):29507-14.
128. Allen-Jennings AE, Hartman MG, Kociba GJ, Hai T. The roles of ATF3 in liver dysfunction and the regulation of phosphoenolpyruvate carboxykinase gene expression. *Journal of Biological Chemistry* 2002;277(22):20020-5.
129. Okamoto Y, Chaves A, Chen J, et al. Transgenic mice with cardiac-specific expression of activating transcription factor 3, a stress-inducible gene, have conduction abnormalities and contractile dysfunction. *The American journal of pathology* 2001;159(2):639-50.
130. Nakagomi S, Suzuki Y, Namikawa K, Kiryu-Seo S, Kiyama H. Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. *The Journal of neuroscience* 2003;23(12):5187-96.
131. Kawauchi J, Zhang C, Nobori K, et al. Transcriptional Repressor Activating Transcription Factor 3 Protects Human Umbilical Vein Endothelial Cells from Tumor Necrosis Factor- α -induced Apoptosis through Down-regulation of p53 Transcription. *Journal of Biological Chemistry* 2002;277(41):39025-34.

132. Yin X, Dewille J, Hai T. A potential dichotomous role of ATF3, an adaptive-response gene, in cancer development. *Oncogene* 2008;27(15):2118-27.
133. Ishiguro T, Nakajima M, Naito M, Muto T, Tsuruo T. Identification of genes differentially expressed in B16 murine melanoma sublines with different metastatic potentials. *Cancer research* 1996;56(4):875-9.
134. Ishiguro T, Nagawa H. ATF3 gene regulates cell form and migration potential of HT29 colon cancer cells. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics* 2001;12(8):343-6.
135. Wolford CC, McConoughey SJ, Jalgaonkar SP, et al. Transcription factor ATF3 links host adaptive response to breast cancer metastasis. *The Journal of clinical investigation* 2013;123(7):2893.
136. Wu Z-Y, Wei Z-m, Sun S-J, Yuan J, Jiao S-C. Activating transcription factor 3 promotes colon cancer metastasis. *Tumor Biology* 2014;35(8):8329-34.
137. Emily H-YC, Wei MC, Weiler S, et al. BCL-2, BCL-X L sequester BH3 domain-only molecules preventing BAX-and BAK-mediated mitochondrial apoptosis. *Molecular cell* 2001;8(3):705-11.
138. Kim K-J, Godarova A, Seedle K, et al. Rb suppresses collective invasion, circulation and metastasis of breast cancer cells in CD44-dependent manner. 2013.
139. Grimshaw MJ, Cooper L, Papazisis K, et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. *Breast Cancer Res* 2008;10(3):R52.
140. Ponti D, Costa A, Zaffaroni N, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer research* 2005;65(13):5506-11.
141. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast cancer res* 2008;10(2):R25.
142. Knowles HJ, Harris AL. Macrophages and the hypoxic tumour microenvironment. *Frontiers in bioscience: a journal and virtual library* 2006;12:4298-314.
143. Fox SB, Generali DG, Harris AL. Breast tumour angiogenesis. *Breast Cancer Res* 2007;9(6):216.
144. Yin X, Wolford CC, Chang Y-S, et al. ATF3, an adaptive-response gene, enhances TGF β signaling and cancer-initiating cell features in breast cancer cells. *Journal of cell science* 2010;123(20):3558-65.
145. Xiang H, Wang J, Boxer LM. Role of the cyclic AMP response element in the bcl-2 promoter in the regulation of endogenous Bcl-2 expression and apoptosis in murine B cells. *Molecular and cellular biology* 2006;26(22):8599-606.
146. van Delft MF, Huang DC. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell research* 2006;16(2):203-13.
147. Fan F, Jin S, Amundson SA, et al. ATF3 induction following DNA damage is regulated by distinct signaling pathways and over-expression of ATF3 protein suppresses cells growth. *Oncogene* 2002;21(49):7488-96.
148. Chambers A, Groom A, MacDonald I. α Dissemination and Growth of Cancer Cells in Metastatic Sites, α *Nat. Rev Cancer* 2002;2:563 α .
149. Kouros-Mehr H, Bechis SK, Slorach EM, et al. GATA-3 links tumor differentiation and dissemination in a luminal breast cancer model. *Cancer cell* 2008;13(2):141-52.

150. Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nature Reviews Cancer* 2009;9(4):274-84.
151. Malanchi I, Santamaria-Martínez A, Susanto E, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature* 2012;481(7379):85-9.
152. Xue Y, Li L, Zhang D, et al. Twisted epithelial-to-mesenchymal transition promotes progression of surviving bladder cancer T24 cells with hTERT-dysfunction. *PloS one* 2011;6(11):e27748.
153. Jackson P, Grimm M-O, Kingsley EA, et al. Relationship between expression of KAI1 metastasis suppressor gene, mRNA levels and p53 in human bladder and prostate cancer cell lines. *Urologic Oncology: Seminars and Original Investigations: Elsevier*, 2002:99-104.
154. Thykjaer T, Christensen M, Clark A, Hansen L, Kunkel T, Ørntoft T. Functional analysis of the mismatch repair system in bladder cancer. *British journal of cancer* 2001;85(4):568.
155. Nguyen LV, Vanner R, Dirks P, Eaves CJ. Cancer stem cells: an evolving concept. *Nature Reviews Cancer* 2012;12(2):133-43.
156. Marotta LLC, Polyak K. Cancer stem cells: a model in the making. *Current opinion in genetics & development* 2009;19(1):44-50.
157. Jaggupilli A, Elkord E. Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity. *Clinical and Developmental Immunology* 2012;2012.
158. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nature reviews Molecular cell biology* 2009;10(7):445-57.
159. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature Reviews Cancer* 2003;3(5):362-74.
160. Peters CM, Jimenez-Andrade JM, Jonas BM, et al. Intravenous paclitaxel administration in the rat induces a peripheral sensory neuropathy characterized by macrophage infiltration and injury to sensory neurons and their supporting cells. *Experimental neurology* 2007;203(1):42-54.