University of Nevada, Reno

miR-25 expression inhibits proliferation and supports a healthy contractile phenotype in a mouse model of allergic inflammation

Senior Thesis in the Biochemistry and Molecular Biology Major

By

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Abstract

Asthma is a respiratory illness that can be primarily characterized by airway remodeling, airway hyperresponsiveness, and inflammation. While current treatments help to ease symptoms, they do little to treat these underlying causes of asthma. This results in poorly controlled symptoms and raises the need for novel therapeutic targets. One such potential target is miR-25, a microRNA demonstrated in human airway smooth muscle cells to inhibit key cell division pathways. This study aims to test the hypothesis that miR-25 regulates airway smooth muscle phenotypes in a mouse model of allergic inflammation. To investigate the role of miR-25 in proliferation, tissue samples were obtained from second and third generation of transgenic mice in which miR-25 overexpression was targeted to smooth muscle cells (TgSM-miR-25) and wild-type (WT) mice treated with ovalbumin or an adjuvant control. Protein samples from tissues were subjected to Western blot analysis using proliferating cell nuclear antigen or cyclin D1 antibodies. To determine the effects of miR-25 on contractile protein expression, blots from the above experiment were re-probed with smooth muscle myosin heavy chain and smooth muscle myosin heavy chain. In lung tissue from ovalbumin-treated TgSM-miR-25 mice, proliferating cell nuclear antigen expression was significantly decreased compared to the wild-type group. Additionally, smooth muscle myosin heavy chain and smooth muscle-α-actin protein levels were significantly increased in the same tissues. Together these data provide evidence that miR-25 mediates airway smooth muscle phenotype in mice by inhibiting airway smooth muscle cell division and contributed to the development of

a contractile phenotype. Thus, miR-25 may be a novel therapeutic target for the development of new asthma therapies.

Introduction

Asthma is a respiratory illness marked by coughing, wheezing, and shortness of breath (1). Currently, there are two types of medication that are used to treat asthma symptoms; bronchodilators that relax constricted airway smooth muscles to open up a closing airway and corticosteroids which lead to reduced inflammation (2). However, these treatments fail to address airway remodeling, an imperative hallmark of the disease in which the airway undergoes structural changes such as increased airway smooth muscle (ASM) cell division, neovascularization and epithelial alterations (3,4). This causes many asthmatics to have poorly controlled symptoms and for the disease to worsen over time as their airways continue to thicken (1,4). It is therefore necessary to develop new therapies for treating asthma that target both the recurring symptoms of wheezing and difficulty breathing, as well as arresting airway remodeling

One potential direction in the development of new therapies for asthma is through targeting regulation of gene expression (2). A key regulator of post-transcriptional regulation is microRNA. These small, noncoding RNAs down-regulate gene expression at the transcriptional level and have been shown to have a wide range of roles, including modulating proliferation of cancer cells, providing immunoprotective functions in infants, and triggering cell death (4-7). Although little is known about microRNA expression in ASM, miR-25 has been shown to target Krüppel-like factor 4 (KLF4), a transcriptional regulator of proliferation and a key component in the airway's inflammatory response (4).

Studies have previously demonstrated that miR-25 plays a significant role in modulating ASM phenotype, potentially through down regulating KLF4 expression, thereby leading to decreased extracellular matrix turnover and modulated contractile phenotype (4). These changes in ASM phenotype can be measured through the expression of protein markers for proliferation, such as proliferating cell nuclear antigen (PCNA), a processivity factor needed for DNA replication, and cyclin D1, a protein needed to progress through the G1 phase of cell division (8,9). In this study, it was predicted that in tissue with an asthma-like phenotype and miR-25 overexpression, PCNA and cyclin D1 expression will be decreased, thereby indicating decreased ASM proliferation. Additionally, in order to further investigate miR-25 involvement in regulating contractile phenotype, this study aim to measure expression of key smooth muscle contractile proteins such as myosin heavy chain (sm-MHC) and α-actin, which expected to increase with miR-25 overexpression (4). This increase in contractile expression would indicate a more mature contractile response in ASM that combined with a reduced proliferative response, would support a role for increased miR-25 expression in the airway as a potential pathway for relieving asthmatic symptoms.

Materials and Methods

Animal and Allergy Challenge

Mice were characterized as either wild-type (WT) or transgenic for targeted smooth muscle miR-25 overexpression (TgSM-miR-25). To produce an asthmatic-like phenotype, mice were first sensitized with 50ug ovalbumin (OVA) intraperitonically on Day 1 through Day 14. On Day 21, mice were challenged intratracheally with 100ug OVA. As a control group, mice were treated with an alum adjuvant using the same process to produce a non-asthmatic phenotype. Mice from the second (15.19) and third (15.19.16) were then used for further analysis (10).

Tissue Homogenization

Kidney and lung tissue samples were harvested from mice having undergone acute treatment with either ovalbumin (OVA, asthmatic-like phenotype) or an alum adjuvant (non-asthmatic phenotype) as described previously. Harvested kidney and lung tissues were frozen with liquid nitrogen and ground with a mortar and pestle. Powdered tissues were resuspended in mitogen-activated protein kinase elution buffer (MAPK EB) containing 60mM Tris-HCl, 2% Sodium dodecyl sulfate, 10% glycerol, and 100mM Halt^{Tm} proteinase inhibitor cocktail (ThermoFisher Scientific) and sonicated with a probe sonicator for approximately 20 seconds each. Sonicated samples were then centrifuged at 4°C for 30 minutes at 13,500 rpm.

Western Blot

Protein concentrations were determined using a bicinchoninic acid assay (BCA) with bovine serum albumin (BSA) as the standard. Sample buffer (4X sodium dodecyl

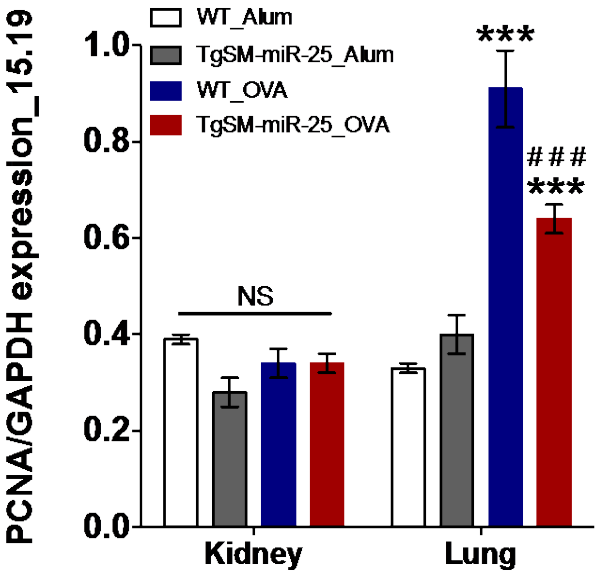
sulfate) was added to each sample to obtain a final total protein concentration of 50 μg per well. Fresh dithiothreitol (DTT)was added to each sample to a final concentration of 50 mM and samples were boiled at 95°C for 5 minutes. Samples were loaded in precast NOVEX NuPAGE 4-12% Bis-Tris Gels (Invitrogen) and run at 150V for 90 minutes. Gels were transferred to nitrocellulose at 19V for 90 minutes in transfer buffer (25 mM Tris Base, 192 mM glycine, 10% methanol). Membranes were stained in ponceau S (0.1% ponceau S, 1.0% glacial acetic acid) to observe total protein transferred using a Bio-Rad Gel Doc system. Membranes were de-stained in distilled water containing 5.0% glacial acetic acid. Membranes were blocked in 50:50 solution of Odyssey blocking buffer and phosphate buffered saline (PBS) for one hour (LiCor Biosciences) and then incubated overnight with primary antibody diluted 1:1000 in a 50:50 solution of Odyssey blocking buffer and PBS + 0.1% Tween20. Anti-PCNA was obtained from Santa Cruz Biotechnologies (#sc7907); Anti-GAPDH from Santa Cruz Biotechnologies (#sc20357); Anti-Cyclin D1 from Invitrogen (#33500); anti-smooth muscle α-actin from Sigma Life Sciences (#A2547), and anti-smooth muscle myosin heavy chain from Alfa Aesar (#J64817). Secondary antibodies used were linked to either AlexaFluor 680 (Molecular Probes) or IRDye800 (Rockland Immunochemicals) and were diluted 1:50,000 in 50:50 solution of LiCor blocking buffer and PBS + 0.1% Tween20. Blots were scanned for fluorometric detection with a LiCor Odyssey scanner.

Results

SM-Specific expression of miR-25 in 15.19 line reduces ASM proliferation in response to OVA- induced allergic inflammation

To determine the expression levels of PCNA, a proliferation marker, 50 μg of total protein were separated on a 4-12% Bis-Tris Gels at 150V for 90 minutes and then transferred. After blocking, blots were probed for PCNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins, for which imaging showed strong bands slightly below and slightly above 37 kDa, respectively. Bands for each antibody were analyzed to obtain PCNA expression normalized to GAPDH for all treatments in both kidney and lung tissue. PCNA expression was significantly increased in lung tissue from mice treated with OVA compared to those treated with alum (Fig. 1). This confirms that OVA-sensitization and challenge successfully induced increased proliferation, a hallmark of asthma, in the lungs of these mice. No significant difference in PCNA expression was found in kidney samples regardless of treatment (Fig. 1). This suggests that miR-25 activity is localized to the SM. As summarized in Figure 1A, line 15.19 (second generation mice) expressing high levels of miR-25 (~142 fold) showed a significant decrease of PCNA expression in the lung for OVA-treated miR-25 transgenic samples (TgSM-miR-25_OVA) compared to wild-type OVA (WT_OVA). This finding indicates that expression of miR-25 in SM decreased proliferation in response to OVA-induced allergic inflammation. PCNA was also measured in 15.19.16 line (third generation mice) that expresses low level of miR-25 (~ 2 fold), In this line, we note slight increase in PCNA expression in lung of TgSM-miR-25_OVA compared to WT_OVA lung (Fig. 1B).

A



B

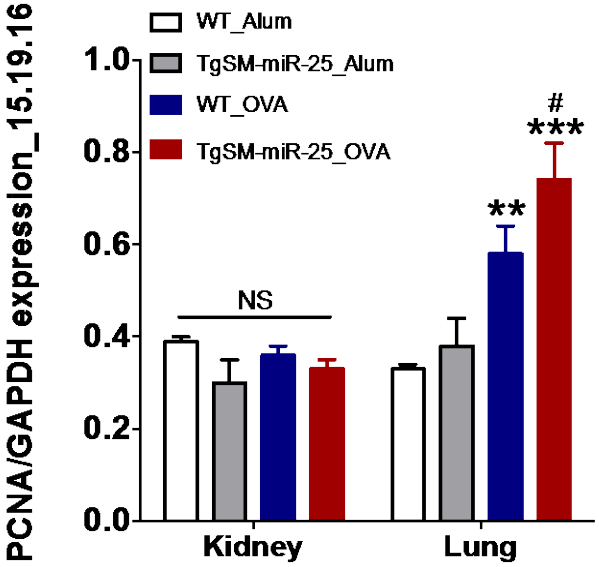


Figure 1: Normalized PCNA expression in kidney and lung tissue. Blots were incubated with anti-PCNA antibody diluted 1:1000 in 50:50 Odyssey blocking buffer and PBS+0.1% Tween20. Blots were re-probed with GAPDH (1:1000), which was incubated and diluted as previously stated. (A) PCNA expression in kidney and lung tissue of second generation mice. ***Statistically significant difference from WT_Alum ($p<0.001$). ###Statistically significant difference from WT_OVA ($p<0.001$). (B) PCNA expression in kidney and lung tissue of third generation mice. **Statistically significant difference from WT_Alum ($p<0.01$). ***Statistically significant difference from WT_Alum ($p<0.001$) and #$P<0.05$ from WT_OVA ($p<0.001$). Replicates (n) = 4-8

OVA treatment does not influence the expression levels of Cyclin D_1 in lung tissues.

In order to further support that miR-25 down regulates cell proliferation in airway smooth muscle, blots were re-probed and analyzed for cyclin D1 expression, a protein involved in G1/S cell cycle progression (9). As shown in Figure 2A, no significant difference in cyclin- D1 expression was observed in 15.19 lung samples regardless of treatment. Conversely, cyclin D1 expression increased in TgSM-miR-25_OVA kidney samples over WT_OVA kidney samples. Mice lungs from 15.19.16 line showed no significant changes in cyclin D1 expression; however, slight decreases in cyclin D1 levels were observed in the kidney samples of TgSM-miR-25 treated with either alum or OVA (Fig. 2B). Furthermore, cyclin D1 expression was unchanged in lung tissues of both transgenic and WT mice in response to OVA treatment (Fig. 2). Taken together, these results indicate that cyclin D1 is not a reliable marker to assess proliferation in an OVA-model of allergic inflammation.

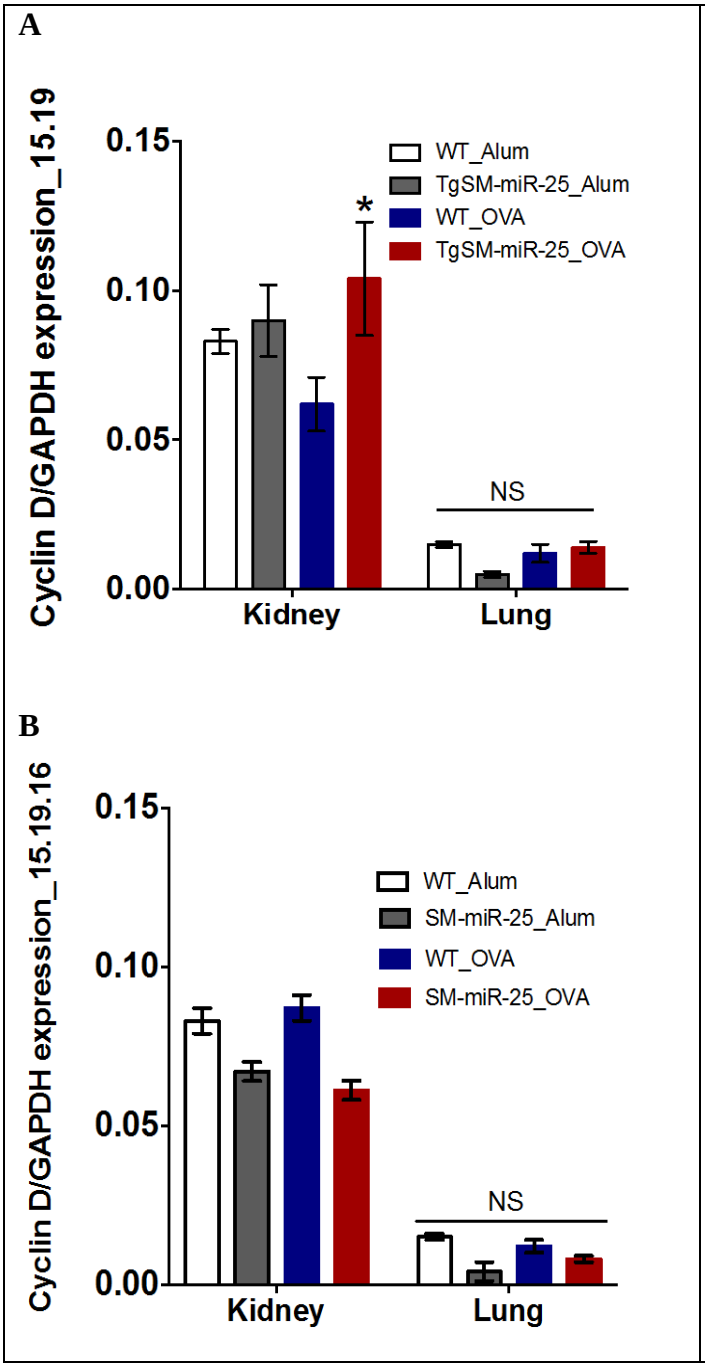


Figure 2: Normalized cyclin D1 expression in kidney and lung tissue. Blots were re-probed with anti-cyclin D1 antibody diluted 1:1000 in 50:50 solution of Odyssey blocking buffer and PBS+0.1%Tween20. Cyclin D1 expression was normalized to GAPDH expression. (A) Cyclin D1 expression in kidney and lung of second-generation mice. *Statistically significant difference from WT_OVA. (B) Cyclin D1 expression in kidney and lung of third generation mice. Replicates (n) = 4-8

Expression of miR-25 enhances the endogenous levels of contractile proteins to support a healthy contractile phenotype in response to OVA-induced allergic inflammation.

As miR-25 has also been shown to regulate select contractile proteins in human ASM cells, blots were also imaged for sm-MHC and sm-α actin. Bands were observed corresponding to these antibodies at approximately 250 kDa and slightly above 37 kDa, respectively. Relative expression levels for sm-MHC in the 15.19 line are summarized in Figure 3A. In both transgenic lung samples treated with either alum or OVA there was an increase in sm-MHC expression compared to wild type mice (Fig. 3A). This suggests that miR-25 may induce the expression of sm-MHC regardless of asthmatic phenotype. Additionally, there were no significant changes in sm-MHC expression in the kidney regardless of treatment (Fig. 3A). This again suggests that miR-25 activity is localized to the SM. Overexpression of miR-25 also produced increased sm-MHC expression in the lung of 15.19.16 line compared to that of WT mice, although the effect was less profound between WT and TgSM-miR-25 alum treated samples (Fig. 3B) than those in the 15.19 line (Fig 3A).

Relative expression for sm-α actin is summarized in Figure 4. As with sm-MHC, sm-actin also showed a significant increase in expression for transgenic lung samples regardless of alum or OVA treatment. This suggests that miR-25 again increased expression of these contractile proteins. Additionally, OVA treated samples also showed decreased α-actin expression overall in comparison to alum treated lung samples. This suggests that the asthmatic animal model may have a less developed contractile response

than that of a non-asthmatic airway. Kidney samples showed low sm-actin expression in general with no significant difference in expression observed regardless of treatment for both the 15.19 line (Fig. 4A) and 15.19.16 line (Fig. 4B) mice. This supports that miR-25 activity is localized to the SM. In 15.19.16 (Fig. 4B), there was again an increase in expression of α-actin in TgSM-miR-25_OVA lung samples compared to WT_OVA samples. This further supports that miR-25 increases the expression of sm-α-actin in response to allergic inflammation. Unlike 15.19 mice, 15.19.16 mice showed a decrease rather than an increase in sm-α-actin expression for TgSM-miR-25_Alum treated mice compared to WT_Alum treated mice.

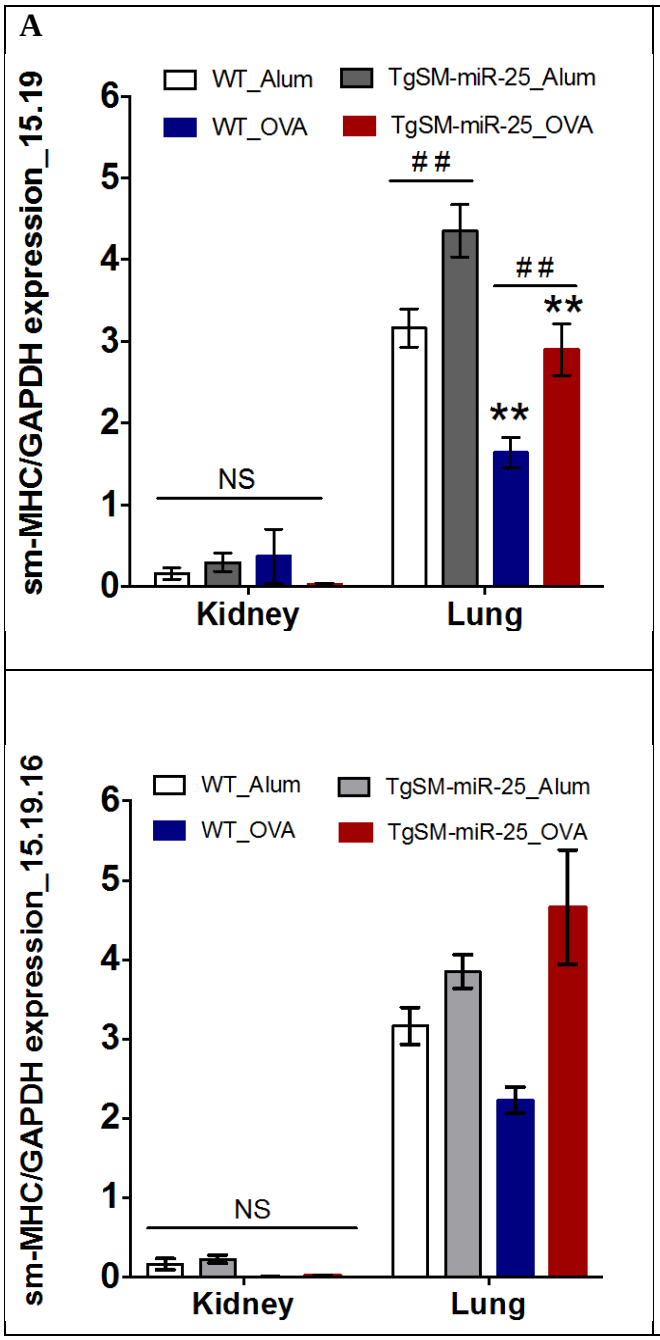


Figure 3: Normalized smooth muscle myosin heavy chain expression in kidney and lung tissue. Blots were re-probed with anti-sm MHC antibody diluted 1:1000 in 50:50 solution of Odyssey blocking buffer and PBS+0.1%Tween20. MHC expression was normalized to GAPDH expression. (A) MHC expression in kidney and lung of second generation mice. **p<0.01 Statistically significant difference versus matched Alum treated mice, ##p< 0.01 Versus matched WT. (B) MHC expression in kidney and lung of third generation mice, No significant (NS). Replicates (n) = 4-8

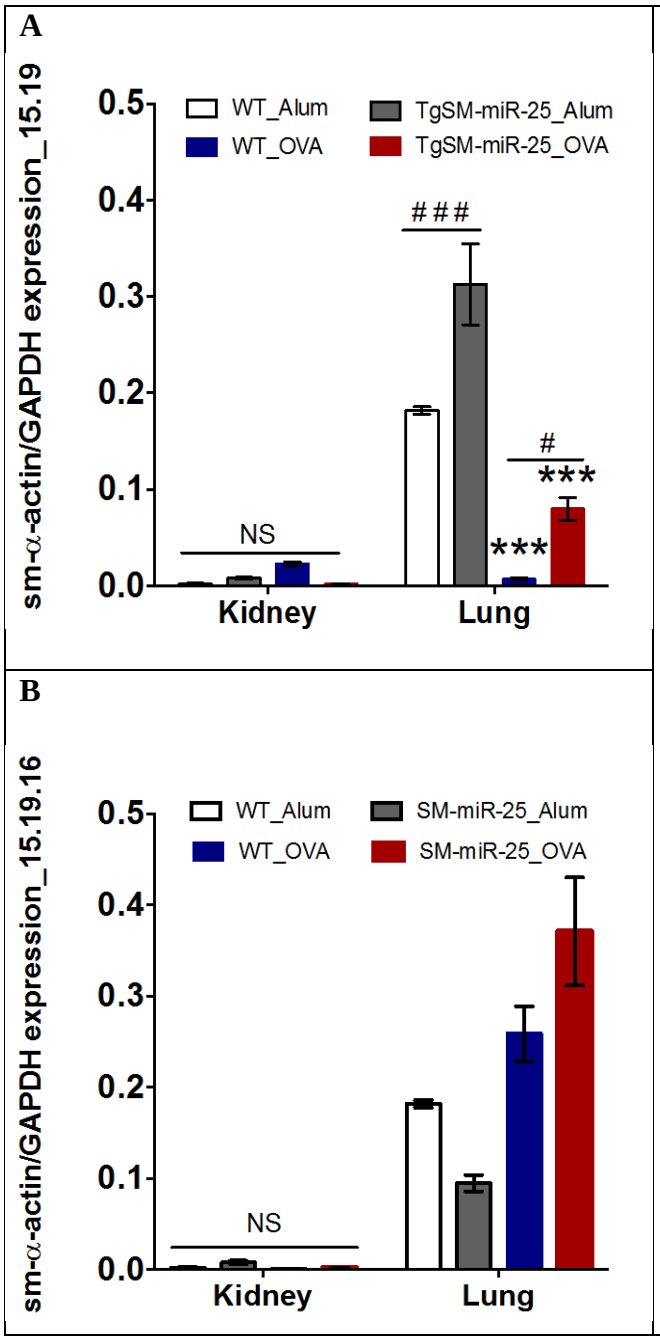


Figure 4: Normalized smooth muscle α-actin expression in kidney and lung tissue. Blots were re-probed with anti-sm α-actin antibody diluted 1:1000 in 50:50 solution of Odyssey blocking buffer and PBS+0.1%Tween20. α-actin expression was normalized to GAPDH expression. (A) α -actin expression in kidney and lung of second-generation mice. ***Statistically significant difference from matched alum control ($p<0.001$), #$p< 0.05$, ###$p<0.001$ Versus matched WT. (B) α -actin expression in kidney and lung of third generation mice, No significant (NS). Replicates (n) = 4-8

Discussion

This study sought to better understand the role of miR-25 in the regulation of ASM phenotypes in response to OVA-induced allergic inflammation in mice, as an animal model of asthma. Previously, miR-25 was shown to post-transcriptionally regulate KLF4, leading to decreased expression of KLF4 and possible modulation of the inflammatory and proliferative pathways (4,11). To investigate the potential for miR-25 to reduce ASM cell proliferation, PCNA expression was measured in lung tissue taken from mice with miR-25 overexpression. As overexpression of miR-25 in asthmatic model mice did show a significant decrease in PCNA expression in lung samples compared to wild type mice of 15.19 line that expresses miR-25 at 142-fold above wild-type levels, suggesting that miR-25 does decrease the proliferation of airway smooth muscle. However, this decrease in PCNA was not observed in the third generation 15.19.16 line with a 2-fold increase in miR-25 expression. This line showed increase in PCNA expression for the transgenic samples with the asthmatic phenotype. This difference in effect on PCNA expression may be due to decreased miR-25 expression in the line 15.19.16 compared to the 15.19 line, indicating that miR-25 expression may not be stable in these animals..

To further demonstrate that miR-25 decreases proliferation of ASM, cyclin D1 expression was also measured in mouse kidney and lung tissues. However, unlike PCNA, no significant difference was found in cyclin D1 expression in lung tissue regardless of asthmatic phenotype. Conversely, cyclin D1 expression was modulated in kidney tissues of mice overexpressing miR-25, a result that is inconsistent with the idea that miR-25 is localized to the airway. However, these results were inconsistent across the 15.19 and 15.19.16 lines. While 15.19 mice showed increased cyclin D1 expression and suggested

that proliferation may be increased, 15.19.16 demonstrated decreased proliferation in miR-25 overexpressing mice. Overall, these results are inconclusive and may suggest that cyclin D1 is too broad and indirect of a target to properly measure proliferation in response to OVA-induced allergic inflammation.

Further evidence has shown that miR-25 up-regulates the expression of certain proteins involved in the contractile phenotype, thereby potentially leading to a more mature contractile response in airway smooth muscle. Consistent with previous studies, altering miR-25 expression in ASM tissue was shown to lead to changes in sm-MHC expression (4). As miR-25 was overexpressed in lung, sm-MHC was shown to increase. Sm-MHC is a key regulator of ASM phenotype and necessary for proper muscle contraction (12); thus, these results suggest miR-25 plays a role in developing the contractile response of airway smooth muscle. Although these results support that miR-25 increases the expression of this contractile protein, there is no binding site predicted for miR-25 in the 3' UTR of MHC (4). This suggests that although miR-25 up regulates sm-MHC expression, it does so indirectly. This reveals possible future research into the pathway by which miR-25 regulates sm-MHC.

Similarly to sm-MHC, sm-α-actin was also shown to increase in airway smooth muscle with miR-25 overexpression. As increased actin availability is key to a well-toned and developed contractile response (13), this again suggests that miR-25 helps regulate development of the contractile response and modulates airway smooth muscle phenotype overall. Like sm-MHC, this apparent regulation of sm-α-actin expression by miR-25 points towards future studies to better identify the pathway by which miR-25 modulates expression.

The data presented here demonstrate that target overexpression of miR-25 in SM effectively modulates airway smooth muscle phenotypes and counteracts the effects of airway proliferation in response of OVA-induced allergic inflammation in mice. Furthermore, this study has further characterized the regulating effect of miR-25 on select contractile proteins, namely sm-MHC and sm- α -actin. In the future, miR-25 inhibition of proliferation and increase in contractile proteins should be further confirmed by immunohistochemistry of airway tissue in order to visually and more directly represent the physiological and phenotypic changes to the airway. With the successful manipulation of ASM phenotype in a mouse model, this study provides valuable insight into the use of miR-25 as a potential novel therapeutic target for asthma and other respiratory diseases.

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