EFFECTS OF CIGARETTE SMOKE CONDENSATE ON MICRORNA EXPRESSION OF HUMAN BRONCHIAL EPITHELIAL CELLS

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Dedication

This thesis is dedicated to my lovely wife Yete

Abstract

Lung cancer is the leading cause of cancer death and accounts for 1.2 million deaths annually world-wide and its five year survival is less than 15%. The primary etiology of lung cancer is genetic and epigenetic alterations caused by tobacco smoke. Although smoking-related genetic changes have been well studied, much is not known about the association between lung cancer and epigenetic changes, in particular deregulation of microRNAs. Therefore, our objective in this study was to examine the association between early phenotypic changes and altered microRNA expression in cigarette smoke condensate (CSC)-treated immortalized human bronchial epithelial cells (HBECs). We hypothesized that extended treatment of HBECs with CSC causes morphological and growth alterations and these effects are mediated, at least partly, through deregulation of microRNA expression. The aims of this study were to: 1. Determine CSC-induced early phenotypic alterations in HBECs; 2. Identify microRNAs whose levels are altered in CSC-treated HBECs.

HBECs were exposed to least-toxic dose of CSC (5 µg/ml) for 4 months and effects on cell proliferation, morphology, transformation, activation of AKT and ERK, and microRNA expression profile were determined. CSC exposure caused HBECs to become round and elongated and their proliferation is increased by 64%. Western blot analysis also indicated activation of ERK1/2 and AKT.

Microarray analysis revealed changes in the expression of 89 microRNAs in CSC-exposed HBECs and 87 of them were down-regulated. Further qRT-PCR analysis revealed altered expression of miR-138, miR-921, miR-293-3p, & IVGN-novel-miR-3526. However only the change in IVGN-novel-miR-3526 was statistically significant (up-regulated by 2.4-fold) (P= 0.03). The expression level of IVGN-novel-miR-3526 in A549 cells found to be 4.6-fold higher

than untreated HBECs. To our knowledge, this is the first report on the altered level of this microRNA in CSC-treated bronchial cells or lung cancer cells.

Overall, this study has provided useful insight on CSC-induced phenotypic alterations and microRNA deregulation in HBECs. Functional assays are required to determine the association between IVGN-novel-miR-3526 deregulation and observed phenotypic changes in CSC-exposed HBECs.

Table of Contents

List of Tables

List of Figures

Chapter 1 Literature review

1.1 Lung cancer

Lung cancer is the most common cause of cancer-related deaths (Garcia et al., 2007). Worldwide, there were more than 12 million new cancer cases in 2007, and lung cancer accounted for 1.5 million new cases, being the leading cause of cancer death in both men and women (Garcia et al., 2008). From the clinico-pathological perspective, lung cancer can be grouped into two major classes: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). SCLC represents approximately 15% of lung cancer cases and shows a very strong correlation with smoking as a risk factor. NSCLC (85% of lung cancer cases) comprises several histological types, the most common being adenocarcinoma, large-cell carcinoma and squamous-cell carcinoma (Lethio & De Petris, 2010)

1.1.1 Etiology

The association between lung cancer and tobacco smoke is unequivocal (Hecht et al., 2006; Jin et al., 2004; Witschi et al., 2005; Doll, 1998). The first evidence of a direct correlation between inhaled tobacco smoke and lung cancer was demonstrated in human studies in Germany between 1930-1940. These reports were later confirmed in Great Britain and the US in the 1950s (Jin et al., 2004; Akopyan & Bonavida, 2006). There are over 60 carcinogens in cigarette smoke for which there is sufficient evidence of carcinogenicity in either laboratory animals or humans, according to evaluations of the International Agency for Research on Cancer (IARC) (Hoffmann et al., 2001). Carcinogens identified in cigarette smoke include polynuclear aromatic hydrocarbons (PAHs), aza-arenes, which are polycyclic aromatic compounds (PAHs) containing a nitrogen in the ring system; nitrosamines; aromatic amines; aldehydes; miscellaneous organic compounds such as benzene, acrylonitrile, vinyl chloride, 2-nitropropane, and ethyl carbamate; and inorganic compounds such as hydrazine and various metals (Hecht, 1997). Among these, the PAHs and nitrosamines have in their families the strongest respiratory carcinogens, while certain

aldehydes and metals are also known respiratory carcinogens. In contrast, some of the other carcinogens such as aromatic amines and benzene are associated with other cancers, such as bladder cancer and leukemia (Hecht, 1997). The role of specific carcinogens of tobacco smoke in human cancers can be assessed by examining the carcinogenic potency of the compounds in laboratory animals, assuming that the mechanisms leading to lung tumorigenesis in rodents are relevant to humans (Hecht, 1997). Although many compounds in tobacco smoke contribute to lung cancer, the most-studied tobacco smoke carcinogens are benzo[*a*]pyrene (BaP), a member of PAH group of compounds, and the tobacco-specific nitrosamine nicotine-derived nitrosamine ketone (NNK, 4-(methylnitro-samino)-1-(3-pyridyl)-1-butanone). Both BaP and NNK induce tumors of the lung, mainly adenoma and adenocarcinoma, independent of the route of administration in both susceptible and resistant strains of mice (Hecht, 1998).

1.1.2 Molecular pathogenesis of lung cancer

Collectively, the available evidence favors BaP and NNK as important compounds responsible for lung cancer induction in smokers. These carcinogens cause the formation of DNA adducts after being metabolically activated by P450 cytochromes, enzymes encoded by the CYP family of genes. Repair of these adducts is linked to adduct excision. If these adducts persist unrepaired during DNA replication, miscoding can occur, leading to permanent mutations in critical genes such as p53 and Ras, which are considered to be important in the initiation of lung cancer (Brambilla et al., 1998) The role of PAHS and NNK in lung cancer is consistent with results of analyses of mutations in the p53 and Ras genes from human lung tumors. These analyses have demonstrated the presence of a large number of GC to TA (G-T) transversions and GC to TA (G-A) transitions in these genes, which is consistent with mutational spectra expected from PAHs and NNK (Brambilla et al., 1998; Jeanmart et al., 2003).

1.1.3 The role of KRAS and p53 signaling pathways in lung cancer

The genetic abnormalities linked to the risk of lung cancer should be regarded in the context of signaling pathways having their main functions altered, rather than focusing on individual proteins.

The complex EGFR signaling pathway consists of a large number of interacting genes and sub-pathways (Fig. 1). The downstream KRAS gene, encoding a small guanosine-5′-triphosphate-binding protein, is one of the well-documented oncogenes, and is frequently activated by missense mutations in many human cancers, making it the most frequent oncogene known to be activated in human cancers. KRAS mutations are detected in ~20% of NSCLCs, especially in adenocarcinoma from smokers. Several studies that analysed both KRAS and EGFR mutation status in the same tumors indicate that EGFR and KRAS mutations are mutually exclusive (Shigematsu et al., 2006). The finding that EGFR and HER2 gene mutations target never-smokers, while KRAS mutations favor smokers, is further evidence that lung adenocarcinoma in smokers and never-smokers arise *via* different pathogenic pathways (Brambilla & Gazdar, 2009).

The p53 pathway includes several genes that belong to multiple upstream and downstream sub-pathways. p53 is the cellular gatekeeper, guarding against genetic instability and abnormality. It functions as a sensor of multiple stress signals, including DNA damage, oncogene activation and hypoxia. This transcription factor has downstream target genes involving cell cycle arrest (G1 and G2), DNA repair or apoptosis, and upstream regulatory genes, including p14 and Mdm2. p53 is the most frequently mutated gene in lung cancer (Oliver et al., 2009). Inactivating mutations in the DNA binding domain are displayed in 90% of SCLC and 50% of NSCLC, of which gain-of-function mutations prevent the p53 protein binding to Mdm2 and subsequent p53 ubiquitin dependent proteolysis. The most frequent mutations are responsible for p53 protein stabilization and are recognized by a simple immunohistochemical test; wild-type protein is not detectable by immunohistochemistry because of its very short half-life of ~7 min.

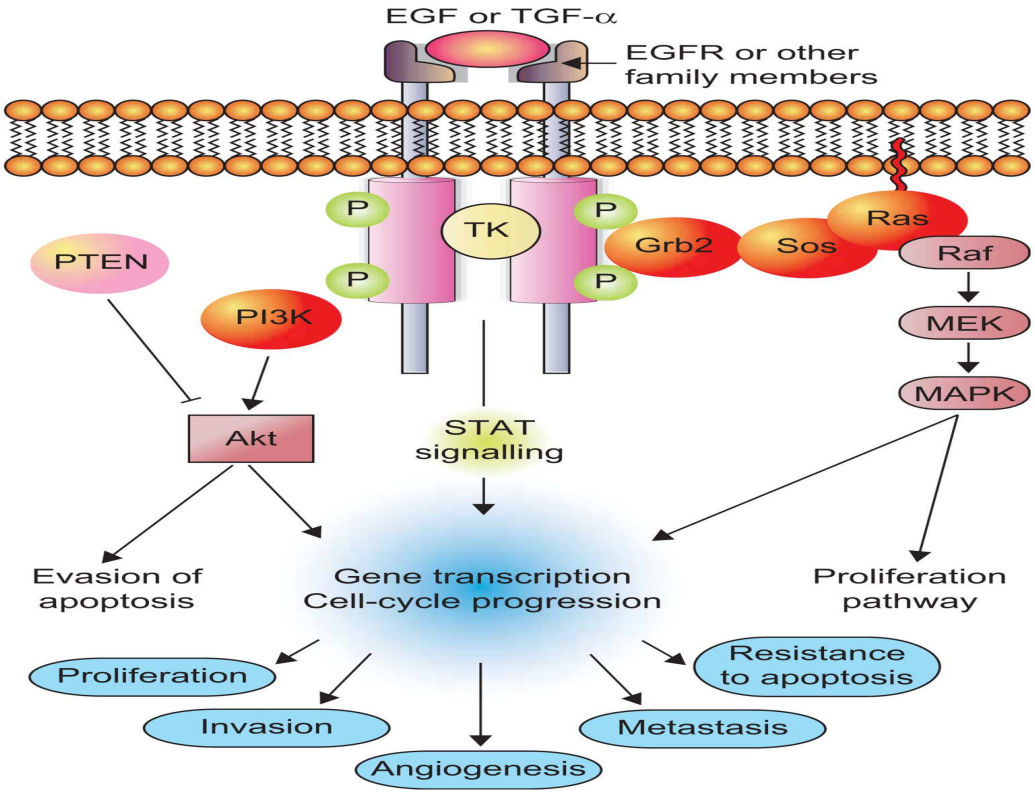

Figure 1. Epidermal growth factor receptor (EGFR) pathway. Ligands, such as epidermal growth factor (EGF), transforming growth factor (TGF)-α, or others, bind to the homo- and heterodimer kinase domain (TK), resulting in activation and receptor transphosphorylation. This creates docking sites for the adaptor proteins, Grb2 and Sos, which recruit Ras and phosphatidylinositol 3-kinase (PI3K), leading to the formation of two major signalling pathway branches, Ras/ MAPK and PI3K/Akt. These networks result in, amongst others, proliferation, evasion of apoptosis and angiogenesis. MAPK: mitogen-activated kinase-like protein. (**Modified from Brambilla E, & Gazdar A. 2009**)

The mutation spectrum of p53 is tightly linked to carcinogen exposure, particularly smoking, which is related to GC to TA (G–T) transversions at CpG sites. Benzo[a]pyrene adducts preferentially induce mutation at guanine in codons 248, 273 and 157; these are the fingerprints of tobacco carcinogenesis. In contrast, GC to TA (G–A) transition at non-CpG sites are associated with lung cancer in never-smokers. p53 alterations and stabilization by mutation are frequent in

proximal pre-invasive lesions of squamous dysplasia type and carcinoma *in situ* (Brambilla et al., 1998; Jeanmart et al., 2003).

1.1.4 Epigenetic changes in lung cancer

Epigenetic modifications refer to a number of molecular mechanisms that regulate gene expression without changing the DNA sequence. These include the following: 1) histone deacetylation; (2) altered methylation of DNA; and 3) aberrant expression of microRNAs (Brambilla & Gazdar, 2009).

1.1.4.1 Histone deacetylation

Modification of the N-terminal group of lysine in histones by acetylation or deacetylation changes the configuration of nucleosomes. The positive charge on unacetylated lysines in the histones is attracted to the negatively charged DNA producing a compact chromatin state that is repressive for transcription. On the other hand, acetylation of the lysines by histone acetylase removes their positive charge and results in an open chromatin structure, which facilitates gene transcription. Histone deacetylase (HDAC) removes the acetyl groups from lysine, which reverses this process and silences gene expression. Aberrant deacetylation of histones in nucleosomes is probably due to dysregulation of the specificity of HDAC and may be associated with neoplastic transformation (Jenuwein and Allis, 2001).

1.1.4.2 DNA methylation

Three forms of abnormal methylation may play roles in tumourigenesis; global hypomethylation, hypermethylation of tumour-suppressor genes and the recent finding that methylation may regulate expression of some microRNAs (Brambilla and Gazdar, 2009). In lung cancer, many genes (perhaps hundreds) have been found to be silenced by promoter methylation (Naoki et al., 2002) and include genes grouped under hallmarks of cancer (Shames et al., 2006).

Among these are *RARB*, *CDKN2A*, *TIMP3*, *MGMT*, *DAPK*, *CDH1*, *CDH13* and *RASSF1* (Hanahan & Weinberg, 2002).

Two recent reports demonstrated the role of CpG island hypermethylation of microRNA gene promoters in the regulation of microRNA expression. First, it has been observed that 5% of human microRNAs are up-regulated by treatment of bladder cancer cells with DNA demethylating agent and HDAC inhibitor (Saito et al., 2006). In particular, miR-127, which is embedded in a CpG island, was strongly induced by a decrease in DNA methylation levels around the promoter region of the miR-127 gene, and the proto-oncogene BCL6, a potential target of miR-127, was translationally down-regulated after treatment (Saito et al., 2006). Second, using a genetic approach that takes advantage of the genomic disruption of DNMT1 and DNMT3b in cancer cells, it has been demonstrated that CpG island hypermethylation is responsible for the down-regulation of microRNAs in human cancer (Lujambio et al., 2007). Most importantly, the methylation-mediated silencing of miR-124a lead to the activation of cyclin-dependent kinase 6 (CDK6) and phosphorylation of the retinoblastoma (Rb) tumorsuppressor gene (Lujambio et al., 2007).

1.2. microRNAs

1.2.1 Introduction

microRNAs are a class of short, 19- to 25-nucleotide-long RNAs that regulate gene expression by binding to sequences in the 3′ untranslated region (3′UTR) of an expressed mRNA, resulting in either modulation of translation efficiency or degradation of the mRNA (Du and Pertsemlidis, 2010; Yekta et al., 2004; Vasudevan et al., 2007). To date, there are around 700 annotated human microRNA loci that form short stem-loop structures when transcribed and produce either one or two mature microRNAs. Mature microRNAs are typically only partially complementary to their target mRNAs, meaning that each microRNA can regulate a broad set of targets. microRNAs have been shown to regulate expression of a variety of genes involved in cell proliferation,

differentiation, apoptosis, stem cell development, and human disease. Recent studies have demonstrated the critical role of microRNAs in cancer pathogenesis (Novotny et al., 2007; He et al., 2007; Hwang et al., 2007; Hebert et al., 2007; Lee et al., 2007; O'Donnell et al., 2007).

1.2.2 Biogenesis of microRNAs

Biogenesis of a microRNA begins with the synthesis of a long transcript known as a pri-microRNA (Fig. 2). In general, pri-microRNAs are transcribed by RNA polymerase II and retain mRNA features such as 5' cap structure and 3' poly (A) tail (Cai et al., 2000; Lee et al., 2004). In the nucleus, pri-microRNA is processed to pre-microRNA by RNase III enzyme Drosha and its interacting partner DGCR8 (DiGeorge syndrome critical region gene 8) (Borchert et al., 2006; Lee et al., 2003; Denli et al., 2004). DGCR8 recognizes the stem and the flanking single stranded RNA (ssRNA) and serves as a ruler for Drosha

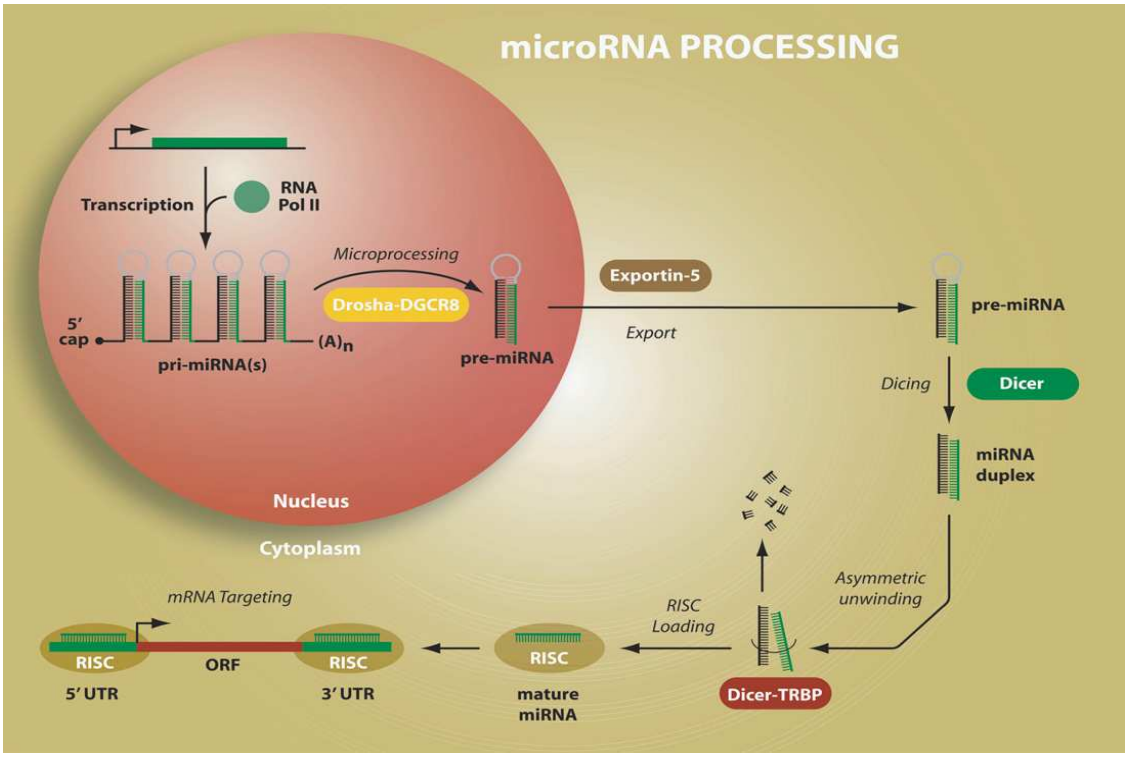

Figure 2. microRNA processing. microRNAs are first transcribed by RNA polymerase II into primary microRNAs (pri-microRNAs). While still in the nucleus, ‘‘precursor microRNA’’ (pre-microRNA) is formed by the action of ‘‘Drosha’. The pre-microRNA is exported from the nucleus to the cytoplasm by Exportin5/RanGTP. Once in the cytoplasm, a second RNase III, termed ‘‘Dicer,’’ in conjunction with a double-stranded RNA binding domain cleaves the pre-microRNA, releasing an approximately 22-nucleotide RNA. A single strand of the duplex is released and incorporated into the microRNA-induced silencing complex (miRISC) while the other strand is degraded. MiRISCs guide microRNAs to the target mRNA to affect either mRNA degradation or translational inhibition. (**Modified from Nana-Sinkam et al., 2009**).

to cut the stem approximately 11 nucleotides away from the stem-ssRNA junction to release the hairpin-shaped pre-microRNA (Gregory et al., 2004). The pre-microRNA is exported by exportin-5 to the cytoplasm (Han et al., 2006; Yi et al., 2003) and is subsequently converted to mature duplex microRNA by another RNase III enzyme, Dicer (Laund et al., 2004). The two strands of the duplex are separated by a RNA helicase (Hutvagner et al., 2004) during the specific assembly of a ribonucleoprotein complex containing microRNA (miRNP). As with the strand

selection of a siRNA (Salzman et al., 2007; Schwarz et al., 2003), the strand whose 5' end forms the more unstable duplex with its partner seems to preferentially survive as the microRNA in the miRNP (Khvorova et al., 2003)

1.2.3 microRNA target prediction and identification

Prediction of microRNA targets is important, given that microRNAs exert their function by regulating target mRNAs. The specificity of microRNA–mRNA interaction is mainly conferred by the first eight nucleotides of a microRNA (known as a seed sequence) (Lewis et al., 2003). The likelihood that a predicted target is a bona fide target is influenced not only by seed pairing but also by other factors such as the number of target sites, the context of surrounding sequence in mRNA (Grimson et al., 2007), and the occlusion of target sites by RNA-binding proteins (Lewis et al., 2003). Currently, several computational algorithms (Maziere & Enright, 2007) can predict the target mRNA(s), but they are far from perfect. The gold standard is experimental demonstration that (*a*) a luciferase reporter fused to the 3' UTR of the predicted target is repressed by over-expression of the microRNA and (*b*) this repression is abrogated by point mutation in the target sequence(s) in the 3' UTR. Many targets are predicted by in silico analyses, but not all of them are confirmed as real targets in this biological assay (Lee and Dutta, 2009).

1.3. microRNA and cancer

1.3.1 Introduction

microRNAs are aberrantly expressed in a variety of cancers. This was first observed in miR-15a and -16-1, which are clustered at chromosome 13q14, a frequently deleted region in B cell chronic lymphocytic leukemia (CLL) and other cancers (Calin et al., 2002). Concomitantly, reduction of these two microRNAs was observed in the cancer samples relative to the normal tissues. Many microRNAs are found to be up- or down-regulated in the cancer samples relative to the normal tissue counterparts. In addition to the distinction of tumors from normal tissue,

microRNA expression is characteristic for a cancer type, stage, and other clinical variables. The first systematic analyses of hundreds of cancer samples and normal tissues (Lu et al., 2005) successfully classified various cancers based on the miRNome. Surprisingly, the miRNome was better at predicting cancer type and stage than the mRNA expression profile; therefore, the miRNome has been proposed as a useful tool for cancer diagnosis and prognosis.

1.3.2 Altered microRNA expression in lung cancer

Our knowledge of microRNAs in lung cancer is just starting to emerge and is summarized in table 1. One of the first microRNA identified (let-7) appears to be important in lung cancer. Over-expression of let-7 inhibits RAS protein expression and let-7 complementary sites are present in human NRAS and KRAS 3'-UTR (Johnson et al., 2005). High-throughput interrogations of the proteome and genome are currently being used to identify the molecular heterogeneity and predictive signatures in lung cancer (Potti et al., 2006; Rahman et al., 2005). The first such study of microRNAs conducted by Yanaihara and colleagues examined global microRNA expression patterns using cDNA array-based technology. The authors compared microRNA profiles in tumor versus adjacent uninvolved lung in 104 cases of non–small cell lung cancer (Yanaihara et al., 2006). The investigators identified 43 microRNAs that were differentially expressed between lung tumors and adjacent uninvolved lung (Yanaihara et al., 2006); in addition, five distinct microRNAs (miR-155, 17–3p, let-7a-2, 145, and 21) predicted prognosis among patients with lung cancer. A more recent study using a RT-PCR– based platform examined microRNA expression in 112 non–small cell lung cancer tumors. They also identified a signature that predicted survival (high risk microRNAs miR-137, miR-182, and miR-372, low-risk microRNAs miR-221 and let-7a) (Yu et al., 2008).

Several of these microRNAs were associated with fragile sites (FRAs). In particular, three microRNAs are located inside FRAs (hsa-mir-21 at FRA17B, hsa-mir-27b at FRA9D, and hsa-mir-32 at FRA9E). Furthermore, many of these microRNAs are located at frequently deleted or

amplified regions in several malignancies. For example, hsa-mir-21 and hsa-mir-205 are located at the region amplified in lung cancer, whereas hsa-mir-126* and hsa-mir-126 are at 9q34.3, a region deleted in lung cancer. Reduced expression of precursor let-7a-2 and let-7f-1 was also found in adenocarcinoma and squamous cell carcinoma (Yanaihara et al., 2006).

1.3.3 Clinical applications of microRNAs

Patterns of microRNA expression appear to be a richer source of pathognomonic tumor information than messenger RNA expression profiling (Lu et al., 2005). Unique patterns of altered microRNA expression provide complex fingerprints that may serve as molecular biomarkers for tumor diagnosis, prognosis of disease specific outcomes, and prediction of therapeutic responses (Lee and Dutta, 2009). Similarly, the presumptive role of microRNAs in tumorigenesis underscores their value as mechanism-based therapeutic targets in cancer that have yet to be fully exploited. Similarly, microRNAs could be used as diagnostic or prognostic tools, since their expression profiles reflect tumor origin, stage, and other pathological variables. Researchers have shown that microRNAs can be isolated and quantified from formalin-fixed paraffin-embedded (FFPE) specimens. qRT-PCR and microarray data were reliably and reproducibly obtained from FFPE samples that had been routinely processed and stored for 10 years (Xi et al., 2007; Lawrie et al., 2007) Also, analysis of microRNAS in the sputum or blood serum/plasma could differentiate lung cancer patients from healthy controls (Xie et al., 2010, Mitchell et al., 2008).

microRNAs have also a great potential as therapeutic agents against cancer as a number of microRNAs affect the growth of cancer cells *in vitro* and *in vivo* when over-expressed or inhibited. Over-expression of microRNAs could be achieved by administering synthetic microRNA mimics; conversely, microRNAs can be inhibited by variously modified antisense oligonucleotides such as 2-*O*-methyl antisense oligonucleotide, and antagomirs (Weiler et al., 2006). Although some preliminary results suggest that microRNAs could be useful for cancer

therapy, (Wiggins et al., 2010). There is still a significant gap between basic research on microRNAs and clinical application. Extensive preclinical and translational research is necessary to increase the efficacy and decrease the side effects of microRNAs *in vivo* (Lee and Dutta, 2009).

Table 1. Experimentally validated microRNAs in lung cancer (**Modified from Du & Pertsemlidis, 2010**)

microRNA	Expression in lung cancer	Function when expressed	Validated targets
miR-21	up	Represses apoptosis Increases proliferation	PTEN, PDCD4 TPM1
miR-17-92	up	Induces angiogenesis Represses apoptosis	PTEN, E2F1-3 BIM
miR-221/222	up	Represses apoptosis Induces proliferation Restricts angiogenesis	TIMP3, c-Kit p27Kip1
miR-34a-c	down	Induces p53-dependent cellular stress responses	CDK4/6, Bcl-2 CCNE2, E2F3
miR-29	down	Induces apoptosis Decreases tumorigenicity	DNMT3A-3B, Mcl-1
let-7	down	Decreases proliferation Decreases apoptosis	RAS, Myc, HGMA2
miR-15/16	down	Induces Rb-dependent cell cycle arrest	Bcl-2, CCND1 CCND2, CCNE1, WNT3A
miR-128b	down	Reduces EGFR-mediated proliferation	EGFR
miR-200/429	down	Increases E-cadherin expression Represses EMT, reduces metastasis	ZEB1/ZEB2
miR-197	up	Increases proliferation	FUS1
miR-93	up	Increases proliferation	FUS1
miR-98	up	Increases proliferation Increases apoptosis	FUS1
miR-126	down	Reduces invasion, migration, and adhesion Decreases angiogenesis	CRK, VEGF-A

1.4 Hypothesis and aims

Although smoking-related genetic changes have been well studied, reports on the association between lung cancer and epigenetic changes, in particular deregulation of microRNAs, remains incompletely understood. Therefore, in this study, our objective was to examine early phenotypic changes and altered microRNA expression in CSC-treated HBECs. We hypothesized that extended treatment of HBECs with CSC causes morphological and growth alterations and these effects are mediated, at least partly, through deregulation of microRNA expression. The aims of this study were: 1. Determine CSC-induced early phenotypic alterations in HBECs; 2. Identify microRNAs differentially expressed in CSC-treated HBECs.

Chapter 2 CSC-induced early morphological and proliferative changes on HBECs

2.1 Summary

The molecular changes induced by cigarette smoke have been studied in several models. One of this is an *in vitro* model where CSC-induced alterations are assessed in respiratory epithelial cells. Hence, in this study, we examined phenotypic alterations and microRNA deregulation induced by CSC in HBECs.

In the cytotoxicity assay, we observed that CSC reduced the viability of HBECs in a dose-dependent manner. Upon continuous treatment with the least-toxic dose of CSC (5 µg/ml) for four months, HBECs became less polygonal, round and elongated. These changes were also accompanied by an increase in cell proliferation rate (64%) as determined by the MTT assay. The proliferation rate remained the same even after discontinuation of CSC exposure. This work suggests the *in vitro* model using HBECs to assess phenotypic changes caused by CSC is relevant as a prelude to functional studies on the effect of CSC.

2.2 Materials and methods

2.2.1 Cells

HBECs, were developed from normal human bronchial epithelial cells immortalized with human telomerase reverse transcriptase (hTERT) and cycline-dependent kinase (cdk) 4. HBECs don't form colonies in soft agar or tumors in nude mice and have epithelial morphology; express epithelial markers cytokeratins 7, 14, 17, and 19, the stem cell marker p63, and high levels of p16INK4a. HBECs have also an intact p53 checkpoint pathway (Ramirez, 2004). In this experiment HBECs were kindly provided by Dr Naomi Fujioka (Medical school, University of Minnesota). Cells were maintained in serum free keratinocyte medium (Invitrogen life Technologies, Carlbad, CA, USA) supplemented with 5 ug/L epidermal growth factor and 50 mg/L bovine pituitary extract in a humidified 5% CO_2 atmosphere at 37°C.

2.2.2 Cigarette smoke condensate (CSC)

CSC (40 µg/µl) stock was purchased from Kentucky Tobacco Research and Development Center, Lexington, KY, USA. The CSC is prepared by smoking University of Kentucky's 3R4F Standard Research Cigarettes on an FTC Smoke Machine. The Total Particulate Matter (TPM) on the filter is calculated by the weight gain of the filter. From the TPM, the amount of DMSO to be used for extraction to prepare a theoretical 4% (40 mg/ml) solution is calculated. The condensate is extracted with DMSO by soaking and sonication, and then packaged as 1 ml/vial in dry vials.

2.2.3 CSC cytotoxicity on HBECs

HBECs were grown at a density of $5x10^3$ cells/well in 96-well plate. After 24 h, cells were treated with different concentrations of CSC dissolved in DMSO (0, 5, 10, 20, 40, 60, 80, & 100 µg/ml) for 24 h, and cell viability determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT salt to formazan dye, giving a purple color. The main

application of this assay is to determine cell viability and cell proliferation (Mosmann, 1983). Briefly, after thawing the MTT assay solution, 10 µl of the reagent was added to each well and mixed by tapping gently on the side of the tray followed by incubation at 37°C for 4 h. Then, the medium was removed and 200 µl DMSO added into each well to dissolve the formazan. The absorbance was measured on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD 570-OD 630) (Biotium Inc., Hayward, California).

Based on the results of the dose range-finding experiment where HBECs were exposed to different concentrations of CSC, we selected the least toxic dose (5 µg/ml) for subsequent chronic exposure intended to study CSC-induced phenotypic changes. Cells were exposed to CSC every other day, split when they became confluent and exposed to CSC again. This was continued for four months. After treatment for four months, cells were harvested and cell proliferation rate determined, using MTT assay, as described above. Also, photomicrogaphs of untreated and CSC-treated (four months) HBECs were taken to show CSC-induced changes in the morphology of these cells.

2.2.4 Anchorage independent assay

To test for soft agar colony growth capacity, untreated and CSC-treated HBECs and A549 cells (positive control) were plated at a density of 2 x 10^4 cells in 4 ml 0.35% agarose over a 0.7% agar base in 60 mm diameter culture dishes, covered with 2 ml complete keratinocyte medium. Medium was replenished every 3 days and growth of colonies was watched for four weeks. At the end of week 4, plates were stained with 0.5 ml of 0.005% Crystal Violet for one h and colonies counted using a dissecting microscope.

2.4 Results and discussion

2.4.1 Survival fraction of cells

We observed a dose-dependent reduction in the viability of HBECs exposed to different concentrations of CSC for 24 h. The survival of HBECs exposed to the highest (100 µg/ml) and lowest (5 µg/ml) concentrations of CSC was reduced by 50% and 5%, respectively, as compared to the viability of DMSO-treated HBECs (Fig. 3). The overall cell toxicity of CSC in this experiment seems to be lower than that reported by Hu et al. (2009). For instance, in our case 20 µg/ml CSC concentration reduced cell viability by 15% only compared to 32% reduction reported by Hu et al. (2009). The variation could be explained by the difference in the cell types and CSC lots used in the two experiments. Hu and his colleagues used HPV-18-immortalized human bronchial epithelial cells, whereas in this experiment we used HBECs immortalized with CDK4 and human telomerase reverse transcriptase.

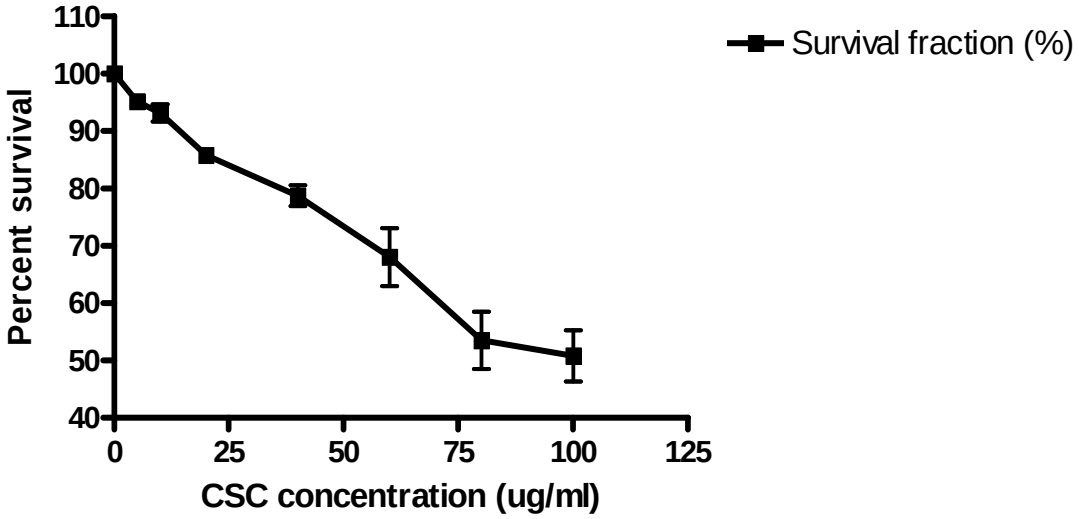

Figure 3. Survival fraction of HBECs exposed to graded doses of CSC for 24 h. Data shown are mean ± SD from three independent experiments.

2.4.2 Changes in cell proliferation

The proliferation rate of HBECs exposed to CSC (5 µg/ml) for over a period of 4 months increased by 64% compared to DMSO-treated cells (Fig. 4). The level of cell proliferation remained the same even after discontinuing CSC exposure of the cells for two months (data not shown). This result is in line with other observations where an increase in the growth rate and a decrease in doubling time were observed in CSC-treated human bronchial cells (Hu et al., 2009; Lemjabbar-Alaoui et al., 2006; Dasari et al., 2006) and an increase in the proliferation of the bronchiolar epithelium and the pulmonary vasculature of CSC-exposed rats (Sekhonn et al., 1994).

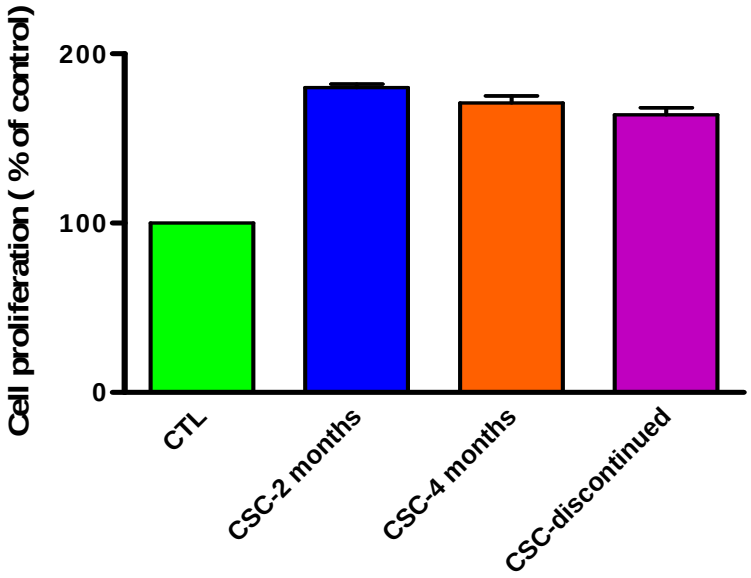

Figure 4. Proliferation rate of HBECs exposed to CSC (5 µg/ml) for different time periods. About 5 x 10^3 HBECs (untreated or treated with CSC for 2 months or 4 months, or treated with CSC for 4 months and kept untreated for two more months) were incubated for 48 and cell proliferation rate determined by MTT assay.

2.4.3 CSC-induced morphological changes

In our experiment in which HBECs were continuously exposed to CSC for four months, the cells became less polygonal and appeared more elongated and round compared to the unexposed

control (Fig. 5). These changes are also in line with previous reports (Liu et al. 2010) where exposure of HBECs to 50-100 µg/ml of CSC induced similar morphological alterations.

F-actin is responsible for controlling cell shape, motility, differentiation, signal-response coupling, adhesion, and the formation of filopodia and lamellipodia (Gardel et al., 2006). In normal cells, F-actin occurs as criss-crossing stress fibers throughout the cell (Carter and Madden, 2000) but toxins can lead to actin fragmentation, and relocalization of F-actin to perinuclear regions and increases in cell surface extensions (Cabello et al., 2003). Our observation of more elongated or round HBECs upon exposure to CSC could be likely attributed to the effect of CSC on F-actin. In fact, in vitro exposure of neutrophils to CSC was found to impair normal F-actin kinetics (Ryder et al., 2002).

2.4.4 Exposure of HBECs to CSC for four months did not induce cell transformation

HBECs exposed to CSC for 4 months did not form colonies on soft agar medium after 4 weeks of incubation, whereas the positive control A549 cells formed colonies within 5 days (data not shown). Liu et al. (2010) reported that HBECs exposed to 100ug/ml CSC for 9 months formed soft agar colonies, but these cells failed to induce tumor growth in nude mice. Similarly, Lemjabbar-Alaoui et al. (2009) reported transformation of SV40-immortalized human bronchial epithelial cells (BEAS-2B) after exposure to CSC (500 µg/ml) for 7 days. In fact, comparison of these studies with ours is difficult, due to major differences in the concentration of CSC as well as duration of treatment used. The variation in the cell lines used could also be another explanation for the difference observed.

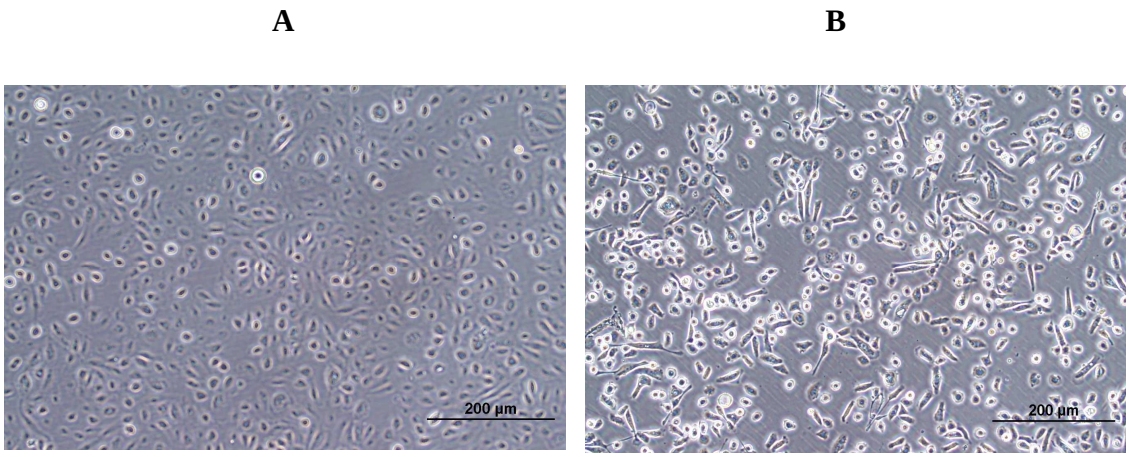

Figure 5. Photomicrographs of untreated (A) or CSC-treated (5 µg/ml for four months (B) HBECs. Cells were grown in serum free keratinocyte medium supplemented with 5ug/l epidermal growth factor and 50 mg/l bovine pituitary extract in the absence (A) or presence (B) of CSC for four months.

Chapter 3 CSC modulates activation of AKT and ERK1/2 in human bronchial epithelial cells

3.1 Summary

The Ras/MAPK and PI3K/AKT pathways are major signaling networks through which cancer cells undergo uncontrolled cell proliferation. Deregulation of these pathways has been observed in multiple tumor types, including non-small cell lung cancer; however direct association of cigarette smoke to activation of these pathways was limited. Hence this experiment was designed to show the link between CSC-induced cell proliferation and activation of these signaling pathways.

We observed activation of both serine/thereonine kinase AKT and ERK1/2 in CSC-exposed HBECs. Treatment of CSC-exposed cells with 5-aza-2-deoxycytidine or histone deacetylase inhibitor caused a reduction in pAKT levels. These results suggest that activation of AKT could be associated with increased expression/activity of methyl transferases and histone deacetlylases. The findings therefore imply there may exist direct or indirect links between CSC-induced cell proliferation and activation of two important cancer-related signaling pathways.

3.2 Introduction

Several pathways with major components have their functions altered in lung cancer, and these pathways are emerging as having considerable importance with regard to targeted therapy. Most stimulatory signaling pathways are led by oncogenes, which drive cells towards a malignant phenotype, proliferation and escape from apoptosis (Weinstein, 2008).

The serine/threonine kinase AKT (or protein kinase B), controls key cellular processes such as glucose metabolism, cell cycle progression, and apoptosis (Lawlor and Alessi, 2001) and active AKT can contribute to tumorigenesis *in vivo* in lymphoid, breast, ovarian, prostate, and brain tissues (Scheid and Woodgett, 2001). Similarly, the Ras-MAPK-signaling pathway has been linked to be responsible for the malignant phenotype, including increased proliferation, defects in apoptosis, invasiveness and ability to induce neovascularization (Slamon et al., 2001). Studies have shown that cigarette smoke extract exerts its biological effects via the MAPK signaling pathway in several cell culture systems (Li et al. 2007). Cigarette smoke increased phosphorylation of residues Thr-202 and Tyr-204 of ERK in airway lining cells and alveolar macrophages in mice. Moreover, analysis of lung tissues from emphysema patients revealed significantly increased ERK activity compared with lungs of control subjects (Mercer et al., 2004). Both PI3K/AKT and RAS/MAPK signaling networks link EGFR activation to cell proliferation, survival, and angiogenesis (Brambilla & Gazdar, 2009).

3.3 Materials & methods

3.3.1 Cell culture

HBECs at $1x10^6$ cell density were seeded on 10 cm petridish and cultured for 24 h and then cells were exposed to 5 µg/ml of CSC every other day for 4 months. Cell lysates from both unexposed control and every 10^{th} passage of CSC exposed HBECs were prepared and stored at -80°C until western blot analysis was performed.

3.3.2 Protein extraction and Western blot analysis

Cell lysates were prepared by suspending the cells with lysis buffer (50 mM Tris--HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride) on ice for 1h. Subsequently, the preparations were centrifuged (14000 g for 25 min at 4ºC), the supernatants collected, aliquoted and stored at -80 ºC. The protein concentration was determined by Bio-Rad protein Assay (Bio-Rad, Hercules, CA).

For Western immunoblotting analysis, 50 µg of total protein per sample was loaded onto a 4–12% on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and run for 60 min at 200 V. The proteins were then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) for 2 h at 30 V. Subsequently, membranes were blocked in 5% Blotto non-fat dry milk in Tris buffer containing 1% Tween-20 for 1 h and probed overnight with the following primary monoclonal antibodies: anti-phospho ERK (1:1000), and anti-phospho AKT (1:1000) from Cell signaling Technology (Danvers, MA). After incubating the membranes with a secondary antibody, anti-rabbit IgG, HRP-linked antibody (1:3000) from Cell signaling technology) for 1 h, chemiluminescent immunodetection was employed. Signal was visualized by exposing membranes to HyBolt CL autoradiography film. Membranes were stripped and probed with anti-total ERK and AKT to check for differences in the amount of protein loaded in each lane.

3.4 Results and discussion

3.4.1 CSC-modulated signaling pathways

We found activation of both serine/thereonine kinase ERK1/2 and AKT (Fig 6) in CSC exposed HBECs. These results are in agreement with previous reports (Gustafson et al., 2010; West et al., 2003). West et al. (2003) reported activation of AKT in non-immortalized human airway epithelial cells *in vitro* by two components of cigarette smoke, nicotine and NNK. Similarly, a significant increase in phosphatidylinositol 3-kinase (PI3K) pathway activation was observed in cytologically normal bronchial airway of smokers with lung cancer and smokers with

dysplastic lesions (Gustafson et al., 2010). Luppi et al., (2005) has found persistent activation of ERK1/2 when NCI-H292 cells were exposed to low concentration of CSC. Similarly, Mercer et al., (2004) has also reported phosporylation of ERK in airway lining cells and alveolar macrophages in mice exposed to cigarette smoke.

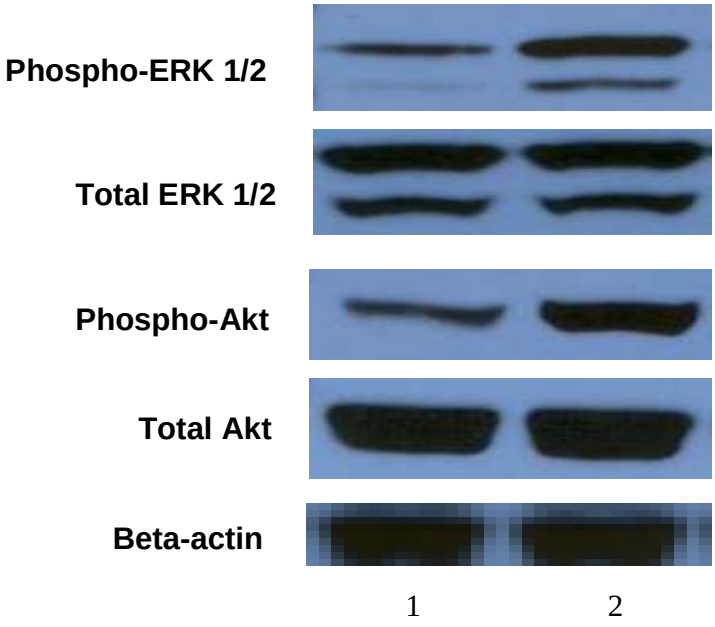

Figure 6. Western blot analysis showing activation of Akt and ERK1/2 in HBECs exposed to CSC (5 µg/ml). Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for beta actin.

3.4.2 Effects of ADC and SAHA, alone or in combination, on Akt activation

Recently, Lin et al (2010) showed an association between activation of Akt and over-expression of methyl transferases which could potentially lead to hypermethylation of tumor suppressor genes. Therefore, we sought to examine if exposure of CSC-pretreated HBECs to ADC or SAHA, inhibitors of DNA methyl transferases and hitstone deacetylases, respectively, could lead to suppression of Akt activation in these cells. Indeed, we observed a dramatic decrease in pAKT level in CSC-pretreated HBECs exposed to ADC (Figure 7). The histone

deacetylase inhibitor, SAHA, also caused a reduction in the activation of Akt but the effect was lower than that of ADC. On the other hand the level of pAkt seems little affected in cells treated with the combination of ADC and SAHA.

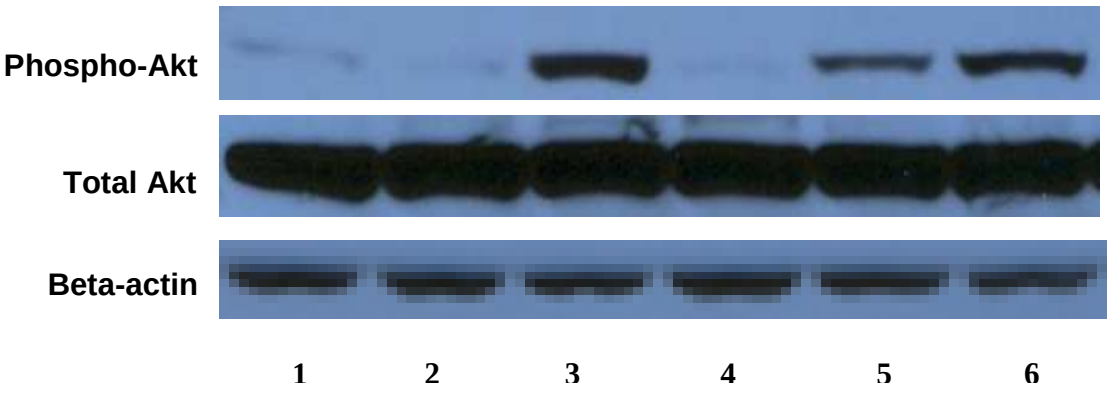

Figure 7. ADC and SAHA attenuated the activation of AKT: (1) un-exposed HBECs, (2) HBECs exposed to CSC for 2 months and then discontinued, (3) HBECs continuously exposed to CSC, (4) HBECs exposed to CSC and ADC, (5) HBECs exposed to CSC and SAHA, (6) HBECs exposed to CSC, ADC and SAHA. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for total AKT.

Chapter 4 CSC modulates microRNA expression in HBECs

4.1 Summary

Despite many reports on altered expression of microRNAs in lung cancer tissues, little is known regarding the microRNA expression response of respiratory epithelia towards environmental carcinogens such as cigarette smoke. Hence, in this study we sought to examine the effect of CSC on the expression of microRNAs, at a global level, in HBECs.

Results from microRNA microarray analysis of RNA samples from control and CSC-treated HBECs revealed the differential expression of 89 microRNAs in unexposed versus CSC-exposed HBECs, and 98% of the altered microRNAs were down-regulated. The expression of 20 of these microRNAs (selected on the basis of magnitude of expression and association with cancer) was further analyzed by qRT-PCR. Of the 20 microRNAs, levels of four microRNAs (miR-38, miR-921, miR-293-3p, & IVGN-novel-miR-3526) were found to be consistently altered in repeated assays, but only the change in IVGN-novel-miR-3526 was significant. To our knowledge, this is the first report regarding the altered expression of IVGN-novel-miR-3526 in CSC-treated cells. Search on Target Scan target gene prediction database revealed that around 280 genes are considered as putative targets of this microRNA. Discontinuation of CSC exposure has resulted in a decline in the expression of IVGN-novel-miR-3526. Furthermore, the level of IVGN-novel-miR-3526 was found to be elevated in human lung adenocarcinoma cell line (A549) as compared to the expression in untreated HBECs.

On the contrary, the expression of IVGN-novel-miR-3526 was not altered in NNK-induced A/J mouse lung tumor tissues relative to lung tissues from vehicle-treated mice, whereas miR-377 and miR-31 were found to be significantly up-regulated, 180- and 13-fold in lung tumors harvested at week 27 and week 52, respectively.

Overall, IVGN-novel-miR-3526 seems to be over-expressed during the different stages of lung tumorigenesis, as revealed by the changes in CSC-treated HBEC (early stage carcinogenesis) and human lung adenocarcinoma A549 cells (late stage lung cancer) and could have a potential to be used as early as well as late stage lung cancer biomarker.

4.2 Introduction

Emerging evidence shows that deregulation of microRNAs might be a primary driver of cancer initiation and progression; hence a greater understanding of the regulatory mechanisms of microRNA expression could lead to novel strategies for the prevention and treatment of cancer (Du & Pertsemlidis, 2010). Recent reports showed deregulation of microRNA expression in lung tissues of mice and rats exposed to environmental cigarette smoke, which was mainly oriented toward down-regulation of a variety of microRNAs involved in important cellular functions (Izzotti et al., 2009). Differential expression of microRNAs was also observed in primary epithelial cells derived from bronchial biopsy samples from never smokers and smokers as well as lung cancer cell line H1299 and normal human bronchial cells exposed to CSC (Schemberi et al., 2009).

4.3 Materials and methods

4.3.1 Treatment of HBECs

In order to study the effect of CSC on microRNA expression, HBECs were exposed to CSC for four months. During this time, HBECs were exposed to fresh solutions of CSC (5 µg/ml) every other day. At the end of treatment total RNA was prepared from CSC-treated and untreated control cells using Trizol reagent.

In order to determine the role DNA methylation and deacetylation on the regulation of microRNA expression, HBECs treated with CSC (5 µg/ml) for four months were exposed to 5 µM of either ADC or SAHA or their combination for 72 h. Subsequently, total RNA was prepared using trizol.

4.3.2 Lung tissues

Lung tissues were obtained from a previous lung cancer chemoprevention study (Kassie et al., 2010). Aliquots of mouse lung tissues (normal lung tissue, 30 mg/mouse or lung tumors, 30 mg/mouse) from three mice were pooled, pulverized using mortar and pestle on dry ice and the tissue powder used for total RNA extraction using Trizol.

4.3.4 RNA preparation

After total RNA was prepared from HBECs treated with the different agents or mouse lung tissues, the RNA samples were treated with DNase I (QIAGEN) according to the manufacturer's protocols. RNA quantity and quality were determined under stringent conditions at the Biomedical Genomics Center of the University of Minnesota. The 260/280 and 260/230 ratios of the RNA samples, which indicate the purity of the RNA samples, were performed by NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Also, RNA integrity was analyzed by capillary electrophoresis using the Bioanalyzer Agilent 2100 equipped with Agilent RNA 6000 nanochips (Agilent Technologies, Waldbronn, Germany). Only samples with a 260/280 and 260/230 value of > 1.9 and RNA Integrity value of > 8 were used for microRNA microarray study. The presence of microRNA was confirmed by the detection of fragments in the 20 -100 bp range.

4.3.5 Microarray analysis

Microarray technique was used to analyze the global microRNA expression profile of unexposed HBECs, CSC-exposed HBECs, CSC-exposed HBECs treated with either ADC, SAHA or their combination following the method of Kalscheuer et al. (2008). Briefly, a microRNA probe set containing 16000 oligonucleotides was purchased from Invitrogen and the oligonucleotides in triplicate were printed on Corning GAPSII-coated slides by the Microarray Facility at the Biomedical Genomic Center, University of Minnesota. In total, 712 microRNAs

(467 human and/or mouse and/or rat microRNAs) were analyzed. For RNA labeling, 25 µg of total RNA was ligated to 0.5 µg of a synthetic linker, pCU-DY547 (Dharmacon, Lafayette, CO). To control for the hybridization process, reference DNA oligonucleotides complementary to a subset of mammalian microRNAs were combined and labeled with a ULYSIS Alexa Fluor 647 Kit (Invitrogen). Labeled RNAs and DNAs were then mixed and hybridized to microarray slides. Afterward, slides were scanned to quantify pixel intensities and individual spots on the slides were further inspected to exclude abnormal spots from subsequent calculations.

4.3.6 QRT-PCR assay of microRNAs

In order to verify the results of the microarray results, quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) assay was performed for selected microRNAs. Briefly, 1 µg of DNase-treated total RNA was reverse transcribed to complementary DNA (cDNA) using miScript Reverse Transcription Kit (QIAGEN). During the reverse transcription step, microRNAs, which are not polyadenylated in nature, were polyadenylated by poly (A) polymerase and subsequently converted into cDNA by reverse transcriptase with oligo-dT and random primers. The cycle parameters for the 20 µl reverse transcription reaction per manufacturer's protocol were 37 ºC for 30 min and 95 ºC for 5 min. The protocol enabled efficient reverse transcription of microRNAs into cDNA in a single step. The cDNA was then used as a template for real-time PCR quantification of microRNAs using the designed specific microRNA primers and the miScript Universal primer in combination with the miScript SYBR Green PCR Kit from QIAGEN.

Complementary primer sequences for the mature forms of selected microRNAs were designed based on miRBase sequence database. All the primer sequences used in this study are presented in Table 2. PCR was performed on an Applied Biosystems 7500 system (Applied Biosystems, Foster City, CA) with aliquots of cDNA equivalent to 25 ng of total RNA in a total PCR reaction mixtures of 25 µl per manufacturer's protocol [initial activation at 95 ºC for 15 min followed by

three-step cycling (45 cycles) of denaturation at 94 ºC for 15 s, annealing at 55 ºC for 30 s and extension at 70 ºC for 30s]. All reactions were performed in triplicate. Differences in levels of microRNAs among samples were converted into fold changes as follows. First, the microRNA data were normalized against the expression of U6 small nuclear RNA by subtracting the Ct value of U6 from the Ct value of the target gene (ΔCt). The ΔΔCt was then calculated by subtracting the ΔCt value of HBECs control from CSC-exposed HBECs or ΔCt value of normal lung tissue from lung tumor tissues of NNK treated mice. These ΔΔCt values were converted to approximate fold differences in gene expression by assuming 100% primer efficiency and using the equation $2^{-\Delta\Delta Ct}$.

4.4 Statistics

The data are expressed as mean ± SEM. Statistical analysis was performed with Student's t test for paired samples following analysis of variance. Differences were considered statistically significant when $p < 0.05$.

4.5 Results and discussion

4.5.1 Microarray analysis

A total of 89 microRNAs were differentially expressed between unexposed control and CSC-exposed HBECs and 98% of them were down-regulated (Fig. 8 & 9). This is in agreement with different reports that showed CS-dependent changes in microRNA expression, predominantly down-regulation, on airway epithelium using both in vitro (Schembri et al., 2009; Mascaux et al., 2009) and in vivo (Izzotti et al., 2009**;** Schembri et al., 2009; Mascaux et al., 2009) models and that microRNAs might therefore play a role in the host response to environmental exposures and the pathogenesis of smoking-related lung disease. The alteration in microRNA expression due to CSC exposure on HBECs was reversed to the expression level of unexposed control by the demethylating agent (ADC) and histone deacetylase inhibitor (SAHA) (Fig. 13). This result

corroborated by previous reports that 5% of human microRNAs were up-regulated by treatment of bladder cancer cells with ADC and SAHA (Saito et al., 2006).

4.5.2 QRT-PCR studies

We picked out 20 microRNAs whose expression levels were found to be altered by ≥ 2-fold in microarrays and their expression level was further assessed by QRT-PCR. Among the 20 selected microRNAs which were further studied by qRT-PCR, miR-138, miR-921, miR-293-3p, & IVGN-novel-miR-3526 have shown altered expression consistently (Figure 9), but only the change in the level of IVGN-novel-miR-3526 was statistically significant (up-regulated by 2.4-fold, P= 0.03). To our knowledge, this is the only report regarding an association between CSC exposure and changes in the level of IVGN-novel-miR-3526. According to target scan gene prediction database, about 280 genes are potential targets for IVGN-novel-miR-3526 and some examples of these are presented in table 3.

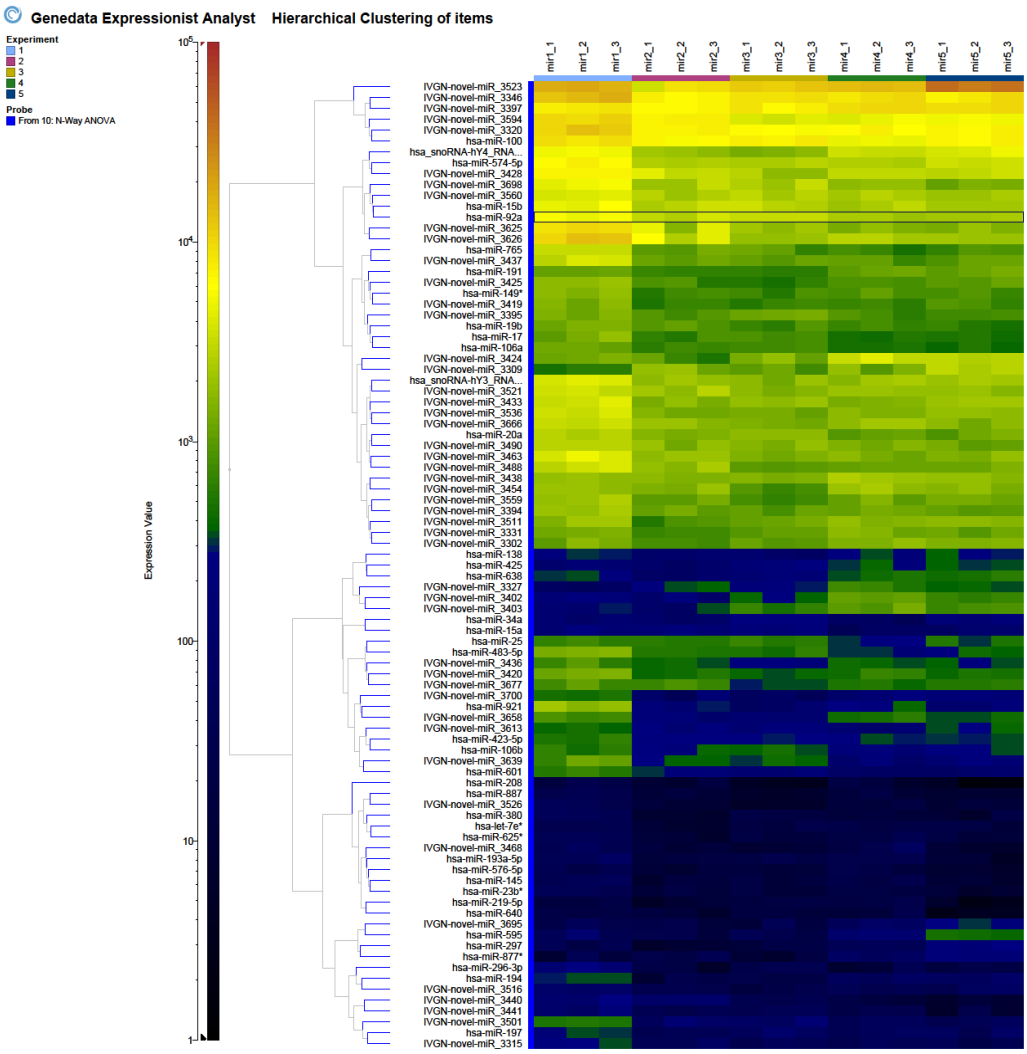

Figure 8. Hierarchical clustering of microRNAs in HBEC cells. Keys for the different treatment groups (each group was processed in triplicates): light blue, untreated; brown, CSC-treated; yellow, CSC + ADC-treated; green, CSC + SAHA-treated; dark blue, CSC+ ADC + SAHA-treated. Key for level of expression: Dark indicates lower expression, orange indicates higher expression.

Various reports have demonstrated changes in microRNA expression in lung tumor tissues, cancer cell lines, and cigarette smoke-exposed lung primary cells (Yanaihara et al. 2006; Nana-

Sinkam et al. 2009; Takamizawa et al., 2004; Du & Pertsemlidis, 2010; Melkamu et al. 2010; Xie et al. 2010). However, not all of these studies reported the same group of microRNAs. Similarly, most of the microRNAs we found to be altered in our microarray studies have not been previously reported in lung tissues or cell lines. This could be explained by the different models used.

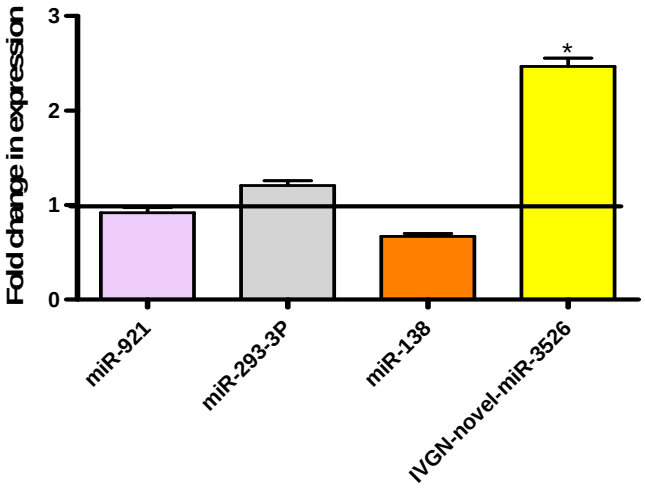

Figure 9. Expression level of selected microRNAs in CSC-exposed HBECs that have shown alteration in the confirmatory qRT-PCR analysis. The changes in microRNA levels were determined by qRT-PCR and normalized against the expression level of U6 and the values are presented as mean fold change ± SEM (n=3). Statistical analysis was performed by the paired one-tailed student's t-test (*$P \leq 0.05$).

We have also tried to see the dynamics of IVGN-novel-miR-3526 expression upon continuous or interrupted exposure (continuous exposure for 6 months or treatment for four months followed by no treatment for two months) of HBECs to CSC. The level of IVGN-novel-miR-3526 increased by 3.3-fold, compared to the expression in untreated cells, upon continuous exposure to CSC for six months, but a relatively weaker expression was observed upon interruption of treatment (1.8-fold increase compared to untreated cells) (Fig. 10). The decline in the expression level of IVGN-novel-miR-3526 after the discontinuation of CSC exposure indicates that the

effects of CSC on microRNA expression, at least in our model, are reversible and this observation is in line with the attenuation of tobacco smoke-induced molecular changes and decreased risk of lung cancer in ex-smokers.

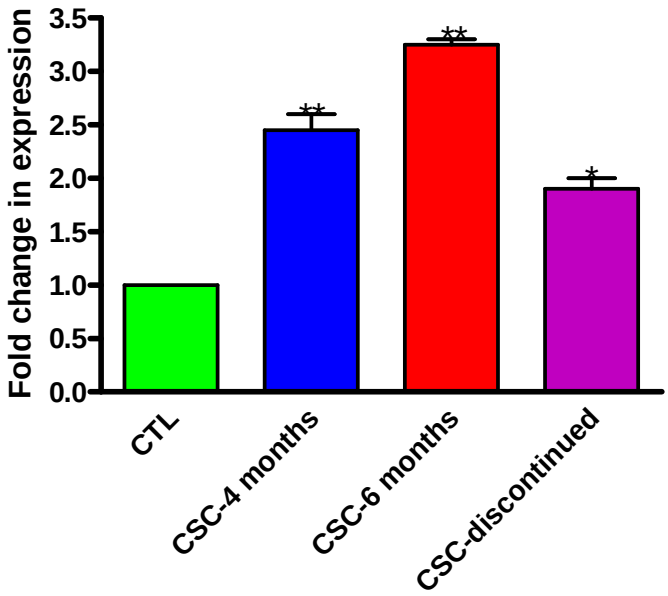

Figure 10. Expression level of IVGN-novel-miR-3526 at different time points. Fold changes of 2.4, 3.25, & 1.9 were observed in HBECs exposed to CSC for 4 months, 6 months and treated for 4 months but discontinued for 2 months, respectively**.** The values are presented as mean fold change ± SEM (n=2). Statistical analysis was performed by the paired two-tailed student's t-test (*$P \leq 0.05$, and **$P \leq 0.005$).

Subsequently, we then checked whether IVGN-novel-miR-3526 is deregulated in human lung adenocarcinoma A549 cells and found out that the level was up-regulated by 4.6-fold (Fig. 11). This indicates that alteration in the level of this microRNA is not limited to the early stager of lung tumorigenesis.

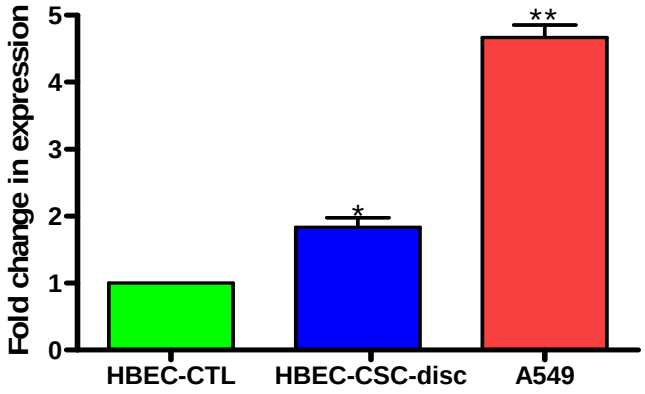

Figure 11. Expression level of IVGN-novel-miR-3526 in different cell lines. Fold changes of 1.8 and 4.6 were obtained in HBECs where CSC exposure was discontinued and A549 cells, respectively. The values are presented as mean fold change ± SEM (n=3). Statistical analysis was performed by the paired two-tailed student's t-test (*P ≤ 0.05, and **P ≤ 0.005).

4.5.3 Expression of IVGN-novel-miR-3526, miR-31 and miR-377 in mouse lung tissues

Altered levels of IVGN-novel-miR-3526 in CSC-exposed HBEC and human lung adenocarcinoma A549 cells incited an interest to look the expression level of this and two other microRNAs known to be involved in lung tumorigenesis, miR-377 & miR-31, (Melkamu et al., 2010). Normal lung tissues from vehicle treated mice and lung tumors (harvested at week 27 and 52) from NNK treated mice were analyzed using qRT-PCR. IVGN-novel-miR-3526 didn't show any change in its expression level between lung tissues from untreated control and NNK treated group (normal and tumorous lung tissues), whereas both miR-377 and miR-31 were up-regulated. This could be explained by the difference in species (human vs. mouse) as well as tumor stage where CSC-exposed HBECs were at early stage whereas tumor samples from mouse were in advanced stage of tumorigenesis (adenocarcinoma). Levels of miR-377 increased by 7.8- and 180- fold at week 27 and week 52 post-carcinogen treatments, respectively; the corresponding changes for miR-31 were 3.3- and 13-fold at week 27- and week 52 post-NNK treatment, respectively (Fig.14). The up-regulation of miR-377 and miR-31 in lung tumor tissues harvested

at week 27 is in agreement with previous reports from lung tumor tissues induced by vinyl carbamate exposure (Melkamu et al., 2010). However, we are the first to show the very high up-regulation (180-fold) of miR-377 in more advanced mouse lung tumor tissues. One report showed the value of this microRNA as a predictive microRNA signature for progesterone receptor status in breast cancer (Wang et al., 2008). On the other hand, studies with mouse and human lung tumors and human lung cancer cell lines showed that miR-31 is an important oncomir (Liu et al., 2010).

Table 2. microRNA primer sequences used for verification of microRNA microarray results by q RT-PCR

Primer	Sequences (5' to 3')	Accession no/reference article	Position
miR-138-2	AGCTGGTGTTGTGAATCAGGCCG	MI0000455	10-32
miR-15b	TAGCAGCACATCATGGTTTACA	MI0000438	20-41
miR-191	CAACGGAATCCCAAAAGCAGCTG	MI0000465	16-38
miR-194-1	TGTAACAGCAACTCCATGTGGA	MI0000488	15-36
miR-293-3p	AGTGCCGCAGAGTTTGTAGTGT	MI0000391	48-69
miR-34	ATGGCAGTGTCTTAGCTGGTTGT	MI0000268	22-43
miR-921	CTAGTGAGGGACAGAACCAGGATTC	MI0005713	2-26
miR-377	ATCACACAAAGGCAACTTTTGT	MI0000785	45-66
miR-31	AGGCAAGATGCTGGCATAGCT	MI0000089	8-28
U6	GGCAGCACATATACTAAAATTGGAA	Kalscheuer et al. (2008) `	
IVGN-novel-miR-3516	GCCGGTTAGCTCAGTTGGT		
IVGN-novel-miR-3526	GCGGCTTCTGTAGTGTAGTGG		
IVGN-novel-miR-3309	CGCGAAGGCCCAAAAA		

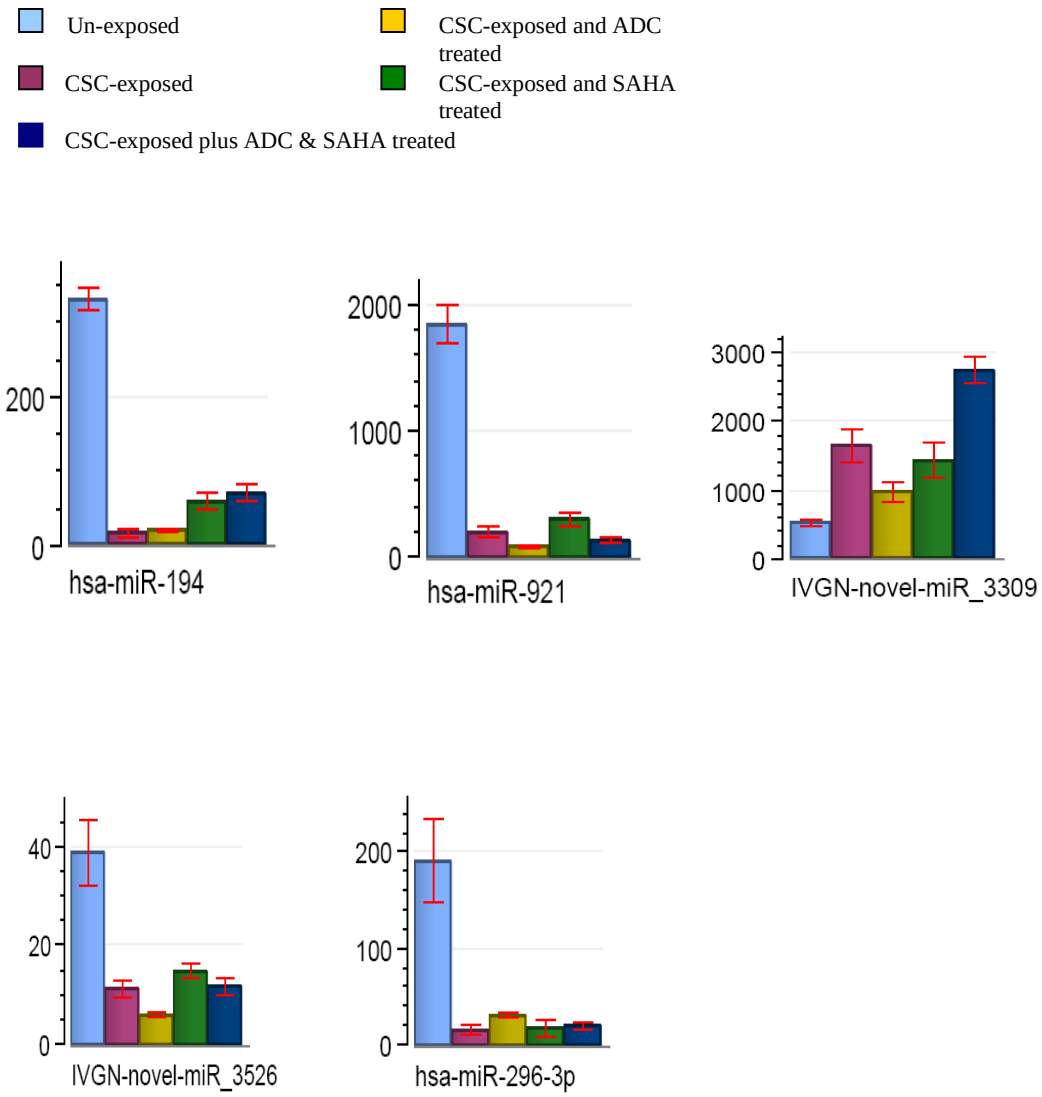

Figure 12. Examples of microRNAs whose expression levels on HBECs were highly modulated by CSC on the microarray analysis. Only IVGN-novel-miR-3309 shows up-regulation due to CSC exposure, the rest are down-regulated.

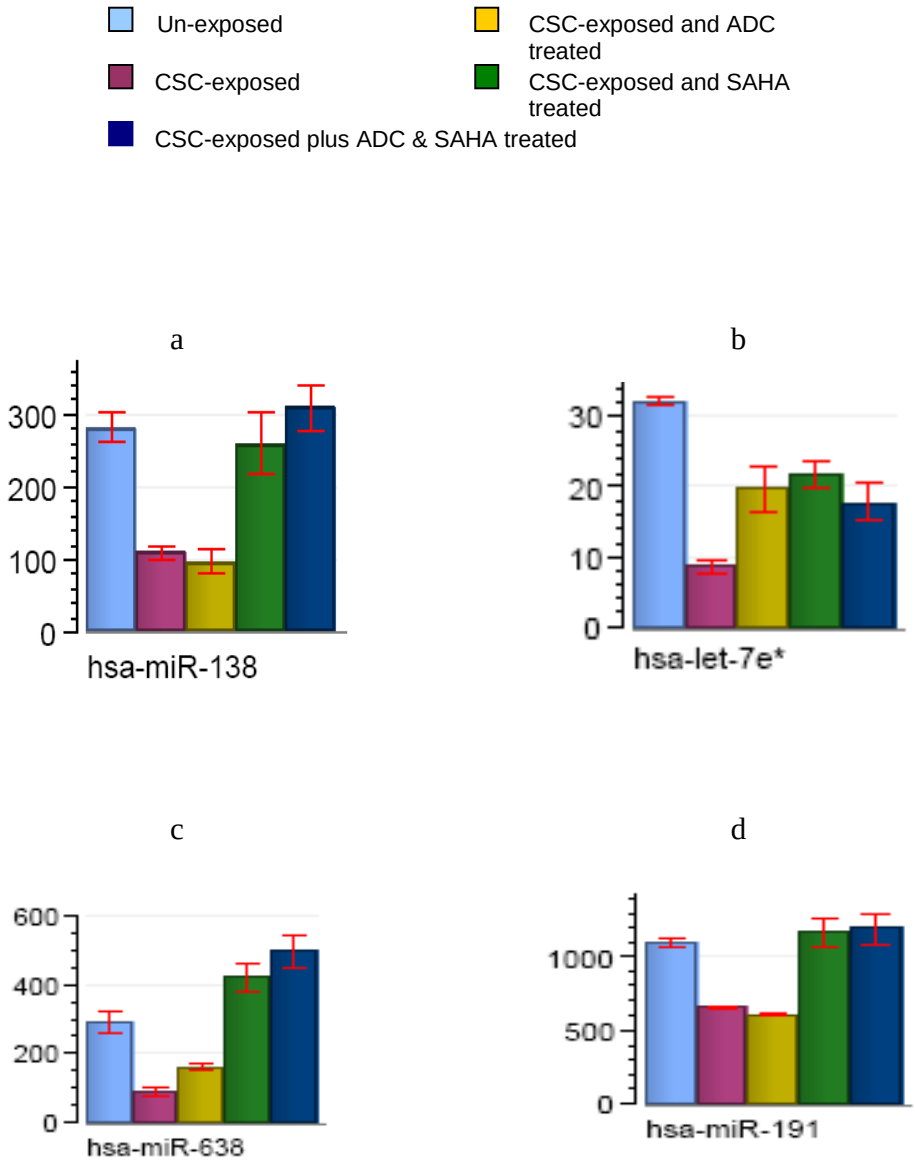

Figure 13 Examples of microRNAs whose expression levels on HBECs were altered by CSC and this alteration was reversed to the expression level of unexposed control by the demethylating agent and histone deacetylase inhibitor. Bar graphs a, c, & d show reversion by SAHA and combination of SAHA & ADC. Bar graph b shows reversion by ADC, SAHA, & combination of the two.

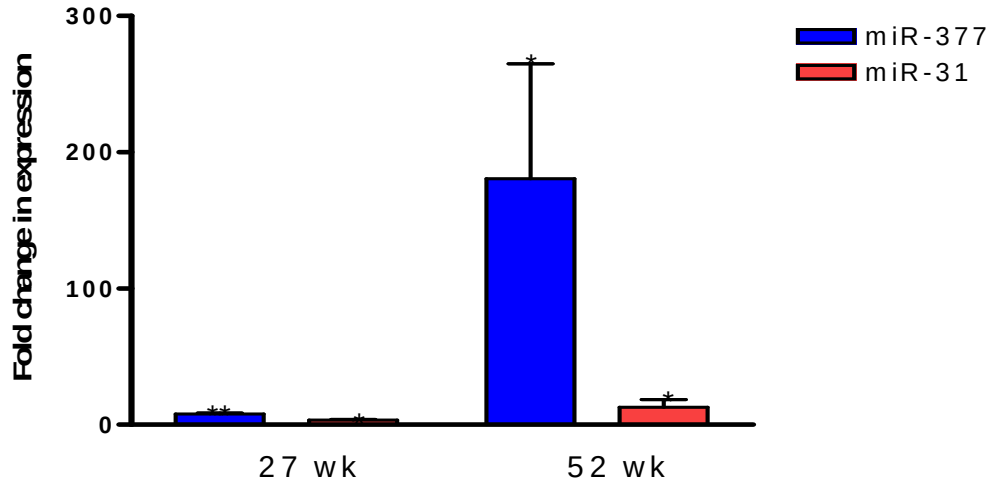

Figure 14. Expression level of miR-377 and miR-31 in lung tumor tissues from mice treated with NNK and sacrificed after 27-and 52-weeks. Fold changes of 7.8 and 180 for miR-377 and 3.3 and 12.9 for miR-31, were observed at 27-and 52-weeks, respectively. The values are presented as mean fold change ± SEM (n=2). Statistical analysis was performed by the paired one-tailed student's t-test (*P ≤ 0.05, and **P ≤ 0.005).

Table 3. Examples of predicted IVGN-novel-miR-3526 target genes from Target Scan database. (Release 5.1: April 2009)

Target gene	Gene name	Conserved sites
ERRFI1	ERBB receptor feedback inhibitor 1	1
BAK1	BCL2-antagonist/killer 1	1
BCL2L13	BCL2-like 13 (apoptosis facilitator)	1
DAPK1	Death associated protein kinase 1	1
NKTR	Natural killer-tumor recognition sequence	1
MYST	MYST histone acetyltransferase 2	1
LTBP1	Latent transforming growth factor beta binding protein 1	1
LTBP2	Latent transforming growth factor beta binding protein 2	1

Chapter 5 Conclusion and future direction

5.1 Conclusion

Exposure of HBECs to CSC caused both morphological and proliferative changes in HBECs. CSC-induced increases in the proliferation of HBECs could be, at least partly, caused by increased activation of ERK1/2 and AKT. The demethylating agent ADC reduced the activation of both ERK and AKT, which is inline with the anticancer effect of the drug.

Microarray analysis revealed a total of 89 microRNAs which were differentially expressed between unexposed control and CSC-exposed HBECs and 98% of them were down-regulated. Among the 20 selected microRNAs, IVGN-novel-miR-3526 was the only one that has shown significant up-regulation by qRT-PCR and its expression was modulated upon continuation or discontinuation of CSC exposure. Furthermore, this microRNA was found to be up-regulated in A549 cells but not in lung tumor tissues from NNK treated mice. However expression of miR-377 and miR-31 was significantly up-regulated in lung tumor tissues.

The fact that IVGN-novel-miR-3526 was found to be deregulated in HBECs exposed to CSC as low as 5 μg/ml and this microRNA is also up-regulated in the established lung adenocarcinoma cell lines (A549) would make it a good candidate for a biomarker and therapeutic target after completing remaining functional assays using gene knock out and knock in experiments.

5.2 Future directions

- It would be important to Identify CSC-modulated tumor suppressor genes in HBECs that are predicted targets of IVGN-novel-miR-3526 and involve in regulation of PI3K/AKT and RAS/MAPK signaling pathways.
- To demonstrate the link between IVGN-novel-miR-3526 up-regulation and observed phenotypic changes (cell morphology and proliferation), it would be necessary to perform

functional assays by knocking down its expression in CSC-exposed HBECs using IVGN-novel-miR-3526 antagomir.

- To further understand the role of IVGN-novel-miR-3526 in lung tumorigenesis, it would be valuable to conduct over- and under-expression of this microRNA in established lung cancer cell lines.

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