Transcriptional regulation of the IL-13Rα2 gene in human lung fibroblasts

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Interleukin (IL)−13 is a type 2 cytokine with important roles in allergic diseases, asthma, and tissue fibrosis. Its receptor (R) α1 is primarily responsible for the biological actions of this cytokine, while Rα2 possesses a decoy function which can block IL-13 signaling. Although the expression of Rα2 is known to be subject to modulation, information about its transcriptional regulation is limited. In this study, we sought to expand the understanding of transcriptional control of Rα2 in lung fibroblasts. We confirmed previous reports that IL-13 elicited modest induction of Rα2 in normal adult human lung fibroblasts, but found that prostaglandin E2 (PGE2) and fibroblast growth factor 2 (FGF-2) – mediators known to influence fibroblast activation in tissue fibrosis but not previously investigated in this regard – led to a much greater magnitude of Rα2 induction. Although both PGE2 (via protein kinase A) and FGF-2 (via protein kinase B, also known as AKT) depended on activation of cAMP-responsive element-binding protein (CREB) for induction of Rα2 expression, they nevertheless demonstrated synergy in doing so, likely attributable to their differential utilization of distinct transcriptional start sites on the Rα2 promoter. Our data identify CREB activation via PGE2 and FGF-2 as a previously unrecognized molecular controller of Rα2 gene induction and provide potential new insights into strategies for therapeutic manipulation of this endogenous brake on IL-13 signaling.

Interleukin (IL)−13 is an important type 2 cytokine best known for its roles in allergic diseases and asthma1, but which also contributes to other inflammatory diseases such as Crohn's disease and ulcerative colitis2, tissue fibrosis3,4, and various forms of cancer5−7. IL-13 recognition is complex, involving heterodimers comprised of three distinct receptor subunits. IL-13 receptor α1 (IL-13Rα1 or Rα1) has low affinity for IL-13, but when associated with the IL-4 receptor (IL-4Rα) subunit, ligand binding results in productive signaling via JAK/STAT68,9. IL-13 receptor α2 (IL-13Rα2 or Rα2) has greater affinity for IL-13, but its impact on signaling upon ligand binding depends on cell type and context10,11. Rα2 lacks a cytoplasmic domain, and accordingly, many studies have reported that Rα2 fails to initiate signal transduction10 and in fact, can act as a decoy receptor capable of binding ligand and thus preventing productive signaling through Rα112–15. On the other hand, more recent reports have identified cytoplasmic interactors for Rα216 and described Rα2-mediated activation of various intracellular signaling pathways17–19. In keeping with such divergent signaling responses, Rα2 has been reported to both mediate and attenuate mouse models of allergic asthma20–22 as well as pulmonary fibrosis23,24. Although Rα1 is exclusively a membrane-bound receptor, mouse Rα2 has been shown to exist in membrane-bound as well as soluble forms, which reflect distinct splice variants25,26. Although the soluble form does not exist in humans, release of soluble Rα2 through matrix metalloproteinase 8-mediated cleavage of membrane receptor has been demonstrated in both mouse and human cells27. Of note, both of these soluble forms of Rα2 have ~ 3-fold greater affinity for IL-13 than does the membrane-bound form, and are capable of inhibiting signaling responses28. In human lung fibroblasts (Fibs), both membrane and soluble forms of Rα2 have been reported to inhibit the IL-13-induced expression of fibrotic genes29,30.

While expression of Rα1 is ubiquitous in most normal tissues29,31, it is not recognized to be subject to substantial modulation. By contrast, while the basal expression of Rα2 in normal tissues is more restricted30, its expression is subject to dynamic regulation. For example, Rα2 expression has been reported to be high in a variety of neoplasms32–34 as well as in lung Fibs derived from patients with the serious fibrotic lung disease,

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idiopathic pulmonary fibrosis (IPF)\textsuperscript{37,38}. On the other hand, its expression was lower in airway Fibs from patients with asthma than from non-asthmatics\textsuperscript{39}. Expression of R\textsubscript{α2} has also been shown to be potentiated by a variety of inflammatory mediators, including IL-13 itself, IL-4, TNF-\textalpha, and lysophosphatidic acid (LPA)\textsuperscript{40–42}. Of these, only induction by IL-13 has been mechanistically characterized, and – unsurprisingly – shown to proceed via STAT6 activation\textsuperscript{43}.

Although R\textsubscript{α2} expression is subject to dynamic control and to dysregulation in a variety of pathological conditions, information about the factors and underlying molecular mechanisms that control transcription of this gene remains quite limited. Understanding the control of its expression is clearly relevant regardless of whether R\textsubscript{α2} serves to mediate or to quench IL-13 signaling. In this study, we have used human lung Fibs to characterize the effects of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and fibroblast growth factor (FGF)\textsubscript{2} on gene expression of R\textsubscript{α2}.

Results

\textbf{Lung Fibs, but not lung epithelial cells or alveolar macrophages, constitutively express Rα2.}

The relative mRNA expression of R\textsubscript{α1} and R\textsubscript{α2} was determined in primary adult normal human lung Fibs from two distinct sources (those isolated at our institution from the lungs of two subjects, and commercially available CCL210 cells). We also studied primary human alveolar macrophages [alv m\textsuperscript{φ}s] and primary human type II alveolar epithelial cells [AEC2s]) isolated at our institution, and human lung epithelial cell lines obtained commercially (Beas-2b and A549). As shown in Fig. 1A, all three human lung cell types exhibited readily demonstrable baseline expression of R\textsubscript{α1}. This was also evident in primary lung Fibs, a lung epithelial cell line (MLE-12), and primary alveolar m\textsuperscript{φ}s derived from mouse (Fig. 1B). In both human and mouse, both epithelial cells and alv m\textsuperscript{φ}s manifested higher levels of R\textsubscript{α1} mRNA than did Fibs from the lung. Conversely, R\textsubscript{α2} transcript was expressed only in lung Fibs, but not in lung epithelial cells or alv m\textsuperscript{φ}s from either humans (Fig. 1C) or mouse (Fig. 1D). Our finding

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\caption{Basal expression of R\textsubscript{α1} and R\textsubscript{α2} in various lung cell types. qPCR analysis of basal expression of R\textsubscript{α1} (A,B) and R\textsubscript{α2} (C,D) in primary human lung cells (Fibs, alv m\textsuperscript{φ}s, AEC2s Beas-2b and A549 (A,C) and in mouse primary lung Fibs, MLE-12 lung epithelial cells, and primary alveolar m\textsuperscript{φ}s (B,D). Expression is relative to that of CCL210 Fibs, represented as open circles. Each bar represents mean values (±S.E.) from three independent samples. *p < 0.05.}
\end{figure}
that among lung cells, basal expression of Rα2 is restricted to Fibs is consistent with single-cell transcriptomic studies reported in human\textsuperscript{44} and mouse\textsuperscript{45} lung tissues. Delta Ct values for human Rα1 and Rα2 (normalized to GAPDH) are provided in Supplementary Table 1.

Upregulation of Rα2 expression in lung Fibs by soluble mediators. Next, we assessed the influence of a variety of soluble mediators with effects on inflammation and fibrogenesis on Rα1 and Rα2 expression in CCL210 Fibs. As shown in Fig. 2A, treatment with IL-13, TNF-α, FGF-2, PDGF, PGE\textsubscript{2}, or TGF-β failed to alter basal Rα1 mRNA expression. In contrast, and consistent with literature reports, Rα2 mRNA expression was modestly but significantly upregulated by IL-13, LPA, and TNF-α (Fig. 2B). Rα2 expression was also significantly upregulated by FGF-2, PDGF, and PGE\textsubscript{2}, but not by TGF-β. Of note, the fold induction of Rα2 by FGF-2 and PGE\textsubscript{2} (~10- to 20-fold) was markedly higher than that elicited by IL-13, LPA, and TNF-α (~2- to 4-fold). The doses of PGE\textsubscript{2} and FGF-2 utilized were previously established as optimal for other actions in human lung Fibs\textsuperscript{46}.

Kinetic analysis of Rα2 induction by FGF-2 and PGE\textsubscript{2} in CCL210 lung Fibs showed its onset at 3–6 h and a plateau reached at 36–48 h after addition (Supplementary Figure 1A). In contrast to their actions in Fibs, neither FGF-2, PDGF, nor PGE\textsubscript{2} showed any effect on Rα2 expression in either A549 lung epithelial cells or primary human alveolar m\textsubscript{φ}s (Supplementary Figure 1B). Although an increase in expression of intracellular Rα2 protein in Fibs by FGF-2 and particularly PGE\textsubscript{2} (Fig. 2C,D) accompaniend that of mRNA (Fig. 2B), the magnitude of induction was substantially less. This prompted us to evaluate the presence of the cleaved and secreted form of Rα2 in Fib conditioned medium\textsuperscript{27}. A modest increase in Rα2 protein in Fib culture supernatant was noted in response to FGF-2 and PGE\textsubscript{2} (Supplementary Figure 1C). To verify the functional effects of upregulated expression of Rα2 on IL-13 signaling, we examined expression of the important R1 target gene periostin. As proof-of-concept, transfection with a CMV promoter-driven Rα2 construct dampened baseline and IL-13-induced periostin gene expression (Supplementary Figure 2A). Likewise, pre-treatment of cells with FGF-2 or PGE\textsubscript{2} also dampened baseline and IL-13-induced levels of this matricellular gene (Supplementary Figure 2B). Co-stimulation with PGE\textsubscript{2} + FGF-2

Figure 2. Stimulated expression of Rα1 and Rα2 in human lung Fibs. CCL210 cells were stimulated with IL-13, TNF-α, FGF-2, PDGF, PGE\textsubscript{2}, or TGF-β for 24 h. Expression of Rα1 (A) and Rα2 (B) were determined using qPCR analysis. In (A), none of the values are significantly different from untreated control. In (B), all treatments except TGF-β are significantly greater than control (p < 0.05); the asterisks indicate values significantly greater than IL-13. (C–D) CCL210 cells were stimulated with IL-13, FGF-2, or PGE\textsubscript{2} for 48 h and expression of Rα2 protein was determined by western blot; the blot shown in (C) is representative of 3 independent experiments, and quantification of western blots by mean densitometric analysis is shown in (D). Each bar represents mean values (±S.E.) from three independent experiments. In (D), all values are significantly greater than control (p < 0.05); asterisks indicate values significantly greater than IL-13. *p < 0.05; ns, not significant.
α-dation. To directly assess the induction of Rα2 by a myristoylated and thus cell permeable PKA inhibitor, PKI 14–22 amide, completely abolished the ability of PGE2 to induce the expression of Rα2. Furthermore, the addition of Act D 24 h after initial treatment with PGE2 and FGF-2 stopped further transcript accumulation but failed to reveal any attenuation of mRNA decay (Fig. 3B). These data suggest that PGE2- and FGF-2-stimulated Fibs. As shown in Fig. 3A, pre-treatment with the transcription inhibitor actinomycin D (Act D) did not affect the baseline expression of Rα2, but completely prevented the ability of both PGE2 and FGF-2 to increase Rα2 transcripts. Furthermore, the addition of Act D 24 h after initial treatment with PGE2 and FGF-2 stopped further transcript accumulation but failed to reveal any attenuation of mRNA decay (Fig. 3B). These data suggest that increases in Rα2 mRNA accumulation by PGE2 and FGF-2 reflect increased transcription rather than decreased degradation. To directly assess the induction of Rα2 transcription by PGE2 and FGF-2 at the promoter level, and to compare their actions to the positive control, IL-1347, CCL210 cells were transfected with Rα2 promoter luciferase construct (pGL3-Rα2), stimulated with IL-13, FGF-2 or PGE2 for 24 h, and luciferase activity determined using Dual-Luciferase reporter assay system. Each bar represents mean values (±s.E.M.) from three independent experiments. All stimulated values were greater than control (p < 0.05). The asterisks depicted in C indicate values significantly greater than IL-13. *p < 0.05.

Figure 3. FGF-2 and PGE2 increase Rα2 expression via new transcription. (A) CCL210 cells were pre-treated ± Act D for 30 min followed by stimulation ± FGF-2 or ± PGE2, for 24 h, and the expression of Rα2 was determined by qPCR analysis. (B) CCL210 cells were stimulated ± FGF-2 or ± PGE2, for 24 h and then treated ± Act D. Samples were harvested at 0 h, 12 h, 24 h (prior to Act D treatment) as well as at 36 h and 48 h, and the expression of Rα2 was determined by qPCR analysis. (C) CCL210 cells were transfected with Rα2 promoter luciferase construct (pGL3-Rα2), stimulated with IL-13, FGF-2 or PGE2, for 24 h, and luciferase activity determined using Dual-Luciferase reporter assay system. Each bar represents mean values (±s.E.M.) from three independent experiments. All stimulated values were greater than control (p < 0.05). The asterisks depicted in C indicate values significantly greater than IL-13. *p < 0.05.

also markedly attenuated IL-13-induced periostin gene expression; however, this reduction was not significantly greater than that seen when cells were pretreated with PGE2 prior to IL-13 stimulation. Taken together, these data clearly show that induction of Rα2 transcript and protein in response to FGF-2 and PGE2 functionally dampens IL-13-induced signaling via Rα1 in lung Fibs.

Induction of Rα2 expression in response to PGE2 and FGF-2 requires new transcription. We next sought to determine the relative roles of new transcription versus increased stability in the increase in Rα2 mRNA in PGE2- and FGF-2-stimulated Fibs. As shown in Fig. 3A, pre-treatment with the transcription inhibitor actinomycin D (Act D) did not affect the baseline expression of Rα2, but completely prevented the ability of both PGE2 and FGF-2 to increase Rα2 transcripts. Furthermore, the addition of Act D 24 h after initial treatment with PGE2 and FGF-2 stopped further transcript accumulation but failed to reveal any attenuation of mRNA decay (Fig. 3B). These data suggest that increases in Rα2 mRNA accumulation by PGE2 and FGF-2 reflect increased transcription rather than decreased degradation. To directly assess the induction of Rα2 transcription by PGE2 and FGF-2 at the promoter level, and to compare their actions to the positive control, IL-1347, CCL210 cells were transfected with a Rα2 promoter luciferase construct (pGL3-Rα2). As shown in Fig. 3C, and consistent with the Rα2 mRNA data shown in Figs. 2 and 3A, stimulation with either PGE2 or FGF-2 increased the Rα2 promoter activity and did so to a greater extent than did IL-13.

PGE2-induced expression of Rα2 proceeds via an EP2/cAMP/PKA pathway. PGE2 can ligate four different G protein-coupled receptors (EP1–4) activating distinct signaling pathways. We previously reported that PGE2 signaling in CCL210 cells is mainly through an EP2 receptor-mediated increase in cAMP signaling46. The role of EP2 and cAMP signaling in Rα2 induction by PGE2 was therefore assessed. As shown in Fig. 4A, Rα2 expression was upregulated by butaprost (an analog of PGE2 that is a selective agonist for EP2) and forskolin (a direct activator of adenylyl cyclase that generates cAMP) in a manner parallel to that of PGE2. We also assessed the role of the classical cAMP effector PKA in the EP2-induced Rα2 induction. As shown in Fig. 4B, inhibition of PKA by a myristoylated and thus cell permeable PKA inhibitor, PKI 14–22 amide, completely abolished the ability of PGE2 to induce the expression of Rα2. Together, these data indicate that PGE2 utilizes an EP2/cAMP/PKA pathway to induce transcription of Rα2.

FGF-2-induced expression of Rα2 proceeds via a PI3 kinase/PDK1/AKT pathway. We previously reported that in human lung Fibs, signaling by mitogens such as FGF-2 critically depends on activation of the phosphoinositide-3-kinase (PI3 kinase)/3-phosphoinositide-dependent protein kinase 1 (PDK1)/AKT pathway46. We therefore interrogated the involvement of this pathway in the induction of Rα2 by FGF-2. First, we verified the sufficiency of this molecular pathway for the induction of Rα2 in Fibs by transfecting cells with a constitutively active form of AKT (myr-AKT). As shown in Fig. 5A, increased expression of Rα2 was observed in CCL210 cells expressing myr-AKT, but not empty vector. Next, we utilized a variety of pharmacologic inhibitors to determine the necessity of this pathway in FGF-2-stimulated cells. As shown in Fig. 5B, pre-treatment of CCL210 cells with three distinct PI3 kinase pathway-specific inhibitors (LY294002, inhibitor of PI3 kinase; GSK 2334470, inhibitor of PDK1; and triciribine, inhibitor of AKT) markedly reduced FGF-2-induced expression of Rα2.

PGE2 and FGF-2 demonstrate synergy in the induction of Rα2 gene expression. The fact that PGE2 and FGF-2 both stimulate Rα2 expression was somewhat unexpected, as we and others have reported that
PGE₂ acts as a negative regulator of a number of FGF-2-induced actions in lung Fibs, including proliferation, migration and survival. It was therefore of interest to examine the effects of co-stimulation with both mediators on Rα₂ expression. As shown in Fig. 6A, co-stimulation of CCL210 cells with PGE₂ and FGF-2 resulted in a markedly synergistic increase of several hundred-fold in Rα₂ mRNA expression. Consistent with the findings shown in Fig. 2A with individual mediators, co-stimulation with PGE₂ and FGF-2 likewise failed to increase Rα₁ expression. Although a synergistic effect was also present at the protein level, it was, as also demonstrated for these agonists individually (as shown in Fig. 2), much less striking than for mRNA (Fig. 6B). As shown in Fig. 6B, even CMV promoter-driven Rα₂ overexpression resulted in no higher level of Rα₂ protein induction than that observed with PGE₂ + FGF-2 treatment. This suggests either that the capacity for translation of Rα₂ mRNA is saturated, or that additional post-transcriptional levels of regulation influence Rα₂ protein induction. Co-stimulation with forskolin (instead of PGE₂) and FGF-2 also resulted in synergy (Fig. 6D). Likewise, stimulation of CCL210 cells expressing myr-AKT (instead of treatment with FGF-2) with PGE₂ also resulted in synergy (Fig. 6D).
CREB activation is necessary for both PGE₂- and FGF-2-induced expression of Rα2. Since phosphorylation and activation of STAT6 has been implicated in IL-13-induced Rα2 expression, we assessed the activation of STAT6 by PGE₂ and FGF-2. Unlike IL-13, PGE₂ and FGF-2 failed to elicit phosphorylation of STAT6 (Fig. 7A). Likewise, pharmacologic inhibition of STAT6 using AS1517499 abolished IL-13-, but not PGE₂- or FGF-2-induced Rα2 expression (Fig. 7B). CREB has long been recognized as an important transcriptional effector downstream of PGE₂/cAMP/PKA, and phosphorylation and activation of CREB have more recently also been documented to participate in FGF-2/AKT signaling. Although CREB has not, to our knowledge, previously been investigated in the transcriptional control of Rα2, it is noteworthy that MatInspector (http://www.genomatix.de/matinpector.html) identified several CREB-binding sites, in addition to STAT binding sites, within the Rα2 promoter (Supplementary Figure 3). We therefore evaluated the role of CREB in Rα2 induction. Consistent with this possibility, both PGE₂ and FGF-2 increased the phosphorylation of CREB, while IL-13 had no such effect (Fig. 7C). As shown in Fig. 7D, forced overexpression of a constitutively active form of CREB (CREB-VP16) resulted in a marked increase in the expression of the well-known CREB target gene c-Fos and a more modest increase in that of Rα2. To assess the necessity of CREB activation in PGE₂- and FGF-2-mediated Rα2 expression, we employed the potent and selective cell-permeable CREB inhibitor 666–15. As shown in Fig. 7E, pre-treatment with 666–15 abrogated the induction of Rα2 by both PGE₂ and FGF-2. Pharmacologic inhibition of CREB activation also abrogated the marked synergistic effect of PGE₂ and FGF-2 on Rα2 induction (Fig. 7F).

**Figure 6.** Induction of Rα2 by PGE₂/forskolin plus FGF-2/myr-AKT is synergistic. (A-B) CCL210 cells were stimulated with PGE₂, FGF-2 or both for 24 h and 48 h and the expression of Rα1 and Rα2 were determined using qPCR analysis (A) and western blot (B), respectively. (B) also depicts protein expression in cells over-expressing Rα2 (mean induction of 6-fold over empty vector). Blot in (B) is representative of 3 independent experiments. (C) CCL210 cells were stimulated with forskolin, FGF-2, or both for 24 h and the expression of Rα2 was determined using qPCR analysis. (D) CCL210 cells were stimulated with PGE₂, transected with myr-AKT, or both for 24 h and the expression of Rα2 was determined using qPCR analysis. In A, C and D, all treatments are significantly greater than control (p < 0.05). Each bar in A, C and D represents mean values (± S.E.) from three independent experiments.
pGe2- and FGF-2-mediated upregulation of Rα2 expression involves transcriptional activation from distinct start sites. Normal human lung Fib RNA-seq data available at the UCSC genome browser (https://genome.ucsc.edu/) reveal the existence of two distinct transcriptional start sites (TSSs) for Rα2 (Fig. 8A). We wished to determine the TSS utilized by IL-13 and to interrogate the site(s) utilized by PGE2 and FGF-2. Therefore, we arbitrarily designated the two distinct TSSs as TSS1 and TSS2. The UCSC genome browser depicts a partial sequence for the TSS1-initiated transcript, and additional data available at the Ensembl browser (http://useast.ensembl.org/Homo_sapiens/Location/View?db=coreg;ENSG00000123496;r=X:115003655-115020297) suggest that the annotated partial transcript from TSS1 represents a long non-coding RNA. In contrast, our data suggest that both IL-13 and PGE2 induce a TSS1-initiated transcript and make Rα2 protein (as shown in Fig. 2C). Moreover, RNA-seq from normal human lung Fibs shows expression of full-length transcript from TSS1 (see Supplementary Figure 4). Therefore, we attempted to characterize the TSS1-initiated transcript by PCR amplification followed by DNA sequencing. As shown in Fig. 8B, using TSS-specific forward primers and a pair of reverse primers (Rα2_Rev1 and Rα2_Rev2 that bind to exon 10 but at different regions (see Supplementary Table 2B), we PCR-amplified the Rα2 transcript. Since PGE2 and FGF-2 show synergistic effects on Rα2 mRNA expression (Fig. 6A), we utilized cDNA from Fibs co-stimulated with FGF-2 and PGE2. As shown in Fig. 8B, amplification of the Rα2 transcript from both TSS1- and TSS2-specific primers was determined by separation in 1% agarose gel yielding PCR amplicons at the expected length (Fig. 8C). Next, DNA sequencing was performed on the PCR...
Figure 8. Transcriptional activation of Rα2 by various stimuli at two distinct transcription start sites. (A) Depiction of TSS1 and TSS2 locations for Rα2 (based on UCSC database). (B) Depiction of exonic positions on Rα2 gene (based on ENCODE RNA-seq data from normal human lung Fibs), and representation of the design of TSS-specific forward primers and a pair of reverse primers (Rα2_Rev1 and Rα2_Rev2) both bind to exon 10 but at different locations. (C) Agarose gel image showing TSS1- and TSS2-specific PCR amplicons and their product size. (D) Schematic representation of the design of TSS-specific qPCR primers with a common reverse primer, Rα2_Rev Primer1 to distinguish distinct TSSs. (E) CCL210 cells were stimulated with IL-13, FGF-2, or PGE2 for 24 h and the expression of distinct Rα2 transcripts were assessed by qPCR using TSS1-specific and TSS2-specific primers. (F) CCL210 cells were stimulated with IL-13, FGF-2, PGE2, IL-13 + FGF-2, IL-13 + PGE2, or FGF-2 + PGE2 for 24 h and the expression of Rα2 was determined using qPCR analysis with the common primer. Each bar in C and D represents mean values (± S.E.) from three independent experiments. In C, the asterisks indicate treatments are significantly greater than control (p < 0.05). In D, all treatments are
either by the fact that the PGE2 dose employed was sufficient for maximal reduction in periostin gene expression, α migration, and differentiation and thereby fibrosis, also strongly enhanced the expression of R2 in Fibs. However, as reported previously, its expression only in Fibs and was undetectable in epithelial cells and alv mφs. 2 was basally expressed in lung cells has never been reported. We found high basal expression of R1 in lung epithelial cells, mαs, and Fibs, whereas FGF-2 preferentially utilized TSS2. As predicted by this pattern of TSS utilization, co-stimulation with IL-13 plus PGE2 (both acting via TSS1) yielded no synergy as compared to either alone, while co-stimulation with IL-13 (acting via TSS1) and FGF-2 (acting via TSS2) demonstrated synergy in Ro2 expression as compared to that observed with either stimulus alone (Fig. 8F). These data indicate that differential utilization of these two TSSs by various agonists may explain interactions in transcription of Ro2. Figure 8G summarizes our findings of differential utilization of distinct Ro2 TSSs by IL-13/STAT6, PGE2/EP2/cAMP/PKA/CREB, and FGF-2/PI3 kinase/PDK1/AKT/CREB pathways.

Discussion

IL-13 is a pleiotropic type 2 cytokine with well-recognized roles in inflammatory, immune, fibrotic, and neoplastic processes. Its receptors Ro1 and Ro2 differ in a number of respects. From a functional perspective, while Ro1 is well known to exert biological actions via JAK/STAT6 signaling, Ro2 in most experimental systems – including lung Fibs43–45 – acts as a decoy receptor dampening the actions of IL-13. Another key difference is that unlike Ro1, expression of Ro2 has been shown to be subject to modulation in various disease states and in response to various mediators. Despite this knowledge about the molecular regulation of Ro2 gene expression is quite limited. In this study, we found that Ro2 gene transcription in human lung Fibs was strongly induced by PGE2, FGF-2, and PDGF – mediators known to exhibit diverse regulatory effects on Fibs. Such induction of Ro2 was not seen in lung epithelial cells or alv mφs. Although the effects of PGE2, and FGF-2 proceeded via distinct and separate signaling pathways, their actions converged on CREB – the first time this transcription factor has been implicated in the control of Ro2 gene transcription.

The UCSC genome browser identifies at least two distinct TSSs in the human Ro2 gene and ENCODE RNA-seq analysis in normal human lung Fibs further confirmed the expression of Ro2 transcripts from both of these TSSs. However, only a partial sequence was deposited at the UCSC database, and it was annotated, based on computational prediction by Havana (Human and Vertebrate Analysis and Annotation), as a lncRNA 218. By contrast, our experimental data clearly demonstrate full-length Ro2 transcript initiated from TSS1. Finally, we identified that synergistic interactions among mediators in Ro2 induction are associated with CREB-mediated transcription initiation at distinct TSSs. Together, these data expand our understanding of the mechanisms governing Ro2 gene transcription.

The actions of IL-13 have been well studied in a variety of lung cell types, including epithelial cells, mφs, eosinophils, Fibs, and dendritic cells46–48. However, the relative expression of its Ro1 and Ro2 subunits among key lung cells has never been reported. We found high basal expression of Ro1 in lung epithelial cells, mφs, and Fibs, consistent with IL-13 responses in these cell types being Ro1-dependent. By contrast, Ro2 was basally expressed only in Fibs and was undetectable in epithelial cells and alv mφs. However, as reported previously, its expression in epithelial cells can be induced by IL-13 to function as a feedback inhibitory loop in these cells49. Basal expression of Ro2 in lung Fibs was modestly enhanced by IL-13 stimulation, as reported previously50. We also verified previous reports that LPA and TNF-α modestly upregulated Ro2 expression. The effects of mitogenic factors FGF-2 and PDGF on Fib Ro2 had not previously been reported, and we found that these markedly increased the expression of Ro2 in Fibs. However, the well-characterized pro-fibrotic factor TGF-β showed no effect on Ro2. These data suggest a potential role for fibrotic drivers other than TGF-β in Ro2 upregulation in lung Fibs during lung fibrosis. Unexpectedly, PGE2, a well-known suppressor of numerous Fib processes including proliferation, migration, and differentiation and thereby fibrosis, also strongly enhanced the expression of Ro2 in Fibs.

Kinetic studies with Act D as well as Ro2 promoter luciferase assays revealed that both PGE2 and FGF-2 increased expression of the Ro2 gene by enhancing transcription, rather than by impeding degradation. The increases in Ro2 mRNA we observed were paralleled by concomitant increases in both intracellular and secreted Ro2 protein, most evident with PGE2. While the increment of protein induction was substantially less than that of mRNA, it was sufficient to be associated with blunted IL-13 induction of its key target gene, the matricellular protein periostin. These results suggest that additional regulation may exist at the levels of protein translation and/or stability. Moreover, the lack of an additive effect of PGE2 + FGF-2 on periostin expression could be explained either by the fact that the PGE2 dose employed was sufficient for maximal reduction in periostin gene expression, or that PGE2 and FGF-2 had no additive effect in this context. Comprehensively analyzing a potential additive effect would require that co-stimulation studies be performed with suboptimal doses of both FGF-2 and PGE2 and then stimulated with IL-13 to measure periostin gene expression. Evaluating these possibilities will require additional studies. Future work might also evaluate the impact of PGE2 and FGF-2 on IL-13 target genes other than periostin.
Studies by our laboratory and others have definitively established that the predominant signaling pathway for PGE\(_2\) in Fibs proceeds through the binding to EP2 with subsequent activation of adenyl cyclase to generate cAMP and resulting activation of PKA. Consistent with an EP2/CAMP/PKA pathway being operative here as well, induction of R\(\alpha\)2 was mimicked by the EP2 agonist butaprost and by the receptor-independent activation of adenyl cyclase by forskolin, while induction by PGE\(_2\) was completely abrogated by the PKA inhibitor PKI 14–22 amide. We and others have reported that the ability of FGF-2 to promote Fib proliferation and migration critically depends on signaling via the PI3 kinase-PDK1-AKT pathway. Since R\(\alpha\)2 induction by FGF-2 was attenuated by inhibitors of all three of these sequential kinases, and was mimicked by expression of constitutively active AKT, we conclude that this pathway is operative in R\(\alpha\)2 transcription as well. PI3 kinase activation is also implicated in the mitogenic actions of PDGF, and it is therefore likely that that the PI3 kinase-PDK1-AKT pathway is required for R\(\alpha\)2 expression by diverse mitogens. Although the well-characterized pro-fibrotic factor TGF-\(\beta\) is also known to activate PI3 kinases, it failed to upregulate R\(\alpha\)2 expression. This may reflect differential activation by distinct growth factors of PI3 kinase isoforms with varying capacities to initiate R\(\alpha\)2 transcription, a prospect that will require experimental evaluation.

Most mediators thus far reported to increase gene expression of R\(\alpha\)2 – including IL-13, TNF-\(\alpha\), and IL-17 – possess predominant pro-inflammatory actions. Among mediators with Fib modulatory actions, only the pro-fibrotic substance LPA has been shown to increase gene expression of R\(\alpha\)2. Increasing expression of this decoy receptor for pro-inflammatory and pro-fibrotic IL-13 thus allows these substances to activate a homeostatic brake on pathologic responses. As mentioned earlier, intrinsic PGE\(_2\) effects on Fibs are largely suppressive, and the same is also the case for its effects on a variety of leukocyte subsets. Induction of R\(\alpha\)2 thereby serves to amplify the anti-fibrotic and anti-inflammatory actions of this lipid mediator. In contrast to PGE\(_2\), the effects of FGF-2 on Fibs are more complex. Its well-known mitogenic and migratory actions, noted above, promote tissue fibrosis, and indeed, FGF-2 has been implicated in the pathogenesis of IPF. On the other hand, FGF-2 has also been reported to possess anti-fibrotic actions in certain contexts. Induction of R\(\alpha\)2 may represent a previously unrecognized mechanism limiting the fibrogenic actions of FGF-2. We have previously demonstrated that PGE\(_2\) strongly blocks the proliferative and migratory actions of FGF-2 in Fibs. For this reason, it was unexpected that these two mediators would exert parallel stimulatory effects on R\(\alpha\)2 transcription. That PGE\(_2\) and FGF-2 would act synergistically to enhance R\(\alpha\)2 transcription was even more surprising. This motivated us to explore the mechanisms responsible for synergistic induction by these substances.

We confirmed findings reported by others that phosphorylation and activation of STAT6 accompanied IL-13-induced R\(\alpha\)2 transcription, and a STAT6 inhibitor abrogated such induction. By contrast, STAT6 activation was not observed with FGF-2 or PGE\(_2\), and the induction of R\(\alpha\)2 by these mediators was unaffected by a STAT6 inhibitor, implicating STAT6-independent mechanism(s) in R\(\alpha\)2 induction. A STAT6-independent mechanism for R\(\alpha\)2 induction has been reported previously for TNF-\(\alpha\), though the mechanism was not elucidated. As a strategy to identify potential transcription factors other than STAT6 responsible for R\(\alpha\)2 gene induction, we performed transcription factor binding site analysis using the transcription factor database MatInspector. This revealed the presence of numerous CREB binding sites within the R\(\alpha\)2 promoter region. To our knowledge, the role of CREB in R\(\alpha\)2 gene regulation has not previously been investigated. Its functional capacity to influence R\(\alpha\)2 expression in Fibs was first confirmed by expressing a constitutively active form of CREB in lung Fibs. Phosphorylation and activation of CREB has been previously reported in response to both PGE\(_2\) and FGF-2, and we confirmed that both of these mediators increased the phosphorylation of CREB in Fibs. The ability of a potent and selective CREB inhibitor to abrogate PGE\(_2\) and FGF-2-induced R\(\alpha\)2 expression provided the critical link between the activation of this transcription factor and its functional role in receptor gene expression. In addition to CREB binding sites, recent studies revealed binding sites for activator protein-1 (AP-1) in the R\(\alpha\)2 promoter region. Of note, prior studies implicated a role for AP-1 in FGF-2-mediated gene regulation. It is thus possible that CREB and AP-1 may act cooperatively in FGF-2-driven R\(\alpha\)2 gene expression, and future studies will be needed to address such a possibility.

The fact that both PGE\(_2\) and FGF-2 acted through CREB made their striking synergy in R\(\alpha\)2 induction even more curious. The STAT6 binding sites implicated in IL-13-induced R\(\alpha\)2 expression are in close proximity to TSS1. On the other hand, the CREB binding sites are positioned close to both TSS1 and TSS2. Experiments using TSS-specific qPCR primers further confirmed that TSS1 was utilized for R\(\alpha\)2 transcription initiated by IL-13. TSS1 was also shown to be the major start site for transcription initiated by PGE\(_2\), whereas TSS2 was exclusively used for transcription initiated by FGF-2. CREB-mediated R\(\alpha\)2 transcription elicited by the combination of an agonist utilizing TSS2 (FGF-2) along with an agonist utilizing TSS1 (either PGE\(_2\) or IL-13) was accompanied by activation of both TSSs, likely explaining the synergistic effects observed for R\(\alpha\)2 expression. The relationship between individual transcription factor binding sites and transcription initiated at the two TSSs remains uncertain. The activation of transcription from both TSS1 and TSS2 also provides a potential explanation for synergistic patterns of induction by various combinations of stimuli. In this regard, it is of interest to note that synergistic induction of R\(\alpha\)2 in lung Fibs was previously observed with the combination of IL-17 and either IL-13 or TNF-\(\alpha\). As the mechanism underlying such synergy was never explored, it will be of interest in future studies to test the relevance of the dual TSS mechanism with these stimuli as well. Similarly, the possibility of functional differences between TSS1- and TSS2-initiated R\(\alpha\)2 transcripts will require further investigation.

R\(\alpha\)2 is unique among IL-13 receptors in that its expression is dynamically regulated and is known to be either increased or decreased in various disease states. Nevertheless, understanding of the mechanisms governing its transcriptional regulation has been limited. Our study expands the range of mediators capable of robustly stimulating R\(\alpha\)2 transcription to include mitogens as well as PGE\(_2\). We also identify CREB as a new transcriptional regulator for R\(\alpha\)2. Finally, we provide evidence for dual TSS utilization as a possible mechanism underlying synergy in R\(\alpha\)2 transcriptional activation. Future studies will be needed to evaluate the relevance of these new mechanistic insights to other transcriptional regulators of R\(\alpha\)2, alone and in combination, and to alterations in its expression in various pathologic states.
Materials and Methods

Cell culture and reagents. CCL210 (CCD-19Lu) primary Fibs isolated from normal adult human lung, A549 human lung adenocarcinoma cells, Beas-2b bronchial epithelial cells, and MLE-12 murine lung epithelial cells were purchased from ATCC. Fibs were cultured in low glucose Dulbecco's modified Eagle's medium and epithelial cells were cultured in RPMI-1640 medium (both purchased from Invitrogen) and supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin (Gibco), and 100 μg/ml streptomycin (Invitrogen). Recombinant human IL-13, FGF-2, PDGE, TNF-α, and TGF-β, as well as LPA, were purchased from Millipore. PGE2, forskolin, butaprost, Act D, LPA, PI3 kinase inhibitor (LY294002) and AKT inhibitor (triciribine) were purchased from Cayman Chemicals. GSK 2334470 was from Tocris Bioscience. PKI 14–22 amide and CREB inhibitor were purchased from Panomics. Unless otherwise specified, the final concentrations of agonists used for cell treatment were: TGF-α (10 ng/ml), PGE2 (50 ng/ml), FGF-2 (50 ng/ml), PDGF (50 ng/ml), IL-13 (10 ng/ml), TNF-α (10 ng/ml), LPA (10 μM), PGE2 (500 nM), forskolin (10 μM), butaprost (10 μM), PKI (10 μM), LY294002 (10 μM), GSK 2334470 (1 μM), triciribine (5 μM), 666–15 (500 nM), AS1517499 (250 nM) and Act D (2 μg/ml). Antibody recognizing Ro2 was purchased from Abcam. Antibodies recognizing CREB, pCREB, STAT6, pSTAT6, and GAPDH HRP conjugate were purchased from Cell Signaling Technologies. High capacity cDNA reverse transcription kit and Fast SYBR Green Master Mix were purchased from Applied Biosystems. Dual-Luciferase Reporter Assay reagents were purchased from Promega.

Harvesting Fib culture supernatant. Supernatant from Fib cultures was collected after 48 h culture. Supernatant was then sequentially centrifuged at 500 × g for 10 min and 2500 × g for 12 min to remove dead cells/debris and apoptotic bodies, respectively. Equal volumes of supernatants from each culture were then concentrated 50-fold using Amicon Ultra-10 centrifugal filters (Millipore) and immediately subjected to western blotting to detect Ro2 protein.

Acquisition of human Fibs, alv mφs, and AEC2s. From the University of Michigan lung tissue biorepository, we obtained IRB-exempted primary type II alveolar epithelial cells and primary lung Fibs from lungs of several subjects lacking lung pathology. Likewise, primary alv mφs were purified from bronchoalveolar lavage samples obtained from subjects undergoing research bronchoscopy at the University of Michigan Hospital Medical Procedure Unit. Subject samples utilized in this study included two with asthma and one non-asthmatic atopic individual; since no differences were noted among these subjects in alv mφ expression of Ro1 or Ro2, they were analyzed as a single group. Informed consent was obtained from each subject prior to sample collection in accordance with the Declaration of Helsinki and with approval of the Institutional Review Board (UM IRB# HUM00136068). Lavage fluid samples were subjected to centrifugation at 500 × g for 10 min (4°C), and pelleted cells were resuspended in complete RPMI 1640 medium (containing fetal bovine serum and other supplements described in Cell Culture and Reagents) and cultured overnight at a density of 0.6 × 106 cells/ml. Non-adherent and loosely adherent cells were washed off with PBS, and the remaining cultures of adherent cells were > 98% alv mφs by Diff-Quik staining.

Isolation of murine lung Fibs and alv mφs. Pathogen-free naïve male C57BL/6 mice aged 6–8 weeks were purchased from The Jackson Laboratory. Mice were housed in groups of 5 and they had ad libitum access to water and food. All methods were carried out in accordance with relevant national and local guidelines and regulations regarding the use of experimental animals and with approval of the University of Michigan Committee for the Use and Care of Animals. Mice were sacrificed and lung lavage and alv mφ isolation and culture were performed as described previously66. Fibs were also outgrown from lung tissue and cultured as described previously46.

RNA isolation and quantitative real-time PCR. Cells were suspended in 700 μl TRIzol reagent (ThermoFischer Scientific) and RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The concentration of total RNA was measured using Nanodrop. Using the high capacity cDNA reverse transcription kit (Applied Biosystems), total RNA was converted to cDNA. Levels of mRNA were assessed by quantitative real-time PCR (qPCR) analysis with a Fast SYBR green master mix (Applied Biosystems) on an ABI Prism 7300 thermocycler (Applied Biosystems). Expression of human Ro1, Ro2, TSS1- and TSS2-specific Ro2, and periostin and murine Ro1 and Ro2 was assessed using sequence-specific primers listed in Supplementary Table 2. Unless specified otherwise, the human Ro2 primer employed was designed to bind downstream of exon 3 and is common to both TSS1- and TSS2-initiated transcripts. Relative gene expression was determined by the ΔCT method, and GAPDH and β-actin were used as a reference gene for human and mouse samples, respectively.

Ro2 promoter activity assay. The Ro2 promoter-luciferase construct (pGL3–Ro2) was a kind gift from Dr. Wei Xu (McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI)71. Cells were grown on 6-well plates and co-transfected at 60% confluence with FuGENE HD (Promega) using 1.0 μg of pGL3–Ro2 or empty (pGL3–Basic) plasmids together with 50-3 Ro2 reference promoter driving Renilla luciferase (pRL–TK) to normalize the data. After 24 h of incubation, cells were stimulated ± PGE2, FGF-2, or IL-13 for an additional 24 h. Cells were then lysed and firefly and Renilla luciferase activities were measured by the Dual-Luciferase reporter assay system using a GloMax 96 microplate luminometer with dual injectors (Promega). Results were normalized by dividing the firefly luciferase activity by the Renilla luciferase activity of the same sample as described previously68.

Plasmid overexpression studies. The Myr-AKT construct was kindly provided by Dr. Philip Tsichlis (Tufts University, Boston, Massachusetts, USA) and the active CREB construct (pCREB–VP16) was a generous
Western blot analysis. Samples were lysed in RIPA buffer (Cell Signaling) supplemented with protease inhibitors (Roche Diagnostics) and phosphatase inhibitor cocktail (EMD Biosciences). Samples were analyzed as previously described[24]. All antibodies were used at a dilution of 1:1,000 except GAPDH-HRP which was used at a dilution of 1:5,000.

Ssp1 restriction digestion. Using TSS-specific primers (listed in Supplementary Table 2C), TSS1 and TSS2 products were amplified by PCR. By running in 1.5% agarose gel, TSS1- and TSS2-products were confirmed, gel extracted, and 500 ng of DNA was digested with Ssp1 enzyme (from NEB) treatment overnight. Samples were then excised from the gel, purified using Qiagen Gel Extraction kit and samples were subjected to DNA Sanger sequencing.

Statistical analysis. Data are presented as means and were analyzed for statistical significance by one-way ANOVA and Dunnett’s multiple comparisons post-hoc test using Prism 7.0 (GraphPad Software). Error bars represent mean values (±S.E.). P-values below 0.05 were considered to be statistically significant.

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Author Contributions
L.R.P. planned and performed experiments, analyzed the data, organized data for presentation, and wrote the manuscript. H.O. and J.M.S. performed experiments. N.L.L., Y.J.H. and S.K.H. provided the patient samples and assistance with their use. M.P.G. planned experiments, analyzed data, and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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