Expression Profiling Identifies *Klf15* as a Glucocorticoid Target That Regulates Airway Hyperresponsiveness

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Glucocorticoids (GCs), which activate GC receptor (GR) signaling and thus modulate gene expression, are widely used to treat asthma. GCs exert their therapeutic effects in part through modulating airway smooth muscle (ASM) structure and function. However, the effects of genes that are regulated by GCs on airway function are not fully understood. We therefore used transcription profiling to study the effects of a potent GC, dexamethasone, on human ASM (HASM) gene expression at 4 and 24 hours. After 24 hours of dexamethasone treatment, nearly 7,500 genes had statistically distinguishable changes in expression; quantitative PCR validation of a 40-gene subset of putative GR-regulated genes in 6 HASM cell lines suggested that the early transcriptional targets of GR signaling are similar in independent HASM lines. Gene ontology analysis implicated GR targets in controlling multiple aspects of ASM function. One GR-regulated gene, the transcription factor, Kruppel-like factor 15 (*Klf15*), was already known to modulate vascular smooth and cardiac muscle function, but had no known role in the lung. We therefore analyzed the pulmonary phenotype of *Klf15*^{-/-} mice after ovalbumin sensitization and challenge. We found diminished airway responses to acetylcholine in ovalbumin-challenged *Klf15*^{-/-} mice without a significant change in the induction of asthmatic inflammation. In cultured cells, overexpression of *Klf15* reduced proliferation of HASM cells, whereas apoptosis in *Klf15*^{-/-} murine ASM cells was increased. Together, these results further characterize the GR-regulated gene network in ASM and establish a novel role for the GR target, *Klf15*, in modulating airway function.

Keywords: asthma; glucocorticoid; *Klf15*

Glucocorticoids (GCs) are among the most effective and widely used therapies to treat asthma and other immune-mediated diseases (1). GCs exert their activity through binding to the GC receptor (GR), causing it to translocate to the nucleus and modulate transcription. Although the basic GC signaling machinery is conserved between cell types, the sets of target genes controlled by GR signaling in different tissues vary widely. This is reflected by the pleiotropic physiologic impacts of GCs, which include beneficial effects, but also encompass the induction of procatabolic side effects (2). A central issue in GR biology and therapeutics, therefore, is to delineate the GR-regulated transcriptome in clinically relevant tissues and to determine whether

CLINICAL RELEVANCE

This research advances our understanding of glucocorticoid (GC) action in airway smooth muscle, one of the key target tissues for asthma therapeutics. This work also uncovers a new role for the GC-regulated gene, *Klf15*, in modulating airway hyperreactivity in murine models of allergic airway disease.

particular target genes and pathways modulate clinically important effects. This is of particular relevance in asthma, as numerous cell types within the lung contribute to the phenotypic manifestations of asthma, and are also involved in the therapeutic response to GCs (3–5).

One important target tissue of GCs in asthma is airway smooth muscle (ASM) (6, 7). A number of key pathologic alterations in ASM have been described in asthma, including hypercontractility, hyperplasia, and hypertrophy (8–10). GR signaling modulates many aspects of ASM dysfunction associated with asthma *in vivo*. In patients, and in animal models of allergen-induced lung disease, treatment with GCs reduces airway hyperresponsiveness (AHR) and reduces expression of smooth muscle actin in ASM (11–14). GCs also prevent increases in ASM mass associated with allergic inflammation in mice (15), and have been implicated in altering ASM growth after acute allergen exposure in subjects with asthma (16).

The molecular mechanisms underlying the effects of GR signaling in ASM have been studied extensively using cultured cell models. GCs can reduce the expression of contractile proteins in cultured ASM cells (17), and also reduce the expression of cytokines and growth factors secreted by ASM, including IL-6 and RANTES (18–22). In addition, GR signaling regulates ASM proliferation in association with altering cyclin-D levels and Rb phosphorylation (23). The effects of GCs on ASM proliferation and apoptosis are modulated by cytokines and growth factors, by β -adrenergic agents, and by components of the extracellular matrix (24). Together, each of these activities of GR signaling in cultured ASM cells—reduced contractile protein expression, altered inflammatory and growth factor gene expression, and regulation of proliferation and apoptosis—likely contribute to the effects of GCs on ASM observed *in vivo*.

Despite the growing understanding of GR activity in ASM, important questions remain unanswered about the global effects of GR signaling on ASM gene transcription and whether specific GR target genes regulate ASM function. Here, we have used transcription profiling at two time points to define a set of early targets of GR signaling in multiple ASM cell lines. We subsequently tested whether a specific GR target in ASM that was identified in this screen, termed Kruppel-like factor 15 (*Klf15*), modulates airway function. Some results presented here have appeared previously in the form of an abstract (25).

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MATERIALS AND METHODS

Cell Culture and RNA Extraction

Primary human ASM (HASM) cells were generated as previously described (26). For RNA extraction, early passage cells were plated at 80% confluence in growth medium (Ham's F12 with 25 mM HEPES, 12 mM NaOH, 17 mM CaCl₂, 2 mM L-glut, 10% FBS [HyClone] and penicillin/streptomycin). After reaching confluence, cells were maintained for 5–7 days. Cells were then induced for 4 or 24 hours with 1 μ M dexamethasone (dex) dissolved in ethanol, or ethanol alone. RNA was extracted with Trizol and Purelink columns (Invitrogen, Frederick, MD). Primary murine tracheal smooth muscle cells were generated as previously described, and assayed between passage 8 and 15 (27).

Proliferation and Terminal Deoxynucleotidyl-Transferase–Mediated dUTP Nick-End Labeling Assays

For proliferation assays, HASM cells in 96-well dishes were infected with Ad-*Klf15* or Ad-GFP, as described. Viable cell number was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining (ATCC, Manassas, VA) after 48 and 72 hours. Terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling (TUNEL) assays (Roche, Pleasanton, CA) were performed on murine ASM cells plated in 96-well dishes after 48 hours of serum deprivation.

Microarrays

Biologic quadruplicate samples from control, 4-hour, and 24-hour dex treatment were used for microarray analysis; one sample was excluded as a technical outlier. False discovery rate (FDR) and adjusted *P* values were used to determine genes that had significant differences in expression levels between samples. Raw data and experimental details are available at the Gene Expression Omnibus Web site (<http://www.ncbi.nlm.nih.gov/geo>).

Quantitative PCR

Arrayed Taqman primers (see the online supplement) and an Open Array Real-Time PCR Platform (Applied Biosystems, Carlsbad, CA) were used for quantitative PCR (qPCR). The template cDNA was prepared as previously described (28). mRNA was generally analyzed in biologic triplicate; a minimum of two interpretable data points was used to determine relative message levels. In most cases, samples were analyzed in biologic triplicate and technical duplicate, with obvious outliers excluded. The normalized relative expression level was calculated by comparing the mean threshold cycle (Ct) value for each gene with the mean RPL19 Ct value (i.e., the $\Delta\Delta$ Ct method). Fold activation or repression was determined by comparing relative expression levels between dex- and ethanol-treated samples. SDs were almost always less than 1. For several cell lines, multiple experiments showed very similar relative expression levels for each gene.

Gene Ontology

Gene ontology (GO) analysis and functional clustering were performed using the DAVID bioinformatics resource (29).

Murine Ovalbumin Challenge

Klf15^{−/−} mice were in a pure C57Bl/6 background (30). Sex-matched control and *Klf15*^{−/−} mice (6–8 wk old) were sensitized and challenged with ovalbumin (OVA), as previously described (31). Briefly, mice were injected with 50 μ g of OVA/1 mg alum on Days 0, 7, and 14. Intranasal challenge with OVA (100 μ g in 40 μ l of saline) or saline alone was performed on Days 21, 22, and 23. At 24 hours after the last intranasal challenge, analyses of serum IgE, bronchoalveolar lavage cell count, and measurements of pulmonary mechanics, using the Flexivent mechanics analyzer (SCIREQ, Quebec, CA), were performed as described previously (32, 33). After Flexivent measurements, lungs were fixed, processed, and stained with hematoxylin and eosin for semiquantitative assessment of inflammation, and with perchloric acid staining to assess mucin positive cells.

RESULTS

In a Time-Dependent Manner, GCs Profoundly Modulate Gene Expression in ASM

To explore the mechanisms of GR signaling in ASM and identify target genes of GR signaling that regulate airway physiology, we performed array-based expression profiling on a primary HASM cell line (HASM1), treated with 1 μ M dex. This dose was selected based on dose–response analysis, in which 1 μ M dex resulted in maximal changes in gene expression for several test genes that are regulated by GR in almost every cell type (data not shown). Because it was not clear whether immediate or delayed targets of GR signaling are responsible for the physiologic effects of GCs in ASM, we profiled expression changes at two time points: 4 and 24 hours after dex-induced GR activation. Using an FDR of less than 0.01 as a cut-off, after 4 hours of dex treatment, 89 transcripts had a greater than fourfold change in expression (Table 1). After 24 hours of dex treatment, however, the number of transcripts with expression changes greater than fourfold increased to 340 (151 up and 189 down). Similarly, with no threshold for the magnitude of expression level change, 1,594 transcripts had altered expression levels after 4 hours, whereas 7,547 had statistically significant (FDR < 0.01) changes in mRNA levels after 24 hours. Thus, sustained activation of GR signaling with a single pulse of ligand induces an expanding program of gene expression changes over time.

The gene expression changes induced by dex in ASM followed various temporally distinct patterns. This is visualized in Figure 1 as a heat map depicting the expression changes at both the 4- and 24-hour time points for 80 genes that had the largest initial changes in expression with dex treatment. Some genes had comparable changes in expression level induced by dex at 4 and 24 hours, seen as heat map rows having the same or a similar color at both time points. Other genes had wide variations in gene expression at the two time points, reflected by strong color differences in a single row of the heat map. Together, these data show that gene expression changes induced by GR signaling are temporally dynamic, with many genes exhibiting only late changes in gene expression and other genes having marked differences in early versus late expression changes induced by GR.

Validation of Expression Profile in Multiple, Independent ASM Lines

Although array-based expression profiling is a standard approach for the global analysis of gene expression, this method can leave unclear whether specific expression profiles are reproducible in independent experiments. We therefore performed high-throughput qPCR with a new set of freshly ob-

TABLE 1. NUMBER OF AIRWAY SMOOTH MUSCLE TRANSCRIPTS WITH STATISTICALLY SIGNIFICANT (FALSE DISCOVERY RATE >0.01) EXPRESSION CHANGES IDENTIFIED BY MICROARRAY AFTER DEXAMETHASONE TREATMENT FOR INDICATED TIME

Expression change	No. of Transcripts	
	4 h	24 h
Increased $\geq$ fourfold	44	151
Decreased $\geq$ fourfold	45	189
Increased $\geq$ 2 $\times$	295	875
Decreased $\geq$ 2 $\times$	249	727
Increased any level	814	4,490
Decreased any level	780	3,057

tained RNA to validate dex-induced gene expression changes that were identified with the microarrays. We analyzed a panel of 48 dex-regulated genes and controls, reasoning that secondary validation of a relatively large panel of genes would strengthen extrapolations from the remainder of the microarray data. The panel was designed to reflect a variety of expression profiles and to include genes with potential relevance to ASM biology. Of 40 putative GR-regulated genes that had detectable qPCR signals in this analysis, 38 showed similar changes in expression measured by qPCR after 4 hours of dex treatment in compar-

ison to the primary microarray data. Data for 16 of the tested genes, including several genes with no reported role in ASM biology, such as *KLF15*, *CMYA1*, *AXIN2*, and *HEYL*, are shown in Figure 2; the entire data set is presented in Table E1 in the online supplement.

We also tested whether the changes in gene expression after 24 hours of dex treatment were reproducible in independent experiments using qPCR. As with 4 hours of dex treatment, the expression changes associated with 24 hours of dex treatment generally followed the pattern of the original array. However, there was greater variability at this later time point in comparison with 4 hours of dex treatment (e.g., *CMYA1* [Figure 2A]). Nevertheless, the central conclusion from these validation experiments is that gene expression changes identified with the array-based expression profiling, especially after 4 hours of dex treatment, are reproducible in independent experiments using

Figure 1. The magnitude of dexamethasone (dex)-induced gene expression changes detected by microarray in airway smooth muscle (ASM) cells varies over time. Heat map showing expression changes after 4 and 24 hours of dex treatment for the top 80 glucocorticoid (GC) receptor (GR)-regulated genes. The color scale is logarithmic (base 2), with the brightest green therefore representing a 128-fold reduction in gene expression and the strongest red representing a 32-fold increase in message levels. Rows with wide changes in color had different expression after 4 and 24 hours of dex treatment, whereas genes represented by rows with similar colors had similar changes in expression detected at 4 and 24 hours.

Figure 2. Quantitative PCR (qPCR) validation of microarray-based detection of gene expression changes in ASM induced by dex treatment. Fold activation (A) or repression (B) of transcript levels for the indicated genes with dex treatment as measured with microarray (gray bars), or with qPCR (black bars), are depicted by bar graphs. Fold activation or repression was determined by comparing relative mRNA levels for a given gene between ethanol-treated cells and cells treated with dex, as indicated. Error bars represent SD. Note the similarity between the expression changes measured using microarray or qPCR, especially after 4 hours of dex treatment. Independent samples were used for the qPCR and microarrays.

Figure 3. Gene expression changes induced by 4 hours of dex treatment are similar in independent human ASM (HASM) lines. *Bar graphs* showing fold activation (A) or repression (B) of transcript levels for the indicated genes with dex treatment in different ASM cell lines. Fold activation or repression for a given gene in each cell line was determined by comparing relative mRNA levels between cells treated with ethanol or dex for 4 hours. *Error bars* represent SD. Note the similarities in the dex-induced gene expression changes between the different cell lines. The first bar in each graph is shown for reference, and is the fold activation or repression measured in ASM1 by microarray. For Kruppel-like factor 15 (*Klf15*), the fold activation with dex was estimated in HASM4 and A-HASM2 by assigning a baseline threshold cycle (Ct) value of 32 for *KLF15*, as *KLF15* expression was below the threshold for detection in those cells without dex treatment.

qPCR. As a relatively large group of genes that covered a range of expression changes were validated with qPCR, we conclude that the majority of the genes that we identified as regulated by GR using microarrays are likely to be reproducible targets of the GR signaling pathway in this ASM cell line.

A difficulty in cell culture-based studies of HASM is that there is no single standardized cell line, which potentially limits the capacity to generalize and compare data from different investigations and laboratories. We therefore tested whether the dex-induced gene expression changes that we had validated with qPCR in a single ASM line also occurred in five additional, independently derived HASM cell lines: HASM2, HASM3, and HASM4 from healthy donors, and A-HASM1 and A-HASM2 from patients with fatal asthma. There was substantial concordance in dex-induced gene expression changes after 4 hours across all of the tested cell lines (Figure 3 and Table E1), although several genes did exhibit variability in some of the cell

lines (e.g., *HEYL* [Figure 3A]). These data suggest that the early transcriptional effects of GR activation are similar in independently derived ASM cell lines.

GO Analysis Predicts that GR Signaling Impacts Many Facets of ASM Biology

The above data established a set of genes that is regulated by GR signaling in cultured ASM cells. These data did not, however, establish whether specific GR-regulated genes modulate ASM function. To gain traction on this question, we performed GO analysis and functional annotation clustering using approximately 200 of the genes most strongly regulated by GR after 4 hours of dex treatment (29). We found nine highly significant clusters of functionally similar terms associated with this group of GR target genes. These included functional clusters of GO terms related to apoptosis, inflammation, transcription, and metabolic processes. An example of a functional cluster of overlapping GO terms related to apoptosis is shown in Tables 2. Genes represented in this functional cluster included Bcl2 modifying factor, *JUN*, *IL12a*, and Fas ligand. All nine clusters identified through this analysis, and the list of genes used to generate the clusters, are shown in the online supplement (Tables E2 and E3).

To explore further the potential for GR-regulated genes to modulate diverse aspects of ASM biology, we investigated the published functions of several GR target genes in greater detail. By virtue of previously described functions in other muscle types, this analysis implicated numerous GR targets as novel candidates to modulate ASM function. For example, *FOXO1* had been shown to regulate skeletal muscle atrophy and vascular smooth muscle (VSM) proliferation (34, 35), but has not been studied in the lung or in ASM. Likewise, the GR targets, *HES1*, *NR4A3*, and *MSX1*, are transcription factors that have been implicated in smooth muscle biology (36–38), but have not been studied in ASM or asthma. Thus, multiple GR target genes without known roles in lung biology appear to be strong candidates to modulate ASM phenotype.

Deletion of *Klf15*, a Noninflammatory GR Target in the Lung, Modulates Airway Contractility in a Murine Asthma Model

One GR target gene with no known role in the lung that emerged from GO analysis as an especially strong candidate to modulate ASM function and airway biology through noninflammatory pathways was *KLF15*. Indeed, *KLF15* is a transcrip-

TABLE 2. GENE ONTOLOGY TERMS RELATED TO APOPTOSIS ARE OVERREPRESENTED IN THE TOP 200 GLUCOCORTICOID RECEPTOR-REGULATED TRANSCRIPTS IN AIRWAY SMOOTH MUSCLE 1 CELLS AND FORM A FUNCTIONAL CLUSTER

Gene Ontology Term	Gene Count*	P Value†
Regulation of programmed cell death	30	1.30E–08
Regulation of cell death	30	1.40E–08
Regulation of apoptosis	29	4.00E–08
Negative regulation of apoptosis	16	7.70E–06
Negative regulation of programmed cell death	16	9.10E–06
Negative regulation of cell death	16	9.40E–06
Antiapoptosis	11	8.90E–05
Positive regulation of apoptosis	11	2.00E–02
Positive regulation of programmed cell death	11	2.10E–02
Positive regulation of cell death	11	2.10E–02

* Total number of genes from the top 200 glucocorticoid receptor targets in airway smooth muscle 1 cells that are annotated with the indicated gene ontology term.

† P value, based on a modified Fisher’s exact test, indicating probability that the number of genes sharing the indicated gene ontology term is due to chance.

Figure 4. Ovalbumin (OVA) challenge induces similar inflammatory changes in wild-type (WT) and *Klf15*^{-/-} mice. (A) Hematoxylin and eosin (H&E) staining of lungs from WT and *Klf15*^{-/-} mice after OVA challenge revealed similar peribronchial/perivascular infiltrates. Representative sections are shown. (B) Bar graph representing semiquantitative averages of inflammatory infiltrates in WT and *Klf15*^{-/-} mice with and without OVA challenge. Lungs from six animals from each group were stained with H&E. Representative sections from each animal were assessed visually for inflammation, which was graded on a semiquantitative scale from 0 to 3. (C) Perchloric

acid staining of lungs from WT and *Klf15*^{-/-} mice treated with intranasal saline or OVA (labeled) revealed similar increases in mucin-positive cells (arrows) associated with OVA challenge. Representative sections are shown. (D) Bar graph indicating total cell counts as indicated from bronchoalveolar lavage (BAL) fluid from WT and *Klf15*^{-/-} mice with and without OVA challenge. Eos, eosinophils; Lymphs, lymphocytes; Macros., macrophages; PMNs, polymorphonuclear leukocytes.

tion factor that has been implicated in skeletal muscle amino acid metabolism, as well as aspects of cardiac and VSM function (30, 39–41). Moreover, *KLF15* had one of the largest relative increases in mRNA levels after 4 hours of dex exposure in ASM cells (Figure 1), was regulated by GR in all six ASM lines that we analyzed (Figures 2 and 3), and was also regulated by GR in whole murine lungs (Figure E1). Therefore, to establish whether *KLF15* exemplifies a noninflammatory GR target gene that modulates airway biology, we analyzed the phenotypes of wild-type (WT) and *Klf15*^{-/-} mice induced to develop AHR through OVA sensitization and challenge.

To accomplish this, groups of age-matched WT and *Klf15*^{-/-} mice were sensitized to OVA and subsequently challenged with either intranasal OVA or saline, after a standardized protocol (33). Both WT and *Klf15*^{-/-} mice developed inflammatory changes typical of this asthma model. Histology revealed qualitatively similar peribronchial and perivascular inflammatory infiltrates in the lungs of WT and *Klf15*^{-/-} mice after OVA challenge (Figure 4A). Although stereologic-based measurements were not performed, semiquantitative analysis of inflammation based on scoring representative sections on a scale from 0 to 3 also indicated similar levels of inflammation in WT and *Klf15*^{-/-} mice (Figure 4B). Mucus production determined by perchloric acid staining was equivalently elevated in the lungs of OVA-challenged WT and *Klf15*^{-/-} mice as compared with saline control animals (Figure 4C). Likewise, OVA-specific serum IgE levels were elevated to comparable extents in the OVA-challenged WT and *Klf15*^{-/-} mice (Figure E2). Finally, although there was a nonsignificant trend toward decreased eosinophilia in the bronchoalveolar lavage fluid of the *Klf15*^{-/-} mice, the profile and number of inflammatory cells in WT and *Klf15*^{-/-} mice with OVA challenge were similar (Figure 4D). Taken together, these data indicate that *Klf15* deletion does not substantially alter the inflammatory response in the acute OVA challenge asthma model.

In contrast, assessment of AHR revealed a striking difference in pulmonary resistance in response to acetylcholine between *Klf15*^{-/-} and control mice (Figure 5). As expected, WT mice challenged with aerosolized OVA had marked increases in

pulmonary resistance in response to acetylcholine in comparison with WT mice treated with the saline control. However, resistances in the *Klf15*^{-/-} OVA challenged mice were significantly less than in WT OVA-challenged mice at several acetylcholine doses (Figure 5). These results indicate that *Klf15* deletion reduces AHR associated with acute OVA sensitization and challenge. Taken together, these data implicate the GR target gene, *Klf15*, in modulating airway resistance in a manner that is largely dissociated from the inflammatory response.

Consistent with this conclusion, even in the absence of OVA challenge (i.e., the saline controls), *Klf15* deletion also resulted in mildly reduced airway resistances in response to acetylcholine in comparison with WT controls (Figure 5). Intriguingly, *Klf15*^{-/-} mice are known to exhibit increased aortic aneurysm formation. These *Klf15*^{-/-} aneurysms are associated with increased VSM apoptosis (40), whereas other forms of vascular injury in *Klf15*^{-/-} animals are associated with increased VSM

Figure 5. *Klf15*^{-/-} mice generate lower airway resistances in response to acetylcholine. Line graph showing mean pulmonary resistance measured at the indicated acetylcholine doses in saline controls and in OVA-challenged WT and *Klf15*^{-/-} mice. Vertical bars around each data represent the SD. Pulmonary resistances were determined for a minimum of six mice in each group.

proliferation (41). In addition, *Klf15* overexpression in cultured VSM reduces proliferation (41). We reasoned that *Klf15* might similarly modulate the balance between apoptosis and proliferation in ASM. Because the ASM layer in normal murine airways is relatively thin, and has not been reported to exhibit altered morphology with the short-term allergic challenge model that we employed (42), we used cell culture approaches to study the effects of varied *Klf15* expression levels on ASM apoptosis and proliferation. First, we used TUNEL to assay apoptosis in cultured tracheal smooth muscle cells generated from a *Klf15*^{-/-} mouse in comparison with two WT control animals. Immunostaining for α -actin was used to verify the expression of smooth muscle markers in the cells (Figure E3). There was substantially increased apoptosis in response to serum deprivation in the *Klf15*^{-/-} cell line compared with the WT cell lines (Figure 6A). Second, we tested whether overexpression of *Klf15* alters proliferation in primary HASM cells. HASM1 cells were infected with a previously described *Klf15*-expressing adenovirus (Ad-*Klf15*) or an Ad-GFP control (30). Adenoviral-mediated overexpression of *Klf15* was verified with qPCR (Figure E4). Infection with Ad-*Klf15* resulted in reduced cell proliferation 48 and 72 hours after infection, as quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining for viable cells (Figure 6B). Thus, similar to observations in VSM, these data suggest that *Klf15* is a mediator of apoptosis and proliferation in ASM. We propose that

regulation of apoptosis and proliferation by *Klf15* in ASM may contribute to the reduction in AHR observed *in vivo* in *Klf15*-deficient mice.

DISCUSSION

The physiologic effects of genes that are regulated by GR signaling in ASM are not fully understood. Here, we used expression profiling and extensive qPCR validation to define the transcriptional response to GR activation in cultured, HASM cells. GO analysis suggested that early transcriptional targets of GCs modulate numerous cell biologic processes in ASM, in addition to altering inflammatory pathways. We therefore tested whether the early GR-regulated gene, *Klf15*, which had a previously defined role in the biology of other types of muscle tissue, also modulated airway function. We found that *Klf15* deletion significantly reduced acetylcholine-induced airway contraction in OVA-challenged mice without substantially altering inflammation. These data thus establish a novel role for *Klf15* in regulating airway contractility. Furthermore, allergen-induced AHR can be uncoupled from allergen-induced airway inflammation. This work also highlights the potential role for GR target genes in regulating airway function independent of the inflammatory response.

Our study complements and expands on two prior publications that reported on aspects of the ASM transcriptional response to GCs. Hakonarson and colleagues (43) used expression profiling to study the mechanism whereby dex limits IL1- β /TNF- α -induced increases in airway contractility. This study, published in 2001, profiled only about 6,000 genes, and did not focus on the primary ASM transcriptional response to GCs. However, in concordance with the results that we have reported here, the prior study showed strong repressive effects of GR signaling on the expression of numerous ASM genes, albeit in the context of inflammatory stimulation. The mechanisms whereby GR represses gene expression both in basal and stimulated conditions are not fully defined, and are likely to be of significant therapeutic importance.

More recently, to investigate the mechanistic basis for cross-talk between inflammatory stimuli and GCs in modulating ASM proliferation and function, Misior and colleagues (44) analyzed the modulatory impact of GR activation and protein kinase A inhibition on EGF and IL-1 β induced changes to the ASM transcriptome. Although there was some overlap with our data, the GC-induced gene expression changes and GO analysis reported by Misior and colleagues (44) mainly focused on the effects of GCs on EGF/IL-1 β -stimulated cells. Accordingly, there also exist substantial differences. For example, *KLF15*, for which we subsequently demonstrated a role in airway function, was not identified as a GR target in their work, even under nonstimulated culture conditions. One possible explanation for the observed differences is that basal culture conditions might exert a strong modulatory effect on GR signaling. In that regard, we studied cells that had been arrested via contact inhibition as opposed to by serum deprivation. A second important difference was that we used two time points to define an expanding program of gene expression changes induced by GR signaling, whereas Misior and colleagues (44) reported on a single intermediate time point. Finally, we used 1 μ M dex in our studies, as opposed to 10 nM of fluticasone propionate. It is possible that different GR agonists and doses have different effects in this cell type, although others have seen similar effects of 10 nM fluticasone and 1 μ M dex on gene expression in ASM (17). In any event, our work substantially expands the set of known, validated GR target genes in ASM.

The substantial concordance with respect to GR-induced changes in gene expression between different primary HASM

Figure 6. Altered apoptosis and proliferation in *Klf15*^{-/-} and *Klf15* overexpressing ASM cells. (A) Bar graph showing average ratio of apoptotic cells to total nuclei in serum-deprived murine tracheal smooth muscle (MTSM) cells isolated from *Klf15*^{-/-} and *Klf15*^{+/+} mice, as indicated; 1,000 cells were plated per well in a 96-well dish, and subjected to serum deprivation for 48 hours before performing terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) and 4',6-diamidino-2-phenylindole staining. Means and SDs from counting eight 10 $\times$ fields from two wells for each cell type are depicted. (B) Bar graph comparing relative viable cell number in HASM cells infected with stocks of GFP expressing adenovirus (Ad-GFP) or *Klf15* expressing adenovirus (Ad-*Klf15*). Cells were infected with the indicated adenovirus and relative viable cell numbers 48 and 72 hours after infection were determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) absorbance. Mean and SD for six wells for each condition are shown.

cell lines was somewhat surprising. These included two cell lines derived from patients with fatal asthma. One contributing factor could be our use of cells that had been arrested through contact inhibition for several days. This would potentially limit cell cycle or circadian variability in the GR response that is likely manifest in clinical samples. Several genes in each of the fatal asthma cell types did, however, exhibit some divergence in expression changes in response to dex treatment (see Table E2). In A-HASM1, *BACH1* and *SOCS3* had diminished responses to dex treatment, whereas, in A-HASM2, the expression levels of *HEYL*, *IGFBP2*, and *FZD5* were relatively unaffected by dex treatment. It remains to be determined whether cell line-specific epigenetic changes, or altered coregulator activity, contribute to subtle alterations in the GR response in the ASM lines generated from patients with fatal asthma.

Our data show that the set of genes regulated by GR signaling expands substantially between 4 and 24 hours. One interpretation is that genes regulated rapidly by GR play a role in controlling gene expression changes that occur at later time points. Interestingly, we identified a number of transcription factors that were strongly regulated by GR after 4 hours, including *FOXO1*, *FOXP1*, *HES1*, *HEYL*, *KLF15*, and *MSX1* (Table E4). We have yet to establish whether any of these factors, including *KLF15*, modulate the GR response either *in vivo* or *in vitro*. Gene knockdown of GR-regulated transcription factors, or exploiting knockout mice when available, should be useful in determining whether specific transcription factors control secondary transcriptional responses to GR activation.

Because GR activation lowers airway resistance clinically and in animal models, the reduction in airway contractility caused by deletion of *Klf15*, the mRNA levels of which are increased with GR activation, initially seemed paradoxical. One possibility is that *Klf15* expression is not regulated at the protein level by GCs in the lung. However, as *Klf15* protein levels are increased by GCs in other cellular contexts (45), we believe that this is unlikely. A second possibility is that *Klf15* deficiency causes an isolated defect in muscarinic acetylcholine receptor expression or function, rather than a general impact on airway physiology. However, we did not observe substantial differences in mRNA expression of muscarinic acetylcholine receptor isoforms (Figure E5), which renders this possibility less likely. Instead, our data are most consistent with a model in which *Klf15* regulates the balance between apoptosis and proliferation in ASM, similar to the proposed role for *Klf15* in VSM. In this model, *Klf15* deficiency promotes apoptosis, whereas increased expression of *Klf15*, which is induced by various stress stimuli, including GCs, reduces ASM proliferation. Intriguingly, altered p53 acetylation is implicated in mediating the effects of *Klf15* on apoptosis and proliferation in VSM (40). The p53 pathway integrates stress signals with proliferation and apoptosis decisions in many cell types, but has not been studied extensively in ASM. It will be of interest to determine whether p53 collaborates with *Klf15* to modulate responses to allergen-induced stress stimuli in ASM. Whether *Klf15* plays a role in the effects of GCs on ASM proliferation, which are currently controversial, also remains to be determined (44, 46).

Although our data favor a model where reduced AHR associated with *Klf15* deficiency is, at least in part, due to altered ASM proliferation and apoptosis, we did not observe obvious differences in ASM morphology or cell number associated with *Klf15* deficiency *in vivo* (see, for example, Figure 4). Thus, the inference that altered cell growth secondary to *Klf15* deficiency plays a role in mediating reduced AHR remains speculative. Notably, however, the absence of visible histologic changes associated with *Klf15* deficiency *in vivo* does not

preclude the validity of this model. Indeed, the 3-day OVA challenge protocol that we employed has not been associated with differences in ASM morphology between WT OVA-challenged mice and saline control animals (42, 47). Various regimens that incorporate at least 2 weeks of OVA challenge are, however, associated with smooth muscle hyperplasia (47, 48). This indicates that visible alterations in smooth muscle growth and morphology in response to allergic stimulus lag behind physiologic alterations, such as increased AHR. We anticipate that histologic differences in ASM between *Klf15*^{-/-} and WT mice will be evident with longer-term OVA challenge protocols.

In conclusion, although GCs remain the single best class of therapeutic agents for controlling asthma symptoms, the mechanisms whereby GCs modulate airway function have not been fully defined. In this work, we have established a framework for studying the transcriptional biology of GR signaling in ASM by describing a validated gene set that is regulated by GR signaling in multiple ASM lines. This will facilitate research on the basic molecular mechanisms underlying activation and repression of gene expression by GR in ASM. We have also established a novel role for the GR target gene, *Klf15*, in modulating airway contractility, possibly through regulating apoptosis and proliferation of ASM. We propose that *in vivo* validation of specific genes regulated by GR in ASM will be fruitful in yielding new candidates for pharmacologic targeting to treat asthma.

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