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HARVARD UNIVERSITY

Graduate School of Arts and Sciences



DISSERTATION ACCEPTANCE CERTIFICATE

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“Exploring the role of microRNAs in airway smooth muscle biology and asthma therapy”

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Date: April 11, 2014

Exploring the role of microRNAs in airway smooth muscle biology and asthma therapy

A dissertation presented

by

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to

Committee on Higher Degrees in Biological Sciences in Public Health

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological Sciences in Public Health

Harvard University

Cambridge, Massachusetts

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Exploring the role of microRNAs in airway smooth muscle biology and asthma therapy

ABSTRACT

The pathophysiology of asthma is characterized by airway inflammation, remodeling and hyper-responsiveness. Phenotypic changes in airway smooth muscle cells (ASM) play a pivotal role in the pathogenesis of asthma. ASM cells promote inflammation and are key drivers of airway remodeling. While airway hyper responsiveness and inflammation can be managed by bronchodilators and anti-inflammatory drugs, ASM remodeling is poorly managed by existing therapies. Therefore, targeting ASM remodeling remains a challenge, and a deeper understanding of the molecular mechanism that regulates ASM phenotypes in asthma pathogenesis will facilitate the search for next-generation asthma therapy. MicroRNAs are small yet versatile gene tuners that regulate a variety of cellular processes, including cell proliferation and inflammation – two phenotypes that are often altered in asthmatic ASM. We thus hypothesized that microRNAs regulate ASM phenotypes in asthma and represent new targets for future therapy. In this thesis, we used a genomic approach that combined next-generation sequencing with functional cellular assays to characterize the role of microRNAs in regulating airway smooth muscle function and drug response to conventional therapies. In Chapter 2, we identified miR-10a as the most abundant microRNA expressed in the primary human airway smooth muscle (HASM) cells. Using an unbiased target identification approach, we identified several novel

potential targets of miR-10a, including the catalytic subunit alpha of PI3 kinase (PIK3CA)—the central component of the PI3K pathway. We demonstrated that miR-10a directly suppresses PIK3CA expression by targeting its 3' Untranslated region (3'-UTR). Inhibition of PIK3CA by miR-10a reduced AKT phosphorylation and blunted the expression of cyclins and cyclin-dependent kinases that are required for HASM proliferation. In Chapter 3, we examined the effect of conventional asthma therapies on miRNA expression. While we did not find significant changes in miRNA levels, it remains to be determined whether microRNAs play a role in ASM tissue response to asthma therapy. Our study is the first to examine the role of microRNAs in ASM proliferation. Results from our study identified a novel microRNA-mediated regulatory mechanism of PI3K signaling and ASM proliferation. They suggest further that miR-10a is a potential therapeutic target to treat airway remodeling in asthma.

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Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

Albert Einstein

CHAPTER 1

Introduction

Asthma

Asthma is a common chronic airway disease prevalent in both developed and developing countries. It affects people of all ages and ethnic backgrounds. The prevalence of asthma is rising and by 2025 it is estimated that 400 million people worldwide will be diagnosed with asthma [1-3].

The clinical features of asthma include intermittent wheezing, cough, breathlessness and airway hyper-responsiveness to external stimuli [2]. These clinical conditions are believed to result from chronic airway inflammation and tissue remodeling [4, 5]. Airway inflammation is associated with a TH2 immune response that stimulates airway infiltration of eosinophils, mast cells and TH2 lymphocytes, as well as elevates IgE levels. Airway remodeling is characterized by epithelial alteration, fibrosis, goblet cell hyperplasia, neovascularization, smooth muscle hypertrophy and hyperplasia [4, 6, 7], and has been found in asthma of all degrees of severity and in both small and large airways.

Conventional asthma therapy

Continuous research efforts have offered great insights that link clinical features of asthma with biological pathways [2]. Two categories of asthma therapies were developed based on the molecular understanding of the disease pathogenesis. The first class, bronchodilators, target airway relaxation and the second class are anti-inflammatory drugs [8-10].

Beta-agonists (β -agonists) are the most commonly used bronchodilators in the treatment of asthma [1-3]. They bind to β 2-adrenergic receptors expressed on airway smooth muscle (ASM) cells to activate G-protein signaling that leads to an increase in cyclic adenosine monophosphate (cAMP) production and bronchodilation [11, 12]. There are two types of bronchodilators: (1)

short acting β -agonists (SABAs), which are used by virtually all asthmatic patients as a rescue treatment in episodes of acute bronchoconstriction; and (2) long acting β -agonists (LABAs), which are usually prescribed together with inhaled corticosteroids (ICS) to control symptoms among severe asthmatics[3].

Inhaled corticosteroids (ICS) are another class of drug that is been widely used to treat asthma. Corticosteroids exhibit powerful anti-inflammatory and immunosuppressive properties and are used to treat a variety of inflammatory diseases such as asthma, COPD and interstitial pulmonary fibrosis [9, 13, 14]. Corticosteroids exert their anti-inflammatory effect by binding to and activating the glucocorticoid receptor (GR). Activated GR mediates anti-inflammatory effects through several different mechanisms. Activated and dimerized GR can bind to DNA directly at sites known as glucocorticoid response elements (GREs) and negative GREs. GREs are found in the promoters of anti-inflammatory genes and binding of GR is associated with increased transcription [15-17]. Conversely, binding of activated, dimerized GR to negative GREs mediates transcriptional repression, and negative GREs are found in pro-inflammatory genes [18]. GR can also exert an anti-inflammatory effect by binding to and inhibiting pro-inflammatory transcription factors, thereby lowering the transcription of inflammatory genes[19].

Limitations of current asthma therapy

Although β -agonists and corticosteroids have been around for more than 50 years, these current treatments are insufficient in managing severe asthmatic symptoms due to treatment-unresponsiveness following prolonged treatment [9, 10]; therefore, identification of new therapeutic targets that improve the efficacy of the existing therapy is needed.

There are two major limitations associated with the β -agonists treatment: (1) high variability in bronchodilator response among patients and (2) the loss of β -agonist efficacy among severe asthmatics due to a potential loss in functional β 2-adrenergic receptors (β 2AR) [10, 20]. Efforts in Pharmacogenetics have focused on studying the β 2-adrenergic receptor gene (ADRB2) to explain the widespread variability in bronchodilator response [21, 22]. The ADRB2 gene is an intron-less gene, which has been sequenced in multiple ethnic groups to identify single nucleotide polymorphisms (SNPs). Earlier bronchodilator association studies have mainly focused on analyzing the association between coding SNPs of ADRB2 and bronchodilator response [23-25]. However, contradictory findings have been reported by different groups and the irreproducibility of coding block SNP associations in the ADRB2 gene have prompted investigation into the regulation of ADRB2 function [20]. Therefore, looking into genetic variants within regulators of β 2AR function may provide new clues to explain the inter-individual variability in bronchodilator response.

Another major limitation associated with β -agonist usage is the loss of efficacy in prolonged treatment, which is most prevalent and problematic in severe asthma management [10]. This loss in efficacy of the treatment is believed to be caused by β 2AR down regulation [12]. β 2AR down regulation is a natural process of the cell to prevent excessive signaling. The activation of β 2AR signaling by β -agonists is attenuated by desensitization of the receptor. Once activated, the receptor is quickly phosphorylated which leads to the recruitment of various adaptor proteins that in turn recruit E3 ligases to ubiquitinate the receptor. Ubiquitinated β 2AR is a target for proteasome degradation. This receptor down regulation process is heavily regulated, and multiple key regulators have been uncovered over the past decade [12, 26-29]. Hence, a targeted inhibition of receptor down regulation could prevent excessive β 2AR loss and improve

the current β -agonist based asthma therapy. However, such strategy would require a thorough mechanistic understanding of the receptor down regulation process.

Similar to the limitations associated with β -agonist treatment, there is also a great degree of individual variability in patient response to ICS [30]. Most of patients with severe asthma are largely resistant to ICS treatments [8, 31]. To better understand the development of ICS resistance and to explain the widely observed variability in drug response, past research has focused on analyzing SNPs along the GR pathway and key regulators in the inflammation pathway. Strong associations have been found in corticotropin-releasing hormone receptor 1 (CRHR1) [32]; T-bet, a key transcriptional factor for naïve T-lymphocyte production [33]; and FCER2, a low affinity IgE receptor [34]. However, only a part of the phenotypic variation is explained by these SNPs [20, 30]. More studies are needed to identify other susceptibility factors that contribute to the heterogeneity in ICS response. Further mechanistic studies on the regulation of GR function are also useful to improve the efficacy of GR treatment within severe asthmatic patients.

The role of airway smooth muscle (ASM) in airway hyper-responsiveness (AHR)

A link between airway smooth muscle (ASM) and airway hyper-responsiveness (AHR) in asthma was first hypothesized two centuries ago [35]: In 1968, it was reported that bronchia are contracted during asthma attack that produces a wheezing noise in expiration which is not dependent on phlegm production. In mid-19th century, it was documented that the bronchial tube is the only possible cause of asthma. The same study documented that bronchial contraction is a consequence of spastic contraction of the fiber like cells which we now refer to as airway smooth muscle (ASM). AHR refers to excessive narrowing of the airway in response to outside stimuli,

is a clinical hallmark of asthma. Now it is well recognized that ASM is the effector cell of the airway narrowing event. Hence, a deeper understanding of ASM biology could identify therapeutic targets in managing AHR.

The importance of ASM in AHR arose from a pivotal finding that ASM obtained from asthmatic patients release more histamine and respond more to contractile agonists, such as house dust and pollen, compared to non-asthmatics' ASM [36]. Following the study, multiple groups have hypothesized that AHR can be caused by structural and functional changes in ASM [37-39]. Structural changes in ASM such as a change in muscle mass and mechanical properties of ASM have been explored extensively as potential contributors to AHR. A landmark study by Lambert *et. al* concluded that the increase in smooth muscle mass was potentially the most important structural change in determining AHR [40]. This conclusion has been supported by many later publications that show an increase in ASM mass is the predominate contributor to AHR and that the underlying causes of an increase in ASM mass are cell hyperplasia and hypertrophy [41-44]. Another branch of research has heavily investigated the mechanical properties of ASM and how they contribute to AHR. Some mechanical properties explored include: the velocity of ASM shortening, ASM force generation, and the effect of deep inspiration on muscle relaxation [44-47]. Comparing ASM cells from asthmatic and normal subjects revealed that asthmatic ASM cells exhibit greater maximal shortening and faster shortening. These characteristics were found to be associated with increased expression of contractile proteins such as myosin light chain kinase, an enzyme that mediates the formation of the actin-myosin cross bridge during muscle contraction [45]. A follow up study showed asthmatic ASM has greater myosin heavy chain isoform (SM-B), which is known to propel faster actin movement and thus a faster contraction [48]. These data suggest that a change in both ASM

mass and the expression of the proteins that make up the contractile machinery in ASM cells can lead to changes in the contractility of ASM cells and ultimately result in a change in AHR.

The role of ASM in airway remodeling

With the accumulation of studies in smooth muscle biology, ASM are now known to be phenotypically very plastic in response to changes in the micro-environment. These phenotypic changes may ultimately lead to the airway remodeling observed in asthma [49]. During asthma pathogenesis, ASM cells are known to constantly undergo changes in its proliferative phenotype [49].

Increased ASM mass is a direct result of a change in ASM's proliferative phenotype and is believed to be the most important component of the airway wall remodeling process in asthma [44,50, 51]. The mechanisms underlying increases ASM mass involve both hyperplastic and hypertrophic changes in ASM cells [42]. An increase in ASM cell number could be a direct result of decreased ASM cell apoptosis or an increase in ASM cell proliferation. Research efforts have mainly focused on the cell proliferation. Mediators of ASM cell proliferation fall into three categories: agonists that activate receptor tyrosine kinase (RTK), agonists that activate G-protein coupled receptors (GPCR), and pro-inflammatory cytokines. Polypeptide growth factors such as platelet derived growth factors (PDGF) and epidermal growth factor (EGF) are mitogens that bind to RTKs. The activation of RTKs then signals through downstream effectors such as phosphoinositide 3'-kinase (PI3K) and mitogen-activated protein kinase (MAPK) to increase DNA synthesis and cell proliferation [43, 52]. Alpha-thrombin (α -thrombin) and endothelin-1 are examples of agonists that bind to GPCRs and increase cell proliferation via GPCR-mediated activation of PI3K [53, 54]. A third group of newly emerged mitogens are inflammatory

cytokines such as interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α). These cytokines signal through surface glycoproteins that are coupled to multiple non-RTK receptors to increase cell proliferation, such as Src family members, mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) systems [55]. Although it is tantalizing to speculate the importance of inflammatory cytokines in regulating cell proliferation, their effects on proliferation is very modest compared to other categories of mitogens [56-58].

As mentioned above, another way to explain ASM hyperplasia is that it could result from dysregulation in apoptosis. However, little is known about the exact role of apoptosis in ASM hyperplasia and remodeling since only a few studies have approached ASM cell survival and their response to apoptotic signals. Endothelin-1 [59], chemokines such as CCL3, CCL5, CXCL8 [60] and some extracellular matrix (ECM) proteins such as fibronectin and collagen I [61] are all known to induce apoptosis in ASM cells. We also know that ASM cells express death receptors like TNFR1, FAS (TNFRSF6) and stimulation of ASM cells with either TNF- α and Fas ligand can induce apoptosis [62, 63]. However, all of these studies were conducted in vitro on healthy ASM cells and the importance of these signaling pathways in asthmatic ASM cell survival remains unknown.

Unlike ASM cell hyperplasia, evidence for ASM cell hypertrophy remains a matter of debate [64, 65]. Increased ASM cell size has been reported from studies looking at ASM samples from intermittent, moderate, severe, fatal asthma. No ASM cell hypertrophy was found among mild to moderate asthma patients [65], and therefore, ASM hypertrophy is believed to be a phenotype only seen in severe asthma patients. Several pathways regulate ASM cell hypertrophy, such as the activation of the mammalian target of rapamycin (mTOR) pathway [66]

and the inhibition of the glycogen synthase kinase 3 beta (GSK-3 β) pathway [67]. However, the pathological relevance of these pathways in the context of asthma requires further investigation.

In addition to increased ASM mass, studies suggest that ASM cells isolated from asthmatics secrete extracellular matrix (ECM) proteins *in vitro* which explains the increased ECM protein depositions of collagen I, III and V, fibronectin and laminin observed in asthmatics [68, 69]. Fibronectin and collagen I have been reported to promote ASM cell growth while laminin inhibits ASM cell growth [70]. The increase in ECM is believed to further contribute to the thickening of the airway wall and remodeling. ECM influences cell proliferation and is postulated to be a positive feedback mechanism for promoting ASM cell growth and further thickening of the airway wall [71]. An *in vitro* experiment suggests that ASM cells proliferate faster on fibronectin or collagen I coated plates than standard polystyrene [71]. Asthmatic ASM cells *in vitro* secrete more perlecan and collagen I and less laminin- α 1, again strengthening the argument of dysregulated ECM production by ASM in airway remodeling [71]. In addition to ECM's effect on cell proliferation, ECM has also been linked to promoting the synthetic phenotype of ASM. ECM substrates like fibronectin and type I collagen were found to enhance IL-13-dependent ASM secretion of eotaxin, a potent chemokine that stimulates the migration of eosinophils to the lung during lung inflammation [72].

The Role of ASM in airway inflammation

While early ASM studies focused on airway remodeling and contractility, more recently attention has shifted to its role in the regulation of airway inflammation. A growing body of evidence consistently demonstrates that ASM is more than a target of the inflammatory process; ASM displays immunomodulatory properties through its synthetic phenotype and its expression

of cell surface molecules such as integrins and toll like receptors [73, 74]. ASM acts in a positive feedback mechanism with mast cell and T lymphocytes to propagate the inflammation cycle.

Growing evidence suggests the asthmatic ASM layer is infiltrated by mast cells and that this specific pathological event is unique to asthma and is absent from other lung inflammatory diseases such as bronchitis [75-78]. The infiltration of mast cells into ASM tissue is believed to be caused by the secretion of chemotactic factors, such as CXCL family proteins, by the ASM cells [75]. Upon activation, mast cells then release tryptase and proinflammatory cytokines such as TNF- α that in return stimulate ASM cells to produce TGF- β 1. In turn, ASM cells differentiate towards a more contractile phenotype, which is coupled with increased expression of α -SMA [79, 80]. The number of mast cells found within the ASM layer of the airway positively correlates with the degree of AHR and the amount of α -SMA. Therefore, there is in vivo relevance of the mast cells' effect on the ASM cell phenotype [80, 81].

T cells alter the contractile phenotype of ASM resulting in changes relating to lung inflammation and AHR [82, 83]. Activated T cells adhere to the surface of human ASM cells via adhesion molecules expressed on the cell surface. The adherence leads to an increase ASM's responsiveness to contractile agonists such as acetylcholine and impairs ASM's relaxation responsiveness to isoproterenol [84]. T cells were also found to drive ASM remodeling by enhancing ASM hyperplasia. Close contact of CD4⁺ T cells with ASM cells causes increased ASM proliferation and DNA synthesis as well as a decreased rate of apoptosis [82]. Furthermore, the number of infiltrated T cells observed in asthmatic ASM correlates with ASM mass in vivo [83]. On the other hand, ASM cells are also known to alter functional properties of T cells. Direct contact between CD4⁺ T cells and ASM cells enhances T cell survival [83]. In addition, ASM cells are able to present super antigens on the cell surface via MHC proteins to activate

resting CD4+ T cells, which results in greater release of IL-13 and a subsequent increase in ASM contractility[85].

Pivotal role of ASM in asthma pathophysiology

The pathophysiology of asthma is characterized by AHR, lung inflammation and airway remodeling [2,86, 87]. AHR is defined by increased airway narrowing to a wide range of stimuli, which is responsible for recurrent asthma attacks and breathlessness. ASM is the main effector tissue that mediates AHR during an asthma attack [88]. It is apparent from evidence presented above that ASM cells undergo constant remodeling and contribute to the increasing severity of AHR-related symptoms [5,83, 89]. Lung inflammation involves the recruitment of various inflammatory cells including mast cells and T lymphocytes. ASM cells themselves are active immunomodulatory cells that promote inflammation and recruitment of inflammatory cells to the airway. ASM cells do this by expressing cell surface adhesion molecules and through its synthetic properties such as secreting ECM proteins [82,85, 90]. Airway remodeling is a dynamic process involving multiple layers of dysregulation, including alterations in epithelial cells, disruption of the complex interactions between inflammatory and ASM cells, enhanced ECM deposition and an increase in ASM cell mass. ASM cells are central to these processes and there are multiple levels of evidence showing that increasing ASM cell mass is the key driver behind airway remodeling and AHR [44, 50, 62, 68, 69]. While AHR and bronchial inflammation can be controlled by conventional therapies, ASM remodeling is not well managed by these same therapies [88, 91]. Therefore, targeting the thickening of ASM is a goal for future asthma treatments. In the future, research needs to focus on ways to prevent and reverse ASM remodeling. Hence, a deeper understanding of the molecular mechanisms that keep airway

remodeling in check represents an area of new opportunity in searching for the next-generation asthma therapy.

Non-coding RNAs and microRNAs

Over the past decade we have learned that protein coding genes only comprise 2% of the entire human genome and that the vast majority of the genome is transcribed into so-called “non-coding” RNAs. [92]. In recent years, it has become increasingly evident that the non-protein coding portion of the genome plays important regulatory roles in development, physiology and disease pathogenesis [93]. The functional evidence of non-coding RNA is best studied in small non-coding RNAs called microRNA (miRNA). However, many other classes of non-coding RNA, such as long non-coding RNA (lncRNA), have also been shown to play important roles in disease progression [94, 95].

MiRNAs are the most widely studied class of non-coding RNAs. They average about 22 nucleotides in length and have profound effects on the transcriptome by mediating post-transcriptional gene silencing [94, 95]. The process of miRNA maturation involves enzymatic processing of primary miRNA transcripts by Drosha to produce a 70-nucleotide stem loop structured precursor miRNA. This precursor miRNA is then transported to the cytoplasm where it is further processed by Dicer into a 22-nucleotide double-stranded RNA molecule [96]. One strand of the double stranded RNA is the mature miRNA that is loaded onto the RNA-induced silencing complex (RISC). As part of RISC, the miRNA mediates post-transcriptional inhibition by the imperfect 5' binding of the miRNA seed sequence to miRNA-recognition elements (MRE) within the 3' Untranslated Region (3'UTR) of target genes. As a result, the complimentary binding of miRNA to its target gene can lead to target mRNA degradation and/or translational

inhibition [95, 96]. Computational analysis indicates that greater than 60% of the human genome may be regulated by miRNAs [97].

Many of the identified miRNAs are evolutionarily conserved, and hence miRNA-mediated gene regulation represents a conserved and complex regulatory network of biological processes [98]. Up to now, miRNAs have been shown to play crucial roles in the regulation of many important cellular functions such as cell proliferation, differentiation, apoptosis, inflammation and stress response [99-102]. Furthermore, expression profiling studies revealed that dysregulated miRNA expression is associated with multiple diseases ranging from cancer to chronic diseases such as cardiovascular disease [99,100, 103]. However, despite increasing awareness of the importance of miRNAs in basic cell functions and diseases, many of miRNA targets, functions and biological relevance are poorly understood.

microRNA expression profiling

In the human genome, there are a limited amount of miRNAs compared to mRNAs: there are believed to be ~1000 miRNAs, compared to ~30,000 mRNAs. Since one miRNA can regulate up to several hundred target genes, a small number of miRNAs may still have substantial influence on a gene expression network overall [104]. Hence, miRNA expression analysis may contain rich biological information as the variation in multiple pathways and genes may all captured in the expression differences in a small set of miRNAs. Expression analysis of miRNAs in different tissues can provide clues about their physiological functions. Identifying tissue specific miRNA can uncover novel biomarkers for diseases and open new therapeutic venues for treatment. It is increasingly evident that miRNAs exhibit tissue-specific expression patterns, which are often highly associated with their function. For instance, miR-1a was found

to account for 45% of all miRNAs identified in the heart and it regulates multiple pathways that control cardiac remodeling [105, 106]. Similarly, miR-122a accounts for 78% of all miRNAs in the liver and controls multiple processes such as cholesterol metabolism, an important component of hepatic function [107].

Over the past decade, a number of molecular biology tools have been developed to assist in miRNA discovery and quantitative expression profiling. Quantitative PCR (qPCR) is used to quantify the relative expression level of a miRNA to a housekeeping small RNA such as U6. This method is highly sensitive and can distinguish difference isoforms of miRNA to a single base pair resolution. However, qPCR is low throughput and suffers from low coverage due to reliance on primer based amplification, which is difficult to scale up and requires a prior knowledge of the miRNA sequence [108]. Microarray-based miRNA profiling offers higher throughput but poorer accuracy, because detection depends on the hybridization efficiency of miRNA to the probes printed on the array. Variations in the measurement can be attributed to many sources such as non-specific binding, temperature and miRNA expression level. Therefore, proper normalization is critical and can often be the biggest source of variations in data [109]. To overcome coverage, specificity and throughput problems, next-generation small RNA sequencing was developed and it is a technology that utilizes massive cDNA sequencing which allows for the direct measure of different small RNA species in a given sample. This new technology offers several advantages over the previous array based approach. First, detection of small RNA is not limited to the known microRNA database, allowing for the miRNAs discovery. Unlike the hybridization based approach, direct sequencing offers single base pair resolution providing for accurate profiling of each variant of the same miRNA species. Lastly, RNA

sequencing offers a larger dynamic detection range: both transcripts that are highly expressed and lowly expressed can be more accurately identified compared to other methods [110].

microRNA target gene identification

Due to the advancement in RNA sequencing technologies, over 1500 miRNAs have been identified. However, the functional characterization of each miRNA remains a challenge because one miRNA can have up to hundreds of evolutionarily conserved gene targets [111]. Unraveling the biological significance of each miRNA is further complicated by the fact that several different miRNAs can target the same gene [112].

Computational algorithms have been the major prediction tool to identify putative miRNA target genes. These algorithms rely on the principle that most miRNAs mediate gene silencing via the binding of its seed sequence (the sequence 2-7 nucleotides from the miRNA 5' end) to the 3'UTR of the target mRNA [113]. The algorithm scans the 3'UTR of all known mRNA and comes up with a list of potential gene targets with the probability of targeting. The probability is determined based on the level of base pair conservation [111]. The most commonly used target prediction software are: PicTar, TargetScan, miRana and miRGen. It is widely recognized that different algorithms yield different miRNA-mRNA pairing predictions and that a large number of predicted target genes are false positives (pairs that are statistically significant but cannot be verified using experimental methods) or false negatives (actual gene targets that are missed from the prediction software). Oftentimes the latter is more difficult to overcome because it requires a better understanding of the mechanism of miRNA target recognition. To test for false positives, an experimental approach is always needed. One common technique for validating miRNA targeting is to either overexpress or inhibit the miRNA and

assess whether the predicted change in the protein level of the target mRNA shifts in the manner expected. To complement this assay, a 3'UTR assay is always required to confirm a direct molecular link between the miRNA and mRNA: the 3'UTR of the target mRNA is cloned into a luciferase reporter, the recombinant plasmid along with the miRNA of interest is co-expressed and luciferase activity is measured to demonstrate the binding of miRNA to the 3'UTR of target gene [114].

Regulation of microRNA expression

It is poorly understood where the primary transcript of each miRNA were first transcribed and what regulates the transcription of primary miRNA transcripts. miRNA transcription is likely regulated in a similar fashion to mRNA: by the binding of positive and negative transcription factors to the promoter region of a miRNA locus. miRNA transcriptional regulated is best studied in the context of cancer. The oncogenic transcription factor *c-myc* binds directly to a stretch of conserved sequence about 1.5kb upstream of the miR-17-5p cluster to regulate the entire mir-17-92 polycistron which is known to be oncogenic and promote tumor growth [115]. Tumor suppressor gene p53 was also found to regulate miRNA expression. P53 can bind to consensus sequences in the promoter region of the miR-34 family and activate their expression to mediate cell cycle arrest and apoptosis [116-119].

miRNA transcription also appears to be regulated by epigenetic modifications and DNA methylation. This is demonstrated by a subset of miRNA genes residing near CpG islands [120]. The transcription of miR-148a, miR-34b/c, miR-9 and *let-7a-3* are carefully regulated by their methylation status and DNA methyltransferases [120-122]. It is estimated that 5-10% of mammalian miRNAs are epigenetically regulated [120]. One dramatic example is miR-127,

which was 50-fold up regulated following methyl transferase inhibitor treatment [123]. Another example includes the *let-7a-3* locus, which was hypomethylated in lung adenocarcinoma tissue and extensively methylated in normal lung tissue. In this case, the miRNA expression is tightly regulated and its dysregulation could contribute to cancer progression [121].

Once transcribed, miRNAs are regulated at the processing level. A mass-spec analysis of flag-tagged Drosha revealed 20 distinct interacting proteins in addition to DGCR8, which binds to Drosha to activate it [124]. The processing activity of Drosha is tightly controlled by positive regulators such as RNA helicase p68/p72 [125], R-smads [126], p53 [127] and negative regulators such as nuclear factor 90/45 [128] and Lin28 [129].

Similar to Drosha, Dicer activity is also carefully regulated. The relatively low levels of pre-miRNA compared to pri-miRNA or mature miRNA suggest that Dicer is very efficient at processing pre-miRNA to mature miRNA. In contrast to Drosha which often requires co-factors to promote processing, Dicer regulation involves more inhibitory factors [130]. The best studied example is the processing of pre-miR-138. The pre-miRNA molecule is efficiently processed by Dicer *in-vitro*; however, the addition of HeLa cell extract inhibits Dicer activity, suggesting that cells express factors that specifically inhibit Dicer activity [131]. The amount of Dicer may serve as another control point to modulate miRNA expression. Altered expression of Dicer is observed in several types of cancer, for example Dicer expression is decreased in non-small-cell lung cancer [132], and increased in prostate tumors [133]. The discovery of Let-7 mediated negative feedback regulation of Dicer activity further demonstrated the complexity of Dicer regulation [134]. Much remains to be learned about the cellular consequences of altered Dicer expression and the biological significance of global change in miRNA expression.

The role of microRNAs in Lung biology

The lung has a specific miRNA expression profile and its miRNAs are shown to be important for lung development [135]. For example, a lung specific deletion of Dicer can lead to abnormal apoptosis and defective airway branching [136]. miR-17-92 is believed to regulate lung development, because its expression is high in embryonic lung and slowly decreases through development from fetus to adulthood [137]. This is further demonstrated by in vivo miR-17-92 knockout mice, which have no terminal bronchioles and have defects in lung epithelium differentiation [138]. In addition to miRNAs role in healthy lung development, a number of studies have implicated miRNA in a range of pulmonary diseases such as lung cancer [139, 140], asthma [141-143] and COPD[144, 145].

The role of microRNAs in asthma

Asthma is characterized by both airway inflammation and remodeling. Several studies have implicated miRNAs in the negative feedback regulation of key inflammatory pathways [142, 146] and miRNAs also contribute to the control of extracellular matrix synthesis and tissue fibrosis [145, 147]. Therefore, it is conceivable that miRNAs play important roles in regulating asthma progression and specific miRNA targeted manipulation may provide new opportunities for asthma treatment.

Although miRNAs have been well studied in inflammation, only a few studies have explored the biological significance of miRNAs in regulating asthma-related inflammation. An animal model of house dust mite induced allergic airway inflammation was used to compare miRNA expression patterns between control and inflamed lung tissue. miRNA-16, -21, and -126 were significantly up regulated in the inflamed group. Interestingly, intranasal administrations of

anti- miR-126 attenuated airway hyper-responsiveness and decreased eosinophil and neutrophil recruitment as well as the secretion of IL-5, IL-13 [142]. This suggests miR-126 mediates airway inflammation. Another in vivo profiling study used the ovalbumin (OVA)-induced allergic asthma model in mice to measure miRNA expression profiles following short, intermediate and long term OVA exposure from whole lung RNA extract. More than 50 miRNAs were differentially expressed at different time points. miR-146b, -223, -29, -483, -574, -672 and -690 were the most likely to participate in asthma pathogenesis according to pathway enrichment analysis [148]. Although most of the miRNA-mRNA target validation has yet to be performed by this study, this is the first large scale in vivo miRNA profiling covering the temporal change in miRNA expression from the initiation of the disease to a state of chronic airway inflammation. This large scale in vivo profiling experiment suggests that the molecular mechanisms underlying asthma pathogenesis are highly complex and it is likely to be regulated by multiple miRNA mediated pathways.

miRNA's role in asthma pathogenesis is also studied using cell-based models. The Human bronchial epithelial cell (HBECs) is a key cell type in airway inflammation. Treatment of HBECs with inflammatory cytokines, such as IL-1 β , TGF- β , TNF- α , and IFN- γ , induced miR-146a expression [149]. miR-146a knockdown and overexpression suggests the miR-146a negatively regulates the release of pro-inflammatory cytokines such as IL8 and RANTES [149]. These results established the role of a miRNA in the negative feedback regulation of inflammation.

The ASM cell, as discussed earlier, is a cell type that is critical to asthma pathogenesis. In asthmatic airways, ASM cells increase in size and number and experience changes in their contractile and secretory phenotypes. In human ASM cells, miR-133a controls smooth muscle

cell contraction by regulating RhoA, one of the key proteins involved in smooth muscle cell contraction [150]. In addition, miR-25 inhibits KLF-4, a potent inhibitor of smooth muscle-specific gene expression [143]. Although human ASM cells are not the primary cell type involved in airway inflammation, cytokines influence their miRNA expression profile. IL-1 β induces a time dependent increase of mir-146a. However, unlike the previous report that inhibition of miR-146a in epithelial cells inhibits cytokine release, mir146a inhibition in ASM cells did not regulate IL-8 and RANTES release [141]. This suggests the function of miRNA may be highly cell type dependent. Besides inflammatory cytokines, mechanical stretch is also known to induce miRNA expression changes. For example, miR-26a is induced by mechanical stress and contributes to airway hypertrophy by suppressing glycogen synthase kinase 3 β (GSK-3 β), which is an anti-hypertrophic protein [151]. As outlined above, miRNAs play crucial roles in controlling airway inflammation and remodeling, both of which are fundamental clinical phenotypes of asthma. Therefore, exploring miRNA-based asthma therapy represents novel opportunities towards better disease management.

microRNA as biomarkers

MiRNAs expression patterns are highly regulated and tissue-specific [105]. It is well accepted that dysregulation of miRNAs may contribute to a variety of human diseases such as cancer, cardiovascular and other chronic diseases [103,106, 152]. Therefore, abnormal miRNA signatures in disease states may be valuable diagnostic markers for early disease detection and prognosis. The role of miRNA as biomarkers is best studied in the context of cancer [152-154]. Aberrant miRNA expression is found to be indicative of tumor progression. Expression levels of miRNAs such as Let-7 and miR-155 are correlated with disease survival in non-small cell lung

cancer [155, 156]. Circulating miRNAs in blood and other human fluid are very stable under most sample storage conditions, making it a convenient biomarker in a clinical setting [157, 158]. So far, only a few studies have looked miRNA profiles in asthma and the results are inconsistent. One study claims differentially expressed miRNAs in lungs from allergen challenged mice compared to controls suggested an important role for miRNA in the pathophysiology of asthma [159]. However, another study based on lung biopsies from normal and mild asthmatic patients found no significant differences in miRNA expression profiles [160]. Other mouse studies have found OVA-induced inflammation caused differential expression in a different set of miRNAs [148, 161]. These conflicting results suggest that more mechanistic studies on miRNA's role in lung biology are needed to establish miRNAs as potential biomarkers for asthma.

microRNA as therapeutics

As miRNAs are powerful regulators in a wide variety of diseases settings, there is great potential in using miRNAs as therapeutic targets to treat diseases. Currently no FDA approved drugs are based on miRNA research, but several promising miRNA targets are under development[162-164]. For example, miR-122 is a hepatic specific miRNA critical to hepatitis C virus replication. Using anti-sense inhibitors of miR-122 can significantly reduce the replication of HCV virus RNA, suggesting that anti-miR-122 can be used as an antiviral therapy [162, 165]. Clinical trials using anti-miR-122 are currently ongoing signifying a new era in which anti-miRs are used as a novel class of drug. Currently, using miRNAs as therapeutic targets to treat asthma is still in its nascent stages as more miRNAs tied to lung biology emerge.

Conclusion

The wide inter-individual variability in responses to β -agonist and ICS and the lack of efficacy of these treatments among severe patients are major problems in the management of

asthma symptoms. miRNAs are naturally occurring small evolutionarily conserved endogenous molecules that orchestrate and regulate the expression of gene networks. miRNAs rely on base pairing to specifically control complex gene regulation networks at single base pair resolution. Gene targets of miRNAs are often central among signaling transduction pathways; and therefore, miRNAs regulate the regulators and are central players in cell signaling themselves. miRNA profiling combined with mRNA gene expression profiling and protein analysis offers a powerful tool for studying global gene regulation networks to greatly enhance our understanding of the pathophysiology of asthma. Ultimately, it may answer some fundamental questions about asthma such as: what drives airway remodeling and inflammation? Much remains unknown regarding the role of miRNAs in lung biology and ASM cell function.

This thesis aimed to explore the role of miRNA in regulating ASM biology and asthma therapy. We have combined the use of various genomic approaches such as next-generation sequencing, microarray analysis and cell based functional assays to define the role of miRNAs in regulating important ASM phenotypes that have the potential to open up new therapeutic venues leading to better asthma management in the future.

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CHAPTER 2

MicroRNA-10a controls airway smooth muscle cell proliferation via direct targeting of the PI3 kinase pathway

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Ruoxi Hu performed all of the experiments.

Wenchi Pan contributed to the graphing of Figure 2-1B.

MicroRNA-10a controls airway smooth muscle cell proliferation via direct targeting of the PI3 kinase pathway

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ABSTRACT

Airway smooth muscle (ASM) cells play important physiological roles in the lung, and abnormal proliferation of ASM directly contributes to the airway remodeling during development of lung diseases such as asthma. MicroRNAs are small yet versatile gene tuners that regulate a variety of cellular processes including cell growth and proliferation; however, little is known about the precise role of microRNAs in the proliferation of the ASM. Here we report that a specific microRNA (miR-10a) controls ASM proliferation through directly inhibiting the phosphoinositide 3-kinase (PI3K) pathway. Next-generation sequencing identified miR-10a as the most abundant microRNA expressed in primary human airway smooth muscle (HASM) cells, accounting for about 25% of all small RNA reads. Overexpression of miR-10a reduced mitogen-induced HASM proliferation by ~50%, whereas inhibition of miR-10a increased HASM proliferation by ~40%. Microarray profiling of HASM cells expressing miR-10a mimics identified 52 significantly down-regulated genes as potential targets of miR-10a, including the catalytic subunit alpha of PI3 kinase (PIK3CA)—the central component of the PI3K pathway. MiR-10a directly suppresses PIK3CA expression by targeting the 3'-UTR of the gene. Inhibition of PIK3CA by miR-10a reduced AKT phosphorylation and blunted the expression of cyclins and cyclin-dependent kinases that are required for HASM proliferation. Together, our study identifies a novel microRNA-mediated regulatory mechanism for PI3K signaling and ASM proliferation and further suggests miR-10a as a potential therapeutic target for lung diseases whose etiology resides in abnormal ASM proliferation.

INTRODUCTION

Airway smooth muscle (ASM) is the critical effector tissue that maintains bronchomotor tone, and phenotypic changes in ASM play a pivotal role in the pathogenesis of a variety of lung diseases [1]. Indeed, an increase in ASM tissue mass is a major driver of airway narrowing associated with asthma and COPD [2-5]. The pathological change in ASM mass is a combined result of hypertrophy (increase in cell size) and hyperplasia (increase in cell number through proliferation). ASM cells proliferate in response to a myriad of growth stimuli, including peptide growth factors (e.g., PDGF and EGF) as well as bronchoconstrictors (e.g., histamine, thrombin and endothelin). These mitogens function by binding to and activating their cognate receptors at the surface of ASM cells. For example, PDGF and EGF bind to and activate their respective receptor tyrosine kinases (RTKs), whereas bronchoconstrictors mostly act through specific G-protein coupled receptors (GPCRs).

Following receptor activation by the mitogens, two main pathways are known to mediate the transduction of the proliferation signals in ASM cells: the MAP kinase (MAPK)/ ERK pathway and the PI3 kinase (PI3K) pathway. While both are needed for maximal ASM cell proliferation [6, 7], these two pathways function mostly independently in ASM cells. Both RTK and GPCR mitogens cause robust activation of the MAPK/ERK pathway as measured by ERK phosphorylation in ASM cells [8]. Chemical inhibition of ERK phosphorylation reduces mitogen-induced DNA synthesis and proliferation in cultured human ASM cells, indicating that the pathway is an important regulator of cell proliferation [8-11]. PI3K is the other major signaling pathway that regulates ASM proliferation [6, 7, 12, 13]. HASM cells express three forms of PI3 kinases: class IA, II and III [12]. RTK and GPCR mitogens both activate class IA

PI3K and its signaling [7]. Inhibition of the PI3K signaling prevents mitogen stimulated cyclin D1 protein expression and DNA synthesis without affecting ERK signaling [13, 14], whereas expression of constitutively active class IA PI3K is sufficient to stimulate DNA synthesis [7]. While it is well recognized that MAPK/ERK and PI3K play important roles in regulating ASM proliferation, little is known about whether and how these pathways may be regulated in the ASM cells.

MicroRNAs (miRNAs) are small (~22 nucleotides long) non-coding RNAs that mediate post-transcriptional gene silencing through binding to the 3'-UTR of target mRNAs to promote mRNA degradation and/or translational inhibition [15-17]. MiRNAs play versatile roles in normal cell physiology and disease pathogenesis [15]. In the airway, miRNAs have been shown to play important roles in regulating inflammation: miR-146 is induced by IL-1 β and suppresses the activation of NF- κ B in alveolar epithelial cells [18], and inhibition of miR-126 suppresses inflammation and the development of allergic airway disease [19]. In ASM cells, miRNAs also regulate inflammation: several miRNAs (miR-25,-140*, -188,-320) were significantly down-regulated in human ASM cells that are exposed to inflammatory cytokines such as IL-1 β , TNF- α and IFN- γ [20], and inhibition of miR-25 up-regulates KLF-4, which is a potent inhibitor of smooth muscle-specific gene expression and a mediator of inflammation [20]. MiRNAs also regulate the contractility of ASM cells. For example, miR-133a down-regulates RhoA, which is involved in regulating mechanical stress and cytoskeleton organization in the ASM [21]; another miRNA let-7f targets β 2-adrenergic receptor, whose activation causes ASM relaxation [22],

Relatively little is known about the potential role of miRNAs in the fundamental ASM phenotype--proliferation. A very recent study implicated a role for miR-221 in the hyper-proliferation of ASM cells in severe asthma [23]. However, how miRNAs may regulate the

signaling pathways that control ASM proliferation remains largely unknown. In this report, we identified a miRNA that is most abundant in ASM as a critical novel regulator of ASM proliferation. Functional studies demonstrated that the miRNA regulates ASM proliferation by specifically suppressing the PI3K signaling pathway.

MATERIALS AND METHODS

ASM cell culturing and transfection

Primary HASM cells were isolated from three aborted lung transplant donors with no chronic illness. The tissues were obtained from the National Disease Resource Interchange (NDRI) and their use approved by the University of Pennsylvania Internal Review Board. HASM cells were maintained and cultured in Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and antibiotics, as described previously [24, 25]. Passages 4 to 7 HASM cells were used in all experiments. MiR-10a mimics and inhibitor (Qiagen) were used to over-express and knockdown miR-10a, respectively. Non-targeting scrambled siRNA (Qiagen) was used as a control for miR-10a mimics experiments; miRNA inhibitor control (Qiagen) was used as a control for miR-10a inhibitor experiments. Transfections of miR mimics and inhibitor were performed using the DharmaFECT 1 reagent (Thermo Scientific) according to the manufacturer's protocol.

Construction and sequencing of TrueSeq small RNA libraries

RNA was extracted with Qiagen miRNeasy kit using the manufacturer's protocol. RNA concentration and quality was measured by Nanodrop (ThermoScientific) and by Bioanalyzer (Agilent Technologies 2100), respectively. Truseq Small RNA Sample Preparation kit (Illumina)

was used to construct DNA libraries from the RNA preparations (1 µg total RNA per sample) according to the manufacturer's protocol. Briefly, 3' and 5' adaptors were sequentially ligated to the extracted small RNA species. Reverse transcriptase PCR amplification was performed in which one primer contains a DNA barcode. The amplified product was then gel purified in order to isolate the small RNA library, which is subsequently validated using the Agilent Technologies 2100 Bioanalyzer. At this point, three samples (each from one HASM cell line) were pooled together (multiplexing) at equal molar concentrations. Quantification of the DNA library samples was done by nanodrop or Qubit. Sequence quality and fragment size in the prepared DNA libraries were verified by running the samples on a 2% agarose gel. Sequencing (single read, 36 bp) of the prepared DNA libraries were done using the Illumina Genome Analyzer IIX platform at the Boston University Illumina Sequencing Core Facility (Boston, MA).

Analysis of deep sequencing data

Data cleaning and alignment were performed using the CLC Genomic Workbench (CLCBio). Raw sequencing reads with an average length of 36 bp in FASTq format were used for 3' adaptor trimming. Trimmed reads were aligned against the human miRNA database (miRBase release 18). MiRNA sequencing count table was generated using the alignment count function of the software, which was set to allow two mismatches. Only perfect matched counts were used the subsequent analysis. The abundance value for each miRNA was derived using the following formula: total percent count for miR-X = (5p mature miRNA reads / total aligned miR reads for the sample) × 100. The percentage of total count reflects the abundance of each miRNA. A pie graph was generated for each donor sample to reflect the miRNA composition. For *in silico* analysis of tissue expression patterns of miRNAs, miRNA enrichment analysis was performed

to compare our dataset with published dataset that contains expression data from 170 cell line/tissues obtained by small RNAs sequencing [26]. Total percent count from 3 different HASM cell lines with total percent count of the published dataset was graphed in R with abundance (total percent count) on y-axis and different cell lines on x-axis.

Quantitative RT-PCR of miRNAs and mRNA

miRNAs and mRNAs were measured using the miScript PCR system with miR-10amiScript Primer Assays (Qiagen) on Light cycler 480 (Roche, Indianapolis, IN), following the manufacturer's recommendations. U6 small RNA and beta-actin were used to normalize miRNA and mRNA quantification, respectively. Expression values and statistical significance were calculated using $2^{-\Delta\Delta Ct}$ method [27, 28].

Microarray-based gene profiling and data analysis

HASM cells from passage 4 were transfected with either miR-10a mimics or control siRNA. About 48 hours post transfection, RNA was extracted from the cells using miRNeasy mini kit (Qiagen). Samples were analyzed on the Human Gene 1.0 ST Array (Affymetrix) which contains 36079 total Refseq transcripts at the Dana-Farber Microarray Core Facility (Boston, MA). Data were subsequently processed and analyzed with dChip Software. Transcripts with fold change (FC) cut off at 0.8 and 1.25; $P < 0.05$ were identified as significant hits.

ASM proliferation assay

For H3-thymidine incorporation, HASM cells were serum starved for 24 hours. Cells were cultured with radio-labeled H3-thymidine (1 μ Ci/well, PerkinElmer) in the presence and absence

of 10% FBS for 24 hours. The cells were then extracted and lysed using a semi-automated PhD cell harvester (Cambridge Technology). Radioactivity was counted using a Tri-carb 2910TR liquid scintillation counter (Perkin Elmer). To measure cell growth, HASM cells were trypsinized, washed two times with PBS and counted with a hemocytometer.

Western blotting

Cells were lysed in Nonidet P-40 lysis buffer (0.5% vol/vol Nonidet P-40, 50 mM Tris-HCL, 150 mM NaCl) supplemented with protease and phosphatase inhibitors (Roche). Lysates were mixed with lithium dodecyl sulfate (LDS) sample buffer and resolved on 4-12% NuPAGE gel (Invitrogen) using MOPS running buffer supplemented with Nu-PAGE antioxidant (Invitrogen). Proteins were transferred onto a nitrocellulose membrane (GE Amersham) and probed with the indicated antibodies. Primary antibodies used in this study include anti-P110 α (#4255), anti-phospho AKT (Ser473) (cat. #4058), anti-AKT (cat. #9272), anti-phospho ERK1/2 (cat. #9101), anti-ERK (cat. #4695) (all from Cell Signaling), and anti-GAPDH (GT239) (Gene Tex).

Luciferase reporter assay

Full length 3'-UTR of PIK3CA was subcloned into a pMir-Target luciferase vector driven by a CMV promoter (Origene). Mutant derivatives of the construct were made through site-directed mutagenesis using the Quick-change Kit (Stratagene). HEK293 cells were co-transfected with the luciferase constructs (wild type or mutant) with miR-10a mimics or control scrambled siRNA for 48 hours using Lipofectamine (Invitrogen) at 2:1 molar ratio (miRNA mimics *vs.* construct reporter) ratio following the manufacturer's protocol. The day before luciferase measurement, cells were counted and re-seeded into 96 well assay plate followed by dual-luciferase assay

according to manufacturer's recommended protocol (Promega). Luminescence was measured by a multi-mode microplate reader (Biotek Synergy 2)

RESULTS

Small RNA profiling identified miR-10a as the most abundant miRNA in primary human ASM cells

To probe the landscape of microRNA expression in the ASM, we performed next-generation sequencing of small RNAs in primary human ASM (HASM) cells from 3 healthy donors. Small RNAs extracted from the cultured HASM cells were used to construct cDNA libraries. Deep sequencing (36 bp single reads) was performed, and sequences were trimmed (to remove library adaptors) and aligned against the miRNA database (miRBase). We obtained around 4-8 million reads for each of the three HASM cell lines. Up to 70% of the reads were aligned to sequences in the miRBase and over 85% of the alignments were perfect matches (Table 2-1).

Table 2-1. Summary of sequencing alignments.

Reads were trimmed and aligned using CLC Genomic Workbench (CLCBio). Trimmed reads were aligned against human miRBASE release 18.

	Total reads	Annotated reads	Percent aligned	Percent of Perfect Match
Donor 1	4078442	1808316	44.3%	87.1%
Donor 2	8018228	6038849	75.3%	90.5%
Donor 3	3958859	2230223	56.3%	89.7%

According to the analyzed deep sequencing data, about 200 miRNAs were detected in HASM cells and half of them were relatively well expressed (with > 100 sequencing reads). Many of the well-known miRNAs are among the best expressed in HASM cells, including miR-10a, miR-21, let-7a, miR-100 and miR-26 (Figure 2-1A). Among them, miR-10a is the most abundant, accounting for over 20% of all aligned reads (Figure 2-1A). To determine if miR-10a is specifically enriched in HASM cells, we performed an *in silico* miRNA analysis by comparing our dataset with a published dataset that contains miRNA deep sequencing data from 170 cell lines/tissues [26]. The expression plot showed that miR-10a was expressed at a much higher level in HASM cells as compared to all other cells/tissues, whereas a similarly abundant miRNA (let-7a) was not differentially expressed in HASM cells (Figure 2-1B). To confirm cellular specificity of miR-10a to ASM cells within the lung, we examined miR-10a expression in primary airway epithelial cells. The expression of miR-10a in airway epithelial cells was more than 30 fold lower than HASM cells (Figure 2-1C). Overall, our data showed that miR-10a is the most abundant microRNA in HASM cells and exhibits an ASM-enriched expression.

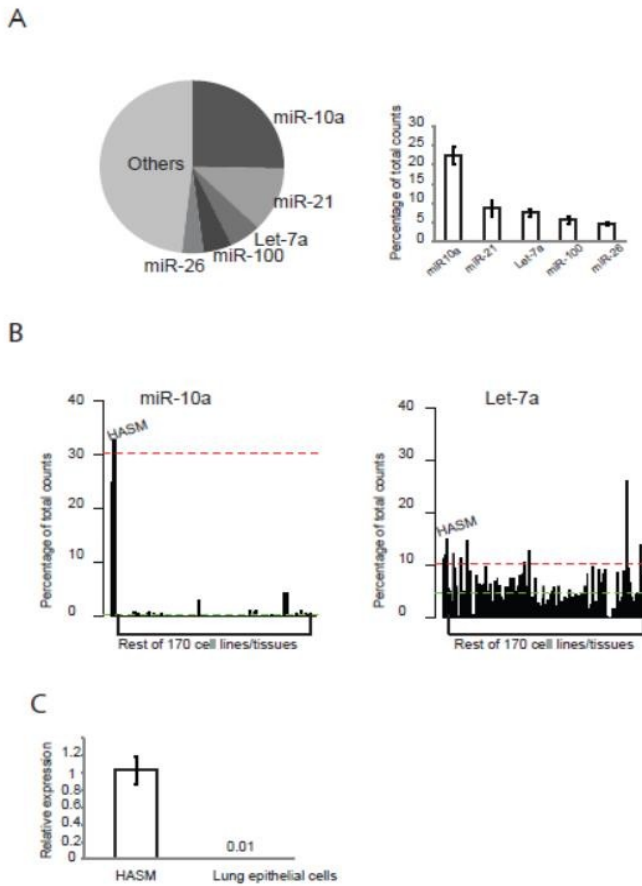


Figure 2-1. Next-generation sequencing identified miR-10a as a top highly expressed miRNA in ASM cells.

A) Profiling of miRNA expression in primary human ASM cells using Next-generation deep sequencing. RNA was extracted from three primary HASM cell lines and sequenced using next-generation sequencing (for details, see Materials and Methods section). Pie chart represents the distribution of the percentages of total sequencing counts in a representative HASM cell line for the top five highly expressed miRNAs. The average percentages for the top five highly expressed miRNAs in three HASM cell lines were plotted in the bar graph. **B)** miR-10a is highly abundant in the ASM as compared to other tissues and cell lines. Expression level of miR-10a (as a

Figure 2-1. (Continued)

percentage of total sequencing reads) in HASM cells was compared with that in 170 other cell lines / tissues. The red dotted line represents the average percentage of total counts for miR-10a in HASM cells; the green line represents the average of miR-10a expression from the other 170 cell lines/tissues. C) qRT-PCR data comparing the level of miR-10a in HASM cells with that in primary airway epithelial cells, RNA was extracted from primary HASM cell lines (3 donors) and primary airway epithelial cell lines (2 donors). miR-10a level was measured using qRT-PCR (for details, see Material and Methods section) and normalized to U6 small RNA. Standard errors are indicated.

Identification of the miR-10a target genes and downstream pathways in ASM cells

MiRNAs function by suppressing the expression of their target genes [15,16, 29]. To identify the genes targeted by miR-10a in HASM cells, we employed an unbiased experimental approach (Figure 2-2A). We first transfected HASM cells with miR-10a mimics or a control scrambled siRNA. We then determined the effect of miR-10a on the HASM transcriptome by whole-genome microarray gene profiling. Differential transcript expression analysis identified over 1,000 significant transcript changes in miR-10a mimics-transfected cells as compared to the scrambled control (Table 2-2). There are many more down-regulated genes (798) than up-regulated genes (304) in the mir-10a overexpressed cells. This is expected given the inhibitory nature of miRNA regulation on gene expression. The widespread impact of mir-10a on the transcriptome is consistent with the notion that a single miRNA regulates expression of many genes through both direct and indirect effects [29, 30]. To identify the direct targets of miR-10a, we cross-compared our microarray dataset with *in silico* predicted targets of miR-10a

(TargetScan 5.1), which all contain putative sequences that match the seed sequence (2-7 nucleotides) of the miRNA. Among the 271 predicted miR-10a targets, 248 (~91%) were detected in HASM cells by microarray and 52 (~19%) of those putative target genes were significantly down-regulated in our microarray dataset (Table 2-3). These 52 genes have a high likelihood of being the direct targets of miR-10a, as they contain miR-10a target sites in their 3'UTR and are down-regulated in miR-10a-transfected HASM cells. Down-regulation of selected genes by mir-10a mimic expression in HASM cells was validated by qRT-PCR (Figure 2- 2B). To identify common biological processes and pathways affected by miR-10a, we performed a gene set enrichment analysis on all transcripts that were significantly changed by miR-10a mimic transfection. Strikingly, 8 out top 10 enriched pathways affected by miR-10a are related to cell cycle (Table 2-4).

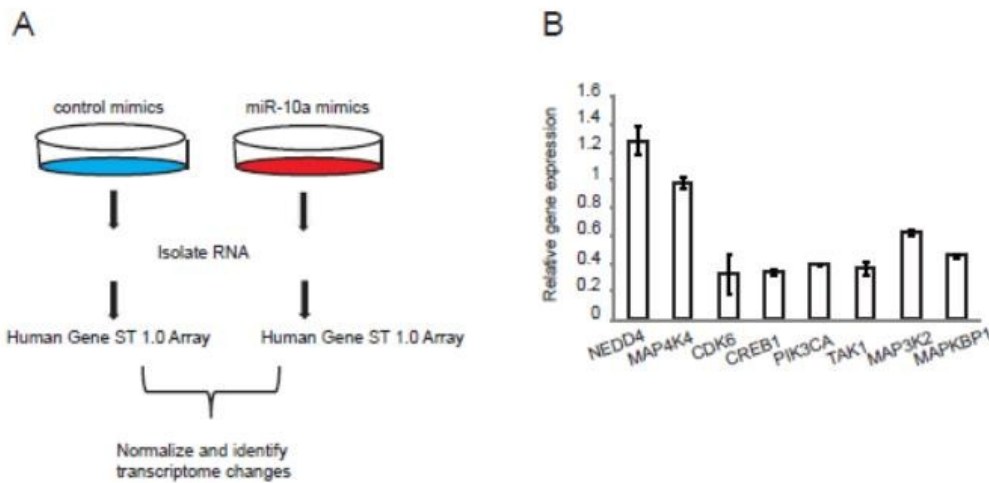


Figure 2-2. Microarray profiling identified putative genes and pathways targeted by miR-10a in ASM cells.

Figure 2-2. (Continued)

A) A microarray-based approach to identify transcriptomic changes resulted from miR-10a mimic expression. HASM cells were transfected with either control scrambled siRNA or miR-10a mimics for 48 hours. RNAs were then extracted and used for hybridization to Affymatrix-based Human Gene 1.0 ST microarray with a coverage of 36079 Refseq transcripts (N=2). **B)** qRT-PCR validation of microarray-identified transcript changes. RNA was extracted from scrambled siRNA control and miR-10a mimics followed by qRT-PCR for selected down regulated miR-10a putative target genes. Genes tested were normalized to beta-actin. Triplicates were used for each condition.

Table 2-2. Summary of microarray analysis.

RNAs from control and miR-10a mimic-transfected HASM cells were analyzed by microarray using the Human Gene 1.0 ST Array (Affymetrix) (N=3). Microarray data were subsequently processed and analyzed using the dChip Software.

		Percent of total genes	Percent of total predicted targets
Total number of genes detected	22021		
Number of significant down regulation (FC<0.8)	798	3.60%	
Number of significant up regulation (FC>1.25)	304	1.40%	
Total number of predicted mir10a targets	271		
Total number of detected predicted targets	248		91.50%
Number of significant down regulated targets (FC<0.8, p<0.05)	52		21%

Table 2-3. Predicted miR-10a target genes that are down-regulated by miR-10a mimics expression in HASM cells.

Genes with fold change (FC <0.8) and P-value<0.05 were shown.

Genes	FC	P-value
CDK6: cyclin-dependent kinase 6	0.37	0.00
USP46: ubiquitin specific peptidase 46	0.39	0.00
RAP2A: RAP2A, member of RAS oncogene family	0.40	0.02
ITGB8: integrin, beta 8	0.40	0.01
CBX5: chromobox homolog 5 (HP1 alpha homolog, Drosophila)	0.41	0.01
H3F3B: H3 histone, family 3B (H3.3B)	0.43	0.00
ST6GALNAC6: ST6 -N-acetylgalactosaminide alpha-2,6-sialyltransferase 6	0.43	0.00
CRLF3: cytokine receptor-like factor 3	0.48	0.02
TMEM183A: transmembrane protein 183A	0.52	0.00
TMEM183B: transmembrane protein 183B	0.52	0.00
LANCL1: LanC lantibiotic synthetase component C-like 1 (bacterial)	0.54	0.01
ANXA7: annexin A7	0.55	0.01
CYTH1: cytohesin 1	0.57	0.00
ARNTL: aryl hydrocarbon receptor nuclear translocator-like	0.58	0.02
ZMYND11: zinc finger, MYND domain containing 11	0.60	0.00
BDNF: brain-derived neurotrophic factor	0.61	0.01
E2F7: E2F transcription factor 7	0.63	0.01
UBE2I: ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	0.63	0.01
CNOT6: CCR4-NOT transcription complex, subunit 6	0.63	0.01
KLHDC10: kelch domain containing 10	0.64	0.01
GATA6: GATA binding protein 6	0.65	0.01
GOLGA3: golgi autoantigen, golgin subfamily a, 3	0.65	0.00
SMAP1: stromal membrane-associated GTPase-activating protein 1	0.66	0.01
MAPRE1: microtubule-associated protein, RP/EB family, member 1	0.66	0.03
TFAP2C: transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)	0.67	0.02
NCOR2: nuclear receptor co-repressor 2	0.68	0.04
MAP3K7: mitogen-activated protein kinase kinase kinase 7	0.68	0.03
HSPC159: galectin-related protein	0.68	0.03
FNBP1L: formin binding protein 1-like	0.70	0.03
PAPD5: PAP associated domain containing 5	0.70	0.04
PIK3CA: phosphoinositide-3-kinase, catalytic, alpha polypeptide	0.71	0.00
SCARB2: scavenger receptor class B, member 2	0.72	0.04
CNNM4: cyclin M4	0.72	0.00
TET2: tet oncogene family member 2	0.72	0.04

Table 2-3. (Continued)

SLC30A4: solute carrier family 30 (zinc transporter), member 4	0.73	0.02
AP4E1: adaptor-related protein complex 4, epsilon 1 subunit	0.73	0.01
ARSJ: arylsulfatase family, member J	0.74	0.01
MTMR3: myotubularin related protein 3	0.75	0.03
JARID2: jumonji, AT rich interactive domain 2	0.75	0.04
MAPKBP1: mitogen-activated protein kinase binding protein 1	0.76	0.03
RORA: RAR-related orphan receptor A	0.76	0.01
BRWD3: bromodomain and WD repeat domain containing 3	0.76	0.03
CREB1: cAMP responsive element binding protein 1	0.77	0.01
SMURF1: SMAD specific E3 ubiquitin protein ligase 1	0.77	0.00
MAP3K2: mitogen-activated protein kinase kinase kinase 2	0.78	0.01
RPRD1A: regulation of nuclear pre-mRNA domain containing 1A	0.78	0.04
BAZ2B: bromodomain adjacent to zinc finger domain, 2B	0.78	0.04
USP25: ubiquitin specific peptidase 25	0.79	0.01
NUP50: nucleoporin 50kDa	0.79	0.02
MTF1: metal-regulatory transcription factor 1	0.79	0.03
WWC2: WW and C2 domain containing 2	0.80	0.02
DOCK11: dedicator of cytokinesis 11	0.80	0.02

Table 2-4. Top 10 enriched pathways regulated by miR-10a

All significant transcriptome changes ($FC < 0.8$ or $FC > 1.25$, $P\text{-value} < 0.05$) were included in the gene-set enrichment analysis using the GeneGo software. The table lists enriched pathways with P-values and FDR (false discovery rate) q-values adjusted for multiple comparisons.

Rank	Pathways	P-value	FDR
1	Cell cycle_ The metaphase checkpoint	3.651E-20	2.140E-17
2	Cell cycle_ Chromosome condensation in prometaphase	1.054E-19	3.089E-17
3	Cell cycle_ Role of APC in cell cycle regulation	2.150E-18	4.200E-16
4	Cell cycle_ Initiation of mitosis	5.129E-13	7.514E-11
5	Cell cycle_ Start of DNA replication in early S phase	4.915E-10	5.534E-08
6	DNA damage_ ATM / ATR regulation of G2 / M checkpoint	5.666E-10	5.534E-08
7	DNA damage_ ATM/ATR regulation of G1/S checkpoint	1.076E-07	9.012E-06
8	Cell cycle_ Spindle assembly and chromosome separation	1.495E-07	1.095E-05
9	Cell cycle_ Cell cycle (generic schema)	3.672E-07	2.391E-05
10	Cell cycle_ Role of 14-3-3 proteins in cell cycle regulation	5.590E-07	3.276E-05

miR-10a inhibits HASM cell proliferation

Because our pathway analysis suggests that miR-10a may function in cell cycle regulation, we next examined the effect of miR-10a on HASM cell growth and proliferation. We assessed HASM cell proliferation by two independent methods: cell counting and thymidine incorporation, which directly measures the rate of DNA synthesis. While the number of control HASM cells (transfected with scrambled siRNA) increased by 3 fold during 72 hours post serum stimulation, the number of miR-10a mimics transfected-HASM cells only increased by 2 fold during the same time period. Such a reduction in cell number in miR-10a mimics-transfected cells was also evident at baseline only 1 day after transfection (Figure 2-3A). Consistent with the result observed on direct cell counting, mir-10a mimics-transfection in HASM cells reduced

FBS-induced thymidine incorporation by ~50% as compared to the control-transfected cells (Figure 2-3B). Conversely, transfection of an inhibitor of miR-10a increased thymidine uptake by ~70% in HASM cells (Figure 2-3C). Together, these results demonstrated that miR-10a is a negative regulator of ASM proliferation.

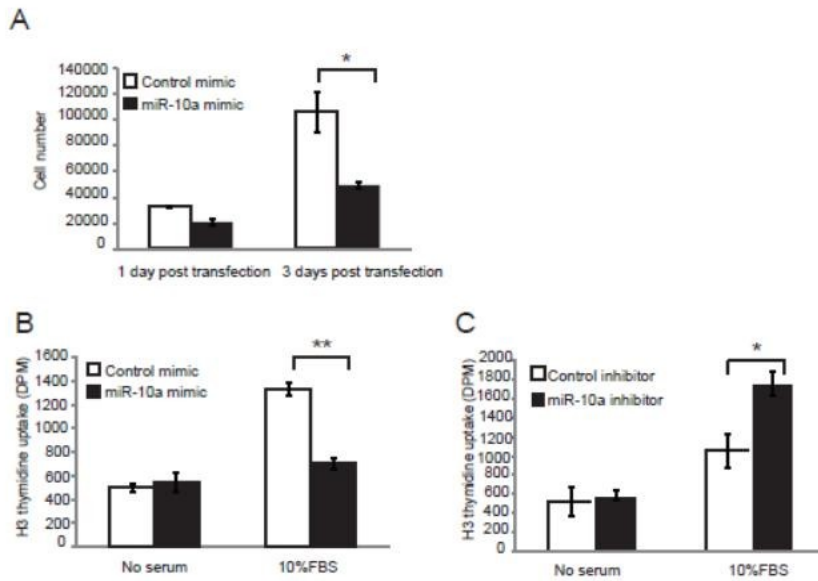


Figure 2-3. Effects of miR-10a on ASM proliferation.

A) miR-10a mimic expression inhibits HASM proliferation. HASM cells were transfected with either scrambled siRNA or miR-10a mimics followed by serum starvation for 24 hours. Cells were then allowed to grow in the presence of FBS for additional 24 or 72 hours, after which cell numbers were counted. Triplicates were done for each condition. Error bars represent standard errors. *, $P < 0.05$. **B)** miR-10a mimics inhibit DNA synthesis in HASM cells. HASM Cells were transfected for 48 hours with either scrambled siRNA or miR-10a mimics and then serum-starved for 24 hours. H3-thymidine incorporation was measured in cells after 24 hours of FBS stimulation. Error bars represent standard error (N=5). **, $P < 0.01$. **C)** miR-10a inhibitor

Figure 2-3. (Continued).

increases DNA synthesis in HASM cells. HASM cells were transfected with either a control miR inhibitor or miR-10a inhibitor for 48 hours, and serum starved for 24 hours. H3-thymidine incorporation was measured in cells after 24 hours of FBS stimulation. Error bars represent standard error (N=5). **, P<0.01. All comparisons were performed using two-tailed student's test.

miR-10a reduces the expression of cdks and cyclins in HASM cells

ASM cell proliferation is directly controlled by cyclins and cyclin-dependent kinases (cdks) (32, 33). To further investigate the effect of miR-10a on HASM proliferation, we examined the expression of several selected cdks and cyclins (Cdk2, Cdk6, Cdc6, cyclin A2, cyclin B1 and cyclin B2), all of which are known to play important roles in cell cycle control: Cdk2 and cdk6 regulate the check point of G1 to S phase [31, 32]. Cdc6 initiates DNA synthesis in the S phase and its activity is tightly regulated by Cyclin A and Cdk2 [33, 34]. Cyclins B1 and B2 regulate G2 to M phase transition [35]. As shown in Figure 2-4A, mRNA levels as measured by qRT-PCR for all of the examined cdks and cyclins were decreased (ranging from 40-70% reduction) in mir-10a mimics-transfected HASM cells. Although down-regulated by miR-10a overexpression, none of these genes except cdk6 was predicted to be direct target of mir-10a, suggesting that miR-10a likely acts directly on genes that are in the pathways upstream of cyclins and cdks.

MiR-10a inhibits PI3K signaling pathway in the HASM cells

Two major signaling pathways (PI3K-AKT and MAPK/ERK) transduce proliferation signals leading to the activation of cyclins and cdks in HASM cells (8-13). Upon growth factor stimulation, PI3K is activated and increases the production of PIP3, which activates PDK1 and causes downstream phosphorylation of AKT [12]; activation of MAPK/ERK pathway leads to the phosphorylation of the kinase. To determine which pathway is regulated by miR-10a, we determined the effect of miR-10a on PI3K and MAPK/ERK signaling pathway by measuring the phosphorylation of AKT and ERK. As shown in Figure 2-4B, EGF treatment activated AKT phosphorylation in both control and miR-10a mimic-transfected HASM cells. However, the extent of activation as indicated by the intensity of pAKT signal was reduced by ~55% in miR-10a mimics-transfected cells. Similarly, the level of AKT phosphorylation induced by FBS was reduced by ~34% in miR-10a overexpression HASM cells (Figure 2-4C). In contrast, we did not observe a consistent significant effect of miR-10a on ERK phosphorylation (Figure 2-4C). These results suggest that the inhibitory effect of miR-10a on HASM cell proliferation is mostly mediated through the suppression of the PI3K-AKT signaling.

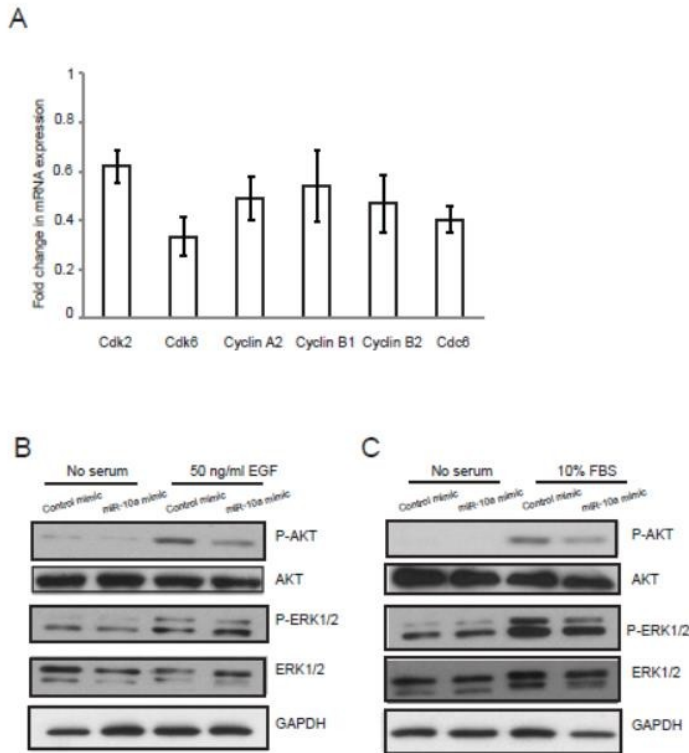


Figure 2-4. Effects of miR-10a on cyclin expression and AKT/ERK phosphorylation.

A) miR-10a mimic expression inhibits the expression of multiple cyclins and Cdks. HASM cells were transfected with scrambled siRNA control or miR-10a mimics for 48 hours, RNA was extracted and analyzed with qRT-PCR to examine the level of different cyclins and Cdks gene expression. Triplicates were used for each condition. **B-C)** miR-10a mimic expression inhibits PI3K-Akt signaling. HASM cells transfected with scrambled siRNA control or miR-10a mimics for 48 hours were first serum starved for 24 hours and then stimulated for 10 min with 50ng/ml EGF or 10% FBS. Lysates from the cells were used to determine the level of AKT and ERK phosphorylation by immunoblotting. Total AKT and ERK levels as well as GAPDH were also determined by immunoblotting using indicated antibodies.

PIK3CA is a direct gene target of miR-10a

We next determined which gene(s) in the PI3K-AKT pathway is the direct miR-10a target that mediates the function of the miRNA. In our microarray experiment and the subsequent qRT-PCR validation (Figure 2- 2B), we found that the *PIK3CA* gene, which encodes the catalytic subunit of class IA form of PI3K, was significantly down-regulated (>60%) following miR-10a mimics expression in HASM cells. To further examine the effect of miR-10a on PIK3CA expression, we re-examined the effect of miR-10a mimics on PIK3CA mRNA along with miR-10a inhibitors. Our result showed that miR-10a mimic expression led to significant down-regulation in PIK3CA mRNA, whereas miR-10a inhibitors caused a modest increase in PI3K mRNA expression (Figure 2-5A). We next used Western blotting to detect PIK3CA protein expression following miR-10a mimic and inhibitor transfection in HASM cells. As shown in Figure 2-5B, miR-10a mimics reduced PIK3CA protein expression by ~60%, whereas miR-10a inhibitor increased PIK3CA protein expression by ~2 fold. These results clearly demonstrated the inhibitory effect of miR-10a on PIK3CA expression. PIK3CA was predicted *in silico* to be a miR-10a target gene, as the 3'-untranslated region (3'-UTR) of the transcript contains a site that is complementary to the seed sequence of miR-10a. To determine if miR-10a suppresses PIK3CA expression by direct targeting of the 3'UTR of PIK3CA, we used a luciferase reporter-based assay. We fused either the wild type or a mutant form (in which we mutated the putative miR-10a target sequence through site directed mutagenesis) of the PIK3CA 3'-UTR to the luciferase gene under the control of a CMV promoter. We then co-transfected the luciferase construct (wild type or mutant) along with either miR-10a mimics or control small RNA into HEK293 cells and measured the luciferase activity. As shown in Figure 2-5C, miR-10a mimic significantly reduced (by ~40%) the luciferase activity of the wild type 3'-UTR construct.

However, this repression of luciferase activity by miR-10a mimics was abolished in cells expressing the mutant 3'-UTR of PIK3CA with changes of two nucleotides in the miR-10a binding site (Figure 2-5C), indicating that the site in the PIK3CA 3'-UTR is required for the inhibitory effect of miR-10a. Together, these data demonstrated that miR-10a directly inhibits PIK3CA expression.

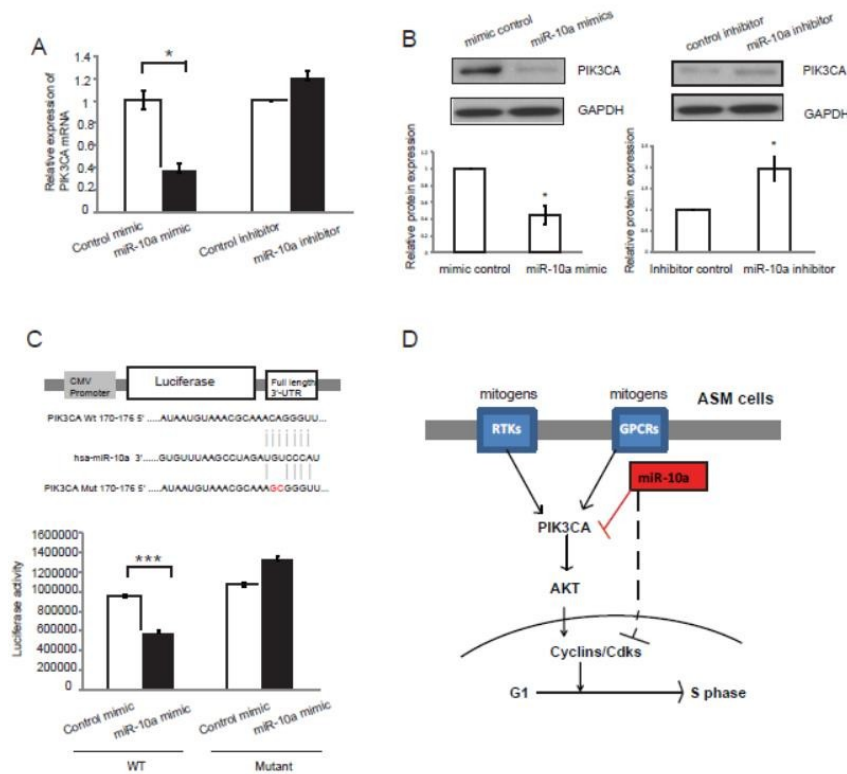


Figure 2-5. Effects of miR-10a on PIK3CA expression.

A) Effects of miR-10a mimics and inhibitor on PIK3CA mRNA expression. RNA was extracted from HASM cells transfected with scrambled siRNA control, miR-10a mimics, control miR-inhibitor, or miR-10a inhibitor and used for qRT-PCR. PIK3CA mRNA level was compared

Figure 2-5. (Continued)

between miR-10 and its control-transfected HASM cells (or between miR-10a inhibitor and its control). Error bars represent standard error (N=3), *, $p < 0.05$. **B)** Effects of miR-10a mimics and inhibitor on PIK3CA protein expression. HASM cells were transfected with scrambled small RNA control, miR-10a mimics, control miR-inhibitor, or miR-10a inhibitor. PIK3CA protein in the transfected HASM cells was detected using immunoblotting. The blots were scanned and PIK3CA protein bands were quantified using the NIH ImageJ software and normalized against GAPDH. Error bars represent standard error (N=3), *, $P < 0.05$. **C)** miR-10a directly targets the 3'-UTR of the PIK3CA gene. Luciferase construct was used to test if miR-10a directly binds to PIK3CA. Full length 3'UTR of PIK3CA was subcloned into the CMV-driven pMir-Target luciferase vector (Origene). A mutant derivative (with two site mutation in the putative miR-10a recognition site) of the construct were made through site-directed mutagenesis. HEK293 cells were co-transfected with the luciferase construct with either miR-10a mimics and control scrambled small RNA for 48 hours, followed by dual-luciferase assays. Error bars represent standard error (N=10), *, $p < 0.05$, **, $p < 0.01$. All statistical comparisons were calculated using two tailed student's t-test. **D)** A model for miR-10a-mediated inhibition on PI3K signaling and ASM proliferation. PI3K mediates the proliferation signals from mitogen-activated RTKs and GPCRs in the ASM. MiR-10a directly inhibits the expression of PIK3CA—the central component of the PI3K pathway—to suppress ASM proliferation.

DISCUSSION

Dysregulated ASM proliferation is a major driver of airway narrowing associated with lung diseases such as asthma. As such, a better understanding of how ASM proliferation is regulated will advance our understanding of lung diseases and may provide mechanistic insights for improving asthma therapy. Using the Next-generation deep sequencing, we identified a highly abundant miRNA (miR-10a) in cultured primary human ASM cells. We demonstrated that miR-10a inhibits the DNA synthesis and proliferation of ASM cells.

Mechanistically, miR-10a modulates ASM proliferation by directly targeting PIK3CA and the associated PI3K pathway. These findings reported here thus revealed a novel miRNA-based mechanism for regulating ASM proliferation (Figure 2-5D). To our knowledge, this study is also the first to experimentally identify a specific miRNA as a regulator of PIK3CA-the central component of the PI3K signaling pathway.

We initially focused on miR-10a largely because of its extreme abundance in ASM cells. While miR-10a is ubiquitously expressed, its expression in ASM is much higher than that in all other tissues including airway epithelial cells. A recent study globally examined miRNA function found that 60% of all miRNAs expressed at relatively low levels exert little effect on gene expression and are thus essentially non-functional [36], suggesting that only miRNAs that are highly abundant can mediate consequential target gene suppression. Moreover, miRNAs exhibiting tissue/cell-enriched expression pattern often play important roles in that tissue or cell type. For example, miR-1a, which is highly enriched and abundant in the heart, regulates cardiac remodeling [26, 37]; miR-122a, which accounts for 78% of all miRNAs in the liver, controls multiple processes critical for hepatic function [38]. Consistent with these studies, our study

demonstrated that the highly abundant miR-10a impacts an important ASM phenotype by inhibiting DNA synthesis and proliferation of ASM cells.

MiR-10a is transcribed from the *Hoxb* locus. Several other miRNAs with similar sequences (miR-10b, miR-99a/b and miR-100) are also transcribed from the vicinity of this *Hox* locus. Interestingly, miR-100 is also among the top five highly expressed miRNAs identified. The *Hoxb* locus is involved in lung branching morphogenesis and may determine cell fate in the lung [39]. In mouse smooth muscle cells, miR-10a is induced by retinoic acid to promote the differentiation of embryonic stem cell into smooth muscle cells [40]. It is conceivable that miR-10a expression increases during smooth muscle development and remains high post differentiation to control the growth of smooth muscle cells.

Previous studies had clearly demonstrated the role of the PI3K pathway in HASM cell proliferation [6, 7, 13]. Our identification of miR-10a as a direct regulator of PI3K provides an additional layer of regulation in ASM proliferation. We established miR-10a as a novel regulator of PI3K signaling and demonstrated that miR-10a inhibits HASM cell proliferation by suppressing PI3K signaling through direct binding to the 3'-UTR of PIK3CA. Interestingly, we note that PIK3CA may not be the only miR-10a target in the PI3K pathway. The cyclin-dependent kinase Cdk6, which is known to regulate cell proliferation through the regulation of cell cycle [32] is also one of the top down-regulated miR-10a target genes. It is possible that miR-10a targets both PIK3CA and Cdk6 to inhibit PI3K signaling (Fig. 5D). At present we cannot exclude the possibility that the observed miR-10a inhibitory effect on ASM proliferation is a combination of inhibition on both PIK3CA and Cdk6. Future experiments aiming to determine the direct targeting of Cdk6 by miR-10a may help strengthen the functional role of miR-10a in regulating the PI3K signaling pathway and ASM proliferation.

PI3K signaling is not the only pathway known to regulate ASM proliferation. Both PI3K/AKT and MAPK/ERK pathways are involved in growth factor-stimulated ASM proliferation [7, 9-11, 13]. Consistent with these studies, our data showed that, upon mitogen stimulation, there is a robust increase in AKT and ERK phosphorylation. Our microarray analysis of miR-10a-transfected HASM cells also showed that multiple genes (MAP3K2, MAP3K7 and MAPBP1) in the MAPK/ERK signaling pathway were down-regulated following miR-10a expression. Because of these results, we also examined the effect of miR-10a mimics on ERK phosphorylation. Despite the fact that multiple genes along the MAPK signaling pathway were down-regulated, miR-10a does not seem to significantly affect MAPK signaling as indicated by ERK phosphorylation. It is possible that the MAP3Ks are several steps upstream of the MAPK signaling pathway and other MAPKKKs may have compensated for the effect of the down-regulation of MAP3K2 and MAP3K7 by miR-10a.

Both MiR-10a and PI3K are highly evolutionarily conserved and are ubiquitously expressed in many other types of cells. We predict that the miR-10a regulation of PI3K play important biological roles in tissues other than ASM proliferation. Indeed, miR-10a has been implicated in multiple cellular processes, such as inflammation, cell differentiation, immune cell function, and tumorigenesis [40-48]. In particular, miR-10a has been implicated in development and cancer, as its expression is frequently altered in several cancers [49] and changes during cancer progression [50-52]. The generation of miR-10a knockdown transgenic mice revealed that miR-10a deficiency increases the frequency of intestine carcinoma [50]. This is highly relevant given that PI3K is one of the most dysregulated signaling pathways in cancer [53]. It is possible that alteration in miR-10a expression may contribute to abnormal PI3K signaling in cancer.

Exploring the miR-10a-PI3K link further will extend our current ASM study and may yield important mechanistic insights into cancer cell proliferation.

In summary, our study established miR-10a as a novel regulator of PI3K signaling and demonstrated that miR-10a inhibits ASM cell proliferation by suppressing PI3K signaling through direct targeting of the central PI3K pathway component (PIK3CA). Our findings raise the possibility of using miR-10a as a novel therapeutic agent to suppress PI3K signaling in order to prevent abnormal cell proliferation including that of the ASM.

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R.H. performed all of the experiments.

W.P. graphed Figure 2-1B.

Q.L. contributed to manuscript editing.

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CHAPTER 3

Effect of asthma treatments on microRNA expression in primary human airway smooth muscle cells

This work is unpublished.

Ruoxi Hu performed all of the experiments for Figure 3-1 to Figure 3-5.

Xiaofeng Jiang performed experiment for Figure 3-6.

ABSTRACT

Asthma is a chronic respiratory disease characterized by airway hyper-responsiveness and chronic lung inflammation. The mainstay therapies for asthma are β -2 adrenergic receptor agonists, which dilate the airway; and corticosteroids, which suppress inflammation. A major limitation associated with these therapies is the widely observed inter-individual variability in drug response. Hence, asthma pharmacogenetics has been an active field examining the association between single nucleotide polymorphisms (SNPs) in the genome and variability in treatment response. However, pharmacogenetic predictions of response to β -agonist and corticosteroid treatments remain unworkable with current genetic variants explaining too little of the overall variation. MicroRNAs (miRNA) are small yet versatile gene tuners that add an additional level of post transcriptional regulation. They are known to play important roles in regulating cellular processes such as cell proliferation and inflammation, which are fundamental biological processes that affect asthma pathogenesis. While miRNAs have been associated with some drug responses, they have not been implicated in asthma therapy. Therefore, we hypothesized that miRNAs are novel asthma drug responsive genetic elements, and they can be combined with future pharmacogenetics studies to better predict asthma treatment response. We used next-generation small RNA sequencing to identify β -agonist and corticosteroid responsive miRNAs. In this study, we examined the effect of albuterol (a β -agonist), dexamethasone (a corticosteroid) and combined treatment (albuterol plus dexamethasone) on the miRNA expression profiles of human airway smooth muscle cells (HASM) from three different donors. We identified around 200 miRNAs in HASM cells among the three donors with similar baseline expression profiles. Surprisingly, we did not detect significant differential expression of miRNAs

under the treatment conditions examined. To test if the treatment conditions were sufficient to induce genome wide transcriptional changes, we found that IL-8, IL-6 and RGS5, genes previously reported to be altered by albuterol, were changed in the same samples. Notably, however, donor to donor variation was high. We also tested several GR responsive genes discovered from a parallel RNA sequencing study looking at the transcriptome effects of dexamethasone conducted by our group and collaborators. CRISPLD2, PTX3, SERPINA3 were induced by ~2-10 fold upon 1 μ M dexamethasone treatment, whereas KCTD12 which is known to contain a negative GRE in its promoter was suppressed by ~80%. In conclusion, our results suggest that albuterol and dexamethasone do not regulate miRNA levels under conditions that are sufficient to induce large mRNA transcriptome changes.

INTRODUCTION

Asthma is a chronic respiratory disease that affects over 300 million people world-wide. It is characterized by airway inflammation, remodeling and hyper-responsiveness [1-3]. Continuous research efforts have offered great insights linking the clinical features of asthma with genetics and novel biological pathways underlying the disease [2]. Based on the molecular understanding of the disease, two mainstay treatments were developed: β 2-adrenergic receptor agonists, which promote airway relaxation; and corticosteroids, which have profound anti-inflammatory effects. β 2-adrenergic receptor agonists are potent bronchodilators that induce cyclic adenosine monophosphate (cAMP) to mediate smooth muscle relaxation [4]. Glucocorticoids exert their anti-inflammatory effect through the activation of glucocorticoid receptors (GRs). Once activated, GRs translocate to the nucleus to bind directly to DNA to modulate transcription or bind to NF- κ B to attenuate NF- κ B-mediated inflammatory gene transcription [5].

These treatments were developed a decade ago and have modest efficacy overall, which is mainly due to the widely observed inter-individual variability in drug response. Hence, asthma pharmacogenomics has been an active area of research in order to develop methods to effectively predict an individual's response to different asthma treatments [6-9]. In the past, the bulk of pharmacogenomics studies have focused on understanding the role of single nucleotide polymorphisms (SNPs) in the genome and mRNA expression level variation. These biomarkers are tested for association with clinical phenotypes such as lung function measurements. In recent years, the discovery of microRNAs (miRNA) furthered our understanding of gene regulation

and opened up a new area of interest to investigate miRNAs as important genetic elements in predicting drug response [10, 11].

miRNAs are small 22-nucleotide long RNA molecules that suppress target gene expression post-transcriptionally. miRNAs function by binding directly to the target's 3'UTR [12-14]. Recent studies have shown that the variations in miRNA expression translate into expression differences of genes that play crucial roles in determining drug responses. This is best demonstrated in cancer therapy where the expression levels of multiple miRNAs are altered by cancer treatments; and these miRNAs, in turn, regulate drug response. Consequently, changes in miRNA expression caused by drug treatment may induce acquired drug resistance [15-17].

miRNAs play versatile roles in normal cell physiology and disease pathogenesis [13]. In the airway, miRNAs play important roles in regulating inflammation: miR-146 is induced by IL-1 β and suppresses NF- κ B activation in alveolar epithelial cells [18]; and inhibition of miR-126 suppresses inflammation and the development of allergic airway disease [19]. In ASM cells, miRNAs also regulate inflammation: several miRNAs (miR-25,-140*, -188,-320) were significantly down-regulated in human ASM cells exposed to inflammatory cytokines such as IL-1 β , TNF- α and IFN- γ [20]; inhibition of miR-25 up-regulates KLF-4, which is a potent inhibitor of smooth muscle-specific gene expression and a mediator of inflammation [20]. miRNAs also regulate the contractility of ASM cells. For example, miR-133a down-regulates RhoA, which is involved in regulating mechanical stress and cytoskeleton organization in the ASM [21]. Notably, microRNA let-7f inhibits β 2-adrenergic receptor, whose activation causes ASM relaxation [22],

Our study employed a synergistic approach by tying together basic miRNA biology and pharmacogenetics to examine the effects of common asthma treatments – β -agonists and

glucocorticoids – on the miRNA expression landscape in order to identify novel drug responsive miRNAs. These drug responsive miRNAs can be used in future association and mechanistic studies to unravel the role of miRNAs in the regulation of β -agonist and corticosteroid responses.

Recent advances in sequencing technologies have made it possible to perform a quantitative and comprehensive survey of small RNAs using next-generation sequencing [23]. Next-generation sequencing utilizes massive cDNA sequencing for direct measuring counts of different small RNA species in a given sample. This new technology offers several advantages over the array based approaches: 1) the detection of small RNA is not limited to the probes printed on the array; 2) unlike the hybridization-based approach, direct sequencing offers single base pair resolution and thus accurate profiling miRNA variants. 3) Lastly, RNA sequencing offers a wider detection range: transcripts highly and lowly expressed are accurately identified [23, 24].

We used next-generation small RNA sequencing to examine the effect of albuterol (a β -agonist), dexamethasone (a corticosteroid) and combined treatment (albuterol plus dexamethasone) on the miRNA expression profiles of human airway smooth muscle cells (HASM) from three different donor cell lines. We identified around 200 miRNAs in HASM cells that have comparable baseline expression pattern among the three donors. Surprisingly, we did not detect significant differential expression of miRNAs under treatment conditions of 1 μ M albuterol, 1 μ M dexamethasone and combined treatment after 18 hours. To test for if the treatment conditions were sufficient to induce transcriptional changes, we found that IL-8, IL-6 and RGS5, genes that were previously reported to be altered by albuterol, were changed, although the donor to donor variation was high. We also tested several GR responsive genes discovered in a parallel RNA sequencing study looking at the poly-adenylated transcriptomic

effect of dexamethasone, which was conducted by our group and collaborators. CRISPLD2, PTX3, SERPINA3 were induced by ~2-10 fold upon 1 μ M dexamethasone treatment, whereas KCTD12 which is known to contain a negative GRE in its promoter was suppressed by ~80%. In conclusion, our result suggests that albuterol and dexamethasone do not regulate miRNA expression under conditions that are sufficient to induce large mRNA expression changes.

MATERIALS AND METHODS

HASM cell culture and drug treatment

Primary HASM cells were isolated from three aborted lung transplant donors with no chronic illness. The tissues were obtained from the National Disease Resource Interchange (NDRI) and their use was approved by the University of Pennsylvania Internal Review Board. HASM cells were maintained and cultured in Ham's F12 medium supplemented with 10% fetal calf serum (FCS) and antibiotics, as described previously [25, 26]. Passages 4 to 7 HASM cells were used in all experiments. HASM cells were treated with 1 μ M albuterol, 1 μ M dexamethasone and 1 μ M albuterol in combination with 1 μ M dexamethasone for 18 hours with corresponding DMSO controls.

Construction and sequencing of TrueSeq small RNA libraries

RNA was extracted with the Qiagen miRNeasy kit using the manufacturer's protocol. RNA concentration and quality was measured by Nanodrop (ThermoScientific) and by Bioanalyzer (Agilent Technologies 2100), respectively. The Truseq Small RNA Sample Preparation kit

(Illumina) was used to construct DNA libraries from the RNA preparations (1 µg total RNA per sample) according to the manufacturer's protocol. Briefly, 3' and 5' adaptors were sequentially ligated to the extracted small RNA species. Reverse transcriptase PCR amplification was performed in which one primer contains a DNA barcode. The amplified product was then gel purified in order to isolate the small RNA library. The library is size-validated using the Agilent Technologies 2100 Bioanalyzer. Three donor cell lines under each condition were pooled together (multiplexing) at equal molar concentrations. Quantification of the DNA library samples was performed by nanodrop (ThermoFisher) or Qubit (Life Technologies). Sequence quality and fragment size in the prepared DNA libraries were verified by running the samples on a 2% agarose gel. Sequencing (single read, 36 bp) of the prepared DNA libraries was done using the Illumina Genome Analyzer IIX platform at the Boston University Illumina Sequencing Core Facility (Boston, MA).

Analysis of deep sequencing data

Data cleaning and alignment were performed using the CLC Genomic Workbench (CLCBio). Raw sequencing reads with an average length of 36 bp in FASTq format were used for 3' adaptor trimming. Trimmed reads were aligned against the human miRNA database (miRBase release 18). miRNA sequencing count tables were generated using the alignment count function of the software, which was set to allow two mismatches. Only perfectly matched counts were used in the subsequent analysis. miRNA differential expression analysis was performed using DESeq in R (Bioconductor) [27]. Data were fitted against the negative binomial distribution for differential analysis [27].

Quantitative RT-PCR of miRNAs and mRNA

miRNAs and mRNAs were measured using the miScript PCR system with miR-10a miScript Primer Assays (Qiagen) on Light cycler 480 (Roche, Indianapolis, IN), following the manufacturer's recommendations. U6 small RNA and beta-actin were used to normalize miRNA and mRNA quantification, respectively. Expression values and statistical significance were calculated using $2^{-\Delta\Delta Ct}$ method [28, 29].

RESULTS

Treatment of albuterol, dexamethasone or combination of the two drugs did not induce significant changes in miRNA expression in HASM cells

To identify β -agonist and corticosteroid responsive miRNAs, we performed next-generation small RNA sequencing on HASM cells treated with 1 μ M albuterol, 1 μ M dexamethasone and 1 μ M albuterol/dexamethasone combined. We obtained around 4-10 million reads from all samples and ~50-90% of reads aligned to miRbase release 18. About 90% of all aligned reads were perfect matches. We performed differential expression analysis using DESeq, which is a statistical package that detects differential expression using RNA sequencing count data [27]. Under the conditions of 1 μ M albuterol, dexamethasone and combined treatment for 18 hours, we did not detect any significant changes between control and the three treatment groups adjusted at a 10% false discovery rate (FDR) (Figure 3-1 to Figure3-3).

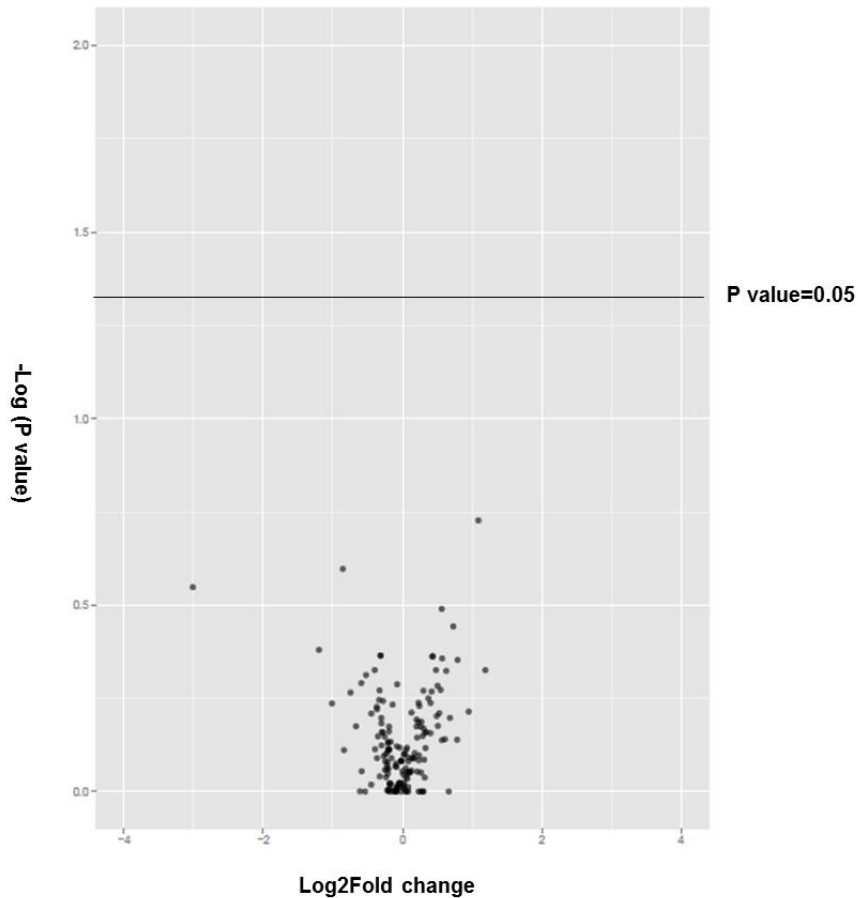


Figure 3-1. Effects of 1 μ M albuterol on miRNA expression in primary HASM cells.

HASM cells from three donors were treated with 1 μ M albuterol. Small RNAs were sequenced using Genome Analyzer II and differential expression analysis was performed using DESeq. Each black dot represents an individual miRNA. Log₂ fold change was calculated based on the normalized sequencing count between control and albuterol treated samples. -Log (P value) was calculated using raw p-values. Most of relative changes in miRNA expression were within two fold and none of these changes reached statistical significance.

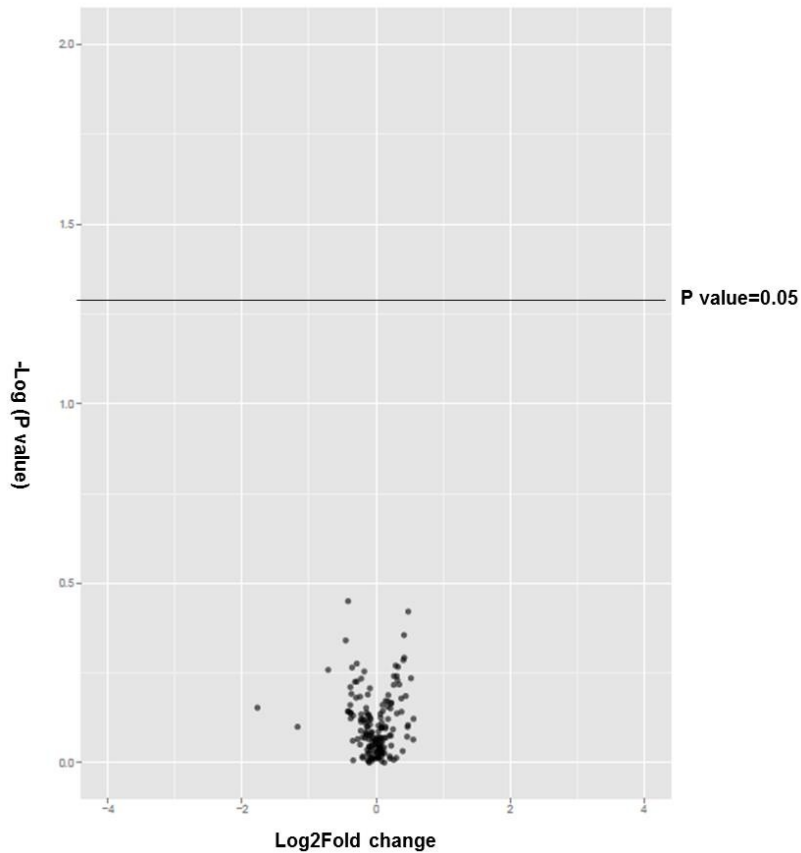


Figure 3-2. Effects of 1µM dexamethasone on miRNA expression in primary HASM cells. HASM cells from three donors were treated with 1µM dexamethasone. Small RNAs were sequenced using Genome Analyzer II and differential expression analysis was performed using DESeq. Each black dot represents an individual miRNA. Log₂ fold change was calculated based on the normalized sequencing count between control and albuterol treated samples. -Log (P value) was calculated using raw p-values. Most of relative changes in miRNA expression were within two fold and none of these changes reached statistical significance.

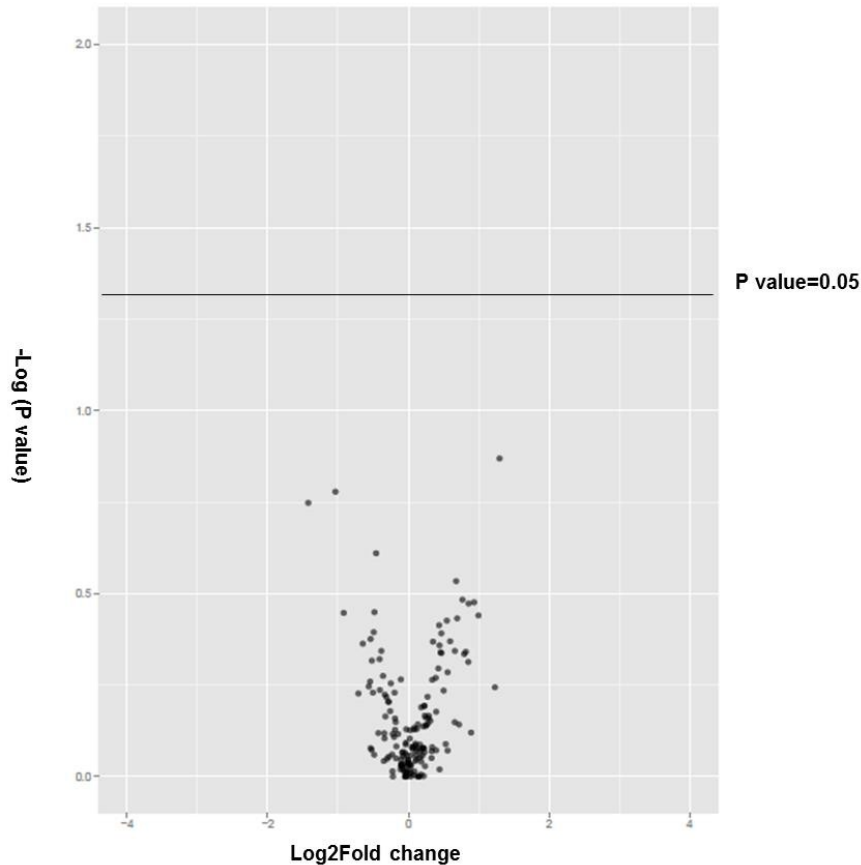


Figure 3-3. Effects of 1µM dexamethasone plus albuterol on miRNA expression in primary HASM cells.

HASM cells from three donors were treated with 1µM dexamethasone and albuterol. Small RNAs were sequenced using Genome Analyzer II and differential expression analysis was performed using DESeq. Each black dot represents an individual miRNA. Log₂ fold change was calculated based on the normalized sequencing count between control and albuterol treated samples. -Log (P value) was calculated using raw p-values. Most of relative changes in miRNA expression were within two fold and none of these changes reached statistical significance.

As shown in Figure 3-1 to Figure 3-3, under all three treatment conditions, most of the changes in miRNA expression are within 2 fold and none of these changes even reached an

uncorrected threshold of $p < 0.05$ before adjusting for multiple comparison at 10% FDR. After we adjusted for 10% FDR, as expected, none of these changes reached statistical significance. To explore what might have contributed to the insignificant miRNA expression fluctuations, we graphed sequencing counts versus fold change for each miRNA (data not shown) and found that most of the changes in miRNA expression occurred at low sequencing counts. In this range, changes are more prone to variation by chance suggesting that the changes were random and most likely were due to error introduced by sequencing count normalization.

We considered the possibility that our experimental design may not offer enough power to detect subtle changes that are around 2-fold given the variations between individual donor cell lines. Therefore, for each condition and donor, we manually set a cut off at 2 fold in both directions (up and down) and selected the miRNAs that were changed more than 2 fold (data not shown). After applying these selection criteria, we realized that none of the changes were consistent among the three donor cell lines; therefore, for downstream validation experiments we selected miRNAs that are changed more than 2 fold in 2 out of the 3 donor cell lines. Interestingly, after we applied the new selection criteria, only samples treated with albuterol had changes that were greater than 2 fold and occurred in 2 out the 3 cell lines (Table3-1).

Table 3-1. Manual selection of miRNA expression changes under albuterol treatment.

Fold change>2					
Donor 1		Donor 2		Donor 3	
	FC		FC		FC
miR-99b	2.6	none		miR-99b	2.6
miR-191	2.2			miR191	2.5
miR-99a	2.3			miR-199	2.2
				miR-4792	2.2
Fold change<2					
Donor 1		Donor 2		Donor 3	
	FC		FC		FC
miR-199a	0.1	miR-199a	0.3	miR-3184	0.5
miR-199b	0.5	miR409	0.4		
miR-190a	0.5				
miR-4792	0.3				

We then performed qRT-PCR on the consistently changed miRNAs (miR-99, miR-199, and miR-191) to validate their changes (Figure 3-4). Figure 3-4 demonstrated that albuterol did not significantly affect the expression of the selected miRNAs, which is consistent with our initial statistical analysis.

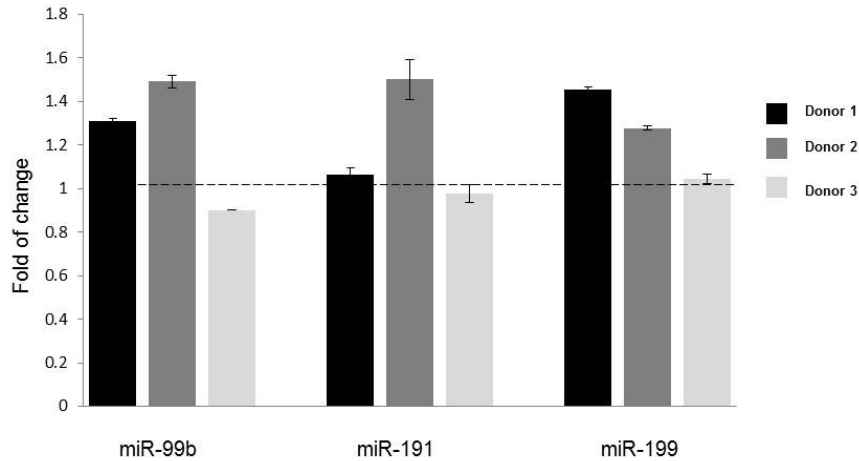


Figure 3-4. qPCR validation of effects of 1 μ M albuterol on miRNA expression.

HASM cells from three donor cell lines were treated with 1 μ M albuterol for 18 hours and RNA was extracted for qRT-PCR analysis of the expression level of miR-99b, miR-191 and miR-199. miRNA expression levels were normalized against U6. Error bars represent standard error (n=3).

Albuterol and dexamethasone induced changes in mRNA gene expression

Another possible explanation for the lack of miRNA expression change could be that the dose used was too mild to produce any change at the transcription level; therefore, we tested the expression of several positive control genes that other studies have reported to change upon dexamethasone and albuterol treatments. For the 1 μ M albuterol treatment, we examined IL-8 and IL-6, which are genes that have a CREB responsive element in its promoter and have been reported to be induced by β -agonists [30, 31]. We also examined RGS5, which is a gene reported to be down regulated by β -agonists [32]. Despite the big cell line to cell line variation, we observed close to 9 fold induction in IL-8 expression, a 1.5-2 fold induction in IL-6 expression and a slight decrease in RGS5. These results suggest the albuterol treatment conditions were

sufficient to cause mRNA level changes; however, the magnitude of change varied significantly between donors (Figure 3-5).

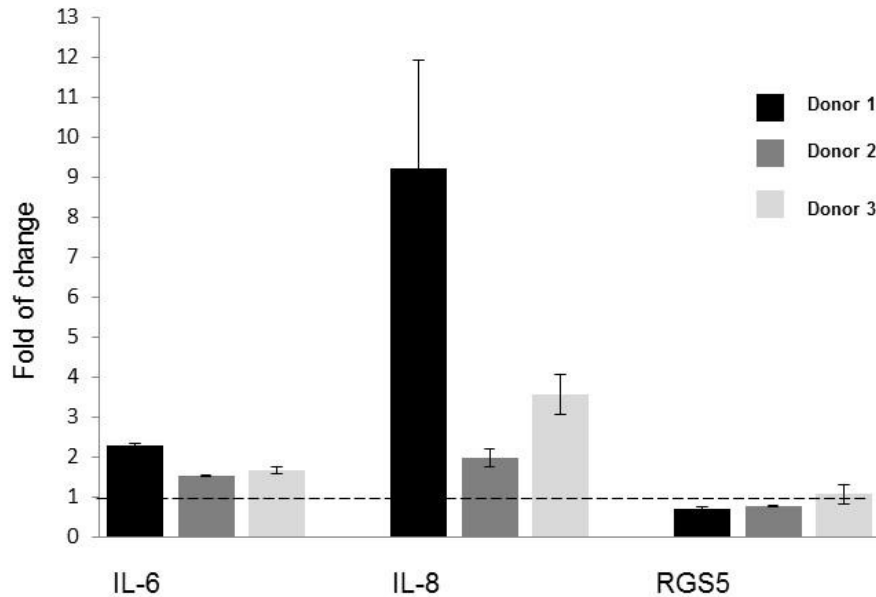


Figure 3-5. Effects of 1 μ M albuterol on mRNA expression.

HASM cells from 3 donor cell lines were treated with 1 μ M albuterol for 18 hours and RNA was extracted for qRT-PCR analysis. mRNA expression was normalized to beta-actin. Error bars represent standard error (n=3).

For dexamethasone, we tested CRISPLD2, KCTD12, PTX3, SERPINA3, and C13orf15, which are top differentially expressed transcripts identified in a parallel RNA sequencing project (Himes *et al.*, submitted). KCTD12, PTX3 and SERPINA3 are canonical GR responsive genes that contain either negative GRE or positive GRE elements in its promoter [33-35]. These genes are known to be either suppressed or induced by dexamethasone treatment. CRISPLD2 and C13orf15 were novel GR responsive genes that were identified through our total RNA

Sequencing project (Himes *et al.* submitted). Similar to the IL-8 finding, we observed big inter-donor variations between cell lines under dexamethasone treatment. CRISPLD2, PTX3, SERPINA3 were induced by ~2-10 fold upon 1 μ M dexamethasone treatment, whereas KCTD12 which is known to contain a negative GRE in its promoter was suppressed by ~80% (Figure 3-6). These data suggest that both 1 μ M albuterol and dexamethasone treatments were sufficient to elicit transcriptional changes at the mRNA level.

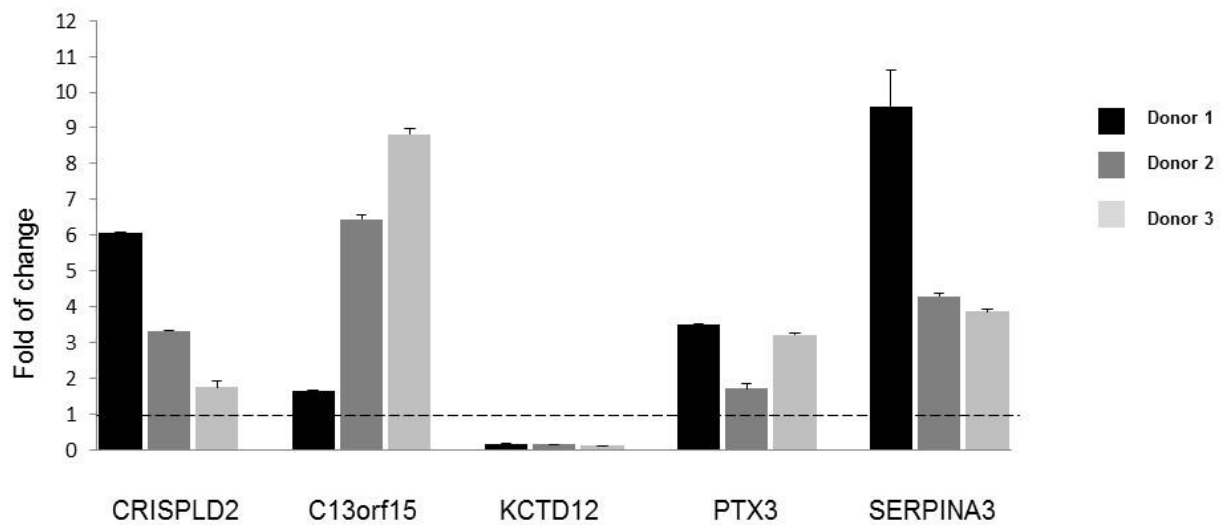


Figure 3-6. Effects of 1 μ M dexamethasone on mRNA expression.

HASM cells from 3 donor cell lines were treated with 1 μ M dexamethasone for 18 hours and RNA was extracted for qRT-PCR analysis. mRNA expression was normalized to beta-actin. Error bars represent standard error (n=3).

DISCUSSION

Individual microRNAs can regulate gene expression by targeting over 100 mRNAs, and thus microRNAs can have vast effects in transcriptome regulation [14, 36, 37]. Although more than 1000 miRNAs have been discovered, the biological functions for a majority of miRNAs remain undetermined [14, 36]. This is especially true regarding the role of miRNAs in the pathogenesis of a complex disease like asthma and the role they play in regulating asthma drug response. Next-generation small RNA sequencing allows for comprehensive and quantitative profiling of miRNAs in a sample. Compared to previous microarray and qPCR based miRNA profiling studies in airway smooth muscle and the lung; this is the first sequencing study of ASM and thus offers better broader coverage of miRNAs in this cell type. Using next-generation small RNA sequencing, we examined the effects of the two mainstay asthma therapies on the microRNA transcriptome in HASM cells.

β -agonists and corticosteroids are both known to have profound effects on gene transcription. Corticosteroids mediate their anti-inflammatory effects through the activation of glucocorticoid receptors (GRs). Once activated, GRs translocate into the nucleus to bind directly to DNA to modulate transcription. GRs can enhance or interfere with NF- κ B mediated gene transcription [38, 39]. β -agonists are potent bronchodilators that activate gene transcription via cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) [30-32]. It is conceivable that when GR and CREB are both activated they interact with each other to modulate both DNA binding and transcription. In our study, 1 μ M treatment of β -agonist, corticosteroid and combined treatment for 18 hours did not cause significant changes in miRNA

expression. However, under the same treatment conditions, we observed significant changes in genes known to be affected by β -agonists and corticosteroids.

The lack of miRNA expression changes is consistent with an existing profiling study that examined the effect of corticosteroids on miRNA expression in the lung, in which miRNA levels before and after a 4-week budesonide (a type of corticosteroid) treatment were compared [40]. Lung biopsy was used to collect airway smooth muscle and 227 miRNAs were interrogated by direct qRT-PCR. No significant differences in miRNA were detected between the before and after the treatment [40]. Compared to the 227 miRNA covered by this previous study, we tested all miRNAs expressed in HASM giving us a much broader picture of miRNA expression. Consistent with the previous study, our findings suggest that miRNA expression is not altered by corticosteroids. Since corticosteroids are known to have profound effects on gene transcription and our parallel mRNA sequencing study indicated the same treatment conditions induced differential expression in 316 mRNAs (Himes et al, submitted), this lead us to speculate that perhaps the control of miRNA processing may have a bigger impact on miRNA expression rather than transcriptional changes of its primary transcript. This theory is consistent with several studies looking at the effect of corticosteroids in lymphocytes. In lymphocytes, corticosteroids were found to suppress several important miRNA processors in the processing pathway – including Dicer, Drosha and DGCR8/Pasha – to cause global repression of miRNAs [41, 42]. Although this effect of corticosteroids on the processing machinery is likely cell type specific and may not be present in HASM cells.

A recent study by Gantier *et al.* determined the half-life of miRNAs at around 5 days, making them more than 10 times more stable than mRNAs[43]. The differences in half-life between miRNA and mRNAs may explain the lack of changes in miRNAs we detected. Perhaps

a longer treatment window would have given observable changes. If an extended time window is needed to observe changes in miRNA expression, then it raises another important question about how miRNAs respond to external stimuli and mediate stress responses if their expression remains stable for days. Perhaps there are other mechanisms (other than expression changes) that allow miRNAs to quickly respond to external cues and mediate their downstream regulation.

In conclusion, our results combined with other studies suggest that unlike mRNAs, miRNAs are much more stable and cannot be easily induced transcriptionally under the treatment conditions we used. It is unlikely that miRNAs comprise novel asthma drug responsive loci for future pharmacogenetic studies.

ACKNOWLEDGEMENT

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CHAPTER 4

Discussion

Summary

The pathophysiology of asthma is characterized by airway inflammation, remodeling and hyper-responsiveness. microRNAs (miRNA) are well recognized important gene fine tuners and they are known to play important role in regulating basic cellular functions such as cell proliferation. The first part of this thesis was aimed to explore the role of miRNAs in the regulation of critical ASM phenotypes and shed lights on potential new asthmatic therapeutic targets. The second half of the thesis was aimed to explore whether miRNAs expression is influenced by conventional asthma treatments.

In chapter 2, we used next-generation sequencing to establish the baseline expression profiles of miRNAs in human airway smooth muscle cells (HASM) followed by functional studies characterizing the role of miR-10a, the most abundant miRNA in HASM cells. Through an unbiased genomic approach, we demonstrated that miR-10a inhibits HASM cell proliferation by inhibiting the PI3K pathway. The significance of this finding is going to be discussed in details below.

In chapter 3, surprisingly, we found that miRNA expression was not changed upon the treatment of conventional asthma treatments (β -agonists and corticosteroids), which both are known to induce transcriptional changes in mRNA expression. Under the same treatment condition, we demonstrated that both treatments induced changes at the mRNA level. This finding suggests β -agonists and corticosteroids do not change miRNA expression. From a microRNA regulation perspective, our result suggest that miRNAs are not transcriptionally inducible and they are regulated differently compare to mRNAs.

The role of miR-10a in regulating airway smooth muscle biology

In chapter 2, we used next-generation sequencing to uncover the baseline miRNA expression profile in primary human airway smooth muscle cells (HASM). We discovered a specifically enriched miRNA, miR10a, whose expression accounts for approximately 25% of all annotated small RNA sequences detected. We then employed an unbiased genomic approach, to assess the potential role of miR-10a in airway smooth muscle cells. We used miRNA mimics as a tool to genetically alter miR10a abundance in HASM cells and whole genome microarray analysis to identify differentially expressed genes resulting from elevated miR10a levels. Gene set enrichment analysis was then performed on the hits to unravel the biological function of miR-10a in HASM cells.

While miR-10a is ubiquitously expressed, its expression in HASM is far higher than in all other tissues tested including airway epithelial cells. A recent study found that 60% of all miRNAs expressed at relatively low levels exert little effect on gene expression and are thus essentially non-functional [1], suggesting that only highly abundant miRNAs can mediate the majority of miRNA-related target gene suppression events. Moreover, miRNAs exhibiting tissue and cell type enriched expression patterns often play important roles in that tissue or cell type. For example, miR-1a, which is highly enriched and abundant in the heart, regulates cardiac remodeling [2, 3]; and miR-122a, which accounts for 78% of all miRNAs in the liver, controls multiple processes critical for hepatic function [4]. Consistent with these findings we discovered that miR-10a regulates HASM proliferation by inhibiting the expression of phosphoinositide 3-kinase (PI3K) and its downstream signaling.

As a follow up study, we have also tested the role of miR-10a in regulating the contractile phenotype of HASM cells. Alpha-smooth muscle actin (α -SMA) is an ASM cell-specific marker

and its expression level correlates strongly with the contractility response of ASM cells in-vitro [5-7]. We used α -SMA as a molecular surrogate for the contractile phenotype of ASM, and have observed that miR-10a inhibitors consistently caused an up regulation of a 2-3 fold increase in α -SMA compared to the inhibitor controls (data not shown). We have also obtained some physiological data suggesting that inhibition of miR-10a led to a 75% greater increase in TGF- β 1 induced ASM contractility as measured by Traction Force Microscopy (data not shown). These preliminary results suggest that miR-10a also regulates the contractile phenotype of HASM through an unknown mechanism. Investigating the molecular mechanism of miR-10a's regulation of contractility could be difficult since the molecular pathways that regulate cell contractility are not as well characterized and multiple parallel pathways appear to control contractility. However, it is interesting to note that a recent study suggests that the activation of AKT1 and its downstream signaling have led to a 6-fold increase in α -SMA expression [8]. This is consistent with our data as it is conceivable that miR-10a also regulates α -SMA expression thorough AKT1 activation by PI3K signaling.

In the future, it will be interesting to characterize the in-vivo function of miR-10a in one of the allergic asthma models. Our in-vitro data suggest that miR-10a is a potential regulator of both the proliferative and contractile phenotypes of ASM, which are the main drivers behind airway remodeling and asthma pathogenesis.

Therapeutic potential of targeting miR-10a to treat asthma

ASM is the critical effector tissue in maintaining bronchomotor tone, and phenotypic changes in ASM play pivotal roles in the pathogenesis of a variety of lung diseases [9]. An increase in ASM tissue mass is a major driver of airway narrowing, which is associated with

asthma [10-13]. The mainstay therapies for asthma mainly involve the use of bronchodilators and anti-inflammatory agents. ASM remodeling remains a symptom not well managed by these conventional therapies [14, 15]. Therefore, the regulatory role of miR-10a in ASM proliferation raises an interesting therapeutic potential for miR-10a targeting.

In a follow up study to explore the possibility that dysregulation of miR-10a levels is involved in asthma pathogenesis, we tested the hypothesis that miR-10a levels is differentially expressed between normal and asthmatic patients. As a pilot study, we used quantitative RT-PCR (qRT-PCR) and examined the expression level of miR-10a in HASM cells from ten healthy donors and ten asthmatics (data not shown). We did not observe significant changes in miR-10a expression between the two groups. The result does not rule out the role of miR-10a regulation in asthma pathogenesis because of several possible reasons including: 1) HASM from asthmatic patients was being cultured in vitro in an environment that lacks inflammatory cytokines and the extracellular matrix environment critical to maintain the asthmatic smooth muscle phenotypes. 2) The ten asthmatic HASM samples were from patients with variable asthma severity, ranging from mild to severe. A detectable difference may be found in a severe asthmatic population. 3) Lastly, just like in any gene expression analysis, there might be big underlying inter-individual variability in miR-10a expression level may mask a trend and necessitate a bigger sample size to detect a significant difference. Because our data did not show any borderline significant changes, the lack of significance result is unlikely to be due to the sample size.

Significance of miR-10a's targeting of PI3K

Our study is the first to experimentally identify a miRNA as a direct regulator of PIK3CA, which is a central component of the PI3K signaling pathway. Both miR-10a and PI3K are highly

evolutionarily conserved and are ubiquitously expressed in many other cell types. We predict that miR-10a regulation of PI3K also plays important biological roles in tissues other than ASM.

With the growing popularity of conducting miRNA expression profiling studies in tumor tissue and cell lines, miR-10a has been implicated in multiple studies on tumorigenesis [16-24]. In particular, miR-10a has been implicated in development of cancer and patient survival; its expression is frequently altered in several cancers including lung cancer, breast cancer and colon cancer, and its expression level correlates with patient survival [25-29]. In a more recent study, miR-10a knockout transgenic mice displayed a greater frequency of intestinal carcinoma [25]. This is highly relevant given that PI3K is one of the most frequently dysregulated signaling pathways in cancer [30]. It is possible that alteration in miR-10a expression may contribute to abnormal PI3K signaling in cancer and thus making miR-10a an attractive drug target for cancer therapy or biomarker for cancer progression. In the future, exploring the miR-10a-PI3K link in different cancer model further will extend the implications of our current study and may yield important mechanistic insights into cancer cell proliferation.

Perspectives in miRNA research

Nearly 97% of the human genome is non-coding and miRNAs are a group of non-coding RNAs that have been intensively studied over the past few year. The biggest obstacle in miRNA research is target identification since one miRNA can have more than 200 predicted mRNA targets. Computational algorithms have been the major prediction tool to identify miRNA target genes. These algorithms rely on the principle that most miRNAs mediate gene silencing via the binding of its seed sequence in the 3'untranslated region (3'UTR) of the target gene [31]. The algorithms typically scan the 3'UTRs of all known mRNAs and populates a list of potential gene

targets with their associated targeting probabilities determined by the level of base pair matching and conservation[32]. It is widely recognized that different algorithms may yield different miRNA-mRNA pairing predictions and a large number of predicted target genes may be false positives or false negatives, which further complicates downstream functional studies. The biggest limitation of relying on computational algorithms is that the predictions are restricted in the 3'UTR; a large proportion of miRNA binding may occur in the coding region of the gene, which is not accounted for in current computational predictions [33]. Lastly, the prediction algorithms do not take into account tissue-specific targeting. It has become clear that miRNAs exhibit both tissue specific expression patterns and their targeting tends to also be highly tissue specific. In our study on miR-10a, we were the first to identify miR-10a's profound effect on PI3K signaling although there have been multiple previous studies on miR-10a linking it to other cell signaling pathways in other cell types [18, 34].

Most recent studies use an experimental-based approach for target identification. Similar to what we did to study miR-10a, transfection of miR inhibitor and mimics followed by high throughput transcriptomic profiling is used. This approach has two serious limitations: first, it cannot differentiate between direct and indirect targets, a careful time course experiment is required to pick up the earliest window for primary change detection. Second, the targets identified through this approach will only include the targets changed at the transcriptional level. Recent progress in high-throughput proteomic studies, such as stable isotope labelling by amino acids in cell culture (SILAC), might offer intrinsic advantages in order to determine the effect of miRNAs on final protein outcome.

Despite of the fact that many miRNAs and their binding sites are highly conserved, which suggests an important biological function, most miRNA effects on target protein

expression is around 2-fold. This is consistent with our observations of miR-10a's effects on PIK3CA and cell proliferation. According to an emerging theory that miRNAs may act as fine-tuning buffers to help the biological system avoid gene expression fluctuations and maintain homeostasis [35]. If this theory holds true, understanding the biological relevance of miRNA is perhaps still in its nascent stages. More functional miRNA studies are needed to help better shape the picture of miRNA-regulated gene networks and to define the big picture biological function miRNA.

It has been over twenty years since miRNA was first discovered in 1993. Despite the growing amount of research in this area since, the cellular functions of miRNAs are just beginning to be uncovered. The rapid development of high throughput and computational tools has facilitated the discovery of many novel miRNAs and has generated a huge amount of in-silico data that have assisted in suggesting biological functions of miRNAs. However, relatively few in silico predictions can be functionally validated using experimental techniques. Therefore, combining computational and experimental approaches would greatly advance the identification of biologically relevant miRNA targets, which is essential for us to better understand miRNA functions and to help us unleash their therapeutic potential.

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Appendix

RNAi screen identifies a novel RNA that regulates β 2-adrenergic receptor expression

This work is unpublished.

The RNAi screen was performed by Hui Pan, Joseph Nabhan and Xiaofeng Jiang.

Ruoxi Hu performed all of the experiments related with hit characterization.

ABSTRACT

The β 2-adrenergic receptor (β 2AR) is a prototypic G-protein coupled receptor (GPCR) that plays a critical role in lung physiology and disease. Agonists of the β 2AR relax airway smooth muscle and are used by virtually all asthma patients to control bronchoconstrictive symptoms. The activation of β 2AR is closely regulated and is attenuated by receptor down regulation, which is believed to be a major cause underlying the loss of efficacy in the prolonged use of β -agonists. Our laboratory has previously performed a genome-wide RNA interference (RNAi) based screen that identified a number of important regulators of β 2AR expression. In this study, we focused on characterizing the top hit from the RNAi screen—AA496068 EST (AAEST), which is a previously un-characterized express sequence tag. We have confirmed the expression of AAEST using RT-PCR and other cloning techniques. We have also established AAEST as an important negative regulator of baseline β 2AR expression in both the screening cell line and physiologically relevant primary airway smooth muscle cells. Overall, our results identify AAEST as a novel RNA regulator of β 2AR expression and suggest a possibility of inhibiting AAEST to improve beta agonist-based asthma therapy.

INTRODUCTION

Asthma is an increasingly prevalent chronic lung disease that affects about 300 million people worldwide [1, 2]. Currently there is no real cure for the disease and most of the treatment options fall into either the rescue or the anti-inflammatory medication categories. All rescue medications are essentially β -agonists that exert their effects by binding to β 2 adrenergic receptors in the lung [3, 4]

The β -adrenergic receptors are members of a family of seven transmembrane receptors that mediates signaling through a heterotrimeric G protein complex. There are three major groups of β -adrenergic receptors: β 1, β 2 and β 3. Compared to other β -adrenergic receptors, the β ARs are primarily found in the lung and are ubiquitously expressed in almost all types of lung cells, such as airway smooth muscle cells, epithelial, and type II cells [3].

Under normal physiological conditions, β 2ARs expressed in the lung are the primary target of catecholamine hormones, such as epinephrine. Binding of agonists to the receptor activates the associated stimulatory G protein, leading to a signaling cascade that includes adenylyl cyclase activation, cAMP formation, and the subsequent protein kinase A (PKA) activation. Activated PKA then phosphorylates multiple downstream target proteins, resulting in reduction of intracellular calcium, ultimately leading to relaxation of airway smooth muscle cells, and bronchodilation [3, 5].

Adopting the physiological function of β 2AR, β -agonists are synthesized and used as a rescue medication to induce bronchodilation during an asthma attack, which is characterized by airway smooth muscle constriction that leads to narrowing of the airways. Activation of β 2AR signaling by β -agonists is usually attenuated by desensitization and down-regulation of the receptor, which are important intrinsic cellular mechanisms that prevent continuous activation of

the signaling pathway and ensures cell homeostasis [5]. Receptor desensitization happens within seconds of β -agonists binding to the receptor: β 2ARs are rapidly phosphorylated by G-protein coupled receptor kinases (GRKs) leading to the recruitment of β -arrestins to the receptor. The interaction between β -arrestins and β 2AR uncouples the activated G proteins and thus effectively turns off the downstream signaling. Prolonged stimulation of β -agonist leads to receptor down-regulation or a loss of functional receptor expressed on the cell surface. β 2AR down-regulation involves clathrin-mediated endocytosis of the receptor. After the receptor being endocytosed, it can either be recycled back to the cell surface, or it can be sorted into the late endosome which will fuse with lysosome, leading to receptor degradation. A key determinant of this process is receptor ubiquitination, which is mediated by an ubiquitin E3 ligase and an adaptor protein [3, 5].

β 2AR down-regulation which leads to permanent loss of receptors on the cell surface can have significant clinical implications especially for severe asthma patients that are regularly treated with long acting β -agonists [6]. The reduced receptor expression can lead to reduced smooth muscle cell relaxation and thus limit the efficacy of β -agonists therapy. Many adverse events could result because of decreased efficacy of treatment such as loss of asthma control and longer duration of exacerbation. Multiple pharmacogenomics association studies also demonstrated the importance of β 2AR receptor regulation [6, 7]. Single nucleotide polymorphisms that display accelerated receptor degradation in vitro are shown to be associated with altered sensitivity of drug treatment. Because β AR regulation plays central role in determining the clinical response of β -agonists, it is critical to fully understand the molecular mechanism underlying the regulation of baseline β 2AR expression and receptor down-regulation.

Genome-wide functional screen offers a powerful approach for unraveling the molecular basis of human diseases and biological processes [8, 9]. RNA interference (RNAi) based gene

silencing relies on the potent and specific gene suppression activity exhibited by small interfering RNA (siRNA) of 19-21nt or by short-hairpin RNAs [8]. The use of RNAi libraries, together with phenotype based functional assays has led to the identification of genes critical for a variety of biological processes, including cell growth, senescence and tumor development[10-12]. Application of a powerful RNAi based screen offers big potential to uncover novel genetic regulatory pathways that are important in the regulation of β 2AR expression and down-regulation.

To explore novel regulators of surface β 2AR levels, we have developed a highly efficient genome-wide RNAi library covering approximately 80% of the entire human genome [13]. Using this RNAi library, together with a robust fluorescence-activated cell sorting (FACS)-based assay for measuring β 2AR amount, we have conducted a surface β 2AR level screen [13, 14]. The highly efficient screen revealed 11 novel genes, which have not been shown to play a role in the regulation of β 2AR [13]. In this study we characterized AAEST which is the top hit from our RNAi screen. Our results identified AAEST as a novel non-coding RNA that suppresses the baseline expression of β 2AR.

MATERIAL AND METHOD

Mammalian cell culture and transfection

Human embryonic kidney HEK293T and 293 β 2AR cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen), 4 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The HEK293 cells stably expressing N-terminal Flag-tagged β 2AR (293 β 2ARWT) and stably expressing N-terminal Flag-tagged recycle-deficient β 2AR (293 β 2AR) were kindly provided by Dr. Mark von Zastrow (UCSF). Primary human airway smooth muscle (HASM) cells were maintained in Ham's F12 medium with 10% fetal calf serum (FCS) and antibiotics, and grown in DMEM supplemented with 1% FBS for isoproterenol(ISO) treatment. Passages 4 to 7 HASM cells were used in all experiments. Transfection of plasmid DNA into 293 β AR cells was performed using TurboFect reagent (Fermentas). Transfection of siRNA was done using DharmaFECT1 reagent (Thermo Scientific). Non-targeting control siRNA and AAEST siRNA were purchased from Sigma-Aldrich.

Flow cytometry analysis

siRNA transfected 293 β 2AR cells (5×10^6) were cultured for 1 day in Opti-MEM (Invitrogen) containing 1%FBS and 10 μ M ISO for 16h to induce the down-regulation of β 2AR. Cells were washed with PBS and incubated with diluted TrypLE Express Enzyme (1:10 dilution in PBS; Invitrogen) for exactly 1 min at room temperature before inactivation with complete medium. Cells were washed once with PBS containing 1%PBS to remove residual traces of TrypLE. Cells (1×10^7) were suspended in 1ml PBS containing 1%FBS and stained with 10 μ g/ml FITC-conjugated anti-Flag M2 antibody (Sigma-Aldrich) on a rotary mixer for 30min. Cells were

washed again and passed through 50- μ m cell strainer to remove cell clumps before being measured using flow cytometry analysis on a FACS Canto II system (BD Biosciences). During flow cytometry analysis, gating was adjusted to measure the FITC (green) signal. Background auto fluorescence was identified by plotting FITC with SSC (side scatter) signal.

Immunoblotting

Transfected cells were washed with PBS and lysed with Nonidet P-40 lysing buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, and 0.5% Nonidet P-40) containing protease inhibitor (Roche).

Protein samples were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad). Immunoblot signals were developed using SuperSignal West Pico/Femto Chemiluminescent Substrates (Pierce) and using X-ray films. Antibody used were β 2AR (H20) (Santa Cruz), HA antibody (Sigma).

3' and 5' RACE (rapid amplification of cDNA ends)

3' and 5' RACE were performed using manufacturer's recommended condition with a kit purchased from Invitrogen. In brief, for 5' RACE, first strand synthesis was performed using gene specific primer (GSP) 92R, followed by dc tailing on the cDNA at the 5' end. The product was further amplified with a nested GSP 114R and the commercial AAP primer for 30 cycles and lastly, the PCR product was enriched by another round of nested PCR reaction using primer 97R and kit provided AUAP primer (30 cycles). For 3' RACE, first strand synthesis was performed using kit provided AP primer, followed by a round of PCR with the primer set 109F and AUAP (30 cycles), the PCR product was further enriched by another round of nested PCR with primer set 110F and AUAP (30 cycles). End PCR products were resolved on a ethidium

bromide stained 1.5% agarose gel at 100 volts for 30 min. DNA bands were cut off and purified using QIAquick gel extraction kit (Qiagen). All sequencing was performed at Genewiz.

Small RNA cloning

Total RNA were extracted from 293 cells using Trizol (Qiagen). RNAs were ligated with customer made adaptors on 3' and 5' adaptor (Epicentre).and PCR amplified for 30 cycles. Amplified DNA products were resolved on SDS-PAGE gel (Invitrogen) in TAE buffer (Invitrogen). Corresponding DNA bands were cut off, purified and sequenced (Genewiz).

Northern blot

Non-radioactive northern blot was performed using DIG-labeled probe system, DIG Northern Starter Kit, according to manufacturer recommended protocol. (ROCHE).

RESULTS

RNAi-based screen identifies AAEST as a novel regulator in β 2AR expression

Our lab has developed a novel shRNA based RNAi library that contains an estimated 600,000 individual shRNA-expressing lentiviral constructs targeting about 28,000 human genes (average over 20 different shRNAs per gene) [13]. We performed the RNAi screen on β 2AR regulation using a mutant HEK293 cell line (PD Cells), in which the β 2AR is stably expressed and can undergo efficient down-regulation upon agonist isoproterenol (ISO) stimulation [13]. In this cell line, the β 2AR is N-terminally Flag-tagged and thus can be easily detected in flow cytometry using anti-FLAG antibodies. This flow cytometry-based assay offers a highly quantitative and efficient way to isolate cells with altered amount of surface β 2AR. PD cells were transduced with a high-titer shRNA lentiviral library and a total of four rounds of FACS (fluorescence activated cell sorting) were performed to isolate cells that contain higher levels of cell surface β 2AR. The FACS-enriched cells, displaying a higher amount of surface β 2AR, potentially contain shRNAs that inactivate genes critically involved in β 2AR regulation. To identify the corresponding shRNAs, we extracted genomic DNAs from these cells and performed genomic PCR [13]. 100 clones were sequenced and the gene hits are summarized below (Appendix-table-1). Most of the shRNAs target known annotated genes. However, strikingly, 34 of the 100 sequenced shRNA clones correspond to AAEST, which is an expressed sequence tag that maps to a region on chromosome 1. The AAEST region of the genome contains no annotated gene.

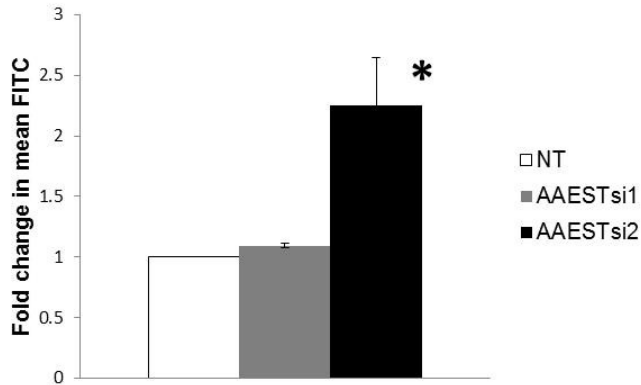
Appendix-table 1. Top hits identified from β 2AR RNAi screen

Gene	# of shRNA clones	Description
ARRDC3	2	arrestin domain containing 3 (Nabhan <i>et al.</i>2010)
FDPS	2	farnesyl diphosphate synthase (Jiang <i>et al.</i> 2012)
AA_EST	34	intergenic region
AI075761	3	Putative lincRNA
LRP5	2	low density lipoprotein receptor-related protein 5
TPPP3	2	tubulin polymerization-promoting protein family member 3
CaMKK2	2	Calcium/calmodulin-dependent kinase kinase 2 (beta)
BZW1L1	1	Basic leucine zipper and W2 domains 1 like 1 , mRNA; Hs.704590
CAMKK2	1	calcium/calmodulin-dependent protein kinase kinase 2, beta
DPM2	1	dolichyl-phosphate mannosyltransferase polypeptide 2
FBXO44	1	F-box protein 44

AAEST knockdown increases β 2AR expression

We next validated the gene hit AAEST from the previously described shRNA-based RNAi screen using a different method of gene knockdown. We used two chemically synthesized siRNAs targeting two different regions of AAEST (AAESTsiRNA1 and AAESTsiRNA2). PD cells were transfected with the siRNAs along with a non-targeting siRNA control for 72 hours. The cells were then labeled with anti-FLAG-FITC and the cell surface β 2AR (FLAG-tagged) amounts were quantified using flow cytometry analysis (signal is typically represented as mean FITC). The fold change in mean FITC in four different experiments was averaged and is shown below in Appendix- figure-1. Cells treated with AAEST siRNA2 showed a two-fold increase in

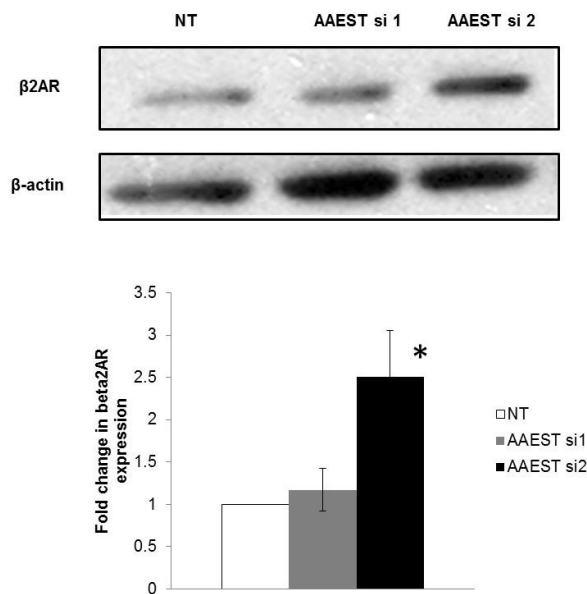
surface β 2AR amount, indicating that AAEST is functioning as an inhibitor of baseline β 2AR expression. Only siRNA 2 had an effect on surface β 2AR, this likely because of siRNA 1 targeted a stretch of duplicated region which has high similarity with a genomic sequence on chromosome 15.



Appendix-figure 1. Effect of AAEST knockdown on surface β 2AR expression. PD cells were transfected with non-targeting siRNA (NT), AAEST siRNA 1 (AAEST si1) or AAEST siRNA 2 (AAEST si2) for 72 hours. Cells were stained with anti-FITC antibody and analyzed with flow cytometry. Error bars represent standard error (N=7). * represent significance compared with student t-test (P<0.05).

We further determined the effect of AAEST knockdown on endogenous β 2AR expression in primary human airway smooth muscle cells (HASM), which are the physiological target of β -agonists. HASM cells were transfected with the two previously described AAEST specific siRNAs and the total endogenous β 2AR protein amount was measured by Western blotting. Consistent with the results obtained from PD cells, cells treated with AAEST siRNA2 displayed the greatest increase in total β 2AR (Appendix-figure-2). About two-fold increase in

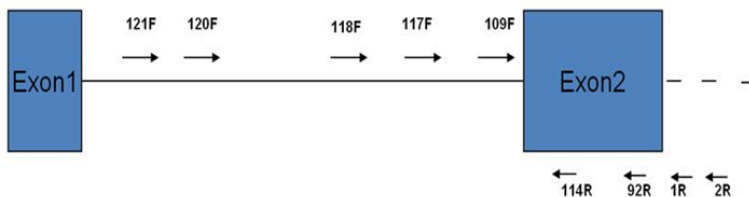
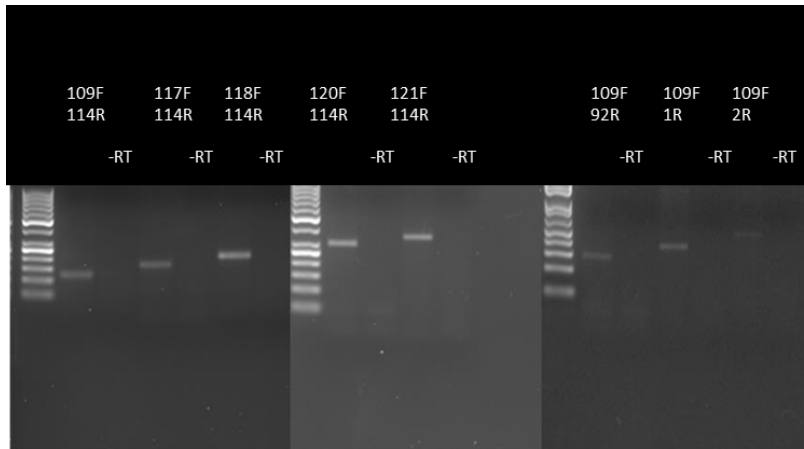
β 2AR expression was observed in AAESTsi2-transfected HASM cells. A representative immunoblot is shown below (Appendix-figure-2). qRT-PCR was also performed to measure the mRNA level of β 2AR. We found no significant difference in β 2AR mRNA levels between the wild type and knockdown cells, suggesting the effect of AAEST on β 2AR expression is not through transcriptional regulation (data not shown).



Appendix-figure2. Effect of AAEST knockdown on total β 2AR protein expression in HASM cells. HASM cells were transfected with non-targeting siRNA (NT) , AAEST siRNA 1 (AAEST si1) and AAEST siRNA 2 (AAEST si2) for 72 hours. Cells lysates were extracted and analyzed with immunoblot with anti- β 2AR antibody. Protein amount was quantified using Image J. Error bars represent standard error (N=4). * represent significance compared with student t-test (P<0.05).

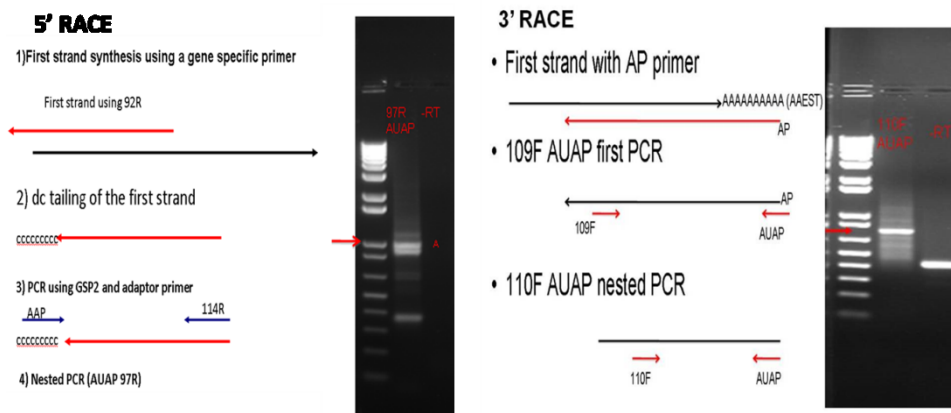
Cloning of the full length transcript of AAEST

AAEST is an unannotated expressed sequence tag, which is likely to be a part of the larger RNA transcript. We next examined the expression of the EST and attempted to clone the corresponding full-length transcript. We first used RT-PCR with multiple primers covering both the predicted intron and exon sequences (based on sequences from UCSC genome browser) to test whether AAEST is expressed in PD cells. Appendix-figure-3 is a sample RT-PCR result with multiple primers covering both predicted intron and exon sequences. All combinations of primers yielded specific amplification. This result not only confirms the expression of AAEST, but further suggests that the predicted sequence that includes AAEST is incomplete and is likely to constitute a much bigger transcript.



Appendix-figure 3. RT-PCR of AAEST. RNAs were extracted from PD cells and amplified using RT-PCR with multiple primer sets spanning cross predicted intron and exon junction of the AAEST sequence.

To further confirm the expression of this transcript, we used quantitative RT-PCR to compare the expression level of this transcript in regular reverse transcribed total RNA, reverse transcribed total RNA treated with RNase I and a without reverse transcriptase reaction control. Treatment of RNA with RNase I completely brought the signal from AAEST transcript to its background signal (data not shown). This result further confirmed the expression of AAEST transcript. We next performed 5' and 3' Rapid Amplification of cDNA Ends (RACE) to determine the 5' and 3' ends of the AAEST encoding transcript. All major amplicons from the reactions were sequenced. The red arrows indicate amplicons corresponding to the specific AAEST sequence (Appendix-figure 4). Combining results from the RACE reactions, we have successfully cloned the full-length 1.85Kb transcript, which includes the AAEST sequence initially identified from the RNAi screen.



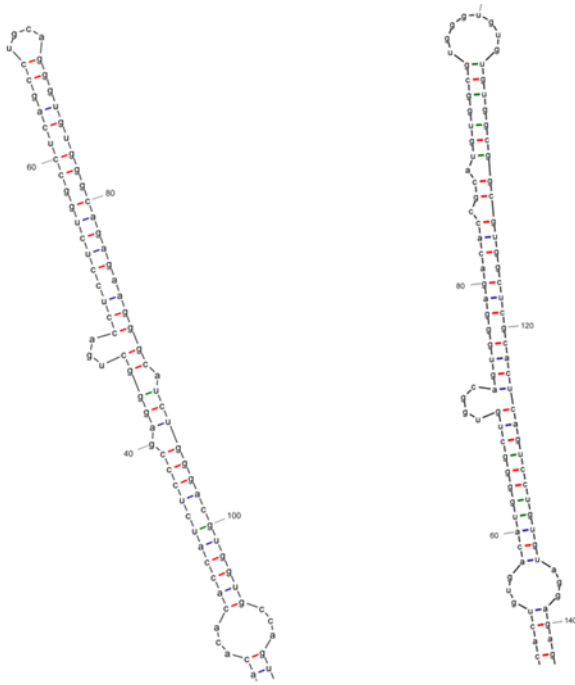
Appendix-figure 4. 5' and 3' RACE of AAEST. RACE experiments at both ends were performed to identify the full length transcript of AAEST. Corresponding bands were cut off and sequenced.

AAEST is unlikely to encode a protein or peptide

Based on our preliminary data, a sequence analysis of the full-length transcript was performed to determine the nature of the transcript, which could potentially provide hints to determine the mechanism of regulation. Open reading frame (ORF) analysis revealed the biggest ORF is 579bp long (start with AUG), which can encode a 22KDalton protein which does not have any homology to known proteins. To test if the ORF is translated, we subcloned the ORF sequence with HA tag into a pCDNA 3.1+ vector and expressed HA-ORF with a positive control HA tagged protein (HA-ARRDC1). HA-ORF was not detected suggesting the ORF is not translated into a protein (data not shown).

AAEST RNA does not encode microRNA

Next, we tested the hypothesis that the AAEST transcript is a non-coding RNA. A secondary structure analysis was performed using the mfold software. Secondary structure analysis revealed that the transcript forms two very stable stem loops on the 5' end, reminiscent of most primary transcripts of microRNA (miRNA) (Appendix-figure 5). Prediction of mature miRNA sequences from the putative stem loops revealed potential seed sequences that are complementary to the mRNA of β 2AR. Most human miRNAs are known to function by inhibiting the translation of their target transcripts [29-31]. Given the secondary structure analysis and our qRT-PCR result that knocking down AAEST does not affect the mRNA level of β 2AR, we hypothesized that AAEST may function as a primary transcript of a novel miRNA, which negatively regulates β 2AR expression through translational repression.

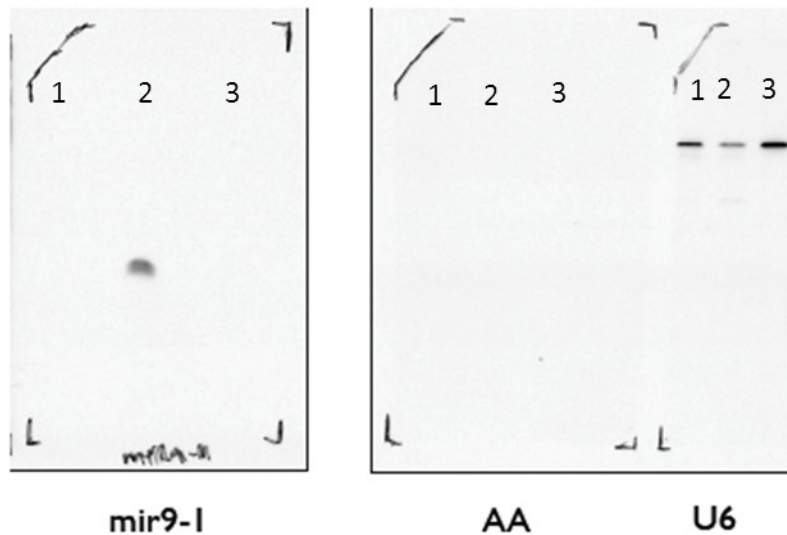


Appendix-figure 5. Predicted stem loop structure of AAEST. AAEST sequence was put into mfold for secondary structure analysis. SL-1 (left) and SL-2 (right).

To test if AAEST is a primary transcript of a miRNA that inhibits β 2AR expression, we transfected PD cells (HEK293 cells expressing Flag- β 2AR) with two plasmids containing the predicted stem loop sequences, SL1 and SL2, both of which could function as precursor miRNAs and also a full length AAEST to reverse the AAEST knockdown phenotype. The SL1, SL2 and full length sequences were subcloned into PCDNA3.1+, which has been previously demonstrated to be suitable to over-express miRNAs [32-34]. Cells were transfected independently with plasmids encoding SL1, SL2 and full length AAEST to test which predicted stem loop functions as a precursor miRNA. The transfected cells were assayed by anti-FLAG immunoblotting (data not shown) and FACS analysis for the total and surface amount of β 2AR. An empty plasmid and miR-9 plasmid were included to serve as a transfection control. None of the overexpression was

able to rescue the knockdown effect of AAEST on β 2AR expression, suggesting neither SL1 nor SL2 are functional forms of AAEST and the full length we have cloned might be incomplete.

We next used northern blot with probe targeting the predicted miRNA to detect its expression. We had probes designed for both SL1 and SL 2. MiR-9 was used as a positive control and U6 was used as a loading control. There was no detection of miRNA encoded by the predicted sequence. Appendix-figure 6 shows a representative blot for SL-1 probe.



Appendix-figure 6. Northern blot analysis of AAEST transcript encoded miRNA. Lane 1 was loaded with a mixture of human kidney, lung and trachea total RNA. Lane 2 was loaded with human brain total RNA. Lane 3 was loaded with 293T total RNA. Probe targeting miR-9, U6, and AAEST-SL1 was used for small RNA detection.

Since miRNA detection using northern blot depend heavily on the expression level of such RNA; therefore, we used 4 different cloning strategies to clone AAEST encoded miRNA

using a PCR based method. In order to take into consideration of the different efficiency in miRNA processing, from its precursor form to the mature form, we have designed multiple primers to cover both forms. Appendix-table 2 summarizes the cloning efforts as well as the different positive controls that were used for each experiment. As shown in Appendix-table 2, we were unable to clone any mature miRNA or miRNA precursors encoded by the AAEST transcript while all the positive controls were successfully cloned. In the deep sequencing of small RNAs in Chapter 3, we did not detect a small RNA or microRNA that originates from the AAEST RNA locus. Taken all the data together, we concluded of AAEST encodes neither a protein nor a miRNA, and thus likely is a novel long non-coding RNA.

Appendix-table2. Summary of cloning strategies used to clone AAEST encoded miRNA

Cloning strategy used	Positive control used
Ligation of 5' adaptor (Epicentre, WI) + AAEST sequence specific primer	miR-21
Ligation of 3' poly-A tail (Epicentre, WI) + AAEST sequence specific primer	miR-21
Ligation of 3' adaptor (Qiagen) + AAEST sequence specific primer	let 7 Pre-Let7
Ligation of a pre-activated 3' linker (NEB) + AAEST sequence specific primer	miR-21 Pre-miR-21 Let7 Pre-Let 7

DISCUSSION

We performed a genome wide genetic screen to identify genes whose inactivation leads to impaired β 2AR down regulation using a novel RNAi platform combined with a robust flow cytometry based β 2AR receptor quantification assay. Our screen has identified many genes that have not been previously recognized to play a role in the β 2AR down regulation process, thus

revealed many novel mechanisms of β 2AR regulation. Several genes from the top hit list was successfully confirmed and characterized [13, 15]. In this study, we focused on characterizing the top hit from the screen- AAEST, an expressed sequence tag. Our results have established that AAEST is a novel transcript and this previously uncharacterized transcript plays an important role in inhibiting the baseline expression of β 2AR. Moreover, we have confirmed the inhibitory effect of AAEST on β 2AR in primary human airway smooth muscle cells, which is a cell type that is more physiologically relevant and directly controls bronchoconstriction. We also performed mechanistic studies to unravel how AAEST regulates β 2AR expression. Our ORF experiment suggested that the predicted ORF encoded by AAEST was not translated and expressed. We also tested the possibility of AAEST gets processed into small RNAs to function as a miRNA and the various cloning results demonstrated it is unlikely that AAEST is processed into small RNAs. Hence, we have excluded the possibility of AAEST being a coding transcript and miRNA, we conclude that AAEST is likely to function as a long non-coding RNA that negatively regulates baseline β 2AR expression.

β 2AR expression level directly affects the efficacy of β -agonist based bronchodilator therapy; therefore, many past studies focused on dissecting the molecular mechanism of agonist induced receptor down regulation which involves receptor ubiquitination, internalization and lysosomal degradation [16-18]. Recently, RNA regulated protein expression has been a popular area of study. Non-coding RNAs has been shown to have profound effect on gene transcription and protein translation [19]. Within non-coding RNAs, miRNAs are predicted to regulate half of the mammalian genes [20]. Distinct from the traditional notion of agonist induced receptor phosphorylation and ubiquitination, Let7f miRNA was the first non-coding RNA shown to regulate baseline β 2AR expression through the binding of its seed sequence to the 3'UTR of

β 2AR, which represents a novel mechanism of how β 2AR protein expression is regulated [21]. Our study again provides another line of evidence highlighting the importance of non-coding RNAs on the regulation of baseline β 2AR expression. Our finding is the first study showing evidence of a long non-coding RNA's regulation of β 2AR; however, the mechanism of how this long non-coding RNA regulates β 2AR protein expression remains elusive.

In the future, it is important to define and confirm the full length transcript of AAEST. Although we used the classical 5' and 3' RACE experiment to define the ends of AAEST transcript, there remains the possibility that this transcript extend further to the previously defined region since the 5' end of AAEST genomic sequence contains a long repetitive sequence that is AT rich which could have interfered with our RACE efficiency. A northern blot experiment will be very useful to confirm the full length of this transcript and examine if there are multiple splice variants of AAEST. However, we think the possibility of AAEST having multiple splice variant is low since only the predominant band from the RACE experiment turned out to match the genomic sequence of AAEST. For follow up mechanistic studies, we believe a microarray experiment on control and AAEST knockdown samples will be very useful to examine the downstream consequence of AAEST's regulation on β 2AR and test if this novel non-coding RNA is a direct regulator of β 2AR expression. In addition to AAEST, we have identified several other new regulators of β 2AR down-regulation. However, we did not identify any of the bona-fide regulators of β 2AR down regulation such as β -arrestin and NEDD4 E3 ligase. This may be partly due to we sequenced only a partial fraction of the sorted cell population from the screen; therefore, sequencing the entire sorted cell population using the increasingly affordable deep-sequencing technology should help us to uncover more genes and reveal more insight into the regulators of β 2AR down regulation.

β -agonist is a mainstay therapy for asthma and there are several limitations to this treatments such as chronic use of long acting β -agonists in severe asthmatic population causes receptor desensitization and down regulation which as a result significantly reduces the efficacy of this treatment. Second, there is significant inter-individual variability in drug response among patients across all levels of severity, which is most likely due to variability in the receptor expression and signaling regulation. Therefore, reduction in functional β 2AR numbers can directly cause loss of bronchodilator effect of β -agonists and result in longer duration of asthma exacerbation. Targeted inhibition of genes regulating β 2AR baseline expression and down regulation provide a novel approach to improve the efficacy of β -agonists in asthma treatment. Our finding on AAEST along with other genes uncovered by the RNAi screen provided the basis for future in-vivo studies to examine the effect of these genes on improving the efficacy of β -agonist based therapy.

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