SNAIL TRANSCRIPTION FACTORS CAN SUPPRESS AMPK GENE EXPRESSION AND REGULATE LIPID METABOLISM

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2017
To all those who love me and I love
ACKNOWLEDGMENTS

I thank Dr. Huachen Luo for initiating this study and performing bioinformatic analysis, qPCR and chromatin immunoprecipitation, as well as helping me with part of the bioinformatic analysis. I thank Prof. Jianrong Lu for providing funding, equipment and guidance for me. I thank all my committee members Prof. Jianrong Lu (Chair), Prof. Jorg Bungert (Cochair), Suming Huang (Cochair), Yi Qiu (Cochair) for help with editing and polishing this thesis.
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SNAIL TRANSCRIPTION FACTORS CAN SUPPRESS AMPK GENE EXPRESSION AND REGULATE LIPID METABOLISM

By
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August 2017

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Major: Biochemistry and Molecular Biology

Epithelial–mesenchymal transition (EMT) is a critical phenomenon involved in many biological events like early embryonic development, adult wound healing and stem cell behavior, and cancer progression. EMT is highly associated with treatment resistance and tumor recurrence. EMT can provide cancer cells with stem-like features and therapeutic resistance ability. The transcription factors of the Snail family are central drivers of EMT.

In this study, we conducted bioinformatic analysis and found significantly enriched binding of Snail proteins at the AMPK family gene transcription start sites. Further qPCR and chromatin immunoprecipitation (ChIP) analysis using the DCIS-Snail-ER cell model showed that Snail transcription factors can bind to the AMPK gene promoters and suppress their expression. In control cells, activation of AMPK signaling reduced cell growth and lipid content, however, such effects were significantly blunted in cancer cells that underwent Snail-mediated EMT. We conclude that the EMT-driving Snail transcription factors can regulate lipid metabolism through modulation of the AMPK signaling pathway.
CHAPTER 1
INTRODUCTION

Epithelial–Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) is a critical phenomenon involved in many biological events like early embryonic development, adult wound healing and stem cell behavior, and cancer progression. During early embryological development, EMT is the primary mechanism for tissue remodeling. To turn an epithelial somite into sclerotome mesenchyme, multiple dramatic changes—decreasing expression of cell junction and epithelial cell-specific genes, and gaining mesenchymal phenotype—are involved. This cell state and behavior switch is mediated by several key transcription factors, including the Snail Family Transcriptional Repressor, Zinc Finger E-box-Binding Homeobox proteins, and Basic Helix-Loop-Helix Transcription Factors. Based on the time, tissue and transcription factors involved, EMT can be classified into three types.

Type-1 EMT, Embryonic Development

Type-1 EMT is highly associated with embryogenesis and tissue development. During early embryo development, to form three germ layers, neural crest cells are required to transform to program locations. All three types of cells-- Epithelial, Mesenchymal, and Mesodermal cells—cooperate with each other during this process. Mesenchymal cells provide migratory and invasive functions. Want signaling is considered fundamental pathway for EMT based on the finding that Wnt-deficient embryos cells are unable to gastrulate and morphological changes for renal fibrosis formation. TGF-β along with Nodal and Vg1, mesodermal posterior 1 and 2, Snail family, and Eomesodermin all have been reported as critical EMT factors.
Type-2 EMT, Fibrosis

Type-2 EMT is activated during injury, which is involved in adult cardiac, kidney and liver disease. In kidney fibrosis studies, fibrosis progress can be monitored or predicted by measuring specific fibroblast markers. Fibroblast-specific protein 1 is the primary marker used for detecting kidney fibrosis cells EMT progress. FSP 1 (or S100 calcium binding protein A4) is a protein of the S100 family which contains 2 EF-hand calcium-binding motifs and high expressed in kidney cells with the EMT phenotype. With the help of FSP 1 antibody, fibrosis related EMT was also reported in lung, and spleen tissues. The direct evidence of conversion of local epithelial cells into fibroblasts observed by Iwano’s group also demonstrates the significance of EMT in fibrosis.

Type-3 EMT, Cell Malignancy

Type-3 EMT is a special catalog for EMT happened during tumorigenesis. It won’t be surprised to see Type-3 EMT sharing many characters with Type-1 or 2. Because, once cells start tumorigenesis progress, they will use all available tools to replicate and survive. Back to 1858, Virchow’s group reported that wound healing mechanism contributes to cancer tissue remodeling. The ability to trigger EMT progress to gain survive advantages has been recognized as one major cancer cell function.

EMT in Cancer

The significance role of EMT played in cancer has been reported and proved both in vitro cell and in vivo animal models in recent studies. But, some functions, especially for how EMT contribute to cancer metastasis, is rife with controversies, because of lacking clinical data support.
In cancer, EMT can be induced by any abnormal changes at genetic, epigenetic, post-transcriptional and post-translation level. Even the tumor microenvironment can also trigger EMT. Dozens of growth factors and transcription factors have been identified as EMT factors. HGF, EGF, PGDF, TGFβ and TNFα have been detected in tumor productions, which can induce EMT progress by active Snail 1 and 2, Zeb 1 and 2, and Twist expression. Multiply signal pathways also be reported involved in the actual implementation of EMT. MAPK, PI3K, Wnt/β-catenin, NFκB, Notch, and Hippo/Warts signaling all of them play an important in the morphogenic process of EMT.

HGF induce EMT progress by activating MAPK pathway to up-regulator early growth response factor-1 (Egr-1). Upon induction by Egr-1, Snail-1 expressed to down-regulator E-cadherin gene expression level. Moreover, the PI3K signaling pathway is critical for induction and maintaining of EMT. Cancer cells can express constitutively active Akt to down-regulate expression of E-cadherin gene by inducing SNAIL. PI3K signaling can also be kicked by Rho family in the morphogenic process of EMT. Also, Wnt signaling pathway is responsible for the β-catenin activity level by phosphorylating tyrosine of β-catenin. And a high level of β-catenin can bind to cadherin to increase cell-cell adhesion. Notch signaling pathway is associated with hypoxia to induce EMT. The released Notch intracellular domain directly Snail-1 promoter to initial Snail-1 gene expression. At the meantime, HIF-1α will active the lysyl oxidase gene expression which will help to stabilize Snail-1 protein to induce EMT.

**EMT and Resistance**

EMT is highly associated with treatment resistance and tumor recurrence. Hijack of any pathway involved in EMT, can provide cancer cells stem-like features and therapeutic resistance
ability. In the LNCaP cell line, Notch signaling pathway is reported associated with increased cell proliferation and survival to form drug resistance to Gemcitabine. Snail-1 and 2 activations associated with radioresistance and chemoresistance, and STAT-3 signaling can block siRNA function in metastatic PC3 prostate cancer cells all have been proved.

**EMT and Tumor Metastasis**

EMT provide cells with migratory and invasive functions is well studied in embryonic development, but whether it is essential for tumor metastasis is full of controversy. Lacking available method to track and detect EMT in patients to gain substantial clinical evidence is a major problem in EMT and tumor metastasis studies.

**The AMPK signaling pathway**

AMP-activated protein kinase (AMPK), a metabolic sensor and regulator, which active when intracellular ATP production level decreases. The AMPK activity depends on the AMP level which will elevate when the cell is lacking energy or under metabolic stress. In mammals, there are seven genes which encoding three types (α, β and γ) of AMPK catalytic subunit.

Besides low energy and cell stresses, multiple pharmacological agents, including Mitochondrial inhibitors, Glycolysis inhibitors, and AICART inhibitors are all able to active AMPK.

The essential role of AMPK is a metabolic checkpoint. One example is, when nutrients are not enough in the microenvironment, AMPK will inhibit cellular growth through suppression of mTOR pathway. From Figure 1-1, we can easily conclude that AMPK is a powerful metabolic checkpoint, and it can regulator most metabolic activity under the help of some certain factors. Some of those factors may be regulator by EMT progress. And it won’t be surprised to see those two events highly overlap.
Summary

Both EMT progress and AMPK signaling pathway are the essential part of cell signaling regulation. Hijack any particular factors involved in those events can elevate cancer cells survival advantages. So, it’s crucial to study the overlap and connection between those two.

In this study, we found extremely enrichment of Snail protein on AMPK gene body. Based on this observation, we designed our experiment to provide evidence to fill the gap between EMT and AMPK signaling by an in vitro cell model.

AMPK pathway. Metformin, one most popular Type 2 diabetes drug, used in this study has been reported as AMPK activator.40
Figure 1-1. Overview of AMPK signaling pathway 41
CHAPTER 2
METHODS

Cell Culture

Human A549 cancer cell line was obtained from ATCC. Mouse neuT and neuTmnt mammary tumor cells were previously established. Both cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS). DCIS-Snail-ER cells were cultured in DMEM/F12 supplemented with 10% horse serum.

DCIS-Snail-ER Cell Construction

DCIS-Snail-ER cell is generated from the MCF10DCIS human mammary epithelial cell line with lentivirus expression of Snail-ER GFP for our lab previous study. This cell line maintains an epithelial phenotype, and only goes through EMT progress after two days 4HT (4-Hydroxytamoxifen) treatment (100nM, two days). In figure 2-1, DCIS-Snail-ER cell was treated with DMSO (negative control) and 4HT for two days and followed an immunoblotting of cell lysates. This western blot result showed dramatic down-regulation of E-cadherin which confirmed this induced EMT cell model works as we designed.

Drug Treatment

All 4HT treatment used 100nM with 48 hours; AICAR treatment used 0.5mM with 24 hours; Metformin used 5mM with 24 hours.

Fluorescence Quantification and Statistical Analysis

For fluorescence staining of treated cells, cells were fixed with Formalin (10% in PBS) for 30 min, and stained with Nile Red for 30 min (10 μg/ml in PBS, ACROS, NJ, USA) and Hoechst 33342 for 5 min (1 μg/ml in PBS, Invitrogen, Eugene, OR, USA). Visualization was carried out under the same microscopic settings with different light wavelength for comparison.
using Leica DMI 6000 B microscope (Leica Microsystems Inc., Buffalo Grove, IL) with the Openlab software (Perkin Elmer, Waltham, MA). Quantification manually by counting Nile Red stained dot number in 3 cells in 3 random visual fields original magnification of 63x fields.

Statistical Analysis was performed by GraphPad Prism 7 (MacOS). P value was calculated through unpaired T-test with Welch’s correction. All data represent mean ± SD; * P ≤ 0.05, **P ≤0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

RNA extraction and real-time RT-qPCR

For RT-PCR, cells were lysed in Trizol reagent (Invitrogen), followed by total RNA extraction. Reverse transcription of RNA was conducted using Moloney murine leukemia virus reverse transcriptase with random primers. The expression levels of selected genes were measured by real-time qPCR using the SYBR Green PCR Kit (Applied Biosystems). Data were normalized against β-actin.

Western Blotting

Whole-cell lysis was denatured by heated 5 min before loaded onto the SDS-PAGE gel. All denatured samples were detected by 8% SDS-PAGE gel, then electrotransferred to the PVDF membrane to perform the immunoblotting. Anti–E-cadherin antibody (Cell Signaling Technology, catalog 3195), anti-tubulin antibody (Sigma-Aldrich, catalog T9026) and AMPK subunit antibody sampler kit (Cell Signaling Technology, catalog 9839) were used as probes.
Figure 2-1. MCF10 DICIS-Snail-ER cell model.
CHAPTER 3
RESULTS

Snai-1 and -2 show enrichment at AMPK associated genes promoters

By further analyzing GSE55421, GSE61475 and GSM1499414, two human and one mouse Snail Protein ChIP-sqe data, we found extremely enrichment of Snail protein on AMPK family gene transcription start sites. (Figure 3-1&2) Considering Snail-1 or -2 protein are working as an EMT regulator, we also analyzed GSE62944, an RNA-Seq data for 9264 tumor samples and 741 normal samples across 24 cancer types from The Cancer Genome Atlas. We also saw correction between AMPK family and Epithelial markers. (Figure 3-3). So, we hypothesized that Snail proteins could bind to AMPK genes loci and suppress their expression.

AMPK genes expression were down-regulated by Snail protein overexpression

To verify our hypothesis. First, we cultured the DCIS-Snail-ER cells with and without 4HT treatment to check their AMPK associated genes expression level. The DCIS-Snail-ER cells maintain an epithelial phenotype and only goes through EMT progress after 4HT treatment because of overexpression of Snail proteins. (Figure 2-1) After two days 4HT treatment, we saw a dramatic decrease on AMPK α1, α2, β1 and γ3 subunits gene expression level (Figure 3-4), which indicated Snail proteins could down-regulate those AMPK genes expression level.

Then, to evaluate the binding of Snail proteins before and after EMT to AMPK gene loci, we performed chromatin immunoprecipitation (ChIP) analysis with AMPK subunit antibody sampler kit, anti-CDH1 antibody, and anti-KRT8 antibody. As expected, compared with the CDH1 signal change before and after EMT, Snail showed similar or even higher enrichment on AMPK α1, β1 and γ3 locus (Figure 3-5). The slight enrichment change on γ2 locus is also consistent with qPCR data. (Figure 3-4&5)
AICAR and Metformin can increase p-AMPK-A1 protein level both before and after EMT

Then, we asked if we put our DCIS-Snail-ER cell model under certain cell stresses, could we weaken or block the regulation of EMT by stimulating AMPK pathway. A previous study showed that, with the treatment of AMPK activators, they could lower TGF-β induced EMT progress.  

So, we treated our cells with 2DG (2-Deoxy-D-glucose), AICAR and Metformin for 24 hours, to calculate cell viability to each treatment, and detected phosphorylated-AMPKα protein level. We saw significant cell viability difference between each treatment before and after EMT (Figure 3-6). AICAR and Metformin can rescue phosphorylated-AMPKα level (Figure 3-7). But, there are no E-cadherin level changes in any treatment setting, except before and after EMT. This indicated, in this Snail-induced EMT model, AMPK activators can rescue phosphorylated-AMPKα level, but cannot influence EMT progress like the previous study.

EMT help cancer cells gain more resistant phenotype and regulate lipid metabolism

To explore the underlying regulation of EMT on AMPK pathway downstream, we performed Nile-Red lipid staining. Nile red is used to localize and quantitate lipids, mainly neutral lipid droplets within cells. Nile red is almost having no fluorescent in water and other polar solvents but undergoes fluorescence enhancement, and large absorption and emission blue shifts in nonpolar environments. DCIS-Snail-ER cells were both cultured on cover slides with 48 hours treatment with 4HT or DMSO and followed 24 hours treatment with or without Metformin. For NMuMT mouse cells treatment, TGF-β was used instead of 4HT to induce EMT. (See details in Method Fluorescence Quantification and Statistical Analysis)

In both DCIS-Snail-ER cells and NMuMG cells Nile red staining, we observed significant lipid dots decrease before EMT which stands for a positive response to Metformin treatment. After EMT, no decrease and even increase of lipid dots were detected when
comparing with and without Metformin treatment (Figure 3-8 and -9). Overall, we saw
significance lipid consumption difference between before and after EMT. This shows EMT play
a significant role in lipid metabolism. But, the mechanism leading to this lipid consumption
deficiency phenomenon and specific factors involved in lipid metabolism difference induced by
two type of EMT factors (TGF-β and Snail) remain unknown. More experiments to dig out the
story behind are necessary.

Figure 3-1. Human Snail-2 protein ChIP-seq binding pike on AMPK α2(Top) and β1(Bottom)
locus
Figure 3-2. Mouse Snail-1 protein ChIP-seq binding pike on AMPK α1, α2, β1, β2 and γ2 locus
Figure 3-3. RNA-Seq data for 9264 tumor samples and 741 normal samples across 24 cancer types from The Cancer Genome Atlas.\textsuperscript{55}

Figure 3-4. AMPK genes expression level in DCIS-Snail-ER cell line with/without 4HT treatment
Figure 3-5. Snai1 ChIP analysis in some AMPK genes sites and Epithelial markers sites
Figure 3-6. DCIS-Snail-ER cell viability under different treatment.

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Figure 3-7. DCIS-Snail-ER cell Western Blotting
Figure 3-8. DCIS-Snail-ER cells Nile Red staining

Figure 3-9. NMuMG cells Nile Red staining
CHAPTER 4
DISCUSSION

In this study, we proved that AICAR and Metformin could promote AMPK signaling pathway even with high enrichment of Snail protein on AMPK gene loci transcription start sites. And combined with some previous clinical study, we hypothesized that, after EMT progress, cancer cells would gain resistance to Metformin.

In adipocyte development studies, it has been reported that, when somatic cells transformed into the adipocyte, the number of lipids is high associated with Cancer cachexia. The adipocyte with small multiple lipids in the tumor is called brown adipocyte, which is much more aggressive compared with white adipocyte which only contains one giant lipid. And Metformin has been studied as a potential antidiabetic drug for years; it functions by suppression of mTOR to arrest cancer cell growth. So, combined those two together, lipid dots number of single cancer cell may relate to drug resistance, for Epithelial Cancer Cells which has positive response to Metformin treatment we saw reduce lipid dots; after EMT, cells gained drug resistance, we saw increase of lipid dots.

Model for lipid metabolism in EMT cancer cell models

Although we saw significant lipid dots number difference between cells before and after EMT, tow EMT cell models induced by different factors response to Metformin differently. In the EMT model induced by Snail, we observed the dramatic elevation of lipid dots; and in the TGF-β induced EMT model, cells have no response to Metformin treatment.

In TGF-β induced EMT model, AMPK has been reported able to degree p300 protein. Once p300 protein being degreed, Smad3&4 complex will lose the regulation ability. In this case, part of AMPK protein will be used for block TGF-β- Smad pathway. But, in our DCIS-
Snail-ER model, AMPK almost has no overlap on Snail-induced EMT pathway. In another word, Metformin-induced AMPK will be consumed to degree p300 in TGF-β induced EMT model which may be the potential mechanism underlying.

**Do AMPK activators work for cancer treatment?**

To answer this question, we further investigated the survival rate of patients who have a higher AMPK-α1 expression level by using Kaplan-Meier Plotter data base. Red curves stand for patients with expression of the high level of AMPK-α1, black curves for the normal level. There is a distinct different between patients with different AMPK-α1 expression level. And multiply studies shows Metformin is an excellent candidate for targeting AMPK/mTOR pathway.\(^{50}\) Metformin treatment usage for cancer therapy has been debated extensively.\(^{51-54}\) Concerns on dosages, the side effect on the special type of cancer and real mechanism behind hasn’t been eased so far. Our data also showed that those activators do have a significant effect on Epithelial Cancer Cells; but once cell goes through EMT, those drugs may make things worse, which suggests that more clinical data are still needed to be collected to find out the mechanism behind.

**Mining in the bioinformatic database**

This study was inspired by our findings in published bioinformatic data. In the last decade, an enormous number of all types of sequence data have been published. Most of them only serve a single experiment. But, the information they contain, may create infinite possibility. How to use they and dig out the hidden treasures, will be a great question to study. And this will be a great treasure for us if we can apply advanced AI to keep analyzing from different perspectives.
Figure 4-1. Model for lipid metabolism in induced EMT cancer cell

Figure 4-2. Model for lipid metabolism in TGF-β induced EMT cancer cell
Figure 4-3. Model for lipid metabolism in Snail induced EMT cancer cell

Figure 4-4. Survival rate of cancer patients: A. Survival rate of breast cancer patients; B. Survival rate of ovarian cancer patients; C. Survival rate of lung cancer patients
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BIOGRAPHICAL SKETCH

Yang Liu was born in Tengzhou, China. He received his Bachelor of Science in biology from Shandong University Life Science School in June 2014. In 2015, he joined Dr. Jianrong Lu's laboratory to work for his Master of Science in biochemistry and molecular biology.