The Roles of Non-Coding RNAs in Solid Tumors

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Non-coding RNAs are functional RNA molecules not translated into protein. There are now different types of non-coding RNAs which have been identified and characterized: 1. Small non-coding (snc) RNAs, 10-40 nucleotides in length. 2. Long non-coding (Inc) RNA, more than several hundreds nucleotides. Recently, a numerous paper has convinced that miRNAs, the largest group of sncRNAs, play a crucial role in tumorigenesis and cancer metastasis. Unlike sncRNAs, the functions of IncRNAs are not well understood although considerable amounts of IncRNAs are expressed in human genome.

Lung cancer is one of the most common causes of cancer related deaths. In particular, Non-small cell lung cancer (NSCLC) is a major type of lung cancer, In spite of some advances in lung cancer therapy, patient survival is still poor. TRAIL is a promising anticancer agent that can be potentially utilized as an alternative or complementary therapy because of its specific anti-tumor activity. However, TRAIL can also stimulate the proliferation of cancer cells through the activation of cell survival signals including NF-κB, but the exact mechanism is still poorly understood. Furthermore, the molecular mechanisms underlying the resistant TRAIL
phenotype is still undefined and little is known on how lung cancer can acquire resistance to TRAIL.

To identify a miRNA involved in innate TRAIL-resistant NSCLC, I characterized miR-34 family, which comprised of tumor suppressor. I found that miR-34a and miR-34c, but not miR-34b target fundamental oncogene, platelet-derived growth factor receptor alpha and beta (PDGFR-α and PDGFR-β) known as a cell surface tyrosine kinase receptors. Consistent with previous observation, I found that miR-34a and miR-34c were downmodulated in lung tumors compared to normal tissues, which consequently showed inverse correlation with PDGFRs. Finally, the ectopic expression of miR-34a and/or -34c or the down-regulations of PDGFRs by siRNA treatment sensitized innate TRAIL-resistant NSCLC and decreases metastatic potentials.

Furthermore, I also characterized how TRAIL-sensitive NSCLC get a resistance. In this study, I showed that chronic exposure to subtoxic concentrations of TRAIL results in acquired resistance. This resistance was associated with the increase in miR-21, miR-30c and miR-100 expression which target tumor suppressor genes fundamental in the response to TRAIL. Importantly, downregulation of caspase-8 by miR-21 blocks RIP1 cleavage and induced the activation of NF-κB, which regulated these miRNAs. Thus, TRAIL activated a positive feedback loop that sustained the acquired resistance and caused an aggressive phenotype. Finally, I proved that combinatory treatment of NF-κB inhibitors and TRAIL was able to revert resistance and reduce tumor growth with important consequences for the clinical practice.
While the biological roles for several miRNAs and mRNAs in cancer progression have been described, miR-224, miR-520c-3p and TUSC3 remain unexplored in lung cancer pathogenesis. Here, I proposed that miR-224 and miR-520c-3p target TUSC3 gene, which was responsible for lung cancer metastases. I observed that TUSC3 expression was downregulated in sites of metastases compared with their matched primary lung tumor tissues. We conducted a series of the experiments validating TUSC3 as a direct target of miR-224 and miR-520c-3p. These miRNAs were upregulated in lymph node involved with metastatic lung cancer and showed in inverse correlation with TUSC3. Both in vitro and in vivo studies showed that TUSC3 reduction contributed to cancer metastases, which were mediated by both NM23H1 and MCAM regulation. These observations supported an unrecognized role for miR-224 and miR-520c-3p as oncogenic miRNAs and TUSC3 as a metastatic suppressor, with implication for the clinical practice in lung cancer metastases.

Like sncRNAs, recent reports showed that IncRNAs were involved in many aspects of tumorigenesis in several cancers. Previously, the former lab member Dr. Taewan Kim first identified a transcription factor, Myc-associated IncRNAs named MYCLos. Among those, I characterized MYCLo-1 and MYCLo-2 to determine whether they were functionally related with Myc functions. In vitro analyses convinced that the downregulation of MYCLo-1 or MYCLo-2 suppressed cell proliferation and transformation abilities of colorectal cancer. Furthermore, by using a series of luciferase assays and qRT-PCR analyses I further characterized that
MYCLo-1 or MYCLo-2 regulated \textit{CDKN1A} or \textit{CDKN2B} known as a Myc-target protein. Finally the oncogenic fuctions of \textit{MYCLo-2} were further confirmed through in vivo xenografted nude mice model.

Taken together, non-coding RNAs plays an important role in several aspects of tumorigenesis and tumor pathogenesis. These mechanistic studies of my current studies will provide a new therapeutic against sold tumors.
Dedicated to my beloved wife, Sunhee Park, for her support and encouragement

To my mother who always prays for me

To my father, the late Mr. Byung-ho Jeon
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# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>vi</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vii</td>
</tr>
<tr>
<td>Vita</td>
<td>ix</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## Chapters:

### 1. Introduction
- 1.1 Lung cancer and Cancer metastasis ........................................... 1
- 1.2 Non-coding RNAs ........................................................................ 1
- 1.3 Apoptosis .................................................................................. 3

### 2. Characterization of TRAIL-resistance in lung cancer

- 2.1 General Introduction for Chapter 2 ............................................ 7

#### 2.2 MiR-34a/c-dependent PDGFR-α/β downregulation inhibits tumorigenesis and overcomes innate TRAIL-resistance in lung cancer.

- 2.2.1 Introduction ........................................................................... 8
- 2.2.2 Materials and Methods ......................................................... 10
- 2.2.3 Results .................................................................................. 13
- 2.2.4 Discussion ............................................................................. 16
2.3 A set of NF-kB-regulated MicroRNAs Induces Acquired TRAIL Resistance in Lung Cancer

2.3.1 Introduction.................................................................26
2.3.2 Materials and Methods..............................................27
2.3.3 Results.................................................................31
2.3.4 Discussion..........................................................42

3. MiR-224 and miR520c-3p-mediated TUSC3 suppression enhances metastatic potential of NSCLC through NM23H1 and MCAM regulation

3.1 Introduction.................................................................64
3.2 Materials and methods..............................................66
3.3 Results.................................................................69
3.4 Discussion..........................................................76

4. Role of MYC-regulated long noncoding RNAs in cell cycle regulation and tumorigenesis

4.1 Introduction.................................................................92
4.2 Materials and methods..............................................93
4.3 Results.................................................................94
4.4 Discussion..........................................................97

5. Conclusions and Future directions

5.1 Characterization of miRNAs involved in TRAIL-resistance.................104
5.2 MiR-224 and miR-520c-dependent TUSC3 suppression is involved in lung cancer metastasis........................................108
5.3 Role of MYC-regulated long noncoding RNAs in cell cycle regulation and tumorigenesis........................................110

Lists of References..........................................................112
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>MiRNA biogenesis</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Two distinct apoptotic pathways</td>
<td>6</td>
</tr>
<tr>
<td>2.1</td>
<td>MiR-34a and -34c directly target PDGFR-α and PDGFR-β 3’UTRs</td>
<td>19</td>
</tr>
<tr>
<td>2.2</td>
<td>PDGFR-α and PDGFR-β are suppressed by miR-34a and miR-34c at mRNA and protein levels</td>
<td>20</td>
</tr>
<tr>
<td>2.3</td>
<td>MiR-34a/c and PDGFR-α/β are inversely correlated in normal and tumor lung tissues</td>
<td>21</td>
</tr>
<tr>
<td>2.4</td>
<td>Co-expression analyses with miRNAs and the target genes in vivo</td>
<td>22</td>
</tr>
<tr>
<td>2.5</td>
<td>MiR-34a/c overexpression or PDGFR-α/β reduction sensitizes the response of NSCLC cells to TRAIL-induced cell death</td>
<td>23</td>
</tr>
<tr>
<td>2.6</td>
<td>MiR-34a/c inhibits migratory and invasive capacities of NSCLC through PDGFR-α/β suppression</td>
<td>25</td>
</tr>
<tr>
<td>2.7</td>
<td>Identification of dysregulated miRNAs in cells with acquired TRAIL-resistance</td>
<td>47</td>
</tr>
</tbody>
</table>

xvi
2.8 MiR-21, -30c and -100 inhibit TRAIL-induced cell death

2.9 Gene Affymetrix microarray of H460S and H460R cells

2.10 Caspase-8, caspase-3, FoxO3a and TRAF-7 3’UTRs are direct targets of miR-21, miR-30c and miR-100.

2.11 Co-expression analysis of miRNAs and target genes

2.12 Caspase-8, caspase-3 and TRAF-7 are involved in acquired TRAIL-resistance

2.13 NF-κB directly activates miR-21, miR-30c and miR-100

2.14 Silencing of upstream activators of NF-κB sensitzes H460R cells to TRAIL-induced cell death

2.15 Caspase-8-dependent RIP1 cleavage blocks NF-κB activation in TRAIL-resistant cells

2.16 Acquired TRAIL resistance induces EMT

2.17 Proposed model showing NF-κB induces acquired TRAIL resistance through miR-21, miR-30c and miR-100 activation

3.1 TUSC3 is suppressed in metastasized lung cancer patient samples

3.2 MiR-224 and miR-520c-3p directly suppress TUSC3 in NSCLC

3.3 Co-expression analyses of miRNAs and TUSC3 showing inverse correlation between miRNAs and TUSC3 in lung cancer patient tissues

3.4 The comparison of expressions of miR-224 and miR520c-3p between primary lung tumors and their matched lymph node metastases of lung tumor tissues

3.5 TUSC3 deficiency confers the cells to be highly metastatic in vitro and in vivo
3.6 The roles of TUSC3 deficiency in migration and invasion are conserved in different cancer cells.

3.7 NM23H1 and MCAM proteins are potential targets for TUSC3.

3.8 MiR-224 and miR-520c-3p enhances metastatic potential of NSCLC through TUSC3 suppression.

4.1 MYCLos are involved in cell proliferation.

4.2 MYCLos mediates Myc-dependent CDKN1A and CDKN1B expressions.

4.3 MYCLos mediates CDKN1A and CDKN1B expressions.

4.4 MYCLO-2 exhibit tumor suppressive phenotypes in CRC and prostate tumors.
Chapter 1

Introduction

1.1 Lung Cancer and Cancer Metastasis

Lung cancer is the most common cause of cancer-related deaths in the world. Non-small cell lung carcinoma (NSCLC) accounts for about 80% of all lung cancers [1]. The reasons for the poor overall 5 year survival in lung cancer are multifactorial including late clinical presentation of disease, occult metastatic disease and few targeted therapeutics. Cancer metastasis is complex, multistep process based in reciprocal interactions between tumor cells and and their microenvironment. Although there have been several studies on this process, the sequence of critical events and molecular mechanisms for cancer metastasis remain poorly understood [2].

1.2 Non-coding RNAs

Non-coding RNA (ncRNA) molecules are defined as a functional RNA including small non-coding RNAs (snRNA) and long non-coding RNA (lncRNA), which consists of more than 98% of human genome. In spite of considerable amount of the transcripts, their functions were not well understood. In a classical point of view, those ncRNAs were considered junk DNA. For the last decays, a numerous
papers, however, have convinced that the ncRNAs are tightly regulated in expression in response to several internal and external stimuli and play critical roles in cell homeostasis and metabolism as well as tumorigenesis [3,4].

SncRNAs, approximately 20-30 nucleotides in length, consist of several classes such as siRNA, miRNA, and piwiRNA. The miRNAs is the largest endogenously expressed group of the sncRNAs in human genome. It has been shown that miRNAs regulate many aspects of cellular metabolism, cell death, cell survival, and tumorigenesis, mainly by silencing effect with respect to their target genes. [5].

The first step of miRNA biogenesis is mainly generated by RNA-polymerase II-dependent transcription, which is further processed by a nuclear enzyme, Drosha [6]. The primary transcript is transported by Exportin 5 dependent manner and is further cleaved by Dicer in cytoplasm, about 22nt pre-miRNAs are loaded into Ago proteins, which make a RISC-loading complex (RLC). In the RLC, mature miRNAs are generated and functions on their corresponding target mRNAs to silence gene expressions [7,8].

In spite of the same purpose that the three classes of the small RNAs induce gene silencing, they have different mechanisms to repress their target mRNAs. siRNAs need to be perfectly matched sequences corresponding to their target mRNAs, which leads to mRNA-cleavage in RNA-induced Silencing Complex (RISC). While miRNAs mainly suppress gene expression by binding on the 3’UTR on target mRNAs as well as the similar mechanism with siRNAs by which miRNA induces mRNA degradation when the seed sequences on miRNAs are perfectly matched with
their target mRNAs [9]. So far, more than two thousand miRNAs have been identified in human, which contributes tumorigenesis, cell homeostasis and stress-responses. Consequently, many miRNAs have been identified as tumor suppressor or oncogene by targeting oncogenes or tumor suppressor genes [4,5].

1.3 Apoptosis

Apoptosis is a programmed cell death, which was firstly characterized at middle of 20\textsuperscript{th} century in multicellular organisms [10-13]. To maintain homeostasis in an organism, life and death of the organism should be well balanced. Consequently, apoptosis should be tightly regulated. Therefore, defects in apoptotic pathways are closely related with several diseases including neuronal aggregate diseases and cancers [14,15].

There are two distinct mechanisms to induce apoptosis when the cells are in unfavorable conditions: Intrinsic apoptosis (the death signal is produced from inside the cell) and extrinsic apoptosis (death signal is initiated on the cell surface in response to several death stimuli from outside cell). Both mechanism is mainly mediated and executed by several caspase (cysteine aspartyl-specific proteases) family. Caspase, known as a zymogen, mediates death signals and induces cell death by cleaving several subcellular proteins. Caspases consist of two groups: initiator caspases are usually activated by death signals and activate down-stream caspases. The executioner capsases are activated by initiator caspases and eventually induce cell death [16].
In intrinsic apoptosis mechanism, mitochondria are a central sensor and hub to initiate intrinsic apoptosis. Various stress conditions such as DNA damage, hypoxia, or ER-stress can induce mitochondrial damage, which consequently triggers the formation of the huge protein complex, called Apoptosome including Apaf-1, caspase-9, cytochrome C and ATP molecule. In the complex, the caspase-9, an initiator caspase, is activated and perform the programmed cell death scenario. The extrinsic apoptosis pathway requires several types of death receptors to mediate outside signal. The death receptors are members of TNF-super family including TNF-receptors, TRAIL-receptors and Fas (CD95). Once death receptors are activated by binding with their corresponding ligands, which also triggers the formation of another apoptosis complexes, called DISC (Death-Induced Stimulating Complex). Another initiator caspases, caspase-8 and/or caspase-10 are activated. In some types of the cells, the activated caspase-8 and/or caspase-10 directly activate executioner caspase without stimulating mitochondrial dependent apoptosis (Type I extrinsic apoptosis). On the other hand, the cells have a chance to amplify death signal by activating mitochondrial apoptotic pathway, if the cell death signal is weak in type II cells [15,17-20].
Figure 1.1 MiRNA biogenesis (modified from Joshi et al., 2014; MicroRNAs in lung cancer. World J Methodol. 2014 June 26; 4(2): 59-72).
Figure 1.2 Two distinct apoptotic pathways. Extrinsic apoptosis is stimulated by death receptors whereas intrinsic apoptosis is triggered by subcellular stresses.
CHAPTER 2

Characterization of TRAIL-resistance in Lung Cancer

2.1 General Introduction for Chapter 2

TNF-Related Apoptosis Inducing Ligand (TRAIL) is a type II transmembrane protein and it was originally discovered by homology search with Fas ligand and TNF [21,22]. After that, numerous approaches have convinced that TRAIL-induced cell death shows high cancer specificity in several tumors [4,23]. Molecular sequence of TRAIL-induced cell death resembles an typical extrinsic apoptotic signals such as TNF- and Fas-induced cell death. The association of TRAIL with one of the two receptors (TRAIL-receptor-1/DR4 and TRAIL-receptor-2/DR5) can trigger extrinsic apoptosis by, eventually, activating caspase-cascade in several solid tumors. The other two receptors, DcR1 and DcR2, are ‘decoy receptors’ and lack the ability to initiate the apoptotic cascade. Currently, recombinant TRAIL and agonistic antibodies directed at DRs are in phase-II clinical trials. However, recent studies have demonstrated that many types of cancer cells possess intrinsic or acquired resistance to TRAIL. Moreover, TRAIL has been found to activate a pro-survival transcription factor, nuclear factor kappa B (NF-κB) and enhance metastasis in apoptosis-resistant cancer cells, which seems to play an important role in TRAIL-resistance [24-27].
As an importance of the nature that TRAIL selectively induces cell death on most tumor cells, many approaches have suggested the mechanism by which the resistant cells escape TRAIL-induced cell death and have identified the several determinants such as c-FLIP, NF-kB, PTEN and c-myc in TRAIL-resistance. In accordance with the studies, the combinatorial drugs are using to clinical trials with recombinant TRAIL or its agonistic antibodies [23,28,29]. However the molecular mechanisms of TRAIL-resistance should be fully elucidated because intrinsic and acquired TRAIL-resistance are hindrance to anti-tumor therapy with TRAIL [30].

Lung cancer is one of the most cause of deaths in the world and the Non-Small-Lung-Carcinoma (NSCLC) is an epithelial lung cancer type, which accounts for more 80% of lung cancer [1]. The observation that most stage-III NSCLC express either or both TRAIL-receptors raises the possibility of TRAIL usage as an anti-cancer drug, indicating that the understanding molecular mechanisms of intrinsic and acquired TRAIL-resistance are required for successful therapy with TRAIL [30,31].

2.2 MiR-34a/c-dependent PDGFR-α/β downregulation inhibits tumorigenesis and overcomes innate TRAIL-resistance in lung cancer.

2.2.1 Introduction

MiR-34 family consists of miR-34a, 34b and miR-34c in mammalians, which are known as tumor suppressors in several solid tumors including lung cancer [32,33].

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1 Results in this chapter were published in: Garofalo and Jeon et al., PLoS One. 2013 Jun 21;8(6):e67581
MiR-34 family is encoded by two different genes. Unlike miR-34a encoded by its own transcripts, miR-34b and -34c are alternative spliced forms from single transcript. Furthermore, it has been known that the expressions of miR-34 family are frequently reduced in several cancers by epigenetic modification such as methylation on the promoter regions [34,35].

Platelet-derived growth factor receptor (PDGFR) is receptor tyrosine kinase and is involved in proliferative and prosurvival signaling pathway, which promotes oncogenic transformation of the cells [36]. PDGFR includes two receptors (PDGFR-\(\alpha\) and PDGFR-\(\beta\)) whereas there are four different PDGF ligands. Several lines of evidence suggest that the PDGFRs play a critical role in tumorigenesis including lung cancer pathogenesis. A study with 150 NSCLC patient samples showed that about 13% of total cases have mutations on PDGFR-\(\alpha\) [37]. In addition, PDGFR-\(\alpha\) expression was strongly correlated with a negative prognostic indicator [38]. Furthermore, the study with PDGF-\(\beta\) knock-out mice showed defective angiogenesis phenotypes [39,40].

In this study, I showed that PDGFR-\(\alpha\) and PDGFR-\(\beta\) are significantly upregulated in NSCLC cells and lung tumor samples, which was mediated by miR-34a and miR-34c. Furthermore, miR-34a/c-dependent PDGFR-\(\alpha\) and PDGFR-\(\beta\) targeting sensitized innate TRAIL-resistance of lung cancer, suggesting that combinatory treatment of TRAIL and PDGFR inhibitors could be effective for anti-NSCLC therapy.
2.2.2 Materials and Methods

Lung cancer cell lines and tissue samples.

Human H460, A549, H1299, H1703 cell lines were grown in RPMI medium containing 10% heat-inactivated fetal bovine serum (FBS) and Calu-6 cells were grown in MEM supplemented with 10% fetal bovine serum. 9 lung tumors (including adenocarcinoma and squamous cell carcinoma) and their normal counterparts were kindly provided by Dr. P. Nana-Sinkam, Pulmonary, Allergy, Critical Care and Sleep Medicine, The Ohio State University Comprehensive Cancer Center, Columbus, OH. 48 lung normal and tumor tissue samples were provided from the Department of Pathology, Ohio State University. All human tissues were obtained according to a protocol approved by the Ohio State Institutional Review Board.

Real-time PCR

Real-time PCR was performed using a standard TaqMan PCR Kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). The 10 μl PCR reaction included 0.67 μl RT product, 1 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.2 mM TaqMan probe, 1.5 mM forward primer and 0.7 mM reverse primer. The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The comparative CT method for relative quantization of gene expression (Applied Biosystems) was used to determine miRNA and genes expression levels. The y-axis represents the $2^{(-\Delta CT)}$, or the relative
expression of the different miRs and genes. MiRs expression was calculated relative to U44 and U48 rRNA (for microRNAs) and to GAPDH and b-actin (for genes). Experiments were carried out in triplicate for each data point, and data analysis was performed by using software (Bio-Rad).

**MiRNA locked nucleic acid in situ hybridization of formalin fixed, paraffin-embedded tissue section.**

In situ hybridization (ISH) was carried out on deparaffinized human lung tissues using previously a published protocol [41], which includes a digestion in pepsin (1.3 mg/ml) for 30 minutes. The sequences of the probes containing the dispersed locked nucleic acid (LNA) modified bases with 5’-conjugated digoxigenin were: miR-34 (5’) ACAACCAGCTAAGACACTGCCA (3’). The probe cocktail and tissue miRNA were co-denatured at 60°C for 5 minutes, followed by hybridization at 37 °C overnight and a stringency wash in 0.2X SSC and 2% bovine serum albumin at 4°C for 10 minutes. The probe-target complexes were observed through the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP). Negative controls included a probe which should yield a negative result in such tissues (scrambled miRNA). No counterstain was used, to facilitate co-labeling for PDGFR proteins. After in situ hybridization with miRNAs, as previously described [42] the slides were analyzed for immunohistochemistry (IHC) using the standard protocols. The percentage of tumor cells expressing miR-34 and PDGFRs was then analyzed with an emphasis on co-localization of the respective targets. Co-expression analysis was done with the Nuance system (Cambridge
Research Institute) per the manufacturer’s recommendations.

**Western Blot Analysis**

Total proteins from NSCLC were extracted with radioimmunoprecipitation assay (RIPA) buffer (0.15mM NaCl, 0.05mM Tris-HCl, pH 7.5, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate and 1% Nonidet P40). Sample extracts (30-50 μg) were resolved on 4–20% SDS–polyacrylamide gels (PAGE) using a mini-gel apparatus (Bio-Rad Laboratories) and transferred to Hybond-C Extra nitrocellulose. Membranes were blocked for 1h with 3% Bovine Serum Albumin in Tris-buffered saline containing 0.05% Tween 20, incubated overnight with primary antibody, washed and incubated with secondary antibody and visualized by chemiluminescence. The following primary antibodies were used: anti-PDGFR-α, anti-PDGFR-β, anti-ERK1/2, anti-p-ERKs, anti-pAKT, anti-total AKT, anti-GAPDH antibodies (Cell Signaling). A secondary anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody peroxidase conjugate (Chemicon) was used.

**Migration assay**

Briefly, Calu-6 cells were transfected with pcDNA-PDGFR-α, pcDNA-PDGFR-β and/or hsa-miR-34a and miR-34c, respectively. 24h after transfection, 2x10⁵ cells in MEM media supplemented with 1%FBS were plated into the upper chambers of the Migration assay and RPMI supplemented with 10%FBS were added into lower chambers to use as a chemoattractant. After 24h, the upper chambers were transferred into a new plate with detaching solutions containing Calcein AM for 1 hour to measure the amount of the migrated cells. The fluorescence was analyzed at an
excitation wavelength of 485 nm and an emission wavelength of 520nm.

**Invasion assay**

Briefly, 2x 10^5 Calu-6 cells transfected with pcDNA-PDGFR-α, pcDNA-PDGFR-β and/or miR-34a and miR-34c in RPMI supplemented with 1%FBS were plated into upper chambers of Invasion assay with a 8-um pore size- polycarbonate membrane. 700 μl of MEM supplemented with 10% FBS were added into the lower chambers as a chemoattractant. After 36-48h, the upper chambers were transferred into a new plate and were incubated with detaching solutions contained Calcein AM for 1 hour to measure the amount of the invaded cells. The fluorescence was analyzed at an excitation wavelength of 485 nm and an emission wavelength of 520nm.

**Statistical analysis**

Student’s t test was used to determine significance. All error bars represent the standard error of the mean. Statistical significance for all the tests, assessed by calculating P-value, was < 0.05.

### 2.2.3 Results

**MiR-34a and miR-34c target PDGFR-α and PDGFR-β 3’UTRs.**

To identify target genes for miR-34 family, I performed a bioinformatic search with Targetscan and Pictar program and found that 3’UTR of human PDGFR-α and PDGFR-β contained regions (PDGFR-α nucleotides 2670–2676; 2699-2705; PDGFR-β nucleotides 1535-1541) that matched the seed sequences of hsa-miR-34a, -34b and -34c (Figure 2.2.1a). To test whether miR-34 family directly target PDGFR-α and PDGFR-β 3’UTR, I conducted luciferase analyses with miR-34 family and the
PGL-3-luciferase vector harboring PDGFR-α or PDGFR-β 3’UTR. Interestingly, miR-34a and -34c, but not -34b decreased luciferase activities of PDGFR-α and PDGFR-β 3’UTR (Figures 2.2.1b and 1c). The targeted luciferase suppression were recovered by same experiments with deleted mutant of seed sequences on PDGFR-α and PDGFR-β 3’UTR (Figures 2.2.1b and 1d), suggesting that miR-34a and -34c directly target PDGFR-α and PDGFR-β.

**MiR-34a and miR-34c are inversely related to PDGFR-α/β expression in vitro and in vivo.**

Next, I analyzed the consequences of the ectopic expression of miR-34a and -34c in Calu-6 and H1703 cells. The overexpression of miR-34a and miR-34c, but not miR-34b suppressed PDGFR-α and PDGFR-β mRNA and protein levels analyzed by qRT-PCR (Figure 2.2.2a) and western blot analyses (Figure 2.2.2b). To verify these inverse correlations between miR-34a/c and the target proteins in vivo, 9 lung tumors (among adenocarcinoma and squamous cell carcinoma) and the adjacent histological normal lung tissues were analyzed for miR-34a and -34c expression, indicating that both miRNAs was suppressed in all tumor samples that I tested (Figures 2.2.3a and 3b). Moreover, miR-34a, 34c and PDGFR-α/β mRNA expression in 24 primary human lung tumor samples were analyzed by qRT-PCR. The expression of both miRNAs was much higher in lung tumor specimens than normal tissues (Figure 2.2.3c). Consequently, there were an inverse correlation between miR-34a/c and PDGFR-α and PDGFR-β (Figure 2.2.3d). To further confirm these findings, in situ hybridization (ISH) analysis was performed using 5’-dig-labeled LNA probes of miR-34a on lung tumors and normal tissues, followed by immunohistochemistry (IHC) for
anti PDGFR-α and PDGFRβ antibodies. Most lung cancer cells showed low expression of miR-34a and high expression of PDGFR-α/β, whereas the adjacent non-malignant lung expressed PDGFR-α rarely and abundantly expressed miR-34a. MiR-34a and PDGFR-α/β expression in the majority of the 107 different tumors analyzed was basically mutually exclusive (Figure 2.2.4).

**Mir-34a and miR-34c sensitizes bona fide TRAIL-resistant NSCLC through PDGFR-α and PDGFR-β downregulation.**

In spite of high therapeutic potential with TRAIL, there are still the tumors showing innate resistance against TRAIL-induced cell death. In addition, PDGFR-α and PDGFR-β are known as a positive regulator of central cell survival signaling pathway such as PI3K/Akt and ERK1/2 pathways [43-45]. Consistently, I found that phosphor-ERKs proteins are suppressed by enforcement of miR-34a or -34c. Likewise, PDGFR-α silencing reduced the activation of both Akt and ERK1/2 (Figures 2.2.5a and 5b). Therefore, I next examined the involvement of miR-34a and -34c in TRAIL-induced cell death. As shown in Figure 2.2.5c, the enforced expressions of miR-34a or -34c increased TRAIL-induced cell death in Calu-6 and H1703 cells. I consistently found that inhibition of PDGFR-α or PDGFR-β enforced by specific siRNA treatment (Figure 2.2.5d) or inhibitor treatment (Figure 2.2.5f) sensitized TRAIL-induced cell death. Conversely, the ectopic expression of PDGFR-α or PDGFR-β makes the cells more resistant against TRAIL-induced cell death (Figure 2.2.5e). The results suggest that PDGFR-α and PDGFR-β play an important role in TRAIL-induced apoptosis and that PDGFR inhibitor can sensitize NSCLC
cells to TRAIL with important therapeutic consequences.

**MiR-34a and -34c-dependent PDGFR-α/β downregulation inhibits migratory and invasive effects of of NSCLC cells.**

To directly test the functional role of miR-34a/c in tumorigenesis, I overexpressed these two miRNAs or siPDGFR-α/β siRNAs in Calu-6 or H1703 cells. Importantly, I observed that the migratory and invasive capabilities of Calu-6 and H1703 cells were significantly suppressed after miR-34a/-34c overexpression (Figure 2.2.6a) or PDGFR-α/-β downregulation (Figure 2.2.6b), confirmed also by wound-healing assay (Figure 2.2.6c). Furthermore, miR-34a/-34c-dependent migration or invasion suppressions were partially recovered by PDGFR-α or PDGFR-β overexpression (Figure 2.2.6d), suggesting that miR-34a/c regulate NSCLC tumorigenesis, at least in part, through PDGFR-α/β.

### 2.2.4 Discussion

MiRNAs are the largest group in small non-coding RNAs, which plays a versatile role in several aspects of tumorigenesis. In particular, it has been shown that miRNAs are used for prognosis of human cancers and plays a critical role in the resistance to chemotherapeutic drugs [37,46]. Although TRAIL or its agonistic antibody has a tremendous therapeutic potential, TRAIL-resistance is still challenging for anti-cancer therapies. In this study, I found that miR-34a and miR-34c strongly suppressed PDGFR-α and PDGFR-β mRNA and protein levels, which sensitized TRAIL-induced cell death and suppressed metastatic potentials of NSCLC.
Several prosurvival signals including PI3K/AKT and MAPK/ERK pathways confer a major hindrance with conventional chemotherapies [47,48]. In particular, several lines of evidence have suggested that small molecule inhibitors of the PI3K/Akt pathway successfully showed in overcoming chemotherapeutic resistance. Furthermore, inhibiting AKT activity may be a valid approach to treat cancer. PDGFRs are known as receptor tyrosine kinases, which provide strong survival signals in several cancers including lung cancer via mainly activating PI3K/AKT and/or MAPK/ERK pathways. In a previous work, our group demonstrated that MET induced tumorigenesis and TRAIL resistance in NSCLC through the activation of the PI3K/AKT pathway [35,43,49]. Therefore, PDGFR-α/β regulations by miRNAs should be able to be very crucial in anti-cancer therapies with TRAIL. Indeed, ectopic expression of miR-34a and miR-34c highly increased the response of semi-resistant NSCLC cells to TRAIL-induced apoptosis, which seems to be, at least in part, mediated by PDGFR-α/β. Moreover, combinatorial treatment of a PDGFR inhibitor/siPDGFR siRNAs with TRAIL enhanced apoptosis.

So far, there have been some reports showing direct association between miR-34 family and PDGFR-α in other cancers and, moreover, tumor suppressive effects of miR-34 family could be mediated by the other factors such as AXL and c-Met proteins [50-52]. The current study also confirmed that PDGFR-α plays an important role in tumorigenesis of lung cancer, which seems to be mediated by miR-34a and -34c but not -34b. Moreover, miR-34a/-34c-dependent PDGFR-β suppression will be also good target for anti-cancer therapies with TRAIL. Taken together, the results of
this section suggest that combined treatment of TRAIL with PDGFR inhibitors could be a good way to sensitize a subset of lung tumors showing TRAIL-resistance.
Figure 2.1 MiR-34a and -34c directly target PDGFR-α and PDGFR-β 3’UTRs. (a) Sequence comparison between miRNAs and the target genes showing perfect matched sequences. (b). PGL3 control-PDGFR-α constructs containing two PDGFR-α binding sites (in red). Deletion of one of the two PDGFR-α sites was used to generate the mutant luciferase plasmids. (c-d) Luciferase activities of PDGFR-α and PDGFR-β 3’UTRs (c) or their mutants (d) upon miR-34 family overexpression in calu-6 cells. Relative repression of firefly luciferase expression was standardized to a transfection control. The reporter assays were performed three times with essentially identical results. *P<0.0001, **P<0.05 by two tailed Student’s t test.
Figure 2.2 PDGFR-α and PDGFR-β are suppressed by miR-34a and miR-34c at mRNA and protein levels. (a) qRT-PCR showing PDGFR-α and PDGFR-β mRNAs downregulation in Calu-6 and H1703 cells after miR-34 and miR-34c but not miR-34b enforced expression (b) miR-34a and miR-34c enforced expression decreases endogenous levels of PDGFR-α/β protein levels in Calu-6 and H1703 cells. Cells were transfected with either scrambled, miR-34a or miR-34c for 72 hr.
Figure 2.3 MiR-34a/c and PDGFR-α/β are inversely correlated in normal and tumor lung tissues. (a) qRT-PCR analyses with 18 lung tumor and normal tissues. MiR-34a and -34c are downregulated in the tumors compared to the normal lung tissues. (c) Box plots showing miR-34a, miR-34c, PDGFR-α and PDGFR-β expression in 52 lung normal and cancer tissues. Two tailed Student’s t test was used to verify the significance. (d) Scattered plots show inverse correlation between miR-34a/-34c and PDGFR-α/-β.
**Figure 2.4 Co-expression analyses with miRNAs and the target genes in vivo.** (a-b) Immunohistochemistry and in situ hybridization on 107 lung cancer tissues samples. MiR-34a (blue) and PDGFR-α/β (brown/red, respectively in RGB and each fluorescent red in Nuance converted image) expression were inversely related in lung cancers and the adjacent normal lung tissues. These serial sections were analyzed for miR-34a expression by in situ hybridization, followed by immunohistochemistry for PDGFR-α/β. (a) Representative example: Co-expression analysis of miR-34a and PDGFR-α. Note lack of expression in the merged image (panel b) (fluorescent yellow = co-expression). (b) Representative example: miR-34a= blue (Panel a), PDGFR-β=red (Panel b), co-expression= yellow (Panel c). RGB= Regular Green Blue image of the ISH/IHC reaction shown in panels a-c. Scale bar indicates 50 µm. The summary tables are shown in the right panel of each figure.
Figure 2.5 MiR-34a/c overexpression or PDGFR-α/β reduction sensitizes the response of NSCLC cells to TRAIL-induced cell death. (a) Western blot analysis in Calu-6 cells after miR-34a, -34b and -34c forced expression. (b) Western blot showing the suppression of the Akt and ERKs pathways after PDGFR-α silencing. (c) Caspase -3/7 assay with miR-34a and -34c. MiR-34a and -34c enforced expression in Calu-6 and H1703 semi-resistant cells, increases the response to TRAIL-induced apoptosis. (d) Caspase -3/7 assay showing that PDGFR-α or PDGFR-β silencing increases the response to TRAIL-induced apoptosis. (e) PDGFR-α or PDGFR-β overexpression in H460 TRAIL-sensitive cells decreases the response to the drug. (f) Combined treatment of PDGFR inhibitor (25 μM) and different TRAIL concentrations (0-50-100 ng/ml) sensitizes NSCLC cells to TRAIL-induced apoptosis. *P< 0.001, ** P< 0.05.
Figure 2.6 MiR-34a/c inhibits migratory and invasive capacities of NSCLC through PDGFR-α/β suppression. (a-b) Migratory and invasive capabilities of Calu-6 cells upon miR-34a/-34c overexpressions (a) or upon PDGFR-α and PDGFR-β silencing (b). (c) Wound-healing analysis with miR-34a/-34c overexpression. (d-e) PDGFR-α and PDGFR-β overexpression partially rescues migratory and invasive capabilities of miR-34a/-34c overexpressing Calu-6 cells. * P< 0.05.
2.3. A set of NF-kB-regulated MicroRNAs Induces Acquired TRAIL Resistance in Lung Cancer.

2.3.1 Introduction

Unlike Fas-downstream signaling, TRAIL-TRAIL-receptor association can also activate a pro-survival transcription factor, nuclear factor kappa B (NF-kB) as a non-apoptotic function of TRAIL, which seems to be a critical factor for TRAIL-resistance [53,54]. A serine/threonine kinase, RIP1 makes a complex with two TRAIL receptors, TRADD, and TRAF-2, which is responsible for TRAIL-induced NF-kB activation and p65 subunit, but not c-rel, of NF-kB complex is mainly involved in cell survival processes against TRAIL-induced cell death [55,56]. In spite of central observation for the TRAIL-induced NF-kB activation, little is known how NF-kB pathway is regulated and consequently contributes to TRAIL-resistance.

For the past years, it has been massively studied that many miRNAs showed in altered expression in cancer, which consequently regulates tumorigenesis [4]. Several lines of evidence suggest that miR-21 is known as an oncomiR by suppressing multiple tumor suppressors in many tumors including Non Small Cell Lung Cancer (NSCLC) and can be used for prognostic marker of NSCLC [5,57]. A study of miR-21 transgenic mice with the loss of function and gain of function allele suggested that miR-21 plays a critical role in tumorigenesis of NSCLC [58]. MiR-30 family seems to have opposite function in each cancer. However we previously showed that miR-30b/c was involved in Epithelial-Mesenchymal Transition (EMT) of

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2 Results in this chapter are now in revision status. Jeon et al., EMBO Journal.
NSCLC in response to EGF and MET stimuli as an oncogenic function [49]. Unlike the obvious roles of miR-21 and -30b/c/e in tumorigenesis of NSCLC, the role of miR-100 in tumorigenesis seems to be context dependent in several tumors and its role in NSCLC is not well understood [59,60]. Furthermore the exact mechanism and contribution of these miRNAs in TRAIL-resistance of NSCLC is largely unknown.

Here I established two acquired TRAIL-resistant cells by repeatedly exposing with TRAIL into TRAIL-sensitive cells for 6 month in NSCLC. I found increased expression of the miR-21, -30c/e, and -100 in acquired TRAIL-resistant cells, which weaken TRAIL-induced cell death but strengthen survival signals by increasing TRAIL-induced NF-\(\kappa\)B activation. Consequently, I propose that the altered and one-sided signal plays a critical role in acquired TRAIL-resistance in NSCLC.

2.3.2 Materials and Methods

Lung cancer samples and cell lines.

69 cancer lung tissues were purchased from US Biomax, Inc. Human H460S/R, H292S/R, HEK293, Calu-1 and A549 cell lines were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) and with 2 mM L-glutamine and 100U/ml-1 penicillin–streptomycin.

Western Blot Analysis

Overall procedure is described in Chapter 2.2.2. The following primary antibodies were used: TRAIL-receptors detection kit (ProSci), PARP-1, caspase-8, caspase-3, Mcl-1, FoxO3a, p-ERK, TRAF-1, TRAF-2, NF-\(\kappa\)B, p-NF-\(\kappa\)B Ser536, I\(\kappa\)B-\(\alpha\), p-IKK-
α, tubulin, GAPDH (Cell Signaling), TRAF-7 (Imegenex), V5 (Invitrogen), NF-κB and cFLIP (Santa Cruz Biotechnology).

**RNA extraction and Northern blotting**

Total RNA was extracted with TRIZol solution (Invitrogen), according to the manufacturer’s instructions and the integrity of RNA was assessed with an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA). Northern blotting was performed as described previously [61]. The oligonucleotides used as probes were the complementary sequences of the mature miRNA (miRNA registry):

- mir-21: 5’ TCAACATCAGTCTGATAAGCTA 3’
- miR-30c: 5’GCTGAGAGTGTAGGATGTTTACA 3’
- miR-100: 5’ CACAAGTTCGGATCTACGGGT 3’

**Human NF-κB Signaling Targets PCR Array**

The overall procedure was conducted according to the manufacturer’s instructions (SABiosciences). Briefly, total RNA was extracted from H460S/R and H292S/R. After generating cDNAs using a cDNA synthesis kit with 4ug of total RNAs, a PCR plate equipped with known probes for 84 NF-κB targets and housekeeping genes used as internal controls was incubated with cDNAs from H460S/R and H292S/R. The samples were then subjected to qRT-PCR analysis.

**MiRNA locked nucleic acid in situ hybridization of formalin fixed, paraffin-embedded tissue section.**

The overall procedure is described in Chapter 2.2.2. The sequences of the probes containing the dispersed locked nucleic acid (LNA) modified bases with 5’-conjugated
digoxigenin were: miR-21 (5’) TCAACATCAGTCTGATAAGCTA; miR-30c -(5’)
GCTGAGAGTGTAGGATGTTTACA (3’); miR-100- (5’)
CACAAGTTCCGATCTACCGGTT3’. After in situ hybridization with miRNAs, as
previously described [42] the slides were analyzed for immunohistochemistry (IHC) using
the optimal conditions for caspase-3 (1:100, cell conditioning for 30 minutes), caspase-8
(1:10, protease digestion for 4 minutes) FoxO3a (1:100, cell conditioning for 30 minutes),
TRAF-7 (1:25, cell conditioning for 30 minutes).

**NF-κB activity**

A pGL4.3-luc2p/NF-κB construct containing five copies of NF-κB response elements was
purchased from Promega. The construct was transfected alone or with miR-21, miR-30c
and miR-100, NF-κB siRNA or dominant active IκB-α plasmid into H460S/R or H292S/R
cells. 24 hours later cells were incubated with TRAIL. 48hr after transfection NF-κB
luciferase activity was tested with Dual Luciferase Assay (Promega) according to the
manufacturer’s instructions. Three independent experiments were performed in triplicate.

**Soft agar colony formation assay**

Overall procedure was performed according to the manufacturer’s protocol. Briefly,
0.5×10^6/ml H460S/R and H292S/R cells were mixed with the agar matrix layer and
added to the solidified base agar matrix layer. RPMI medium including 10% FBS with or
without 50 ng/ml of TRAIL and Bay-117085 was added to the layers. After 7 days, the
cell-matrix mixture was dissolved with a matrix solubilization buffer and colonies were
quantified by standard MTS assay protocol. The absorbance of each sample was measured
at 495 nm in a Multilabel Counter (Bio-Rad Laboratories).
**Generation of Stable Clones with miR-21, miR-30c and miR-100 downregulation.**

H460R cells were stably infected with the Human anti-miR-21, anti-miR-30c, anti-miR-100 and the GFP gene under the control of two different promoters (System Biosciences) or an anti-miR control. Anti-miRs and control constructs were packaged with pPACKH1 Lentivector Packaging Plasmid mix (System Biosciences) in a HEK293TN packaging cell line. Viruses were concentrated using PEGit Virus Precipitation Solution, and titers were analyzed using the UltraRapid Lentiviral Titer Kit (System Biosciences). Infected cells were selected by FACS analysis (FACScalibur; BD Bioscience). Infection efficiency >90% was verified by fluorescent microscopy and confirmed by real-time PCR for miRNAs downregulation.

**In vivo studies**

Animal studies were performed according to institutional guidelines. 1×10^6 H460S and H460R cells were subcutaneously injected into the right flanks of 6-wk-old male nude mice (Charles River Breeding Laboratories). Treatments started 5 days after the injections by daily i.p. administration of TRAIL/Apo2 (10 mg/kg/day) or vehicle (PBS) for 8 days and/or Bay-117085 (5 mg/kg) twice per week for 2 weeks. Tumor size was assessed every 2 days by a digital caliper. Tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume (V = lw^2/2). 20 days after the injections, mice injected with H460R cells and treated with TRAIL were sacrificed due to significantly enlarged tumors. The other four groups of mice were sacrificed 27 days after inoculations. Animal experiments were conducted after approval of the Institutional animal care and use committee, Ohio State University.
Bioinformatics analysis

Bioinformatics analysis was performed by using these specific programs: Targetscan\(^1\), Pictar\(^2\), RNhybrid\(^3\).

3. http://bibiserv.techfak.uni-bielefeld.de/

Statistical analysis

Student’s t test and one-way analysis of variance (ANOVA) were used to determine significance. All error bars represent the standard error of the mean. Statistical significance for all the tests, assessed by calculating \(p\)-value, was < 0.05.

Accession Numbers

The Geo accession number for the Nanostring and Affymetrix data reported in this paper is GSE55860.

2.3.3 Results

MicroRNA dysregulation in cells with acquired TRAIL resistance.

Although the many molecules that regulate the TRAIL signaling cascade are known, the mechanisms by which tumor cells become more resistant to TRAIL is still not clear. To this end, I generated TRAIL-resistant cells (H460R and H292R) by exposing H460 and H292 sensitive cells (H460S and H292S) to stepwise increases in TRAIL concentrations (1-500 ng/ml) over a period of 6 months to select cells capable of growing at high concentrations of TRAIL (Figure 2.3.1a). The establishment of the
resistant cells was verified by counting the apoptotic nuclei after TRAIL treatment (Figure 2.3.1b). TRAIL receptor isoforms analyzed by western blot or quantitative RT-PCR (qRT-PCR) revealed comparable levels of expression in TRAIL-sensitive and resistant cells. Moreover, sequencing did not show any mutations, possible due to chronic exposure to the drug, in TRAIL-receptors of both H460R and H292R compared to the sensitive parental cells (data not shown).

We have previously reported the involvement of miRNAs in the innate resistance of NSCLC cell lines to TRAIL [43, 62]. Therefore, I analyzed the miRNA expression profile in H460R versus H460S, using nanoString technology. Several miRNAs were found dysregulated (Figure 2.3.1c) and I focused on three miRNAs with the highest fold change in expression: miR-21, miR-30c and miR-100. qRT-PCR and Northern blot analyses confirmed the overexpression of the three miRNAs in H460R compared to H460S cells (Figures 2.3.1d and 1e).

**MiR-21, miR-30, and miR-100 modulate the response to TRAIL by targeting fundamental tumor suppressor genes.**

To investigate the role of miR-21, miR-30c and miR-100 in acquired TRAIL-resistance, I overexpressed these miRNAs in H460S cells. Enforced expression of miR-21, miR-30c and miR-100 drastically reduced the sensitivity to TRAIL-induced apoptosis, as assessed by MTS and caspase-3/7 assays (Figures 2.3.2a and 2b) and western blot analysis for PARP-1 cleavage (data not shown). Conversely, knockdown of miR-21, miR-30c and miR-100 by anti-miR oligonucleotides in H460R cells showed a very low proliferation rate and increased caspase-3/7 activity (Figures 32
2.3.2c and 2d). Identical results were obtained in the H292S and H292R cells (data not shown), suggesting that miR-21, miR-30c and miR-100 are involved in the resistance to TRAIL-induced cell death.

A gene expression profile was analyzed to highlight the global changes in gene expression in H460R compared to H460S cells. Downregulation of important tumor suppressor genes such as *caspase-8*, *caspase-9* and upregulation of oncogenes, such as *PI3K* and *Cyclins* were observed (Figure 2.3.3). The search for mRNA targets of miR-21, miR-30c and miR-100 using Targetscan and Pictar computational tools revealed that 3′ UTRs of human *caspase-8*, *caspase-3*, *TRAF-7* and *FoxO3a* genes contained evolutionarily conserved binding sites specific for these miRNAs (data not shown). I focused on these genes since they are tumor suppressors with defined roles in apoptosis and in the TRAIL pathway. TRAF-7, the unique, noncanonical member of the TRAF family, physically associates with RelA/p65 (p65) member of the NF-κB transcription factor family inducing its polyubiquitination which results in lysosomal degradation of the protein, repressing NF-κB activation [27,63]. The forkhead transcription factors FoxOs play pivotal roles in tumorigenesis and in mediating chemotherapy sensitivity [64].

To verify that *caspase-8*, *caspase-3*, *FoxO3a* and *TRAF-7* are direct targets of miR-21, miR-30c and miR-100, 3′ UTRs were cloned downstream of the luciferase open reading frame. These reporter constructs were cotransfected along with the respective miRNA in HEK293 cells. Increased expression of these miRNAs significantly reduced luciferase expression, measured as relative luciferase activity.
Target gene repression was rescued by deletions of the complementary seed sites (Figure 2.3.4b), confirming that caspase-8, caspase-3, FoxO3a and TRAF-7 are direct targets of miR-21, miR-30c and miR-100. To determine whether these miRNAs affect the target gene expression in the H460S cellular environment, I analyzed the consequences of their ectopic expression in H460S cells. MiR-21, miR-30c or miR-100 enforced expression significantly reduced the endogenous levels of caspase-8, caspase-3, FoxO3a and TRAF-7 (Figure 2.3.4c). Intriguingly, by qRT-PCR, I found that only caspase-3 and caspase-8 (Figure 2.3.4d) and not FoxO3a and TRAF-7 mRNAs (data not shown) were downregulated in H460S cells after miRNAs overexpression, indicating that miR-21 and miR-30c induce the degradation of caspase-8 and caspase-3 mRNAs whereas FoxO3a and TRAF-7 are regulated at the translational level. Conversely, stable knockdown of miR-21, miR-30c and miR-100 by lentivector-based anti-microRNAs in H460R cells, confirmed by qRT-PCR (Figure 2.3.4e), increased protein levels of the target genes (Figure 2.3.4f). To verify these findings in vivo, in situ hybridization analysis was performed using 5'-dig-labeled LNA probes on lung cancer tissues, followed by immunohistochemistry for the target proteins. These analyses were done initially on serial sections so as to compare the results on the same groups of cells, followed by co-expression analysis. These data showed that in about 90% of the cores expression of the miRNA and the protein was mutually exclusive. Co-expression analysis indicated that when both the miRNA and protein were found in the same core, they were being expressed in different groups of cancer cells. Aggressive lung cancers
(defined by lymphonode metastases) showed high expression of miR-21, miR-30c and miR-100 and rarely expressed caspase-8, caspase-3, FoxO3a and TRAF-7. Levels of the miRNAs and their target genes were also inversely related in the majority of lung cancer cases (Figure 2.3.5).

Next, to evaluate the contribution of the target genes in acquired TRAIL resistance I compared their endogenous levels in H460R and H292R versus H460S and H292S cells. Western blot analysis clearly indicated a downregulation of the target genes in the resistant cell lines. As expected, caspase-3 and caspase-8 (Figure 2.3.6b) but not FoxO3a and TRAF-7 mRNAs (data not shown) were also downregulated in H460R and H292R cells. Furthermore, enforced expression of caspase-8, caspase-3, TRAF-7 and Foxo3a in H460R and H292R cells, decreased cell viability (Figure 2.3.6c; data not shown for H292R), increased caspase-3/7 activity (Figure 2.3.6d; data not shown for H292R) and enhanced PARP-1 cleavage (Figure 2.3.6e) compared to the cells transfected with an empty vector. These data suggest that these genes play a fundamental role in TRAIL-induced cell death.

**NF-κB transcriptionally activates miR-21, miR-30c and miR-100.**

The transcription factor NF-κB is broadly associated with oncogenesis including control of metastasis and angiogenesis [65,66]. It has been shown that primary TRAIL-resistant cells usually have higher NF-κB/p65 activity [30,56,67-69]. Therefore I examined NF-κB activation in acquired TRAIL-resistant cells. A pGL4.3-luc2p/ NF-κB construct containing five copies of an NF-κB response element
that drives transcription of the luciferase reporter gene was transfected into H460S and H460R cells stimulated with TRAIL at different times. Basal NF-κB activity was higher in H460R compared to H460S cells with the highest peak after 3 hrs of TRAIL treatment. A drop in NF-κB activity was observed after NF-κB silencing or the overexpression of dominant active IκB-α (Figure 2.3.7a). Moreover, western blot confirmed a higher phosphorylation of NF-κB and IKK-α in H460R cells, whereas IκB-α was downmodulated in H460R cells after TRAIL stimulation compared to control cells (Figure 2.3.7b). Next, I analyzed NF-κB nuclear translocation in both H460S and H460R cells. Cells were incubated with TRAIL for 3 hrs and then nucleo-cytoplasmic separation was performed.

Western blot showed higher NF-kB expression in the nuclei of H460R compared to the H460S cells (Figure 2.3.7C). Hundreds of NF-κB target genes have been identified by chromatin immunoprecipitation (ChIP) as well as bioinformatic analyses based on predicted binding sites. I used a qRT-PCR Array (Sabioscience) to profile the expression of 84 key genes responsive to NF-κB in H460S and H460R (Figures 2.3.7d and 7e). I found that anti-apoptotic genes such as BCL2A1, BIRC2, XIAP and genes involved in inflammation and immune responses were upmodulated in H460R and H292R cells (Tables A and B). In addition, western blot analysis showed increased expression of NF-κB-transcriptionally-activated TRAIL antagonists such as Mcl-1 and c-FLIP [70,71] in H460R compared to H460S cells (Figure 2.3.7F; data not shown). Then, I addressed whether NF-κB could be involved in miR-21, miR-30c and miR-100 transcriptional activation. First, endogenous levels of miR-21,
miR-30c and miR-100 were higher in TRAIL-resistant cells following TRAIL-stimulation and consistently decreased after NF-κB knockdown (Figure 2.3.7g). By bioinformatics search (PROMO database: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3; TESS database: http://www.cbil.upenn.edu/cgi-bin/tess/tess), I found that miR-21, miR-30c and miR-100 had NF-κB binding sites located ~ -1280 bp (miR-21), ~ 3730 bp and ~ 4180 bp (miR-30c), ~ 3600 bp (miR-100) upstream of the pre-miRNAs 5’ ends, respectively. To determine if the identified NF-κB sites were located in promoter regions, I cloned the NF-κB binding sites into the pGL3basic vector, which harbors the promoterless luciferase gene. These constructs were cotransfected along with NF-κB siRNA in H460R cells. Subsequent luciferase assays showed that NF-κB downregulation gave rise to a 40-50% reduction in luciferase activity (Figure 2.3.7h). Since I noticed that the promoter regions were responsive to NF-κB modulation, to verify a direct binding of NF-κB on the miR-21, miR-30c and miR-100 promoters I carried out chromatin immunoprecipitation (ChIP) assays. As expected, ChIP assays of H460R cells showed considerable NF-κB binding at the predicted analyzed regions for miR-21, miR-30c and miR-100 (Figure 2.3.7i). Together these data led me to conclude that NF-κB is the transcription factor responsible for miR-21, miR-30c and miR-100 transcriptional activation in NSCLC. Finally, since caspase-8 and TRAF-7 inhibit NF-κB activation and they are directly downregulated by miR-21 and miR-100, I questioned if miR-21 and miR-100 overexpression could induce NF-κB activation. In this regard, the pGL4.3-
luc2p/NF-κB construct was co-transfected along with miR-21 and miR-100 in H29 2S cells. Luciferase assays showed an increased luciferase activity, compared to the cells transfected with a scrambled miRNA (Figure 2.3.7j). Moreover, immuno blot analysis displayed an increase in NF-κB phosphorylation after miR-21 and miR-100 enforced expression (Figure 2.3.7k), confirming the activation of NF-κB by miR-21 and miR-100 and the presence of a positive feedback loop.

**NF-κB and TRAIL signaling**

It is known that the TRADD-TRAF-NF-κB signaling cascade acts downstream of TRAIL receptors. TRADD has a survival role in TRAIL signaling and deficiency of TRADD sensitizes cells to TRAIL-induced apoptosis in innate TRAIL-resistance [72]. TRAF-2 has recently been shown to have a positive role in the canonical pathway that activates NF-κB [68]. I found an increased phosphorylation of ERKs and increased expression of TRADD and TRAF-2 in H460R cells in response to TRAIL stimulation compared to H460S (Figures 2.3.8a and 8b). Knockdown of TRAF-2, TRADD or NF-κB in H460R cells decreased cell survival rate, increased caspase-3/7 activity and induced PARP-1 cleavage (Figures 2.3.8c-8e), suggesting that NF-κB-dependent cell survival signal is conserved in acquired TRAIL-resistant cells.
Caspase 8-dependent RIP1 cleavage plays an important role in acquired TRAIL-resistance

The death domain kinase RIP1, a key component of the TNF signaling complex, is cleaved by caspase-8 in TNF-induced apoptosis, resulting in the blockage of TNF-induced NF-κB activation [55,73-76]. Therefore, I investigated the role of caspase-8-dependent RIP1 cleavage in the acquired TRAIL-resistant system. First, H460S cells were exposed to TRAIL at different times for up to 6 hrs and, as expected, a decrease in NF-κB phosphorylation and an increase in cleaved RIP1 and caspase-8 activation starting 4 hrs after TRAIL-treatment were observed (Figure 2.3.9a). Conversely, RIP1 cleavage and caspase-8 activation were not detected in H460R cells in response to TRAIL stimulation (Figure 2.3.9b).

Remarkably, I observed a decrease in NF-κB phosphorylation and enhanced RIP1 cleavage in H460R cells transfected with anti-miR-21 in response to TRAIL treatment (Figure 2.3.9c). In addition, phosphorylation levels of NF-κB were higher in caspase-8 knock-down H460S cells (Figure 2.3.9d). Conversely, ectopic expression of pCMV-caspase-8 in H460R cells suppressed NF-κB phosphorylation and increased RIP1 cleavage (Figure 2.3.9f). Also, NF-κB promoter activity was enhanced in H292S cells transfected with caspase-8 siRNA and reduced in H292R cells overexpressing caspase-8 (Figures 2.3.9e and 9g). Identical results were observed in H460R cells (data not shown). Finally, I cloned RIP1 death domain into a pcDNA4-V5 vector (tRIP1-V5). Overexpression of this construct in H460R cells significantly decreased NF-κB phosphorylation (Figure 2.3.9h). Moreover, NF-κB promoter activity was
suppressed in H292R cells after enforced expression of cleaved RIP1 compared to the cells transfected with an empty vector (Figure 2.3.9i). MTS and caspase-3/7 assays showed that overexpression of RIP1 death domain induced apoptosis in both H460R and H292R cells (data not shown). Taken together these data strongly suggest that caspase-8-dependent RIP1 cleavage driven by miR-21 blocks NF-κB activation in NSCLC cells with acquired TRAIL-resistance and the use of an anti-miR-21 oligonucleotide can sensitize acquired resistant cells to TRAIL-induced apoptosis.

**Acquired TRAIL-resistance induces EMT and aggressive cancer phenotype.**

Epithelial-mesenchymal transition (EMT) is associated with reduced sensitivity to many chemotherapeutic drugs [77]. I observed an impressive change in cellular shape of H460R cells from an epithelial polarized phenotype to a spindle-fibroblastoid morphology (Figure 2.3.10a), inducing me to further investigate whether this morphological change could be due to a EMT. I assessed the expression of key EMT-associated markers and observed increased expression of mesenchymal markers and decreased E-cadherin expression (Figure 2.3.10b). In addition, H460R and H292R showed higher migratory and invasive capabilities compared to the parental cells (Figures 2.3.10c and 10d). Anchorage-independent growth is one of the hallmarks of cell transformation. Therefore, H460R and H292R were plated in soft agar to verify their colony formation ability. H460R and H292R cells presented higher growth rates than their control cells. Interestingly, they displayed increased colony growth in response to TRAIL but the colony growth was dramatically reduced when they were co-treated with TRAIL and the irreversible NF-κB inhibitor Bay-
117085 (Figures 2.3.10e and 10f). Finally, to analyze tumor growth and the response to TRAIL-induced apoptosis in vivo, I injected H460S and H460R in the right side of nude mice. Mice injected with H460R cells presented tumors slightly but significantly enlarged compared to those injected with the H460S cells. Furthermore, tumor size dramatically increased when H460R-injected mice were treated with TRAIL (Figure 2.3.10g). Of note, combinatory treatment of TRAIL and Bay-117085 showed a highly anti-tumor effect compared to the mice treated only with the inhibitor (Figure 2.3.10h). MiR-21, miR-30c and miR-100 expression in the tumor samples was analyzed by qRT-PCR. MiRNA expression levels were higher in H460R-derived tumors and reduced in Bay-117085 or Bay-117085 + TRAIL treated groups (Figure 2.3.10i). Consistently, NF-κB phosphorylation was reduced in the Bay-117085 or Bay-117085 + TRAIL treated mice (Figure 2.3.10j). These results indicate that acquired TRAIL-resistance induces a more aggressive cancer phenotype in vitro and in vivo and the use of NF-κB inhibitors can improve the response of cancer cells to TRAIL-induced apoptosis.

Figure 2.3.11 details a model that summarizes the results of this study. In the TRAIL-sensitive cells caspase-8 cleaves RIP1, therefore NF-κB activation is weak and apoptosis is triggered after TRAIL treatment. In the cells with acquired resistance continuous TRAIL treatment induces the accumulation of miR-21, miR-30c and miR-100 which are transcriptionally activated by NF-κB. Caspase-8 and TRAF-7 downmodulation by miR-21 and miR-100 further strengthens the NF-κB signal, establishing a positive feedback loop that leads to TRAIL resistance and EMT.
2.3.4 Discussion

Drug resistance is a major obstacle in cancer therapy and it can be either intrinsic or acquired during therapy. So far, no strategy has been found to overcome resistance, which is based on highly complex and individually variable biological mechanisms. Lung cancer still represents a very deadly disease in strong need of new, effective therapeutic approaches. The long-term survival for patients with advanced high-grade lung cancer has been limited by the frequent occurrence of resistance to chemotherapeutic drugs. In this context, TRAIL may represent an alternative therapeutic molecule. Several TRAIL agonists have recently entered clinical trials and seem to be effective in a small fraction of lung cancer patients [78,79]. However, the majority of NSCLC patients are resistant or, as with other molecularly targeted agents, resistance is likely to develop later during therapy. In spite of massive studies for intrinsic TRAIL-resistance, the molecular mechanisms underlying the acquired TRAIL-resistance is still unclear and little is known on how lung cancer can acquire resistance to TRAIL [30,80]. Therefore, it is crucial to identify biomarkers to predict patient response and to improve the therapeutic efficacy through drug combinations that not only synergize with TRAIL but that can also overcome resistance as it arises. To understand the mechanisms involved in acquired TRAIL resistance I established TRAIL-resistant human isogenic cell lines (H460R and H292R) from the parental TRAIL-sensitive (H460S and H292S) cells. Alterations in the expression levels of TRAIL-R1 and TRAIL-R2 or decoy receptors have been associated with the modulation of TRAIL-induced cell death in
different systems [81-83]. Both cell lines H460R and H292R had similar TRAIL receptor cell membrane expression levels and did not show any mutation in their sequences, suggesting that the resistance may be linked to the alteration of downstream pathways.

MiRNAs, single stranded RNAs of 19–25 nt in length, are key players in cancer onset and progression. Recent data demonstrate that selective modulation of miRNA activity can improve the response to chemotherapy [49,84]. Therefore, I compared the miRNA expression profile of H460R and H460S cells. Several miRNAs, including miR-21, miR-30c and miR-100 were found drastically upregulated in TRAIL-resistant compared to the sensitive cells. Noteworthy, up-regulation of miR-21, miR-30c and miR-100 was also observed in NSCLC cell lines with de novo TRAIL resistance (data not shown) suggesting that these miRNAs may play a role in both the primary and acquired resistance to TRAIL-induced apoptosis.

Furthermore, I demonstrated that enforced expression of miR-21, miR-30c and miR-100 in TRAIL sensitive cells is sufficient to induce resistance to the drug by directly targeting fundamental tumor suppressor genes and effectors of the TRAIL pathway, such as caspase-8, caspase-3, TRAF-7 and FoxO3a.

Besides inducing apoptosis through the activation of the caspase signaling pathway, TRAIL is also known to activate NF-κB in innate TRAIL-resistant cells [18,53]. Furthermore, the role of caspase-8 in NF-κB activation is still controversial. Caspase-8 is required for NF-kB activation in HeLa and HEK293 cells in response to TNF stimulation [85]. However, active caspase-8 cleaves RIP1, which prevents
sustained NF-κB activation, and activates downstream caspases [55,73,76,86]. In TRAIL-sensitive cells, RIP1 is cleaved in a caspase-8-dependent process and is unable to activate NF-κB signaling. I proved that in acquired TRAIL resistant cells downmodulation of caspase-8 by miR-21 induces the cleavage of RIP1, and consequently NF-κB activation, leading to acquired TRAIL resistance. Of note, miR-21 knockdown in H460R cells enhanced RIP1 cleavage and increased cells sensitivity, demonstrating that modulation of miRNAs is able to revert the sensitivity of cancer cells to TRAIL treatment. TRAFs were initially discovered as adaptor proteins that couple the tumor necrosis factor receptor family to signaling pathways. A recent study showed that TRAF-7 induces cell death by repressing NF-κB activation [63]. I proved that miR-100, by downregulating TRAF-7, induces NF-κB activation in the cells with acquired TRAIL resistance. Therefore, TRAIL triggers apoptosis through caspase activation in sensitive cells and NF-κB signaling is weak because of the cleavage of RIP1 by caspase-8. In the resistant cells, downmodulation of caspase-8 and TRAF-7 by miR-21 and miR-100 activates NF-κB, which, in turn, in a positive feedback loop, induces the transcriptional activation of miR-21, miR-30c and miR-100. In a previous study we reported that miR-221 and miR-222 were involved in primary TRAIL resistance by targeting PTEN and TIMP3 [43]. Intriguingly, I found an upregulation of miR-221 in H460R cells (Figure 2.3.1), suggesting that it could play a role also in acquired TRAIL resistance.

Unlike TNF-induced cell death, RIP1 and TRAF2, however, are dispensable for TRAIL-induced cell death [55,87], which seems that there are compensatory
survival signals such as mTOR-S6K1, PI3-AKT, and survival MAPK signals [19,23].
The dispensable role of RIP1 and TRAF2 in TRAIL-induced cell death is not consistent with my findings that an abrogation of NF-kB activity makes the acquired TRAIL-resistant cells TRAIL-sensitive. I believe that this inconsistency is due to the nature that the acquired TRAIL-resistant cells have addicted to NF-kB-dependent cell survival signals rather than the other known cell survival signals. Likewise, a translational blocker such as cyclohexamide to inhibit TNF-induced NF-kB activation efficiently induces TNF-induced cell death in some tumor cells [73,74,88,89].
Furthermore, it is known that there is a strict correlation between acquired drug resistance and EMT. Recently it has been shown that NF-κB constitutive activation is involved in EMT of NSCLC cells through the upregulation of master-switch transcription factors [90]. I found a morphological change and a more aggressive phenotype in H460R and H292R cells (Figure 2.3.10). Analysis of epithelial and mesenchymal markers confirmed that acquired TRAIL resistance induced epithelial to mesenchymal transition in both cell lines. Genetic inhibition of the NF-κB pathway affects both the initiation and the maintenance of lung cancer, identifying this pathway as a promising therapeutic target [91]. Remarkably, TRAIL-treatment to acquired TRAIL-resistant cells make the cells more aggressive and the combination of TRAIL and NF-κB inhibitors has shown, *in vitro* and *in vivo*, increased apoptosis and reduced cell proliferation in acquired TRAIL resistant cells compared to treatments involving TRAIL or the NF-κB inhibitor alone. Taken together these data identify NF-κB as a potential companion drug target, together with TRAIL, in
l lung cancer and provide insight into the mechanisms by which tumour cells become resistant to TRAIL-induced apoptosis. In addition, the results not only suggest that, miR-21, miR-30c and miR-100 expression levels could be used as prognostic tool to predict TRAIL sensitivity or resistance but also that in the near future, the delivery and modulation of specific miRNAs could improve the response of lung cancer patients to TRAIL.
Figure 2.7 Identification of dysregulated miRNAs in cells with acquired TRAIL-resistance. (a) A schematic diagram showing generation of acquired TRAIL-resistant cells. (b) Cell death measurement of H460R and H292R cells after TRAIL treatment. H406S/R and H292S/R cells were exposed to TRAIL at the indicated time and concentration. Cell death rates were determined by counting apoptotic nuclei after staining with Hoechst dye. (c) MiRNA heatmap showing dysregulated miRNAs in H460S versus H460R cells. *-values were obtained by analysis of variance (ANOVA) test (*p < 0.001). (d) qRT-PCR analysis of miR-21, miR-30c, and miR-100 in H460S/R and H292S/R cells. (e) Northern blot analysis showing miR-21, miR-30c and miR-100 upregulation in H460R compared to H460S cells. Bars indicate mean ±S.D (n=3). *-values were obtained by two tailed student t-test (*p < 0.01).
Figure 2.8 MiR-21, -30c and -100 inhibit TRAIL-induced cell death (a-b) Increased cell survival (a) and decreased caspase-3/7 activates (b) of TRAIL-sensitive cells after miR-21, miR-30c and miR-100 overexpression in response to TRAIL. (c-d) Decreased TRAIL survival (c) and increased caspase-3/7 substrate activates (d) of TRAIL resistant cells after miR-21, miR-30c and miR-100 knockdown by anti-miR lentiviral particles in response to TRAIL.
Figure 2.9 Gene Affymetrix microarray of H460S and H460R cells showing increased expression of anti-apoptotic genes compared to the H460S cells.
**Figure 2.10 Caspase-8, caspase-3, FoxO3a and TRAF-7 3’UTRs are direct targets of miR-21, miR-30c and miR-100.**

(a-b) Luciferase assays with miR-21, -30c and -100. pGL3-caspase-3, pGL3-caspase-8, pGL3-FoxO3a and pGL3-TRAF-7 luciferase constructs, containing wild-type (a) or mutated (b) 3’UTRs, were transfected into HEK293 cells.

(c) Down-regulation of caspase-8, caspase-3 FoxO3a, and TRAF-7 by ectopic expression of miR-21, miR-30c and miR-100.

(d) qRT-PCR showing caspase-3 and caspase-8 downregulation after miR-21 and miR-30c enforced expression.

(e) qRT-PCR analyses showing the expression of the miRNAs and their target genes in H460R cells infected with anti-miR-21, anti-miR30c and anti-miR-100. Error bars indicate mean ±SD (n=3) and the p-values were calculated by two-tailed student t-test (*p < 0.01, **p < 0.005).

(f) Restored expression of the target genes in lenti-anti-miR-21, anti-miR-30c, and anti-miR-100 H460R stable infected cells.
Figure 2.11 Co-expression analysis of miRNAs and target genes. (a) The top panel shows co-expression analysis of capase-8 and miR-21 (first two panels) and miR-100 and TRAF-7 (last two panels). The first and third images are the regular light microscopy views after co-expression of the protein (red) and the miRNA (blue). The small arrows point to the stroma and large arrows to the cancer cells. The second and fourth panels are the Nuance converted images where co-expression of the miRNA (fluorescent blue) and protein is seen as fluorescent yellow. The bottom panels show the regular light microscopy images for miR-30c and caspase-3 (RGB, middle row, 2nd panel from left) as well as miR-30c and FoxO3a (RGB, middle row, last image on right). The other panels show the Nuance converted individual protein expression (fluorescent red), the individual miR-30c (fluorescent blue) and the merged images of miR-30c/caspase-3 and miR-30c/FoxO3a. Note the lack of co-expression in the merged images (no fluorescent yellow). (b) The summary table shows the inverse relation between miRNAs and target expression in 69 lung tumors. Each core of the TMA was read blinded to the corresponding data. The score (0-100) was based on the signal intensity and % of + tumor cells – if a case had a score of 0-15, it was scored as negative. Any core with a score > 15 was scored as positive.
### a

![Lung tumors images](image)

### b

**Total 68 cases for miR-21 and caspase-8**

<table>
<thead>
<tr>
<th>miR21+ / Casp8+</th>
<th>miR21+ / Casp8-</th>
<th>miR21- / Casp8+</th>
<th>miR21- / Casp8-</th>
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<td>2 (0.03%)</td>
<td>29 (42.0%)</td>
<td>10 (14.7%)</td>
<td>27 (39.7%)</td>
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**Total 69 cases for miR-30c and caspase-3**

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<th>miR30+ / Casp3-</th>
<th>miR30- / Casp3+</th>
<th>miR30- / Casp3-</th>
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<td>21 (30.4%)</td>
<td>15 (21.7%)</td>
<td>25 (36.2%)</td>
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**Total 69 cases for miR-30c and FoxO3a**

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<th>miR30+ / FoxO3a-</th>
<th>miR30- / FoxO3a+</th>
<th>miR30- / FoxO3a-</th>
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<tr>
<td>11 (15.9%)</td>
<td>18 (26.1%)</td>
<td>18 (26.1%)</td>
<td>22 (31.9%)</td>
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</table>

**Total 69 cases for miR-100 and TRAF7**

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<th>miR100+ / TRAF7-</th>
<th>miR100- / TRAF7+</th>
<th>miR100- / TRAF7-</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 (26.1%)</td>
<td>14 (20.3%)</td>
<td>19 (27.9%)</td>
<td>18 (26.1%)</td>
</tr>
</tbody>
</table>
Figure 2.12 Caspase-8, caspase-3 and TRAF-7 are involved in acquired TRAIL-resistance. (a) Down-regulation of caspase-8, caspase-3, FoxO3a and TRAF-7 in TRAIL-resistant cells. (b) qRT-PCR showing caspase-3 and caspase-8 mRNAs downregulation in H460R compared to H460S cells. (c-e) Proliferation (c), caspase-3/7 assay (d) and western blot showing PARP-1 cleavage (e) after caspase-3, caspase-8, and TRAF-7 overexpression in TRAIL-resistant cells. Error bars indicate mean ±SD (n=3) and the p-values were calculated by two-tailed student t-test (*p<0.02, **p < 0.01).
Figure 2.13 NF-κB directly activates miR-21, miR-30c and miR-100. (a) Increased NF-κB promoter activity in H460R cells in response to TRAIL stimulation. NF-κB activity was assessed by measuring firefly luciferase activity. (b) Western blot analysis showing NF-κB activation in H460R after TRAIL treatment. (c) Cytosol-Nucleus fractionation of H460S and H460R cells to determine NF-κB localization. H460S and H460R cells were treated with 50 ng/ml of TRAIL. After 3 hrs a cytosol-nucleus extraction kit (Cell Biolabs, Inc.) was used to separate nuclear and cytosolic proteins. Anti-tubulin and PARP-1 antibodies were used for cytosolic and nuclear marker, respectively. (d-e) Volcano plots of NF-κB target genes. Total RNAs of H460S/R (d) and H292S/R (e) were subjected to qRT-PCR in plates equipped with primer sets for 84 NF-κB target genes. p-values were obtained by three independent experiments. Fold difference is reported as H460R/H460S and H292R/H292S. (f) Western blot analysis of NF-κB anti-apoptotic targets in control and NF-κB knockdown H460S and H460R cells. (g) NF-κB silencing reduces miR-21, miR-30c and miR-100 expression levels. The values were normalized with a negative control. Error bars represent mean ±SD (n=4) and p-values were calculated by two-tailed student t-test or ANOVA test (*p<0.001). (h) NF-κB activity on miR-21, miR-30c and miR-100 promoters. The promoter regions of miR-21, miR-30c or miR-100, containing NF-κB binding sites were sub-cloned into pGL3-luciferase vectors. The constructs were transfected into H460R cells along with a scrambled or NF-κB p65 siRNA. After 48 hrs, promoter activity was assessed by measuring firefly luciferase activity. (i) Chromatin immunoprecipitation (CHIP) showing a direct interaction between NF-κB p65 and miR-21, miR-30c and miR-100 promoter regions. (j) MiR-21 and miR-100 activate NF-κB in H460R cells. pGL4.32-luc2-luciferase NF-κB vector was co-transfected with a scrambled miRNA, miR-21 or miR-100 in H460R cells. After 48 hrs, cells were subjected to a luciferase assay to measureNF-κB activity. Error bars represent mean ±SD (n=3) and p-values were calculated by two-tailed student t-test (*p<0.05). (k) Increased phosphorylation of NF-κB p65 in H460S cells overexpressing miR-21 and miR-100.
Figure 2.14 Silencing of upstream activators of NF-κB sensitzes H460R cells to TRAIL-induced cell death. (a) Western blot analysis showing upregulation of p-ERK1/2, TRADD and TRAF-2 in H460R cells. (b) qRT-PCR showing up-regulation of TRAF-2, TRADD and RIP1 mRNAs in H460R cells. (c-d) Proliferation (c) and caspase-3/7 (d) assays showing increased sensitivity to TRAIL-induced apoptosis in H460R cells after TRAF-2, TRADD and NF-κB silencing or overexpression of dominant active (DA)-IκB-α. (e) Increased PARP-1 cleavage in H460R cells following TRAF-2, TRADD and NF-κB silencing. The results were verified by performing each western blot three times. Bars indicates mean ±SD (n=4) and p-values were calculated by ANOVA test (*p<0.01, **p<0.005).
Figure 2.15 Caspase-8-dependent RIP1 cleavage blocks NF-κB activation in TRAIL-resistant cells. (a-b) Increased NF-κB phosphorylation in H460R cells (b) and not in H460S (a) cells after TRAIL treatment. The sensitive and resistant cells were exposed to 50 ng/ml of TRAIL at the indicated time points. Cells were harvested and analyzed by western blot for p-p65, RIP1 cleavage and caspase-8 activation. (c) MiR-21 knockdown by anti-miR oligonucleotides in H460R cells reduces p-p65 phosphorylation and increases RIP1 and PARP-1 cleavage. (d-e) Increased NF-κB phosphorylation in H460S (d) and activity in H292S (e) after caspase-8 knockdown. (f-g) Increased RIP1 cleavage and reduced NF-κB phosphorylation in H460R (f) and activity in H292R (g) cells overexpressing caspase-8. (h-i) Enforced expressing c-terminal fragment of RIP1 (tRIP1) inhibit NF-κB activation. pcDNA4-tRIP1-v5 vector were transfected into H460R cells. After 24 hrs, cells were stimulated with 50 ng of TRAIL for 2 and 3 hrs and analyzed by western blot (h) and luciferase assay to assess NF-κB activity (i). Error bars indicate mean ±SD (n=4) and p-values were calculated by ANOVA test (*p<0.001).
Figure 2.16 Acquired TRAIL resistance induces EMT. (a) Morphological changes of H460R compared to H460S cells. Scale bar indicates 20µm. (b) Western blot analyses showing EMT markers in acquired TRAIL-resistant cells. (c-d) Enhanced migration and invasion capacity of acquired TRAIL-resistant cells. Bars show mean ±SD (n=4) and p-values were calculated by paired student t-test (*p<0.02, **p<0.01). (e-f) Reduction of transformation capabilities of TRAIL resistant cells in response to TRAIL and the NF-κB inhibitor, Bay 117085 treatments. Bars show mean ±SD (n=3) and p-values were calculated by paired student t-test (*p<0.01). (g) Growth curve of engrafted tumors in nude mice injected with H460S and H460R cells. One group of the H460R injected mice was treated intraperitoneally with 10 mg/kg of TRAIL for 8 days. Error bars indicate mean ±SD (n=4) and p-values were calculated by ANOVA test or paired student t-test (*p<0.022 and **p<0.005). (h) Tumor regression in a combined treatment of TRAIL and Bay-117085. H460R cells were subcutaneously injected into three groups of nude mice. 5 mg/kg of Bay-117085 were administered intraperitoneally twice per week for two weeks. 10 mg/kg of TRAIL were injected intraperitoneally daily for two weeks. Combination of TRAIL and the NF-κB inhibitor, Bay-117085 reduced tumor volumes as compared with a control group treated with PBS and DMSO only. Error bars show mean ±SD (n=4) and p-values were calculated by ANOVA test or paired student t-test (***p=0.027 and ****p=0.006). (i) qRT-PCR showing miR-21, miR-30c and miR-100 expression in the tumors. Three tumors were isolated from each group and prepared for qRT-PCR analysis. p-values were calculated by ANOVA test or paired student t-test. (*p<0.001, **p<0.005, and ***p=0.011). (j) Significant phospho-p65 (p-p65) downregulation in the xenograft tumors treated with TRAIL plus Bay-117085. Tumors from three mice of each group were minced and analyzed by western blot with the indicated antibodies.
Figure 2.17 Proposed model showing NF-κB induces acquired TRAIL resistance through miR-21, miR-30c and miR-100 activation. In TRAIL-sensitive cells, binding of TRAIL to TRAIL receptors induces cell death through the canonical caspase-cascade and FoxO3a up-regulation. In cells with acquired TRAIL-resistance cell death is blocked by miR-21, miR-30c and miR-100-mediated down-regulation of caspase-8, caspase-3, FoxO3a and TRAF-7. As a consequence of caspase-8 and TRAF-7 knockdown by miR-21 and miR-100, NF-κB activation is enhanced. Activated NF-κB increases miR-21, miR-30c and miR-100 expression, which strengthens the positive feedback loop inducing acquired TRAIL-resistance.
Sensitive Cells

Death

Resistant Cells

Survival

Aggressive Cancer
Chapter 3

MiR-224 and miR520c-3p-mediated TUSC3 suppression enhances metastatic potential of NSCLC through NM23H1 and MCAM regulation³.

3.1 Introduction

In spite of drastic studies, the sequence of the events and molecular mechanisms for cancer metastasis are not well understood because of its nature that cancer metastasis is an inefficient multistep process based on complicated reciprocal interaction between tumor cells and the microenvironment [2,4]. Tumor Suppressor Candidate 3 (TUSC3) was first determined in metastatic prostate cancer to be a deleted gene in the chromosome 8p22 locus [92]. TUSC3 is known to be an endoplasmic reticulum membrane protein. Its expression is frequently suppressed by various mechanisms including chromosomal deletion or epigenetic modification of the promoter region of the TUSC3 gene in various tumors including colon and pharyngeal carcinoma [93,94]. Several lines of investigation suggest that TUSC3 is a

³ The results in this chapter are Jeon et al., manuscript in preparation.
potential tumor suppressor. TUSC3 suppression in a zebrafish model demonstrates that TUSC3 is responsible for intracellular magnesium uptake, which is critical to embryonic development [95]. The deletion of TUSC3 is associated with poor cancer survival in head and neck carcinoma [96]. Furthermore, downregulation of TUSC3 in ovarian carcinoma appears to correlate with tumor progression [97]. TUSC3 is also known to be a member of the N-linked glycosylase complex with its glycosylase function contributing to tumor suppressive functions in ovarian carcinoma and prostate cancer [98-100]; however, the expression, regulation, and role for TUSC3 in cancer metastasis including lung cancer progression are largely unknown.

Several lines of evidence suggest that miR-520c-3p and its functional cluster miR-373 suppress multiple tumor suppressors such as CD44 in breast cancers [101]. Additionally, the roles for miR-224 have also been studied in several tumors. MiR-224 is one of the most up-regulated miRNAs in hepatocellular carcinoma (HCC) and cervical carcinoma while contributing to both HCC migration and proliferation [102-104]. However, miR-224 expression is down-regulated in other malignancies such as prostate cancer and metastatic colorectal tumors suggesting tumor type dependency of expression [105,106]. Despite these few functional studies of miR-224 and miR-520c-3p in other cancers, both the expression status and contribution of these miRNAs to NSCLC pathogenesis are unclear.

Here I showed that expression of TUSC3 gene, a bona fide target of miR-224 and miR-520c-3p, is downregulated in lymph node metastases of lung tumors. Furthermore, I showed that miR-224 and miR-520c-3p-dependent TUSC3 down-
regulation contributes to the metastatic potential of NSCLC through regulation of NM23H1 and MCAM proteins.

### 3.2 Materials and Methods

**Plasmids and siRNAs**

TUSC3, NM23H1, and MCAM plasmids were purchased from Origene. For the TUSC3 gene, the cDNAs were subcloned into pcDNA4.1-v5 vector with EcoR I and Xho I restriction sites. The 3’UTR of TUSC3-1 and TUSC3-2 plasmid were purchased from Origene and the 3’UTRs were subcloned into psiCHECK2 dual luciferase vector with Xho I restriction site. On-Target Smart Pools containing 4 different siRNAs on each target gene for TUSC3, MCAM and NM23H1 were purchased from Thermo Scientific. For knocking down TUSC3, shRNA TUSC3 plasmids and recombinant shTUSC3 lentivirus containing a pool of three target-specific siRNA sequences were purchased from Santa Cruz Biotechnology, Inc.

**Cell culture and nucleic acid delivery**

The H460, A549, HeLa, and HCT116 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and HEK293 cells were grown in DMEM supplemented with 10% FBS (SIGMA). The transient expression of the plasmids and shRNAs was obtained by using Lipofectamine 2000, RNAiMAX, or Lipofectamine Plus reagents according to the manufacturer’s protocol (Invitrogen). For transient expression of TUSC3, TUSC3 cDNAs were transfected to cells. 48 hrs
after transfection, the cells were used for experiments after removing dead cells by washing with PBS.

**Stable cell generation**

For TUSC3 knockdown cells, H460 or A549 cells were transfected with shTUSC3 plasmids. The transfectants were selected using puromycin treatment for 2 weeks. After that, the cells were sorted into each single cell and grown for 2 weeks. Each knockdown cell line was checked for TUSC3 expression by qRT-PCR analyses and/or IF assays. To make stable miR-224 or miR-520c-3p knockdown cells, the recombinant lentiviruses containing GFP gene and anti-miR224 or anti-mi520c sequences were transduced into A549 or H460 cells. Two weeks after transduction, GFP-positive cells were isolated using an ARIA FACS cell sorter. The reduced expressions of miR-224 or miR-520c were confirmed by a standard Taqman assay using miR-224 or miR-520c-p3 specific probe. To make the double knockdown cells of miR-224 and miR-520c-3p, the anti-miR-520c-3p oligonucleotide was transfected into miR-224 knockdown cells.

**Quantitative RT-PCR**

The procedure was followed as previously described in Chapter 2

**Migration and Invasion assays**

Overall procedure is described in Chapter 2
**Antibodies**

Anti-TUSC3 antibody was purchased from SIGMA. Anti-NM23H1 and anti-GAPDH antibody were purchased from Cell Signaling. Anti-MCAM antibody was purchased from EMD Millipore and Santacruz Biotech, Inc.

**Recombinant Lentiviral production**

The lentiviral vector (pmiRZIP lentivector) containing anti-miR-224 and anti-miR-520c-3p was purchased from System Biosciences. The recombinant lentivirus was generated as described in the manufacturer’s protocol (System Biosciences). Briefly, the lentiviral vector was co-transfected with pPACK packaging plasmids into HEK293 cells. After 48hrs, the supernatant was collected and enriched for the recombinant viruses by incubating with a virus precipitation solution, PEG-iT™ (System Biosciences) for 24hrs at 4°C. The recombinant lentiviruses were recollected by centrifuging the samples at 1500G for 30min at 4°C.

**Soft agar colony formation assay**

The procedure was followed as previously described in Chapter 2

**In vivo xenograft experiments**

The animal experiments were followed by institutional protocol. Two groups of three nude mice each were intravenously injected with $5 \times 10^7$ TUSC3 knockdown H460 cells or control cells (Jackson laboratory). Four weeks after injection with H460 TUSC3 knockdown cells, the mice were sacrificed and their lungs were biopsied. The
samples were prepared for H&E staining and the number of foci was counted under light microscope (Nikon, Eclipse 50i). For the IVIS in vivo imaging, the stable A549 TUSC3 knockdown and GFP+/luc+ cells were generated and two groups of three nude mice each were injected (i.v.) with the 1x10⁷ A549 TUSC3 knockdown and GFP+/luc+ cells. From week 1 of the injection, luciferase activity was monitored using the IVIS in vivo imaging system weekly for 6 weeks by i.v. injecting in vivo luciferin reagents (Promega). Animal experiments were conducted after approval of the Institutional Animal Care and Use Committee, the Ohio State University.

MiRNA locked nucleic acid in situ hybridization and immunohistochemistry for lung cancer tissue.

The overall procedures for co-expression analysis and IHC were described previously [42,107]. The optimal detection of miR-224 and miR-520c-3p was determined in proteinase K with a concentration of the digoxigenin-tagged LNA probe of 0.5 pmol/ul. For IHC of MCAM and NM23H1 protein, each 5 ug/ml of anti-MCAM antibody and 1:100 (vol/vol) of anti-NM23H1 (D98) antibody were used.

Statistical analysis

The results were analyzed using ANOVA and/or two paired student t-test. Only p-values < 0.05 were considered significant.

3.3 Results

TUSC3 is suppressed in lymph nodes of advanced stage lung cancer patients

Based on previous observations showing that TUSC3 is down-regulated in certain cancer types, I decided to test the expression patterns of TUSC3 in primary
and metastatic lung cancer patient tissues compared to normal. I tested 20 normal, 14 primary and 21 lymph node metastasized tumor tissues and observed that there were no significant differences in TUSC3 mRNA expression in primary lung cancer patient tissues compared to normal lung tissues. TUSC3 expression, however, was suppressed in metastatic cancer patient tissues (Figure 3.1a). In order to confirm the observed downregulation of TUSC3 in metastatic tumor samples, immunohistochemistry were performed with 80 primary tumor tissues and their matched lymph-node metastases of lung cancer tissues. Among 46 cases showing TUSC3 expression, I observed that 28 cases showed decreased expression of TUSC3 in metastatic samples compared to the primary tumor tissues (Figure 3.1b and 1c). Moreover, the overall expression of TUSC3 was decreased in metastatic tumor tissues that I tested (Figure 3.1d), suggesting that TUSC3 is down-regulated in metastatic tumor tissues.

**miR-224 and miR-520c-3p suppress TUSC3 expression in NSCLC cells**

A target search using two separate in silico programs (TARGETSCAN and PICTAR) predicted that miR-224 and miR-520c-3p are likely to target TUSC3 at 102-108bp and 22-28bp downstream of the 3’UTR respectively (Figure 3.2a). I performed luciferase assays to determine whether miR-224 and miR-520c-3p can suppress TUSC3 expression. The TUSC3 3’UTR was cloned into a psiCHECK-2 dual luciferase vector and the construct was transfected in combination with miR-224 or miR-520c-3p into both A549 and HEK293 cells. The resulting luciferase activity was measured and observed to be decreased by both miR-224 or miR-520c-3p
overexpression (Figure 3.2b). However, upon transfecting cells with 3’UTR mutants harboring either a miR-224 or miR-520c-3p binding site deletion, the luciferase expression remained unchanged upon miRNA overexpression (Figure 3.2c).

Immunofluorescence (IF) analysis with an anti-TUSC3 antibody showed that ectopic expression of miR-224 or miR520c-3p decreased intensity of TUSC3 in both H460 and A549 cells (Figure 3.2d; data not shown). Moreover, TUSC3 mRNA expression was suppressed when these miRNAs were overexpressed in A549 and H460 cells (Figure 3.2e), suggesting that miR-224 and miR520c-3p directly target the TUSC3 3’UTR at the mRNA level. I next sought to determine if there was an inverse correlation between these miRNAs and TUSC3 gene expression in vivo. We performed in situ hybridization analysis with 5’-dig-labeled LNA probes for these miRNAs followed by immunohistochemistry for TUSC3 in 80 cases of lung cancer patient tissues. Among 58 cases that expressed miR-224 and/or TUSC3, 71% (41 cases) showed an inverse correlation between miR-224 expression and TUSC3 protein. Similarly, in 59 cases expressing either miR-520c or TUSC3, 68% (40 cases) showed an inverse correlation between miR-520c-3p and TUSC3 (Figures 3.2f and 3.3). Importantly, the expression of miR-224 was increased in 22 tissue samples of lymph node metastasis (55%) and miR-520c was increased in 19 (47.5%) metastatic tissue samples (Figures 3.2g and 3.4). These initial observations suggested that a biological relationship may exist between miR-224, miR520c-3p and TUSC3.
To understand the roles of TUSC3 in tumorigenesis of NSCLC, I generated TUSC3 knock-down cell lines of H460 and A549 cells (#2A1 and #2C1 for H460 and #3B1 and #3C1 for A549). Migration and invasion assays showed that TUSC3 down-regulation led to increased migratory and invasive capacity in both H460 cells and A549 cells. However, the introduction of smTUSC3, which has silent mutations that correspond to the shRNA target sequences in TUSC3, into knockdown H460 cell, reduced their migration and invasion (Figures 3.5a and 5b). Similar results were also observed in TUSC3 knock-down A549 cells (data not shown). Furthermore, the wound-healing ability of TUSC3 knockdown cells is higher than that of control cells but transient expression of smTUSC3 inhibits the wound healing capacity of TUSC3 knockdown cells (Figure 3.5c), suggesting that TUSC3 downregulation enhances the migratory and invasive potential of H460 and A549 cells in vitro. Furthermore, the results from ectopic expression of the TUSC3 gene into HeLa and HCT116 cells, which do not express TUSC3, showed that TUSC3-dependent migration and invasion suppression is conserved in different cancer types (Figures 3.6a and 6b). To further confirm the effect of TUSC3 on metastasis in vitro, I performed a PCR-array with RT-PCR probes for 84 known metastasis-related genes using RNA from two different H460 TUSC3 knock-down cell lines (#2A1 and #2C1). The volcano plots show that several metastatic suppressors including APC and CDH11 are suppressed and several metastatic inducers such as MMPs and e-MET are increased in both the TUSC3 knock-down H460 cells (Figures 3.6c and 6d; Tables 3 and 4).
To confirm the involvement of TUSC3 in metastasis in vivo, I performed an orthotopic xenograft with nude mice using H460 and A549 TUSC3 knockdown cells. 5x10^7 shTUSC3-H460 knockdown cells (sh#2C1) were intravenously injected into three nude mice. After 4 weeks the mice were sacrificed and the foci in lung tissue were counted. I observed that the number of foci were higher in the lung from mice injected with H460 knockdown cells as compared to those injected with control cells (Figure 3.5d). In addition, three nude mice were intravenously injected with GFP^+/luc^+ TUSC3 knockdown A549 cells or control cells. Bioluminescence was first detected at 3 weeks post-injection in GFP^+/luc^+ TUSC3 knockdown A549 but not in the control injected mice (data not shown). Moreover, 5 weeks after the injections, the intensity of luminescence in #sh3C1-injected mice was significantly stronger than the control (Figure 3.5e). These observations indicate that TUSC3 deficiency increases the metastatic potential of NSCLC in vivo.

**MCAM and NM23H1 are target proteins in response to TUSC3 reduction**

The observations that TUSC3 is suppressed in metastatic patient samples and is involved in metastatic processes led us to analyze for potential downstream target genes that respond to TUSC3 reduction. Immunofluorescence (IF) and western blot analysis showed that NM23H1 (Non-Metastatic Cell 1) protein, a metastatic suppressor [108], was downregulated whereas a Melanoma Cell Adhesion Molecule (MCAM) protein was increased in TUSC3 knockdown H460 cells (Figures 3.7a and 7b). To further confirm the relationship between TUSC3 and two target proteins in vivo, I analyzed the expression patterns in 80 lung cancer patient samples by
performing immunohistochemistry (IHC) and observed that TUSC3 expression showed correlation with NM23H1 whereas MCAM expression showed inverse-correlation with TUSC3, consistent with our previous observation (Figures 3.7c-7e). Furthermore, 60% of the cancer patient tissues that we tested showed decreased expression for NM23H1 but increased expression for MCAM protein in lymph node metastasis of lung tumors compared to matched primary tumor tissues (Figures 3.7f and 7g). These observations indicate that NM23H1 and MCAM genes have an inverse relationship to the TUSC3 gene. To understand the contribution of altered NM23H1 and MCAM expression in TUSC3 knockdown cells, I transfected these cells with MCAM siRNA (siMCAM) or NM23H1 plasmids (pc-NM23H1). When MCAM was reduced in TUSC3 knockdown cell lines, the increased migratory and invasive abilities were repressed. Similarly, ectopic expression of NM23H1 in TUSC3 knockdown cell lines decreased migratory and invasive activities (Figures 3.7h and 7i), suggesting that TUSC3 dependent regulation of metastasis involves NM23H1 and MCAM activity.

**miR-224 and miR-520c-3p enhance metastatic potential of NSCLC through TUSC3 suppression**

To test whether miR-224 or miR-520c-3p regulates NM23H1 and MCAM, I conducted western blot analyses for NM23H1 and MCAM in A549 and H460 cells. NM23H1 was decreased whereas MCAM was increased upon the ectopic expressions of miR-224 or miR520c-3p (Figure 3.8a). In addition, IF analysis showed direct correlation between TUSC3 and NM23H1 and an inverse correlation between TUSC3
and MCAM proteins in miR-224 or miR-520c-3p overexpressing H460 cells (Figure 3.8b). The overexpression of miR-224 and miR-520c-3p was confirmed by qRT-PCR (data not shown). According to PICTAR and TARGET SCAN, neither NM23H1 nor MCAM are predicted as targets for miR-224 and miR-520c-3p (data not shown), indicating that the altered NM23H1 and MCAM protein expression is not directly regulated by miR-224 and miR-520c-3p. Conversely, the western blot analysis with miR-224 and miR-520c-3p double knockdown A549 cells showed a decreased MCAM expression and an increased NM23H1 expression; however, when TUSC3 was knocked down by siRNA treatment into the double knockdown cells, the altered expression of MCAM and NM23H1 was recovered (Figure 3.8c). These data suggest that miR-224 and miR-520c-3p regulate MCAM and/or NM23H1 through TUSC3 downregulation.

To understand the function of miR-224 and miR-520c in TUSC3-dependent migration and invasion, I performed migration and invasion assay by overexpressing the miRNAs in A549 cells. The migration and invasion rates were increased (Figure 3.8d; Figure 3.8e, left panels); however, these effects were diminished by TUSC3 overexpression in A549 cells (Figure 3.8e, right panels). Similar effects were also observed in H460 cells (data not shown). Conversely, the knockdown of miR-224 and miR-520c decreased transformation, migration and invasion capacities of A549 and H460 cells. However, these abilities were recovered in response to TUSC3 knockdown in miR-224 and miR-520c-3p knockdown A549 and H460 cell lines (Figures 3.8f-8h). Taken together, these data indicate that TUSC3 suppression
mediated by miR-224 and 520c-3p enhances migratory and invasive capacity in NSCLC.

3.4 Discussion

While expression patterns for TUSC3 have been studied in several cancers, very little is known regarding the expression and biological function of TUSC3 in lung cancer. Similarly, the function and expression of miR-224 and miR-520c-3p remain unknown in lung cancer. In the current study, I found that TUSC3 is suppressed in metastatic tumor tissues compared to primary lung tumor tissues. Furthermore, I made the novel observation that TUSC3 down-regulation in NSCLC by miR-224 and miR-520c-3p enhanced metastatic abilities of NSCLC by regulating both a metastatic suppressor, NM23H1 and a cell adhesion molecule, MCAM.

MiRNAs can target multiple genes, which consequently control various key regulators in tumorigenesis [4,5]. Recent studies show the plausibility of in vivo studies of miRNAs in several types of cancers [109,110]. In this study, I first determined that miR-224 and miR-520c-3p are upregulated in lymph node metastasis of lung tumors and suppress TUSC3. MiR-520c-3p is well characterized oncogenic miRNA in breast cancer, wherein it works cooperatively with miR-373 [101]. The current study also show that miR-520c-3p also harbored oncogenic properties in NSCLC through the inhibition of TUSC3; however, I did not observe any significant expression of miR-373 in several NSCLC cell lines such as H460, A549, H1299, and H292 (data not shown). Unlike miR-520-3p, miR-224 has an opposite role in breast, colon, HCC, or prostate cancer. Here, I showed that miR-224 and miR-520c are up-
regulated in metastatic lung tumors, and seem to function as oncogenic miRNAs in NSCLC. The results in this chapter suggest that miR-224 and miR-520c-3p could be biomarkers and/or therapeutic targets for NSCLC.

Previous studies have shown that TUSC3 gene is frequently deleted or silenced by several mechanisms such as methylation of the 5’ regulatory sequences of TUSC3 in colon, prostate, head and neck and ovarian tumors [92,94,99,111]. The IHC results show that TUSC3 gene is frequently absent in both primary and metastatic lung cancer patient tissues, nevertheless TUSC3 is still expressed in many tissues and cell lines in NSCLC and its expression is decreased in metastatic lymph node tumors. Here I propose a mechanism by which TUSC3 expression is mediated by miR-224 and miR-520c in vitro and in vivo. I show that miR-224 and miR-520c directly target the TUSC3 3’UTR and are overexpressed in metastatic NSCLC tissues where they have an inverse correlation with TUSC3 expression. Furthermore, the increase in miR-224 and miR-520c-dependent metastatic potential was abrogated by TUSC3 expression and vice versa, suggesting a functional association between miR-224 and miR520c-3p-dependent metastasis and TUSC3.

The role of TUSC3 in lung cancer metastasis seems to be dependent on the regulation of Non-metastatic cells 1 protein A (NM23A or NM23H1) and Melanoma cell adhesion molecule (MCAM) protein. Through PCR array, IF, and western blot analysis I show that NM23H1 and MCAM proteins seem to be regulated at post-translational levels. NM23H1 is known as a metastatic suppressor in many cancer types including NSCLC [108]. MCAM induces metastasis in several cancers.
including melanoma by activating oncogenic signals such as Src family kinase [112-114]. A study using IHC analysis in NSCLC patient tissues showed that MCAM is highly upregulated in NSCLC compared to the normal lung tissue [115]. Based on IHC analysis I further observed that MCAM is increased in metastatic lung tumor patient tissues compared with their matched primary lung tumor tissues. Inhibition of MCAM enforced by specific siRNA treatment and ectopic expression of NM23H1 decreased the capabilities of cell migration and invasion in TUSC3 knockdown cells.

Interestingly, IHC results also show that TUSC3 is strongly expressed in other stromal cells including infiltrating lymphocytes in most NSCLC tissue samples independent of TUSC3 expression in tumor cells. Therefore the metastasis mediated by TUSC3 suppression may be due to cell autonomous effects in the lung cancer. Moreover, overexpression of TUSC3 gene was observed to trigger cell death in 24 - 48 hrs after transfections, which could be partially rescued by a pan-caspase inhibitor, z-VAD (data not shown). I believe that this cell death could be a TUSC3-mediated tumor suppressive function although downstream events should be further addressed. Recently, it was discovered that TUSC3 knockdown enhanced N-linked glycosylation, which plays an important role in prostate cancer progression [100]. In addition, MCAM is known as a highly glycosylated protein involved in cancer [114]. Furthermore I showed by western blot that glycosylated MCAM protein was increased in TUSC3 knockdown cells, suggesting that TUSC3-dependent MCAM suppression may be related with the function of TUSC3 as a subunit for N-linked glycosylase complex.
Taken together, I present a novel study demonstrating that miR-224 and miR520c-3p-mediated TUSC3 deficiency plays a critical role in NSCLC metastasis by regulating NM23H1 and MCAM. These target proteins and miRNAs may have the potential as clinically relevant biomarkers and therapeutic targets in lung cancers.
Figure 3.1. TUSC3 is suppressed in metastasized lung cancer patient samples. (a) Box plots showing the RNA expression of TUSC3 in normal, primary, or metastatic lung tumor samples. The RNA samples were purified from 20 normal, 14 primary, or 21 metastatic lung tissue samples and subjected to qRT-PCR with a TUSC3 probe. The expressions were normalized using GAPDH expression. (b-c) Expression analyses of TUSC3 by immunohistochemistry (IHC) showing suppressed expression in matched metastatic samples. 80 sets of primary lung tumors and their matched lymph node metastases of lung tumor tissues were analyzed by IHC with anti-TUSC3 antibody (b) and the scattered blot shows the expressional difference in TUSC3 between primary and its metastatic lung tumors (c). (d) Box plots showing the comparison of the expressions in 80 sets of lung tumor samples. For qRT-PCR experiments, each sample was triplicated for each reaction and the reactions were also triplicated. The $p$-values were calculated by ANOVA t-test or paired student t-test.
Figure 3.2. MiR-224 and miR-520c-3p directly suppress TUSC3 in NSCLC.

(a) Schematic diagram showing matched sequences between the seed sequences of miRNAs and 3’UTR of TUSC3. (b-c) Luciferase activity with psiCheck2-3’ UTR of TUSC3 in miR-224 or -520c-3p-overexpressing Human Embryonic Kidney 293 (HEK293) cells. The psichek2-TUSC3-3’UTR plasmids (b) or mutants with deleted target sequences corresponding with miR-224 or miR-520c-3p binding region (c) were co-transfected with miR-224 or miR520c-3p in HEK293 cells. After 48 hrs, the samples were subjected to dual luciferase analysis. The relative values were obtained by normalizing to internal Firefly luciferase values. (d) Immunofluorescence (IF) assay of TUSC3 in miR-224 or miR-520c-3p overexpressing H460 cells. The miR-224 or miR-520c-3p was ectopically expressed in H460 cells. After 48 hrs, the cells were harvested and prepared for the immunostaining with anti-TUSC3 antibody. (e) qRT-PCR analyses of TUSC3 in miR-224 or miR-520c-3p overexpressing NSCLC. The H460 or A549 cells were transfected with miR-224 or miR-520c-3p for 48 hrs. The cells were harvested and prepared for the qRT-PCR with TUSC3 probe. (f) Co-expression analysis of TUSC3 and miR-224/-520c. The images in the top left panel in the miR-224 and miR-520c section show a lung adenocarcinoma after co-expression with miR-224 (lower section, blue) / miR-520c (upper section, blue) and TUSC3 (brown). The tissue was then analyzed with a Nuance-based system that isolates the miRNAs signal (top right panel in each section, fluorescent blue) and the TUSC3 signal (bottom left, fluorescent red). The signals are then mixed (bottom right); the lack of fluorescent yellow shows the lack of co-expression of those two targets. (g) IHC results showing increased expression of miR-224 and miR520c in lymph node metastasis of lung tissues. Bars indicate means ±SD (n=3) and the p-values were addressed by two-tailed student t-test (*p < 0.005, **p < 0.01).
Figure 3.3. Co-expression analyses of miRNAs and TUSC3 showing inverse correlation between miRNAs and TUSC3 in lung cancer patient tissues. The expressions were scored and summarized. (a-b) Summary of co-expression results for TUSC3 and miRNAs in primary lung tumors (a) and their corresponding lymph node metastasized lung tumors (b).
Figure 3.4. The comparison of expressions of miR-224 and miR520c-3p between primary lung tumors and their matched lymph node metastases of lung tumor tissues. (a-b) The summary tables show increased expression of miR-224 and miR-520c-3p in lymph node metastases of lung cancer samples.
Figure 3.5. TUSC3 deficiency confers the cells to be highly metastatic in vitro and in vivo. (a-b) Increased migratory and invasive capabilities of TUSC3 knockdown cells. Two TUSC3 knockdown cell lines were transfected with pcDNA4.1-v5 empty or pcDNA4.1-smTUSC3-v5 plasmid containing silence mutations for three shTUSC3 RNA target sequences, and then incubated with a pan-caspase inhibitor, z-VAD. After 48 hrs, the cells were washed with PBS and then, were introduced into migration (a) and invasion (b) chambers for 20-40 hrs. The migratory and invasive cells were quantified by live-dead cell staining with Calcein AM. Relative folds were obtained by dividing with values from scrambled shRNA expressing H460 cells transfected with pcDNA4.1-v5. (c) Quantification of the gap area in wound-healing assay. The relative gap areas at 0 hr time points of each experiment were considered as 100% of the gap area. The relative areas were calculated by dividing the area values by the area at 0 hr. (d) Increased tumor burden in orthotopic xenograft mice model with H460 TUSC3 knockdown cells. 5x10^7 H460 control (shCtrl) or TUSC3 knockdown H460 cells (sh#2C1) were intravenously injected into three mice of each group. After 4 weeks, the lung tissues were obtained and prepared for H&E staining (upper panel) and the number of foci was counted under light microscope (lower panel). (e) IVIS in vivo imaging results showing increased metastatic ability of TUSC3 knockdown A549 cells. 1x10^7 GFP+/Luc+ control A549 or TUSC3 knockdown A549 cells were inoculated intravenously. After 3 weeks, the bioluminescence was obtained by intraperitoneal injection of luciferin and the photon flux rates were calculated under IVIS in vivo imaging system. Bars indicate means ±SD (n=3) and the p-values were addressed by two-tailed student t-test (*p < 0.03, **p < 0.01).
Figure 3.6. The roles of TUSC3 deficiency in migration and invasion are conserved in different cancer cells. (a-b) Migration and invasion abilities of cells with TUSC3 reduction. HeLa and HCT116 cells were transfected with pcDNA4.1-TUSC3-v5 plasmids and the cells were incubated with pan-caspase inhibitor z-VAD. After 48 hrs, the cells were washed by PBS and placed to migration and invasion chambers for 20-40 hrs. The migratory or invasive cells were quantified by Calcein AM staining. Bars indicate means ± SD (n=3) and the p-values were calculated by two-tailed student t-test (*p < 0.005, **p < 0.02). (c-d) Volcano blots of TUSC3 knockdown H460 cells showing differentially expressed metastasis-related genes. Total RNAs purified from two different knockdown or control cells were prepared for qRT-PCR and added into plates containing the probes for 84 genes known as metastasis regulators. The volcano plots were analyzed and generated by RT²-profiler PCR Array analysis software (Sabiosciences). The expressional fold difference of each target RNA is listed in Table 3 and 4.
Figure 3.7 NM23H1 and MCAM proteins are potential targets for TUSC3.
(a-b) Altered expression of NM23H1 and MCAM protein in TUSC3 knockdown cells. TUSC3 knockdown H460 cells were prepared for immunofluorescence (IF) (a) and western blot analysis (b) with indicated antibodies. (c-e) Immunohistochemistry (IHC) of TUSC3 and the target proteins showing direct correlation with NM23H1 (d) and showing inverse correlation with MCAM proteins (e) in lung cancer patient samples. The 80 lung patient samples were prepared for IHC with indicated antibodies. The expression scores were obtained under 20 different grades between 0 and 100. R-factor values were calculated by Spearman correlation coefficients method and p-values were obtained by two-paired student t-test. (f-g) Altered expression of NM23H1 and MCAM proteins in primary and their matched lymph node metastases of lung tumor samples. 40 sets of primary and matched metastatic tumor tissues were purchased (Biomax, Inc) for immunohistochemistry (IHC) with anti-NM23H1 (f) or anti-MCAM antibodies (g). The expression scores were obtained under 20 different grades between 0 and 100. The summary tables and scattered plots show decreased patterns of NM23H1 expression and increased patterns of MCAM expression in the matched metastatic tumor samples compared to primary tumor samples. (h-i) Reduced migratory (h) and invasive (i) abilities of TUSC3 knockdown cells by MCAM reduction and NM23H1 overexpression. TUSC3 knockdown cells were transfected with the mixture of four different MCAM siRNAs or pCMV-NM23H1 plasmid for 48 hrs. After that, the cells were harvested and placed into migration chamber (h) for 20 hrs or invasion chamber (i) for 40 hrs. The number of migratory cells were calculated by Calcein AM staining. Error bars indicate means ±SD (n=4) and the p-values were calculated by two-tailed student t-test (*p<0.03, **p < 0.001).
Figure 3.8 MiR-224 and miR-520c-3p enhances metastatic potential of NSCLC through TUSC3 suppression. (a-b) Altered expressions of NM23H1 and MCAM proteins in response to miR-224 or miR-520c-3p overexpression. H460 cells were transfected with miR-224 or miR-520c-3p for 48 hrs and the cells were prepared for western blot analysis (a) and IF analysis (b) with indicated antibodies. (c) The reduction of miR-224 and miR-520c-3p led to increased NM23H1 and decreased MCAM protein expression in A594 cells. The miR-224 stable knockdown A549 cells were generated by transducing with recombinant anti-miR-224 lentivirus. These knockdown cells were then transduced with recombinant anti-miR-520c-3p lentivirus to generate double knockdown of miR-224 and miR-520c. 24 hrs after transduction, the cells were transiently transfected with shControl vector or shTUSC3 plasmid for 48 hrs and the cells were harvested for western blot analysis. (d-e) Enhanced migratory and invasive capacities as a result of miR-224 or miR-520c-3p overexpression. The miR-224 or miR-520c-3p was co-transfected with either pcDNA4.1-v5 (d) or pcDNA4.1-TUSC3-v5 plasmid (e) and the cells were incubated with z-VAD. After 48 hrs, the cells were placed into migration and invasion chambers to measure the migratory and invasive capabilities of the cells. (f-h) The reduction of miR-224 and miR-520c-3p decrease transformation, migratory, and invasive capacities. The miR-224 and miR-520c-3p double knockdown cells were transfected with shControl (sh-ctrl) or shTUSC3 (sh-tusc3) plasmid for 48hrs. After that, the cells were placed into an Agar Matrix Layer for 5 days (f) or into migration (g) for 20 hrs and invasion (h) for 40 hrs chambers. The numbers of colonies were quantified with standard MTS assay (f) or the migratory and invasive cells were quantified with Live-dead cell staining with Calcein AM (g and h). Bars indicate means ±SD (n=4) and p-value were calculated by ANOVA test and/or paired student t-test (*p<0.005 and **p<0.001)
Chapter 4

Role of MYC-regulated long noncoding RNAs

in cell cycle regulation and tumorigenesis

4.1 Introduction

Mammalian genomes encode a large number of long-noncoding RNAs (lncRNAs), a non-coding RNA having longer than 200bp in length, as well as small non-coding RNAs from small [116,117]. Although more than 30,000 lncRNAs have been identified, a few lncRNAs have been characterized [118-121].

The MYC transcription factor is known as a proto-oncogene and is frequently amplified in several cancer including Colorectal tumors [122,123]. Its downstream genes are involved in various aspects of tumorigenesis such as cell cycle progression and cell transformation. In particular, CDKN1A and CDKN2B are critical intermediators of MYC-mediated tumorigenesis [124,125].

Previously, our former lab member Dr. Taewan Kim identified a novel lncRNAs differentially regulated in CRC by profiling CRC cells and tissues. Among those candidates, he identified MYC-associated lncRNAs and named MYCLos. Through a

4 The results in this chapter were published. Kim and Jeon et al., 2015 J Natl Cancer Inst. In-press
series of gene micro arrays, he found that several MYC target genes such as \textit{CDKN1A} and \textit{CDKN2B} are likely to be functionally associated MYCLos. Moreover, he also found that MYCLO-1 physically interacts with HuR and that MYCLO-2 does with hnRNAPK to mediate MYC-dependent oncogenic functions. Here I characterized that MYCLO-1 and -2 regulated MYC-dependent cell proliferation and also propose that MYCLO-2 has an oncogenic property \textit{in vivo}.

4.2 Materials and Methods

\textit{Xenograft and tumor-free survival analysis.}

Animal experiments were approved by the Ohio State University animal care and use committee and conducted following the Ohio State University animal policy in accordance with NIH guidelines. 0.5x10^6 cells transfected with indicated siRNAs 24hr before injection were subcutaneously injected into the right flanks of 5-week-old female nude mice (Jackson laboratory). Tumor initiation was observed and counted when a palpable tumor (approximately 50-60 mm$^3$) formed. Tumor volume was determined by measuring the length and the width of the tumor mass and calculating the volume (Volume = (width)$^2$(length)/2).

\textit{Cell culture and nucleic acid delivery}

All cell lines were purchased from ATCC and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). For each MYCLO, 2 kinds of custom siRNAs were designed and used. The siRNAs targeting MYC were purchased from
Dharmacon (siMYC) or Santa Cruz Biotechnology (siMYC-2). Cells were transfected with Lipofectamine RNAiMAX (Invitrogen) for oligonucleotides, Lipofectamine LTX for plasmids and Lipofectamine 2000 for oligonucleotides/plasmids cotransfection in accordance with manufacturer’s instructions. In all transfections of siRNAs, final concentration of siRNAs was 50nM for transfection of 1-3 oligonucleotides or 100nM for transfection of more than 3 oligonucleotides.

**Quantitative RT-PCR**

The procedure was followed as previously described in Chapter 2

**Antibodies**

All antibody used in the chapter 4 were purchased from Cell signaling Company.

**Soft agar colony formation assay**

The procedure was followed as previously described in Chapter 2

**Statistical analysis**

The results were analyzed using ANOVA and/or two paired student t-test. Only *p*-values < 0.05 were considered significant.

**4.3 Results**

*MYC-induced MYCLos enhance cell proliferation and repress expression of MYC target genes such as CDKN1A and CDKN2B*

To determine whether three MYCLos are involved in the MYC-dependent cell proliferation, I performed the cell proliferation assay with MYCLo-1, -2 or -3 in HCT116 (colorectal cancer) and PC3 (prostate cancer) cells. The knock-down of each
MYCLos enforced by specific siRNA treatment showed in decreased cell proliferation (Figures 4.1a and 1b). Conversely, the ectopic expression of the MYCLos enhanced cell proliferation (Figure 4.1c), suggesting that three MYCLos that I tested are involved in cell proliferation.

To characterize how the MYCLos mediate Myc-functions, a collaborator Dr. Kim found that analyzed 183 cell cycle regulator genes by using nCounter Virtual Cell Cycle Gene Sets of the NanoString Gene Expression Assay. As a result of the experiments, he isolated 19 genes regulated by MYCLo-1, 49 genes regulated by MYCLo-2 and 13 genes regulated by MYCLo-3 (See the paper Kim and Jeon et al., 2014 for the details). Among those putative positive clones, I confirmed the altered expressions of CDKN1A and CDKN2B by knockdown of MYCLo-1 or -2 as well as MYC knockdown (Figures 4.2a and 2b). The down-regulations of MYCLo-1 and -2 were confirmed by qRT-PCR analyses (Figures 4.2a and 2b, right panel). These elevations are consistent with the effect of MYC knockdown, however, independent of MYC, Miz-1 and p53 expression levels (Figure 4.2c).

**MYCLo-1 and -2 repress transcription activities of CDKN1A and CDKN2B in the distal promoter regions**

To understand how MYCLo-1 and -2 regulate CDKN1A and 2B expression, Dr. Kim analyze putative candidate by using a set of data base (data base of open chromatin TFBS by ChIP-sequencing from ENCODE/Open Chromatin at UT Austin). He found that there are multiple Myc binding sites on CDKN1 and 2B promoter regions. Therefore, we decided to determine whether there are MYCLo-associated
promoter regions at Myc Binidng site (MB) and its flaking region (Figures 4.3a and 3b).

Dr. Kim found that there was no any promoter activity for MYCLo-1 and -2 in MB regions when the MYCLo-1 and -2 were reduced by specific siRNA treatment (data not shown). In addition, I analyzed the possibilities of promoter region by a series of luciferase assays with specific siMYCLo-1 and -2 siRNAs. I subcloned the distal promoter regions into pGL-luciferase vectors and conducted luciferase assays, indicating that when the MYCLo-1 was knocked-down, luciferase activities were increased (Figure 4.3c). As shown in Figure 4.3d, when I addressed luciferase activities with a series of deleted mutants on distal promoter regions, CDKN1A promoter region approximately between -2768bp and -3980bp is critical for MYCLo-1-dependent CDKN1A regulation (Figure 4.3d). Furthermore, the similar experiments were also conducted for CDKN2B promoter regions for MYCLo-2. I found that CDKN2B promoter region approximately between -840bp and -570bp plays an important role for CDKN2B regulation by MYCLo-2 (Figures 4.3e and 3f). Interestingly, the distal regions of CDKN1A and 2B promoter were also regulated by Myc protein. Therefore, I believe that Myc is able to regulate those genes not only the MB regions but also the distal promoter regions. Furthermore, MYCLo-1 and -2 regulate CDKN1A and 2B genes as an independent manner with Myc protein by, at least, distal promoter regions for those genes.

**MYCLo-2 is an oncogenic IncRNA**

Previously, Dr. Kim found that MYCLo-2 is more highly expressed in CRC and prostate tumor samples confirmed by qRT-PCR (data not shown), indicating that
MYCLO-2 is likely to be involved in tumorigenesis. Therefore, transformation abilities were addressed with MYCLO-2 in vitro and in vivo. Anchorage-independent assays were performed with siMYCLO-2 siRNA treatment, showing that the reduction of MYCLO-2 significantly reduced the number of cells and showed in defects of colony formation ability (Figure 4.4a), suggesting that down-regulation of MYCLO-2 is likely to inhibit transformation ability. To further confirm the effects in vivo, I performed xenograft experiments with a CRC cells or prostate cancer cells by enforcing siMYCLO-2 siRNAs. 5x10^6 cells of control or siMYCLO-2 siRNAs-expressing cells were introduced into nude mice, subcutaneously and the tumors were monitored at indicated time periods. Consistent with in vitro experiments, the tumors with knock-downed HCT116 or PC3 cells showed a defect in tumor development (Figures 4.4b and 4c). These findings suggest that MYCLO-2 is strong tumor suppressor candidate.

4.4 Discussion

In spite of massive amounts of lncRNAs existed in the cells, so far, there are a couple of CRC-associated lncRNAs which have been identified and characterized [126-128]. Here I first identified and characterized several lncRNAs showing reduced expression in CRC, which possibly functions as a tumor suppressor. In particular, MYCLO-2 was validated in vitro and in vivo, suggesting that it is likely to function as a tumor suppressor.

Myc transcription factor plays a versatile role in tumorigenesis [122]. It has been known that Myc contributes cell stemness as well as tumotigenesis [129]. Moreover, the studies with dicer-deficient mice embryonic cells and computational
approaches showed that considerable amounts of lncRNAs are dependent on dicer and Myc transcription factors in expression [130]. Therefore, Myc-dependent lncRNAs regulations or its functional significance should be further addressed. Furthermore, $CDKN1A$ and $CDKN2B$ are negatively regulated by Myc proteins, which mediates Myc-dependent cell cycle progression, tumorigenesis, epithelial-mesenchymal transition and stemness [124,131-134]. The current findings in this chapter consistently showed that Myc-dependent MYCLo-1 and -2 expressions antagonize $CDKN1A$ and $CDKN2B$ expressions, which seems to be involved in tumorigenesis.

Interestingly, $MYCLo$-1 or -2 stable cell lines were not properly generated. The knock-downing effects were diminished at 1-2 weeks after the mixed populations were established. In addition, transient expression of siMYCLo-1 or -2 siRNAs induced weak but significant cell death. Therefore, it suggests that the knockdown of $MYCLo$-2 inhibits cell cycle progression or induces cell death by unknown mechanism at early stage.

Taken, together, the current results in this chapter support a notion that Myc-dependent lncRNAs play a critical role in tumorigenesis, which provides the clue of new strategy and targets of cancer and Myc-associated pathogenesis.
Figure 4.1. MYCLos are involved in cell proliferation. (a-b) Cell proliferation assays. HCT116 (n=5, a) or PC3 (n=4, b) cells were treated with corresponding siRNAs as indicated and subjected to proliferation assay every 24 hr (†, \( P < .013 \); ‡, \( P = .0048 \); ¶, \( P = .009 \); *, \( P < .001 \)). (c) Cell proliferation assay with MYCLO-overexpressing cells. CCD-18 cells were transfected with pcDNA3.3 plasmids (EV) expressing MYCLO-1, -2 or -3 and subjected to a cell proliferation assay every 24 hr (n=3) (*, \( P = .001 \); †, \( P = .0045 \); ‡, \( P = .0025 \)). Bars indicate means ±SD and the \( p \)-values were addressed by two-tailed student t-test.
Figure 4.2. MYCLos mediate Myc-dependent CDKN1A and CDKN1B expressions. (a) qRT-PCR analyses showing increased expressions of CDKN1A (left) and CDKN2B (middle) expression by MYCLo-1 in HCT116 cells. The cells were transfected with each siRNAs indicated in the figure for 72 hrs and subjected for RT-PCR analysis with indicated probes. Data are mean ± S.D. of three independent experiments and each measured in triplicate (††, \( P = .003 \); §, \( P = .0017 \); ‖, \( P = .013 \); †, \( P = .013 \); ‡, \( P = .001 \); #, \( P = .002 \); *, \( P < .001 \)). (b) qRT-PCR analyses for CDKN2B by MYCLo-2 knockdown (right). Data are mean ± S.D. of three independent experiments and each measured in triplicate **, \( P = .03 \); ††, \( P = .002 \); ‖, \( P = .003 \); §, \( P = .01 \); †, \( P = .001 \); ‡, \( P = .005 \); #, \( P = .016 \); ¶, \( P = .004 \); *, \( P < .001 \)). (c) Western blot analyses with known Myc-targeted proteins.
Figure 4.3. MYCLos mediates CDKN1A and CDKN1B expressions. (a-b) Schematic figures showing Myc-binding regions (MB) and its distal promoter region of CDKN1A and 2B done by Dr. Taewan Kim. (c,e) Luciferase assays with siMyclos and siMyc siRNAs showing increased luciferase activity for MYCLo-1 and Myc protein for CDKN1 promoter region (c) and for MYCLo-2 and Myc protein for CDKN2B promoter region (e). (d,f) Luciferase assays with a series of deleted mutants as indicated figure.
Figure 4.4. MYCLo-2 exhibit tumor suppressive phenotypes in CRC and prostate tumors. (a) Anchorage independent assays with MYCLo-2 knock-downed cell lines. HCT116 and PC3 cells were subjected for soft agar colony formation assay kit. The number of cells were quantified by MTS assay and the number of colony were counted by a microscope. Bars indicate ± SD (n=3; **, P=.003; †, P=.0055; ¶, P=.0063; ‡, P=.0092; *, P<.001). (b-c) Kaplan-Meier (KM) plot of tumor-free survival analysis in xenografted nude mice with siMYCLo-2 transfected HCT116 (b) or PC3 (c) cells. The KM method was used to estimate survival curves, and the log-rank test was used to test for differences between curves using SPSS Statistical Software (SPSS Inc., Chicago, IL).
CHAPTER 5

Conclusions and Future Directions

5.1 Characterization of miRNAs involved in TRAIL-resistance

Resistance to cytotoxic agents is a major problem in the treatment of many tumors that clinicians have to face on a regular basis. Drug resistance may be intrinsic or acquired during a course of therapy. Intrinsic resistance is observed when tumors are first exposed to anticancer agents, whereas acquired drug resistance is seen in tumors that no longer respond to the drugs to which they were initially sensitive. The long-term survival for patients with advanced high-grade lung cancer has been limited by the frequent occurrence of resistance to chemotherapeutic drugs. In this context, TRAIL may represent an alternative therapeutic molecule for this type of cancer. The molecular mechanisms underlying the resistant TRAIL phenotype in human lung carcinoma cells is still unclear and little is known also on how lung cancer can get bona fide resistance or acquire resistance to TRAIL.

Since the identification of TRAIL, it has been highlighted to be used for anti-cancer drug due to high selectivity of many cancer cells and the lack of toxicity. Now it is in phase I and II clinical trials against several solid tumors
In spite of the massive studies for TRAIL-resistance, the intrinsic and acquired TRAIL-resistances are still a hindrance in cancer therapeutics [30,80]. Furthermore, little is known what factors are involved in acquired TRAIL-resistance.

For the above reasons, many researchers have tried to elucidate the mechanism what specific factors drive TRAIL-resistance or how the sensitive cells get more resistance with continuous exposure with TRAIL. As the results of the efforts, several key signaling pathway have been proposed such as the regulation of TRAIL-receptors, NF-κB and/or the lack of intrinsic cell death machinery with c-myc deficiency, and some specific factors involved in innate TRAIL-resistance such as a HDAC inhibitor, O-glycosyltransferase, GLAN14 and Par4 [43,69,70,135-138].

In particular, miRNAs have been considered an attractive molecule involved in tumorigenesis due to their natures that single miRNA family is able to block several signaling pathways by suppressing multiple target genes [4,5], which consequently regulates several key regulators in tumorigenesis. Recent studies also showed the plausibility of in vivo application with miRNAs [109,110]. In addition, the former lab members, Dr. Garofalo and her colleagues first characterized that miR-221/-222 are involved in innate TRAIL-resistance in lung cancer [43]. Furthermore, the results in the chapter 2 consistently suggest that miR-34 is able to play a key role in overcoming TRAIL-resistance in lung cancer by suppressing a receptor tyrosine kinase, PDGFRs. However, those studies are still needed to show clinical relevance in lung cancer patients. In particular, I need to confirm how PDGFRs actually
contributes to establish or maintain innate TRAIL-resistance in cancer patient samples showing innate TRAIL-resistance. Moreover, I need to further characterize the other targets for miR-34 family because I believe that PDGFRs are not only protein involved in miR-34-dependent innate TRAIL-resistance.

Among several key oncogenic pathways, NF-κb has been believed most responsible for innate TRAIL-resistance, which consequently increases several key anti-apoptotic molecules such as c-FLIP and anti-apoptotic Bcl-2 family. Now combinatorial therapeutic potentials have been addressed in vitro and in vivo [23,70]. However, the involvement of any oncogenic signaling including NF-Kb signaling pathway is largely unknown in acquired TRAIL-resistance. To this end, I established acquired TRAIL-resistant cells from the TRAIL-sensitive H460 and H292 lung cell lines as described in chapter 2.2. I found that a set of miRNAs, miR-21, -30c and -100 was found drastically upmodulated in acquired TRAIL-resistant cells and our data clearly show that miR-21, -30c and -100 induce acquired TRAIL resistance by targeting important tumor suppressor genes and mediators of the TRAIL pathway, such as caspase-8, caspase-3, TRAF7 and FoxO3a. Furthermore, it also demonstrated that the propensity of TRAIL-sensitive cells that caspase-dependent cell death dominates cell survival signals is changed to opposite manner in acquired TRAIL-resistant NSCLC. It is evident that the enhanced cell death signals by reinforcing targeted genes enforced by lentiviral transduction of anti-miR-21, -30c, or -100 efficiently made the resistant cells TRAIL-sensitive, suggesting that one sided signals can be a determinant in acquired TRAIL-resistance between NF-κB-dependent cell
survival and caspase-dependent cell death signals. Taken together, TRAIL-stimulation triggers a typical extrinsic apoptosis in TRAIL-sensitive NSCLC. However the cells become more resistant, enhanced NF-κB activation and suppressed caspase-dependent cell death mainly enforced by miR-21, 30c, and -100 escape the sensitive cells from TRAIL-induced cell death, which eventually showed higher aggressive cancer phenotype like the side effects of the other acquired drug resistance systems, providing a new insight in anti-cancer therapeutics with TRAIL.

However, this study lacks of validation for in vivo approaches that those miRNAs-dependent NF-κB regulations are really conserved in lung cancer patient samples experienced chemotherapy with TRAIL. Unfortunately, those kinds of TRAIL-resistant patient samples are not available at this point. Therefore, I need to generate the model close to actual cancer patient samples. I have a plan to modify a patient-derived xenograft model system to get acquired TRAIL-resistant model. In particular, I will perform the xenograft with cancer-patient derived cells and inject TRAIL into the nude mice. After several rounds of selection, the cells will be harvested and cultured to analyze how the patient-derived tumor cells get an acquired TRAIL-resistance based on my finding described in chapter 2.2.

Interestingly, I also observed that PI3K-AKT signaling pathway was highly upregulated in the acquired resistant cells. In addition, the cells were dramatically sensitive when the cells were treated a PI3K-ATK inhibitor with TRAIL (data not shown). It can be caused by the fact that AKT protein is known to be a prosurvival protein and it mediates NF-κB-dependent cell survival signal in TRAIL-induced cell...
death. However, it has been proven that PI3K-AKT and MAPK signaling pathway play a crucial role in lung cancer [44,71,139]. Therefore, I need to further analyze the contributions of increased AKT phosphorylation in acquired TRAIL-resistant NSCLC.

5.2 MiR-224 and miR-520c-dependent TUSC3 suppression is involved in lung cancer metastasis.

Lung cancer is the largest cause of cancer-related death in the world. The major type of lung cancer (more 80% of lung cancer) is non-small cell lung carcinoma (NSCLC), which is the most dangerous epithelial cancer, and shows a very poor prognosis [1,2]. However exact mechanisms for tumor development and metastasis in lung cancers are not fully understood. In this chapter 3, I first characterized the functions of miR-224 and miR-520c as an oncomiRNA in lung cancer, which eventually regulate a novel gene, TUSC3. I also found that miR-224 and miR-520c-dependent TUSC3 suppression enhanced metastatic potentials of lung cancer by decreasing a metastatic suppressor, NM23H1 protein and by increasing a cell adhesion molecule, MCAM protein expression in vitro and in vivo.

Endoplasmic Reticulum (ER) stress in commonly observed in transformed cells, which plays a critical role in cellular metabolism as well as tumor cell adaptation. Imbalanced homeostasis inside or outside of a cell such as transformation of the cell can trigger the accumulation of unfolded protein, resulting in induction of ER-stress [140]. Although it has been known that ER-stress frequently triggers pro-apoptotic or anti-apoptotic signaling pathway in cancer, the exact mechanism of ER-stress induced cell death is not fully understood [30,141].
Although, I confirmed the relation between TUSC3 and its two target gene (NM23H1 and MCAM protein) in lung cancer patient samples, it still fails to show how TUSC3 gene regulation those two protein. One plausible explanation is that TUSC3 is an ER-membrane protein and is responsible for ER-stress responses in prostate cancer [100]. TUSC3 is ER-membrane protein and its expression is suppressed in several cancers [93,94,96,97]. And TUSC3 is also known to be a member of N-glycosylated complex in ER, which seems to be required for cancer progression in prostate cancer [99]. In addition, MCAM is known as a highly glycosylated protein involved in cancer [114] my preliminary data showed that the expression of TUSC3 gene is increased in responses to ER-stress induction (data not shown). Therefore, I hypothesize that ER-stress induced TUSC3 affects MCAM functions by regulating its glycosylation status and perform the experiments to prove the hypothesis.

Hypomagnesaemia is related with many diseases such as diabetes mellitus and ischemic cardiac disease (Toril et al., 2009). In metastatic cancers, hypomagnesaemia is also commonly observed. Consistently magnesium treatment has been used as a cancer therapy. The several physiological evidences have suggested that hypomagnesaemia is related to cancer progression. In tumorigenesis, primary cancer cells have normal levels of magnesium. However, the progressed cancers show hypomagnesaemia, which seems to have a role in cancer metastasis. Furthermore the primary cancers having hypomagnesaemia are defective in tumor growth and
angiogenesis. However, the molecular mechanisms by which the cancers undergo magnesium deficiency are largely unknown [142].

It is possible that TUSC3 deficiency causes hypomagnesaemia. Interestingly, many previous reports suggested that magnesium transporter, N33 is down-regulated or suppressed by various mechanisms in several cancer and cancer cell lines [92,93,95,97,143]. Furthermore, as tumors severity progresses from grade 1 and 2 to grade 3, the expression of N33 is down-regulated in ovarian carcinoma [97]. Therefore the studies that TUSC3 deficiency inhibits cell growth and induces apoptosis by decreasing magnesium uptake in response to hypoxia in this proposal will be provide the mechanism for this issue.

Therefore I believe that TUSC3 deficiency-induced metastatic potential is likely to be associated with intracellular magnesium deficiency. Consistently, the intracellular magnesium level measured with a fluorogenic marker, Mag4-flor in TUSC3 knockdown H460 cells was slightly, but significantly decreased (data not shown). Therefore, I will analyze how intracellular magnesium were changed in TUSC3 deficient cell lines and/or mice, which eventually provide a clue to explain how the hypomagnesaemia is frequently observed in cancer patients.

5.3 Role of MYC-regulated long noncoding RNAs in cell cycle regulation and tumorigenesis

Compared to small non-coding RNAs, the functions of IncRNAs are not well understood although they are extensively transcribed in genome [144-146]. Previously, a former lab member, Dr. Taewan Kim identified IncRNAs differentially
expressed associated with Myc transcription factor and suggests the potential roles of them as a Myc mediator [147]. By using lncRNA nanostring analysis with Myc alteration, he identified another sets of Myc-associated lncRNAs named with \textit{MYCLos}. Among those positive clones, I characterized \textit{MYCLo-1} and \textit{-2} that negatively regulates Myc-associated \textit{CDKN1A} and \textit{CDKN2B} proteins involved in Myc-dependent cell cycle regulation. I further characterized that \textit{MYCLo-2}, in particular, has a potent oncogenic effects \textit{in vitro} and \textit{in vivo}. This study provides first evidence that Myc-associated lncRNAs mediates Myc functions in cancer cells.

Although I propose that \textit{MYCLo-1} and \textit{-2} possibly suppress \textit{CDKN1A} and \textit{CDKN2B} by using a series of luciferase assay and qRT-PCR analyses, it still needs to be further addressed how the lncRNAs regulates the promoter activities. One plausible hypothesis is that the MYCo-1 and -2 physically interacts the promoter region to make a huddle against RNA polymerase functions. Therefore, I need to analyze the mechanism of lncRNA in terms of target gene suppression. Recently, several reports have proposed the possibility of small non-coding RNAs as a therapeutic drug [109,110]. Therefore, \textit{MYCLo-1} and \textit{-2} also should be addressed for therapeutic potential \textit{in vivo} as well.
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