

## Kisspeptins inhibit human airway smooth muscle proliferation

Niyati A. Borkar, ... , Y.S. Prakash, Venkatachalem Sathish

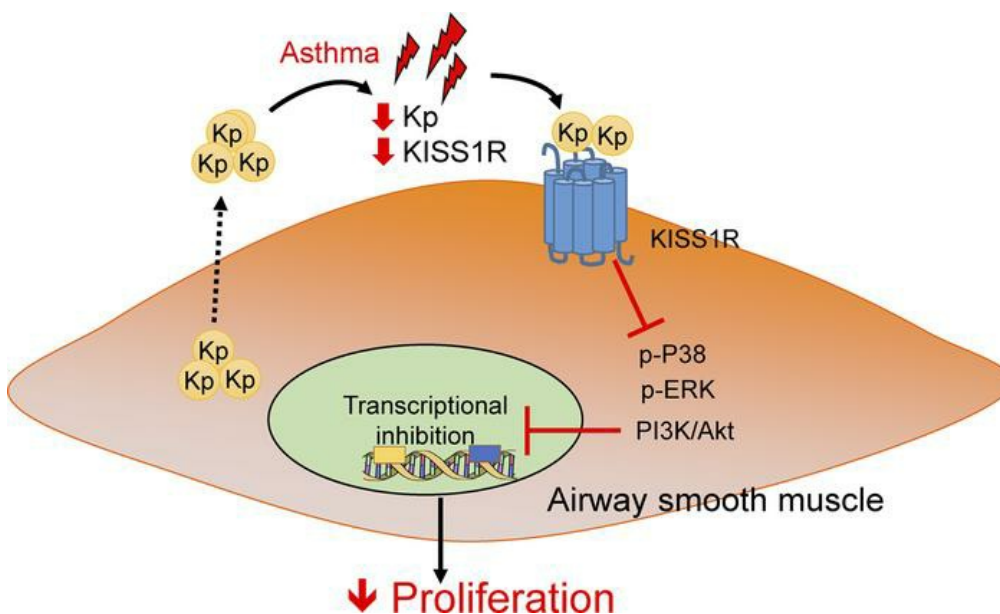
*JCI Insight.* 2022;7(10):e152762. <https://doi.org/10.1172/jci.insight.152762>.

Research Article

Muscle biology

Pulmonology

### Graphical abstract



Find the latest version:

<https://jci.me/152762/pdf>



# Kisspeptins inhibit human airway smooth muscle proliferation

Niyati A. Borkar,<sup>1</sup> Nilesh Sudhakar Ambhore,<sup>1</sup> Rama Satyanarayana Raju Kalidhindi,<sup>1</sup> Christina M. Pabelick,<sup>2,3</sup> Y.S. Prakash,<sup>2,3</sup> and Venkatachalem Sathish<sup>1</sup>

<sup>1</sup>Department of Pharmaceutical Sciences, North Dakota State University, Fargo, North Dakota, USA. <sup>2</sup>Department of Anesthesiology and Perioperative Medicine and <sup>3</sup>Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota, USA.

Sex and gender disparity in asthma is recognized and suggests a modulatory role for sex steroids, particularly estrogen. However, there is a dichotomous role for estrogen in airway remodeling, making it unclear whether sex hormones are protective or detrimental in asthma and suggesting a need to explore mechanisms upstream or independent of estrogen. We hypothesize that kisspeptin (Kp)/KISS1R signaling serves this role. Airway smooth muscle (ASM) is a key structural cell type that contributes to remodeling in asthma. We explored the role of Kp/KISS1R in regulating ASM proliferation. We report potentially novel data indicating that Kp and KISS1R are expressed in human airways, especially ASM, with lower expression in ASM from women compared with men and lower in patients with asthma compared with people without asthma. Proliferation studies showed that cleaved forms of Kp, particularly Kp-10, mitigated PDGF-induced ASM proliferation. Pharmacological inhibition and shRNA knockdown of KISS1R increased basal ASM proliferation, which was further amplified by PDGF. The antiproliferative effect of Kp-10 in ASM was mediated by inhibition of MAPK/ERK/Akt pathways, with altered expression of PCNA, C/EBP- $\alpha$ , Ki-67, cyclin D1, and cyclin E leading to cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase. Overall, we demonstrate the importance of Kp/KISS1R signaling in regulating ASM proliferation and a potential therapeutic avenue to blunt remodeling in asthma.

## Introduction

Asthma is a chronic inflammatory disease of the airways involving both intrinsic and extrinsic (i.e., environmental) factors (1–4) and affecting more than 339 million people worldwide (5). The key features of asthma include exaggerated airway inflammation in conjunction with airway hyperresponsiveness (AHR) and remodeling, resulting in narrowing of the airways (6–10). Among the multiple cell types involved, the airway smooth muscle (ASM) is an important structural cell well known for contributing to the contractile and AHR aspects of asthma (11–16). However, increased ASM mass is a commonly found pathological feature of asthma, suggesting a role for enhanced ASM proliferation (13, 17–19), which may further contribute to the increased extracellular matrix production within asthmatic airways. Thus, factors that modulate ASM structure and function become important to understanding asthma pathophysiology and, in turn, for developing novel therapeutic strategies. Here, mechanisms that influence airway remodeling are particularly appealing, given that current therapies including corticosteroids are not effective in targeting remodeling in asthma (6).

Asthma shows age- and sex-related differences in epidemiology and clinical manifestation: prepubertal boys are more likely to have asthma than are girls, but after puberty, adult women have greater incidence, frequency, and severity of asthma than do men, a difference that decreases after menopause (1–4, 7, 20). Some female patients with asthma have exacerbated asthma symptoms during premenstrual or menstrual phases (21, 22). These clinical data suggest a functional role for sex steroids in asthma, especially estrogen. However, the role of estrogen per se in asthma appears paradoxical, because some studies suggest that estrogens enhance inflammation (23), whereas others associate estrogens with an asthma-mitigating role (24). Specific to ASM, we and others have found that differential effects 17 $\beta$ -estradiol depend on the contributions of estrogen receptor subtypes (specifically, ER- $\alpha$  vs. ER- $\beta$ ) in regulating ASM structure and function (8, 9, 13, 14, 16, 25, 26), thus adding to the complexity of sex steroid signaling in asthma pathophysiology.

**Authorship note:** NAB, NSA, and RSRK contributed equally to this work.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Copyright:** © 2022, Borkar et al. This is an open access article published under the terms of the Creative Commons Attribution 4.0 International License.

**Submitted:** June 29, 2021

**Accepted:** April 12, 2022

**Published:** May 23, 2022

**Reference information:** *JCI Insight*. 2022;7(10):e152762.  
<https://doi.org/10.1172/jci.insight.152762>.

Furthermore, estrogen fluctuations in perimenopausal women suggest a role for other pathways during the luteal period (27). This raises the possibility that other mechanisms upstream to or independent of estrogen may play a role in the observed sex differences in asthma. Several pieces of evidence in the CNS point to kisspeptin (Kp) being a potential modulatory mechanism upstream of sex steroids: (a) Kp is critical for initiating puberty and regulating ovulation via controlling the hypothalamic-pituitary-gonadotropic axis (28); (b) Kp regulates gonadotropin-releasing hormone and gonadal steroids (29); (c) administration of kisspeptin receptor (KISS1R) agonist has been investigated as a potential treatment for sex steroid-dependent diseases (30). Accordingly, Kp/KISS1Rs in the airways may be important for identifying pathways that contribute to asthma, per se, and potentially the noted sex differences.

Kp is encoded by the *KISS1* gene on chromosome 1, which generates a 145-amino acid Kp, which is further cleaved to produce multiple peptides (31), including Kp-10, Kp-13, Kp-14, and Kp-54, which share a common, functional, C-terminal, 10-amino acid sequence (32). Kp is an endogenous ligand for KISS1R (GPR54/AXOR12/hOT7T175) (33), a member of the rhodopsin family of G protein-coupled receptors with sequence homologies to the galanin receptor family (31). Studies in endocrinology and oncology suggest a crucial role for Kp/KISS1R signaling in different cell types (34) with a pivotal contribution to the onset of puberty (28, 35) and suppression of cancer metastasis via inhibition of proliferation (36). Kp/KISS1R influences p38 mitogen-associated protein kinases (MAPK) signaling pathways and can thus modulate inflammation and proliferation (37), 2 aspects also important for ASM in asthma. However, to our knowledge, there is no information on Kp/KISS1R in the lung, although data in other systems raise the question of whether Kp/KISS1R can regulate airway remodeling in the context of asthma. We hypothesized that Kp/KISS1R signaling has a protective role in regulating ASM-mediated airway remodeling, an effect lost during inflammation and/or asthma. To address our hypothesis, we designed the present study to determine (a) the expression of Kp and KISS1R in human ASM; (b) Kp/KISS1R expression levels with respect to sex and asthma; (c) the role of Kp and KISS1R signaling in regulating ASM proliferation; and (d) underlying mechanisms of Kp/KISS1R signaling.

## Results

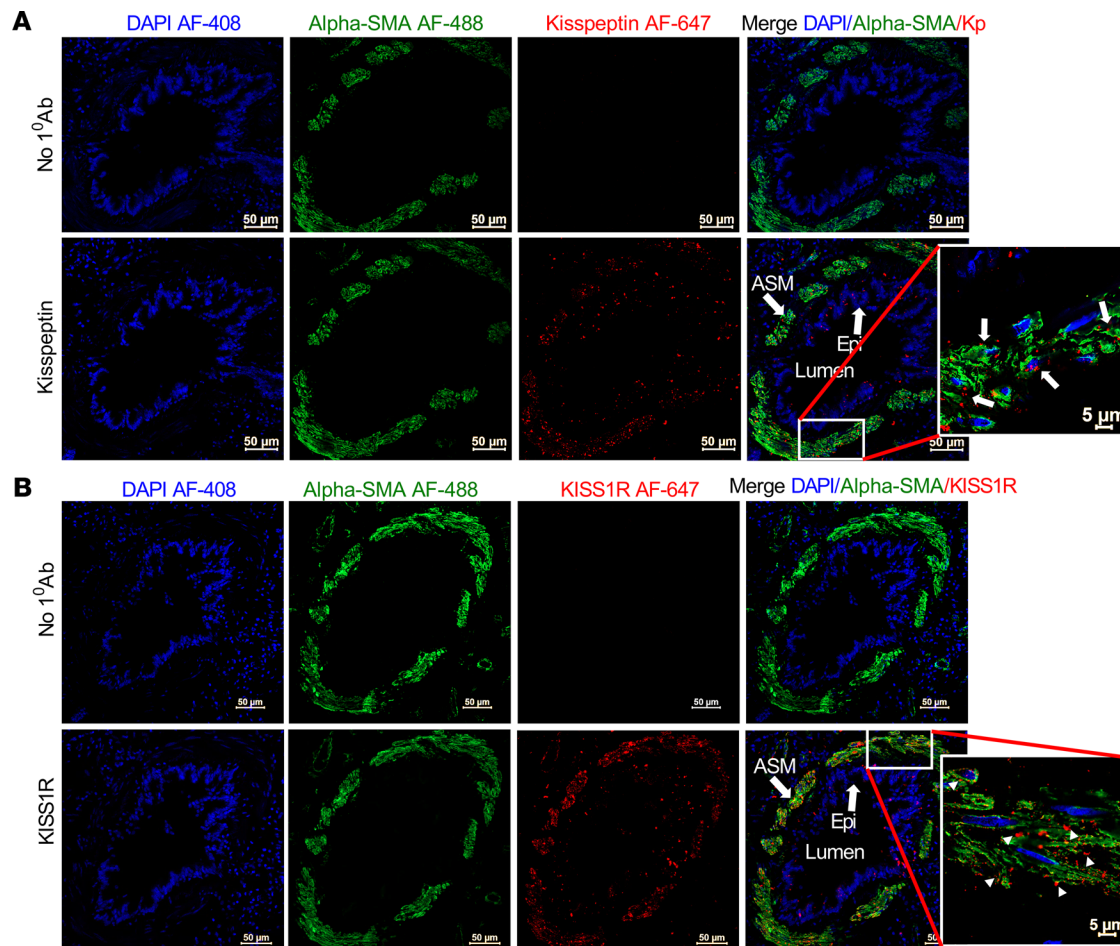
*Expression of Kp and KISS1R in human lung tissue and ASM cells.* IHC of lung tissue sections revealed that both Kp and its receptor KISS1R are expressed in human airways, especially in ASM as determined by colocalization using  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in lung sections (Figure 1, A and B) and by multicolor immunofluorescence staining of isolated human ASM cells (Figure 2, A and B).

*Sex differences in ASM expression of Kp and KISS1R.* Baseline mRNA and protein expression of *KISS1* (mRNA)/Kp (protein) and KISS1R in primary ASM cells from male and female donors without asthma showed both *KISS1* ( $P < 0.01$ ; Figure 3A)/Kp ( $P < 0.001$ ; Figure 3C) and KISS1R were significantly lower in women than in men for both mRNA ( $P < 0.01$ ; Figure 3B) and protein ( $P < 0.001$ ; Figure 3D).

*Kp and KISS1R in ASM patients with asthma.* mRNA expression of *KISS1* was significantly lower in ASM from men ( $P < 0.01$ ) and women ( $P < 0.05$ ) with asthma compared with their counterparts without asthma (Figure 4A). This was confirmed by Western analysis for Kp protein expression ( $P < 0.001$ ; Figure 4C). A significant difference was noted for *KISS1R* mRNA in ASM from men and women with asthma ( $P < 0.001$ ; Figure 4B) compared with individuals without asthma, again confirmed by analysis for KISS1R protein ( $P < 0.001$  for men;  $P < 0.01$  for women; Figure 4D).

*Cleaved Kps and ASM proliferation.* Previous studies have shown that all cleaved forms of Kps have comparable KISS1R binding affinity but have different downstream potency in terms of cellular effect (38). Thus, Kp effects may be cell and context specific. To determine which of the cleaved Kps may be involved in regulating ASM proliferation, we evaluated the effects of 1  $\mu$ M Kp-10, Kp-13, Kp-14, and Kp-54 on basal and PDGF-stimulated proliferation of ASM cells from individuals without asthma, using MTT assay (Figure 5A). At baseline (without PDGF), Kp-10, Kp-14, and Kp-54 did not significantly alter ASM proliferation, but Kp-13 showed a small but significant ( $P < 0.01$ ) increase compared with vehicle. PDGF substantially increased ASM proliferation ( $P < 0.001$ ) compared with vehicle. However, PDGF's effect on proliferation was significantly blunted by treatment with Kp-10, Kp-14, or Kp-54 ( $P < 0.001$ ), with a lesser effect of Kp-13 ( $P < 0.05$ ) compared with PDGF alone.

Based on these data, we selected Kp-10 as the cleaved form of Kp to further explore. We first performed a concentration-dependence study at 100 nM, 1  $\mu$ M, and 10  $\mu$ M for PDGF-induced human ASM proliferation using ASM cells from people without asthma and found 1  $\mu$ M and 10  $\mu$ M Kp-10

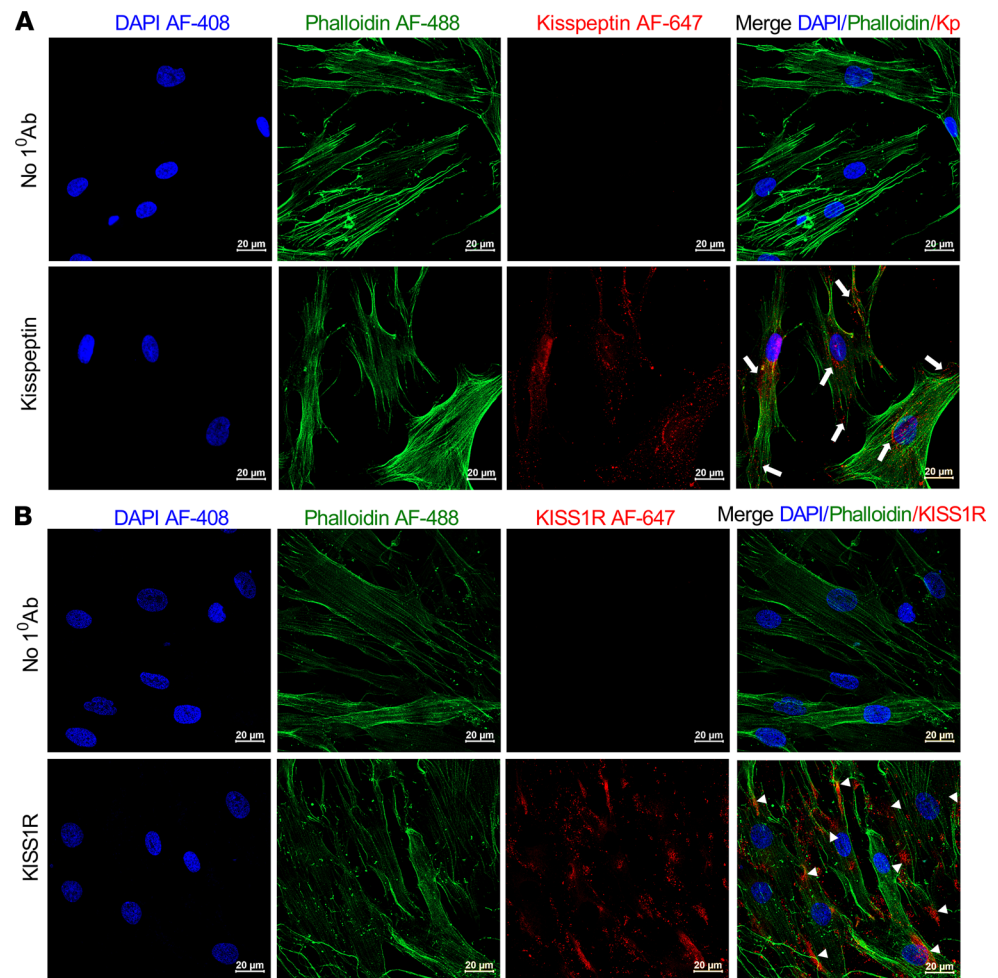


**Figure 1. Kp and its receptor, KISS1R, are expressed in the human airway, as shown by IHC.** Human lung sections were immunostained for (A) Kp (AF-647) and (B) KISS1R (AF-647) with  $\alpha$ -SMA (AF-488) as a reference marker for smooth muscle-specific colocalization. DAPI was used to stain the nucleus (AF-408). Z-stack images were taken on a Zeiss LSM900 confocal microscope with Airyscan2 settings. Scale bar: 50  $\mu$ m; insert: 5  $\mu$ m. Representative images are from 5 independent samples from donors without asthma. Epi, epithelium.

significantly ( $P < 0.001$ ) inhibited proliferation (Figure 5B). Accordingly, we selected 1  $\mu$ M Kp-10 for subsequent studies. To verify the lack of cytotoxicity from Kp-10, we performed a lactate dehydrogenase (LDH) assay (Figure 5C) with serum-free medium as a negative control and 0.1 % Triton-X 100 as positive control; we found no cytotoxicity with any of the treatment groups.

**Kp-10 effects on proliferation.** We performed bright-field (BF) and MTT assays in both ASM cells from people with and without asthma with and without PDGF (Figure 6, A and B). In addition, we incorporated a KISS1R inhibitor (KI; Kp-234 trifluoroacetate; 100 nM) as a treatment group to confirm the role of this receptor. As expected, we observed significantly higher proliferation in ASM cells from patients with asthma compared with those from people without asthma ( $P < 0.05$ ). At baseline, Kp-10 did not significantly alter proliferation in either ASM cells from either group. Interestingly, inhibition of KISS1R with KI significantly increased the basal proliferation of ASM cells of people without asthma ( $P < 0.01$  in BF assay;  $P < 0.05$  in MTT assay) and those with asthma (BF assay,  $P < 0.01$ ; MTT assay,  $P < 0.05$ ). Furthermore, ASM cells exposed to PDGF had a significant ( $P < 0.001$  for both BF and MTT assays) increase in proliferation of ASM cells from people with asthma and those without, with a greater effect on ASM cells from the former ( $P < 0.05$  in BF assay;  $P < 0.001$  in MTT assay). Pretreatment with Kp-10 significantly blunted the pro-proliferative effect of PDGF in ASM cells of individuals without asthma and those with asthma (both groups,  $P < 0.001$ ). Interestingly, PDGF enhancement of proliferation was unaltered by pretreatment with KI. Similarly, ASM cells first treated with KI and then exposed to Kp-10 did not influence PDGF enhancement of proliferation in cells from either individuals with asthma or those without, overall highlighting the importance of KISS1R in mediating Kp-10 effects.



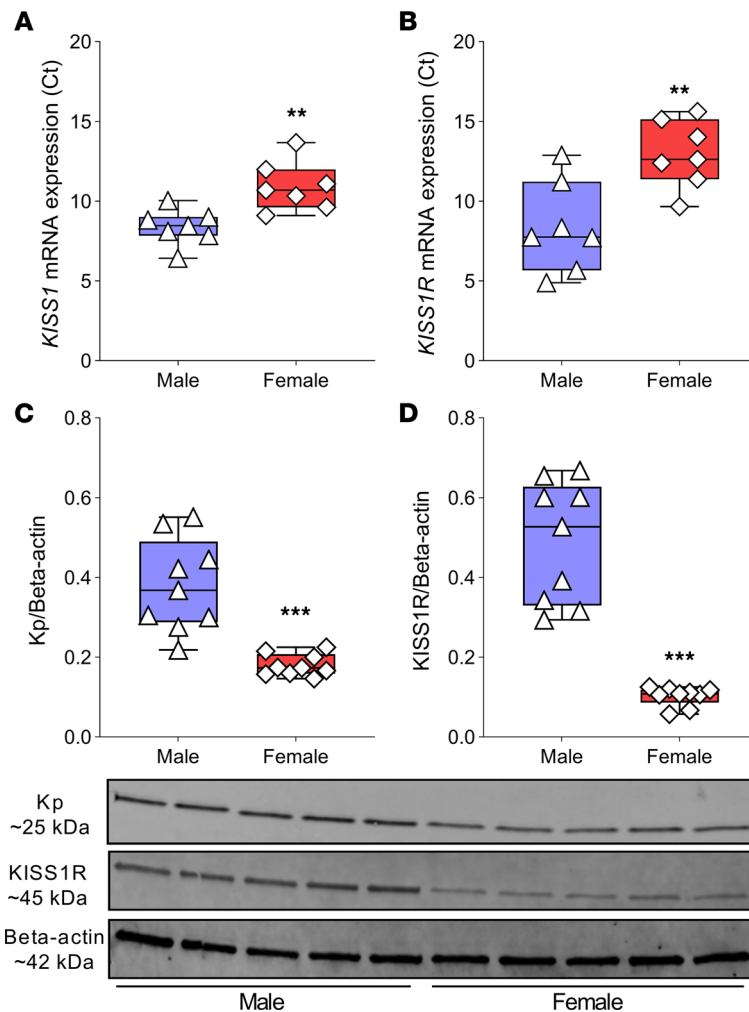


**Figure 2. Kp and KISS1R expression in isolated human ASM cells.** Paraformaldehyde-fixed human ASM cells were immunostained with Kp (A) and KISS1R (B). Kp and KISS1R were probed with AF-647 secondary Abs and actin filaments with phalloidin (AF-488). DAPI was used to stain the nucleus (AF-408). Z-stack images were taken on a Zeiss LSM900 confocal microscope with Airyscan2 settings. Scale bar: 20  $\mu$ m. Representative images are from 5 independent samples from donors without asthma.

To further confirm the role of KISS1R in the Kp-10 effect, we used KISS1R shRNA knockdown (with a scrambled negative shRNA as a control; Figure 6, C and D). The efficiency of shRNA transduction was confirmed by Western analysis ( $P < 0.001$ ; Figure 6E). Kp-10 alone did not significantly alter basal cell proliferation in both negative and KISS1R knockdown cells. PDGF significantly increased ASM proliferation in negative shRNA-transduced cells from individuals without and those with asthma ( $P < 0.001$  for both BF and MTT assays); effects that were amplified in KISS1R knockdown ASM cells from individuals without and those with asthma ( $P < 0.001$  both BF and MTT assays). Pretreatment with Kp-10 significantly lowered PDGF-induced ASM proliferation in negative shRNA transduced ASM cells from individuals without and those with asthma ( $P < 0.001$  for both BF and MTT assays), but not in KISS1R shRNA knockdown cells.

These data suggested an autocrine effect of ASM-generated Kps on proliferation. To confirm an autocrine Kp effect, we measured and found endogenous ASM Kp secretion in the conditioned media of ASM samples from individuals without and those with asthma (by ELISA; Figure 6F), with significantly lower secretion found in patients with asthma ( $P < 0.05$ ).

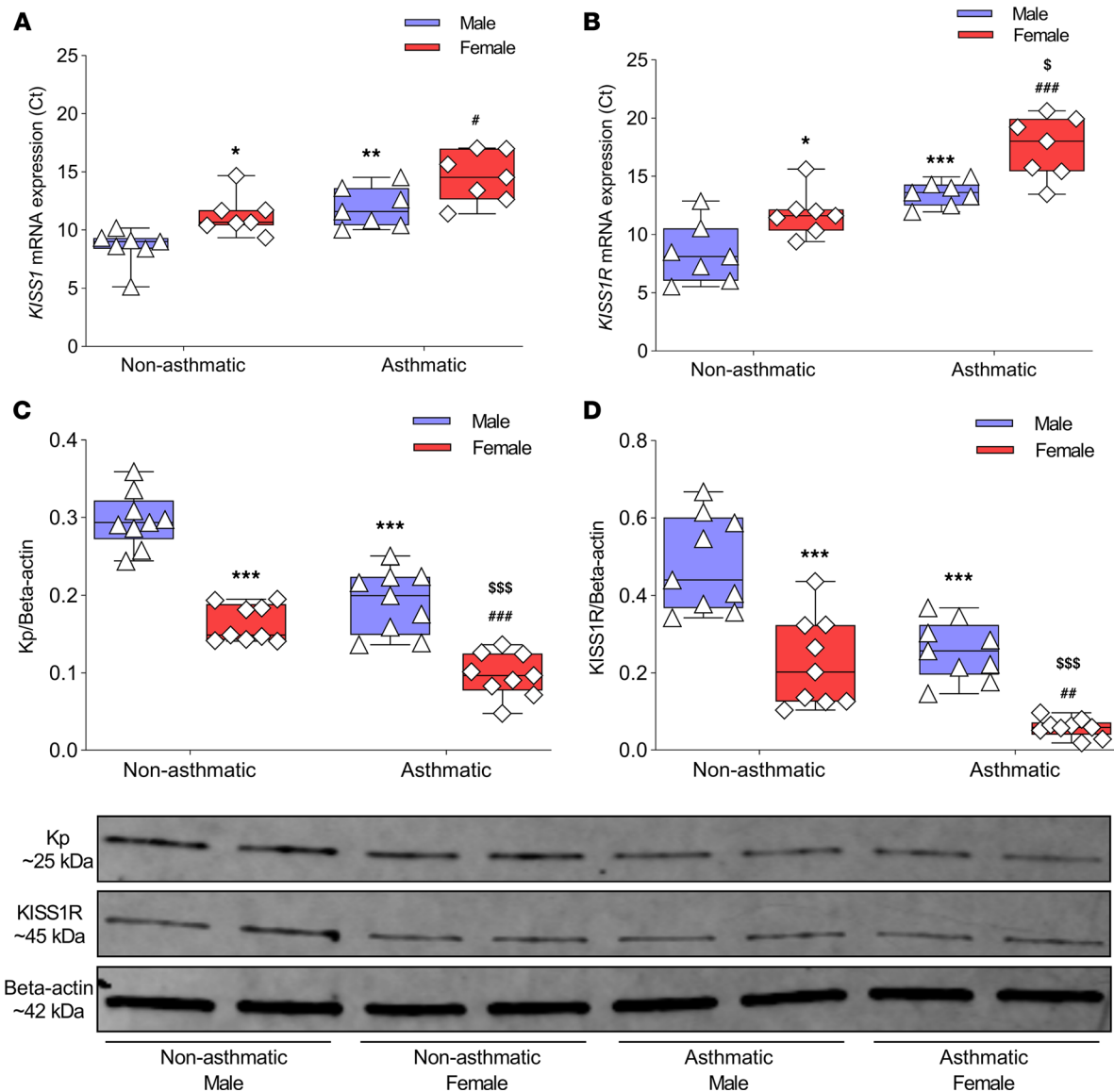
*Effect of Kp-10 on markers of proliferation.* Cell cycle analysis was performed by flow cytometry using propidium iodide (PI)-stained human ASM cells. PDGF significantly increased the number of cells in the S phase compared with vehicle in ASM cells from individuals without asthma ( $P < 0.05$ ) and those with asthma ( $P < 0.01$ ; Figure 7B), consistent with an increase in proliferation. This mitogenic effect of PDGF was significantly inhibited by Kp-10 pretreatment in ASM cells of individuals without asthma ( $P < 0.05$ ) and



**Figure 3. Kp and KISS1R expression in primary human ASM cells from men and women.** qRT-PCR and Western blot analysis data show lower baseline Kp (A and C) and KISS1R (B and D) expression in ASM cells from women compared with those from men. mRNA expression of *KISS1* (A) and *KISS1R* (B) was normalized with housekeeping gene *s16* and is represented as Ct. Western blots of Kp (C) and KISS1R (D) are representative results from independent experiments. All proteins were normalized to  $\beta$ -actin. Data are reported as a minimum to maximum of 7 to 9 individual samples from male or female donors without asthma and analyzed using a 1-tailed unpaired *t* test. \*\**P* < 0.01, \*\*\**P* < 0.001 vs. male samples.

those with asthma ( $P < 0.01$ ). Furthermore, pretreatment with Kp-10 significantly downregulated PDGF-induced cell entry into the  $G_2/M$  phase in ASM of individuals with and without asthma ( $P < 0.01$ ; Figure 7C). Furthermore, Kp-10 pretreatment increased the numbers of cells in  $G_0/G_1$  phase compared with PDGF in ASM cells from both groups ( $P < 0.01$ ; Figure 7A), suggesting the arrest of cell cycle progression in the  $G_0/G_1$  phase and, again, being consistent with inhibition of proliferation (Figure 6). The representative images of cell cycle distribution profiles by various treatments in individuals with and without asthma are shown in Figure 7, D and E.

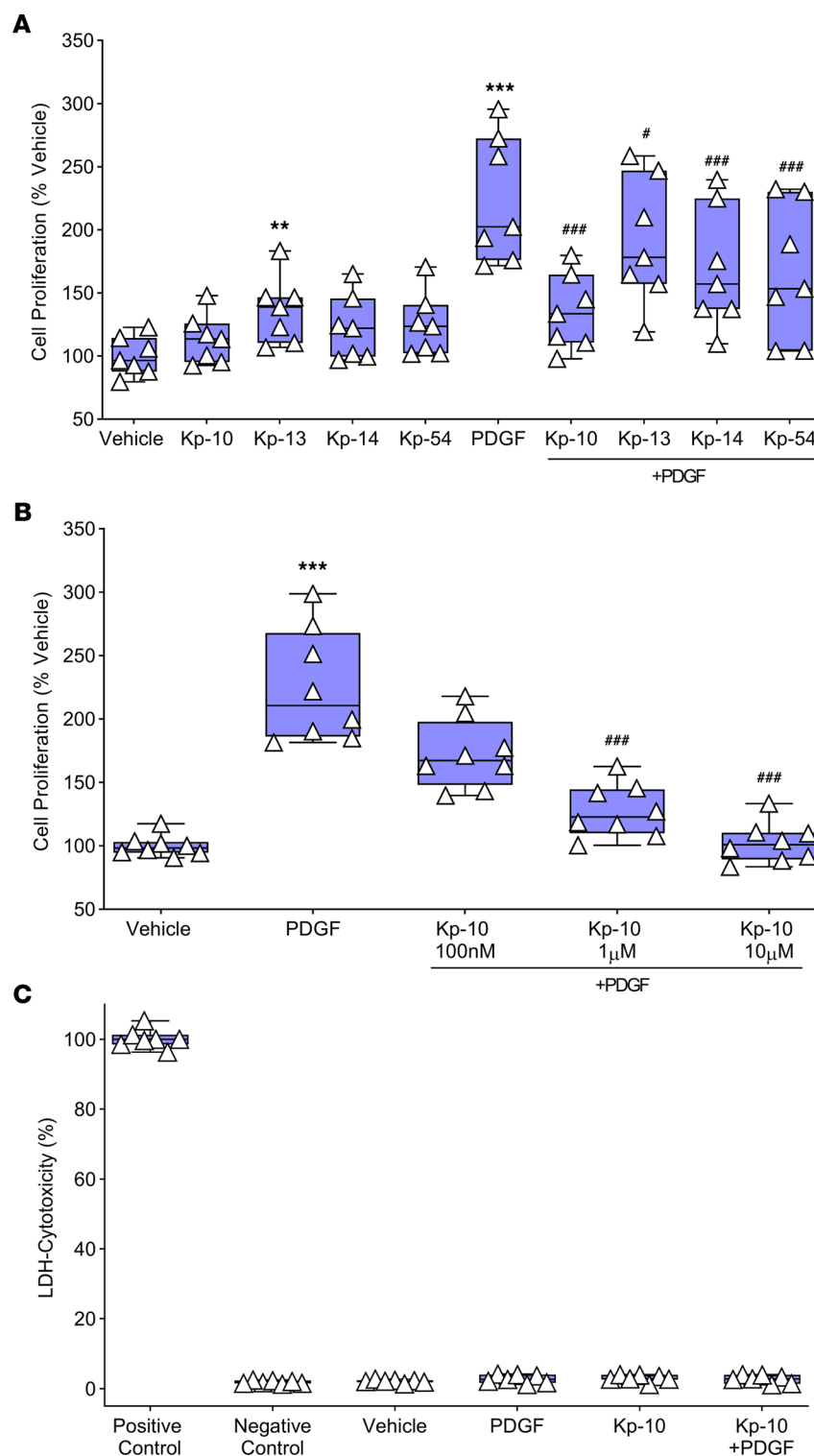
We evaluated the expression of proliferative markers PCNA and C/EBP- $\alpha$  using qRT-PCR and Western blot analyses, and we evaluated changes in Ki-67 expression by qRT-PCR and nuclear localization by immunofluorescence analysis. PDGF significantly increased the mRNA expression of PCNA ( $P < 0.01$ ), C/EBP- $\alpha$  (individuals without asthma,  $P < 0.01$ ; patients with asthma,  $P < 0.001$ ), and Ki-67 (for both groups,  $P < 0.001$ ; Figure 8, A, B, and E). Kp-10 treatment inhibited PDGF-induced increase in mRNA expression of PCNA (individuals without asthma,  $P < 0.05$ ; patients with asthma,  $P < 0.01$ ), C/EBP- $\alpha$  (individuals without asthma,  $P < 0.01$ ; patients with asthma,  $P < 0.05$ ), and Ki-67 ( $P < 0.001$ ). Consistently, PDGF significantly upregulated protein expression for PCNA (individuals without asthma,  $P < 0.01$ ; patients with asthma,  $P < 0.05$ ; Figure 8C) and C/EBP- $\alpha$  (individuals without asthma,  $P < 0.001$ ; patients with asthma,  $P < 0.05$ ; Figure 8D).



**Figure 4.** Kp and KISS1R expression in human ASM cells from individuals with and without asthma. qRT-PCR and Western blot analysis data showed lower baseline Kp (**A** and **C**) and KISS1R (**B** and **D**) expression in ASM cells from donors with asthma than in ASM cells from individuals without asthma. Interestingly, Kp and KISS1R expression was low in ASM cells from both men and women with asthma compared with ASM cells from individuals without asthma. mRNA expression of *KISS1* (**A**) and *KISS1R* (**B**) was normalized with housekeeping gene *s16* and is represented as Ct. The protein expression of Kp (**C**) and KISS1R (**D**) was determined by Western blot analysis in human ASM cells and normalized with  $\beta$ -actin as a loading control. Data are reported as a minimum to maximum of 7 to 9 ASM samples from individual male and female donors and analyzed using 2-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. men without asthma; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs. women without asthma; \$ $P < 0.05$ , \$\$\$ $P < 0.001$  vs. men with asthma.

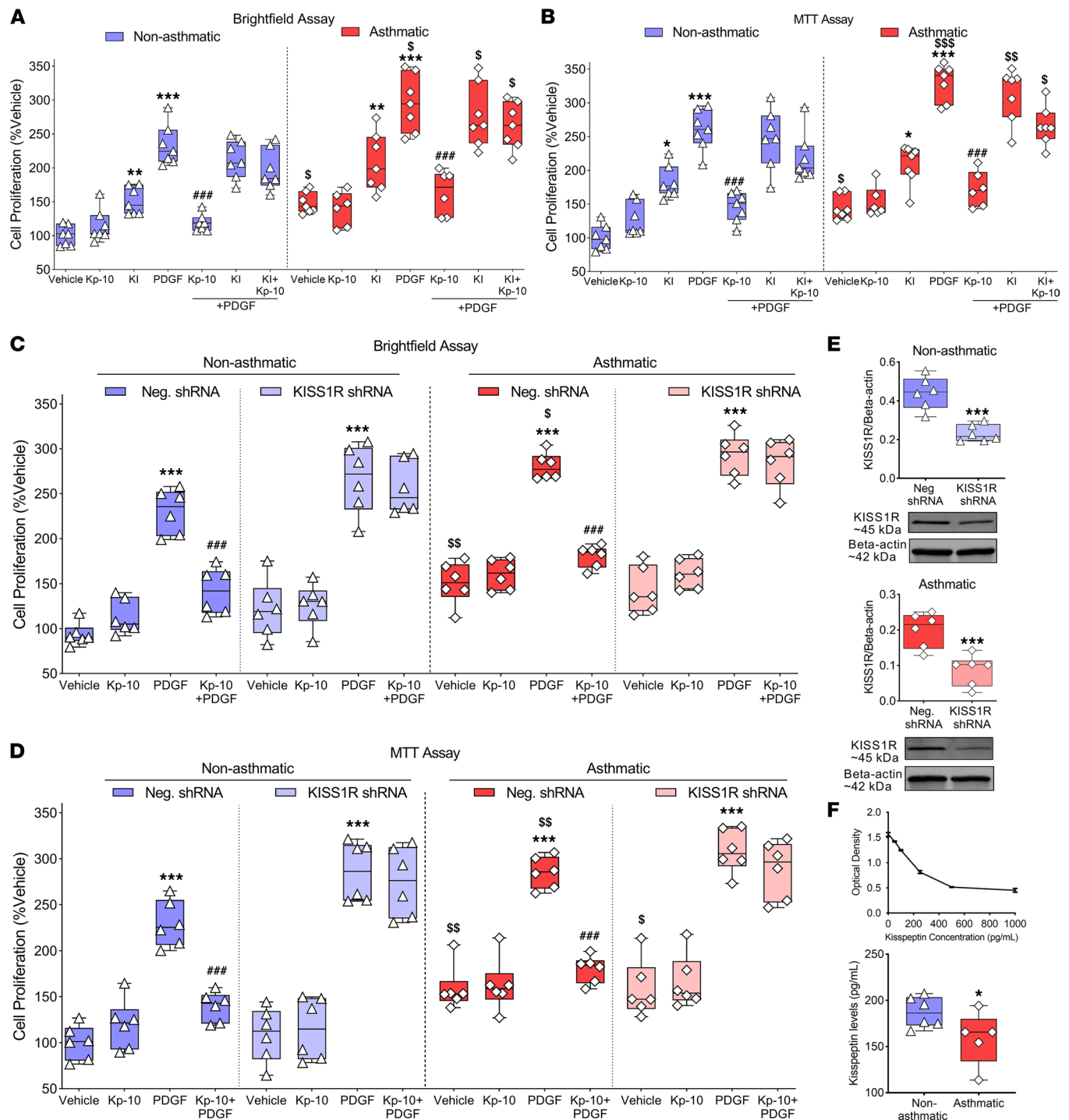
Nuclear localization of Ki-67 was significantly increased with PDGF ( $P < 0.001$ , Figure 8F, which was further inhibited by Kp-10 (individuals without asthma,  $P < 0.05$ ; patients with asthma,  $P < 0.001$ ). Representative images of Ki-67 nuclear localization are shown in Figure 8, G and H.

PDGF also significantly increased mRNA expression of the cell cycle proteins cyclin D1 ( $P < 0.05$ ) and cyclin E (individuals without asthma,  $P < 0.001$ ; patients with asthma,  $P < 0.01$ ; Figure 9, A and B)—effects significantly inhibited in both ASM of individuals without asthma ( $P < 0.05$ ) and those with asthma ( $P < 0.01$  for cyclin D1;  $P < 0.05$  for cyclin E) pretreated with Kp-10. Consistent results for cyclin D1 and cyclin E proteins were observed (Figure 9, C and D). PDGF effects on the cell cycle proteins were significantly blunted with Kp-10 pretreatment in both ASM cells of individuals without asthma ( $P < 0.001$  for cyclin D1;  $P < 0.01$  for cyclin E) and those with asthma ( $P < 0.05$  for both cyclin D1 and cyclin E).



**Figure 5. Kps and human ASM cell proliferation.** The effect of different Kps (namely, Kp-10, Kp-13, Kp-14, and Kp-54) on basal and PDGF-induced cell proliferation in ASM cells of people without asthma was evaluated using MTT assay (**A**). Among the 4 forms of Kps (1  $\mu$ M), Kp-10 significantly blunted PDGF-induced ASM cell proliferation. Furthermore, the effect of different log concentrations of Kp-10 (100 nM, 1  $\mu$ M, and 10  $\mu$ M) on regulating PDGF-induced ASM proliferation was determined (**B**). The relative level of LDH was measured in ASM cell supernatants to evaluate the cytotoxicity of the selected concentration (1  $\mu$ M) of Kp-10 (**C**). The treatment groups did not show cytotoxicity after 24 hours of exposure when compared with the negative control group. Data are reported as a minimum to maximum of 7 to 8 individual ASM samples from donors without asthma and analyzed using 1-way ANOVA followed by Tukey's post hoc test. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. respective vehicle; # $P < 0.05$ , ### $P < 0.001$  vs. PDGF-exposed groups.





**Figure 6. KISS1R activation and PDGF-induced proliferation of human ASM cells.** KISS1R agonist, Kp-10, significantly blunted the mitogenic effect on PDGF in ASM cells from individuals with and without asthma, as evaluated by high-contrast bright-field (**A**) and MTT (**B**) assays. KI (Kp-234 trifluoroacetate) showed a significant increase in basal ASM proliferation compared with vehicle and did not show any changes in PDGF-induced proliferation in ASM cells from either individuals with asthma or those without. Human ASM cells transduced with KISS1R-specific shRNA had increased basal ASM proliferation compared with those transduced with negative shRNA. Furthermore, we did not observe any significant reduction in PDGF-induced proliferation after Kp-10 treatment in KISS1R shRNA-transduced cells as measured by high-contrast bright-field (**C**) and MTT (**D**) assays. Western blot analysis was performed to confirm the transduction efficacy of KISS1R shRNA in ASM cells from individuals with and without asthma (**E**). The endogenous Kp secretion of ASM was measured by ELISA using conditioned media. The calibration of ELISA was confirmed by a standard curve of Kp (**F**, top panel). ASM cells from donors with asthma had reduced Kp secretion in conditioned media compared with those from donors without asthma (**F**, bottom panel). Data are reported as a minimum to maximum of 5 to 7 individual ASM samples from donors with and without asthma and analyzed using 1- or 2-way ANOVA followed by Tukey's post hoc test or 1-tailed unpaired *t* test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs. vehicle from donors without asthma or negative shRNA vehicle; ###*P* < 0.001 vs. respective PDGF-exposed group; \$*P* < 0.05, \$\$*P* < 0.01, \$\$\$*P* < 0.001 vs. respective group without asthma or negative shRNA group.

*Effect of Kp-10 on ASM proliferative signaling pathways.* We evaluated phosphorylated and total p38 MAPK, Akt, and ERK1/2 in human ASM cells (Figure 10, A–C). PDGF significantly increased phosphorylation of p38 MAPK, Akt, and ERK1/2 ( $P < 0.05$ ) compared with the vehicle group. Pretreatment with Kp-10 significantly reduced PDGF-induced phosphorylation of p38 MAPK, Akt ( $P < 0.05$ ), and pERK1/2 ( $P < 0.01$ ).

## Discussion

Dysregulated ASM proliferation contributes to airway remodeling and AHR in asthma (2, 13, 39–45). Exploring the mechanisms underlying ASM remodeling is important, given that current therapies including corticosteroids are not effective in alleviating or reversing airway remodeling in asthma (6, 12, 46–51). In this study, we highlight what we believe is a novel and potentially targetable mechanism that modulates ASM proliferation in the context of asthma: Kp/KISS1R signaling.

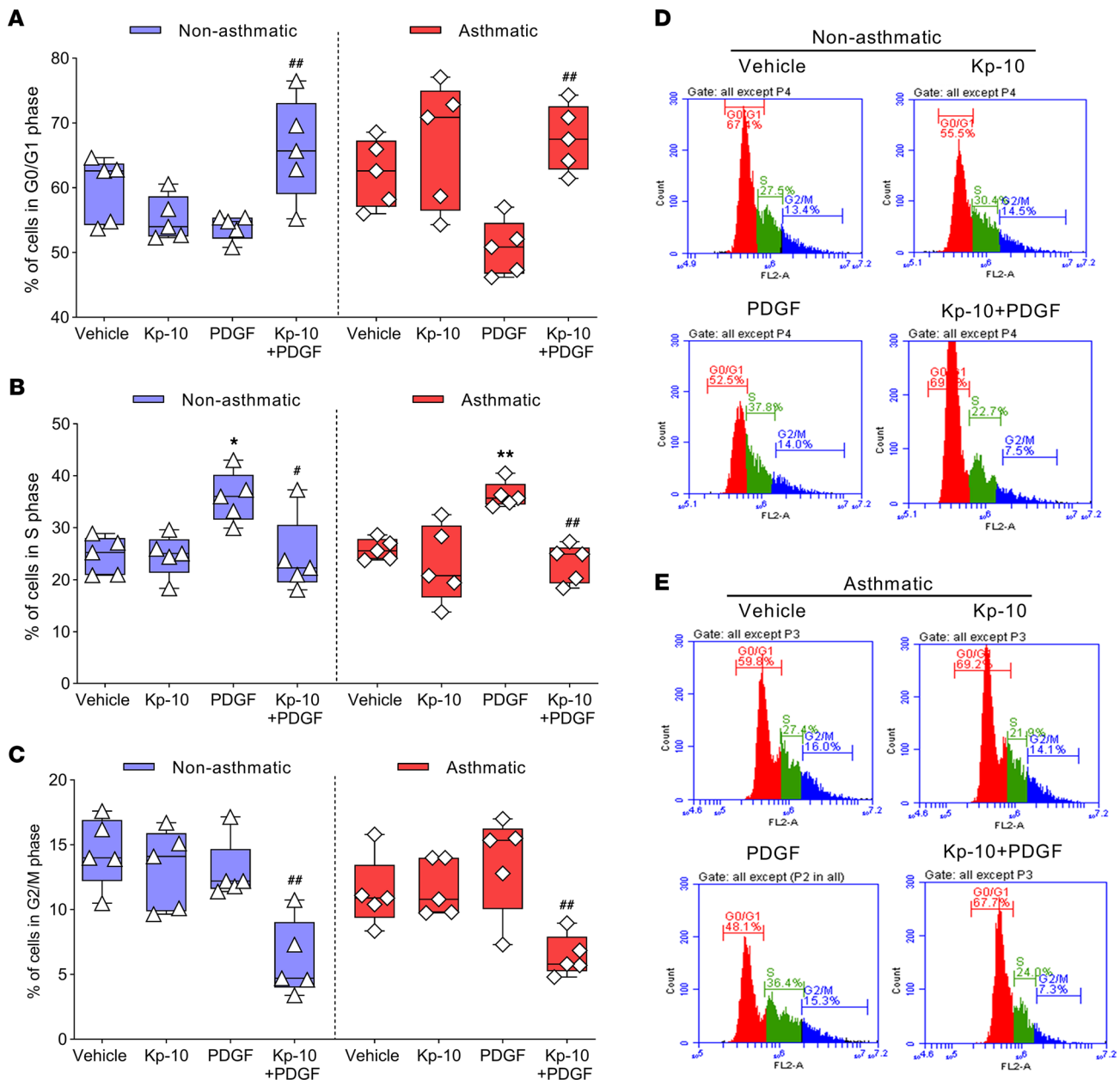
A majority of information regarding Kp derives from studies of the CNS, where Kp regulates puberty and hormonal function (28, 35). Earlier studies using rat receptors suggested that all the cleaved Kps (i.e., Kp-10, Kp-13, Kp-14, and Kp-54) possess similar affinity and efficacy in activating the KISS1R (32). However, data from endocrinology and oncology suggest that the differential effects of Kp fragments are a major aspect of understanding Kp biology (52). In this regard, although much attention has been given to metastin (a term used interchangeably with Kp), our data suggest that Kp-10 is a potent Kp in the context of ASM and remodeling. Interestingly, other Kp fragments were less effective in modulating PDGF-induced proliferation. Although other Kp fragments were effective, our data clearly highlight the greater potency of Kp-10, justifying further exploration of this fragment.

To our knowledge, there has been no information on the expression of Kp or KISS1R in the airways. Our findings show that Kp and its receptor, KISS1R, are expressed in human lung tissue, especially in ASM, suggesting a plausible role for Kp/KISS1R signaling in airway biology. Interestingly, we observed less expression of Kp and KISS1R in ASM of individuals with asthma, suggesting possible reduced or loss of intrinsic Kp/KISS1R signaling in asthma. Here, increasing evidence from other cell types shows a crucial regulatory role for Kp/KISS1R signaling in cell proliferation and migration by inhibiting NF- $\kappa$ B and MAPK signaling pathways (53–55), which are also relevant to ASM remodeling. The observed reduction of Kp/KISS1R expression and signaling in patients with asthma aligns with the increased ASM proliferation observed in airways of these patients.

In cell proliferation studies, Kp-10, Kp-14, and Kp-54 had no significant mitogenic effect at baseline per se, suggesting that any modulatory effect of Kps occurs in the presence of extrinsic mitogenic stimuli, as would occur in inflammation or asthma. To stimulate proliferation in ASM cells, we used PDGF as a mitogenic agent, given its well-known effects in human ASM (13, 56–58). PDGF and its receptor (PDGF-R) play significant roles in airway proliferation and remodeling (56, 57, 59). Additionally, in earlier studies, we established that PDGF, at a concentration of 2 ng/mL, significantly induces ASM proliferation via activation of intracellular signaling pathways such as ERK1/2, p38, and Akt (13) that are relevant to both asthma and Kp biology. The increased proliferation observed with PDGF is consistent with findings of our earlier studies (13).

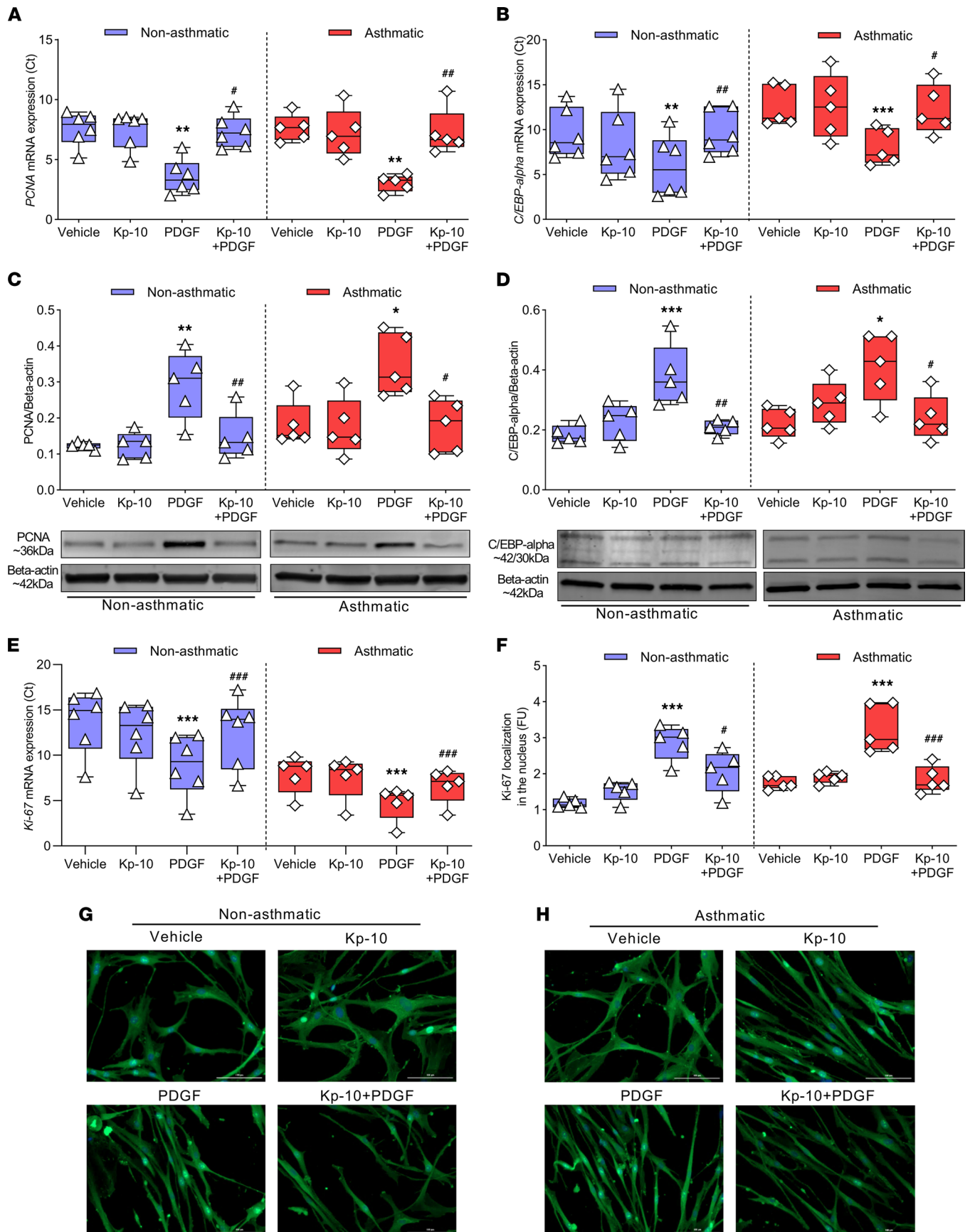
An interesting observation was that Kp-10 effects on proliferation were similar between ASM of individuals without asthma and those with asthma despite differences in KISS1R expression. This could be due to an enhanced inhibitory effect of Kp-10 via KISS1R in highly proliferative ASM cells or differential signaling of ASM of patients with asthma. Regardless, what we believe is novel and potentially relevant are the data showing that ASM from both groups secretes Kp with autocrine effects on proliferation. Here, KISS1R inhibition by KI significantly increased baseline ASM proliferation, suggesting activation of KISS1R by an autocrine mechanism. KI prevented PDGF from further increasing ASM proliferation, which might be due to a ceiling effect. Kp-10 did not reduce PDGF-induced ASM proliferation in KISS1R shRNA knockdown cells, indicating KISS1R-dependent activation. Kp-10 via KISS1R activation downregulated PDGF-induced expression of cyclin D1 and cyclin E, thereby initiating cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase, consistent with reports about other cell types, which indicate that Kps initiate cell cycle arrest via KISS1R/GPR54-dependent mechanisms (60).

PCNA and C/EBP- $\alpha$  are established markers for proliferation because they play a crucial role during the DNA synthesis phase of mitosis (61, 62). PDGF upregulated the expression of PCNA and C/EBP- $\alpha$  in ASM cells, which is prevented by Kp-10 pretreatment, further supporting the modulatory role of Kp/KISS1R signaling in ASM proliferation. Notably, previous studies reported a decreased C/EBP- $\alpha$  expression



**Figure 7. Effect of Kp-10 on different phases of human ASM cell cycle studied using flow cytometry.** Cell cycle analysis showed decreased cell population in G<sub>0</sub>/G<sub>1</sub> phase (A) and increased cell numbers in S and G<sub>2</sub>/M phases (B and C) with PDGF treatment. Kp-10 treatment significantly reversed the PDGF effect and resulted in reduced cell populations in S and G<sub>2</sub>/M phases, suggesting the arrest of cell cycle progression in the G<sub>0</sub>/G<sub>1</sub> phase. The representative profiles of ASM cell cycle distributions of individual without asthma (D) and those with asthma (E) by various treatment groups are depicted. Data are reported as a minimum to maximum of 5 individual samples from donors with and without asthma and analyzed using 1-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$  \*\* $P < 0.01$  vs. respective vehicle; # $P < 0.05$ , ## $P < 0.01$  vs. PDGF-exposed group.

in asthma (63, 64), whereas our observed data show increased C/EBP- $\alpha$  expression with PDGF exposure in ASM cells from individuals with and without asthma. The reason for this discrepancy is unclear and warrants further exploration. Ki-67 is a crucial marker for mitosis because it is exclusively detected in the nucleus during the G<sub>1</sub>, S, and G<sub>2</sub> phases of the cell cycle and is absent in the G<sub>0</sub> phase (65). PDGF-induced nuclear translocation of Ki-67 in the nucleus was blunted by Kp-10 pre-exposure, further strengthening our findings from ASM cell cycle analysis. Furthermore, the importance of MAPK/ERK signaling pathways in cell growth, proliferation, and differentiation of human ASM cells is well established (13, 66–68). In other cell systems, KISS1R activation inhibits the phosphorylation of MAPK signaling via  $\beta$ -arrestin-1 (69). Additionally, KISS1R via regulating the  $\alpha$  subunit of Gq/11 inhibits the phosphorylation of PI3K/Akt and





**Figure 8. Effect of Kp-10 on PDGF-induced human ASM cell proliferative marker proteins.** ASM cells from human donors with and without asthma showed increased mRNA and protein expression of PCNA (**A** and **C**) and C/EBP- $\alpha$  (**B** and **D**) after PDGF exposure, an effect substantially blunted by Kp-10. Similarly, PDGF-induced mRNA expression and nuclear localization of Ki67 (**E–H**) were significantly reduced by Kp-10 treatment. Scale bar: 100  $\mu$ m. Data are reported as a minimum to maximum of 5 to 6 individual samples from donors with and without asthma and analyzed using 1-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. respective vehicle; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs. PDGF-exposed groups.

subsequently regulates cell growth (69, 70). Similarly, we found increased phosphorylation of ERK1/2 and p38 MAPK upon PDGF exposure, which was inhibited by Kp-10 pretreatment. Furthermore, PDGF exposure upregulated the PI3K/Akt signaling, which activates mTOR signaling, which functions as a serine/threonine protein kinase that regulates ASM cell proliferation (71–73). These effects of Kp-10 via KISS1R activation are consistent with data from other cells types (53, 74). The inhibition of Akt phosphorylation by Kp-10 further indicated the importance of Kp/KISS1R regulation of ASM proliferative pathways.

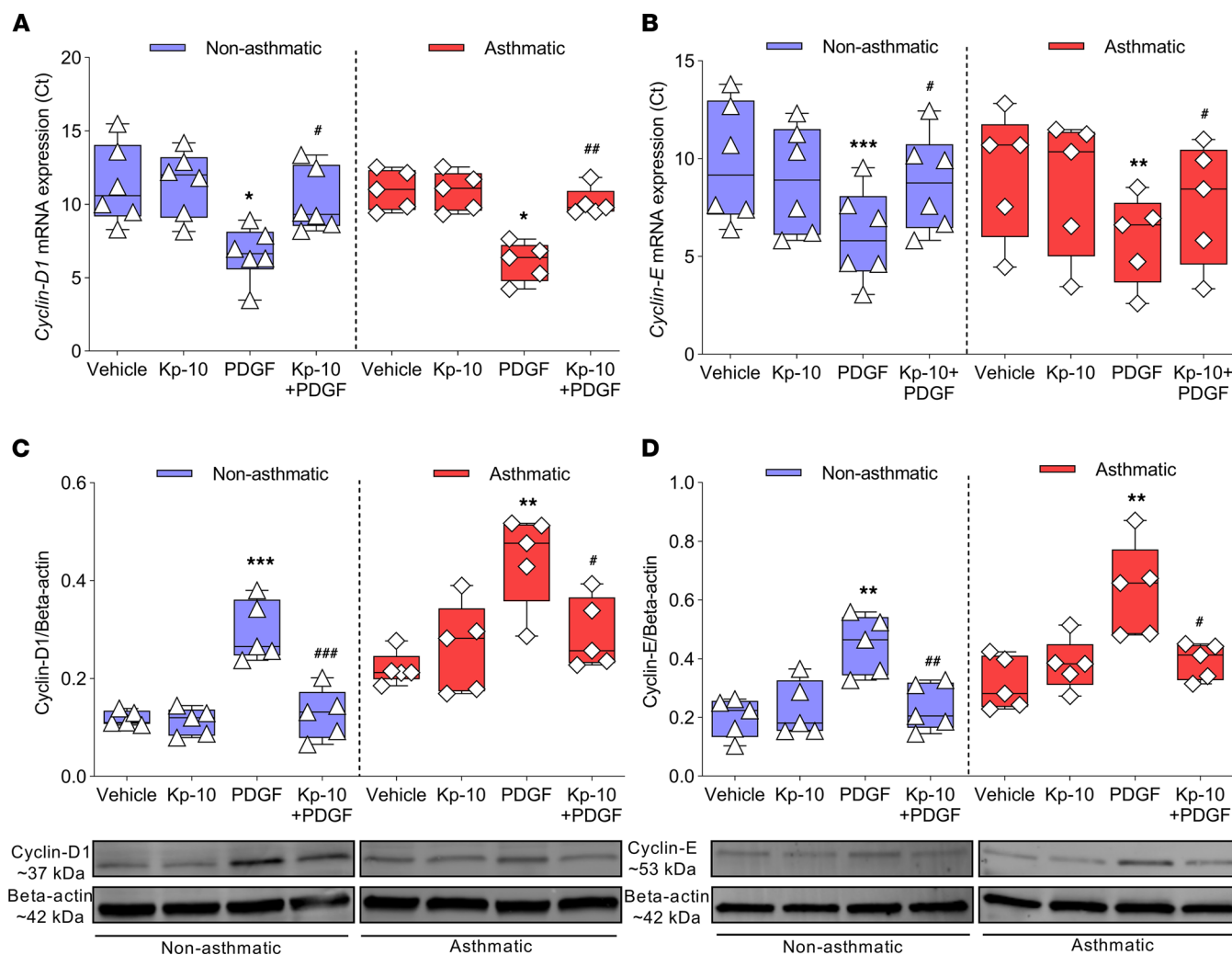
The relevance of Kp/KISS1R expression and signaling in airways also lies in the known sex differences of asthma (1, 3, 4, 75). Although there has been much exploration of sex-steroid effects, particularly estrogens and their effects on ASM proliferation (13), intracellular calcium handling (16, 76, 77), extracellular matrix dynamics (14), and cell migration (43), it remains unclear whether female sex steroids are protective or detrimental in asthma, given clinical observations of catamenial asthma, and some laboratory studies showing estrogens enhance inflammation (23, 78) but others associating estrogens with an asthma-mitigating role (13, 24, 79–84). Accordingly, it becomes important to consider if mechanisms upstream or even independent of sex hormones play a role or, alternatively, if asthma involves loss of intrinsic protective mechanisms. Here, we found sex differences in Kp and KISS1R expression in human ASM, particularly in people with asthma. How such differences contribute to the sex differences in airway structure and function or to change in asthma, particularly in the concurrent presence of sex steroids, remains to be explored.

Overall, our findings suggest that Kp/KISS1R is abundantly expressed in the human airways but is lower in ASM of individuals with asthma. KISS1R activation plays a protective role in regulating ASM proliferation, which is lost during asthma, thereby potentially permitting exacerbated ASM remodeling. This proliferation modulatory effect of cleaved Kp (Kp-10) in ASM is via KISS1R-mediated inhibition of p38 MAPK/ERK/Akt signaling pathways, thereby limiting the transcription of PCNA, C/EBP- $\alpha$ , Ki-67, cyclin D1, and cyclin E. Although not the primary focus of this study, we show for the first time to our knowledge the differential expression of Kp/KISS1R in women compared with men, which may underlie intrinsic differences and endogenous effects in vitro. Therefore, we lay the foundation for further exploratory studies on Kp/KISS1R signaling in the airways.

## Methods

### Chemicals, drugs, inhibitors, Abs

Cell culture reagents, DMEM/F12, antibiotic-antimycotic (AbAm), and 0.25% trypsin-EDTA were obtained from Invitrogen. Charcoal-stripped FBS was procured from Sigma-Aldrich. Human recombinant PDGF-BB (PHG0046) and protease and phosphatase inhibitor cocktail (P178445) were purchased from Thermo Fisher Scientific. Kp-10 (Metastatin 45-54, KISS1R agonist; sc-221884), control shRNA lentiviral particles-A (sc-108080), GPR54 shRNA lentiviral particles (sc-60747-V), primary Abs for Kp (sc-101246), PCNA (sc-25280), C/EBP- $\alpha$  (sc-365318), cyclin D1 (sc-8396), cyclin E (sc-377100), p38 (sc-271120), Akt1/2/3 (sc-81434), and p-ERK1/2 (Tyr204; sc-7383) were obtained from Santa Cruz Biotechnology, Inc. KI Kp-234 trifluoroacetate (3881) was purchased from Tocris Bioscience. Primary Ab for KISS1R (PA5-27179) was obtained from Thermo Fisher Scientific. Kp-13 (50-194-6708) was procured from Bachem. Kp-14 (crb1000930) was purchased from Discovery Peptides. Kp-54 (1443) was obtained from R&D Systems. Human Kisspeptin ELISA Kit (MBS3803580) was procured from MyBiosource and the LDH-Cytotoxicity Colorimetric Assay Kit (K311) from BioVision. p-p38 (Thr180/Tyr182) (4511S), p-Akt (Ser473; 4060S), ERK1/2 (9102S), and  $\alpha$ -SMA (19245T) were procured from Cell Signaling Technologies.  $\beta$ -Actin Ab (G043) was purchased from Applied Biological Materials. IRDye goat anti-mouse and goat anti-rabbit secondary Abs were used for scanning in Li-Cor Odyssey CLX (Li-Cor Systems). Other chemicals and reagents were procured from Sigma-Aldrich unless otherwise indicated.



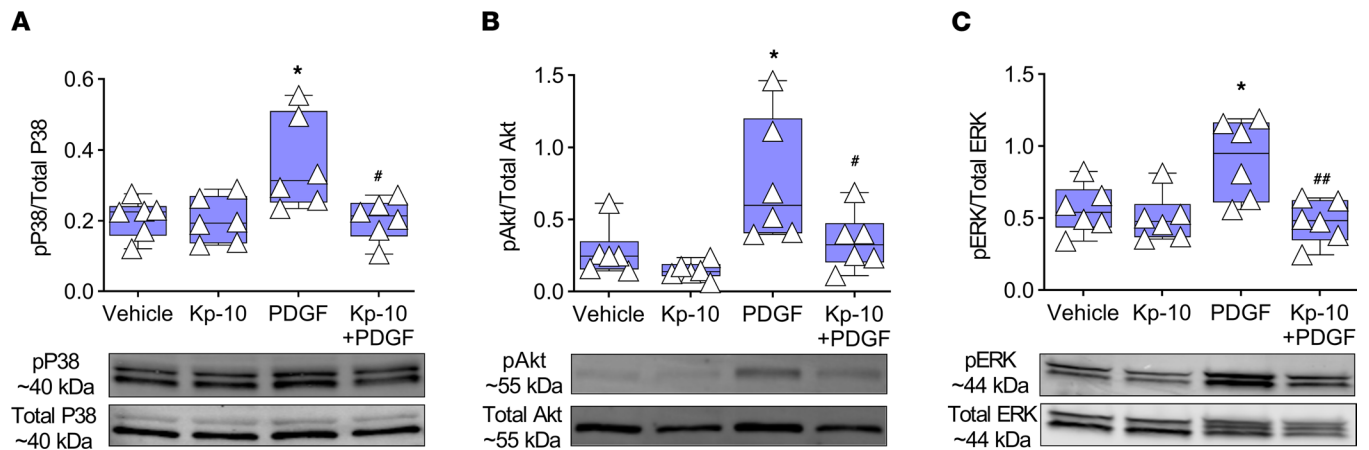
**Figure 9. Effect of Kp-10 on PDGF-induced human ASM cell cycle progression proteins.** mRNA and protein expression of cyclin D1 (A and C) and cyclin E (B and D) were significantly increased in ASM cells of individuals with and without asthma upon PDGF exposure. Pretreatment with Kp-10 significantly decreased PDGF-induced mRNA and protein expressions of cyclin D1 and cyclin E. Data are reported as a minimum to maximum of 5 to 6 individual samples from donors with and without asthma and analyzed using 1-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. respective vehicle; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs. PDGF-exposed groups.

### Tissue and ASM cells

The procedure for acquiring human lung samples and isolating primary human ASM cells has been described previously (85, 86). FFPE human lung tissue sections were used for immunofluorescence studies. Airway samples denuded of epithelium and ASM tissue were enzymatically dissociated per the manufacturer's instructions (Worthington Biochemical) to generate ASM cells. For cells, cultures (before the fifth passage) were maintained under standard conditions of 37°C (5% CO<sub>2</sub>, 95% air) using DMEM/F12 supplemented with 10% FBS and 1% AbAm.

### Immunofluorescence studies

Standard immunofluorescence techniques were applied to 6 µm thick human lung sections. Briefly, sections were baked (56°C for 2 hours) and deparaffinized using xylene and ethanol. Sodium citrate buffer (pH 6.0) was used for antigen retrieval by steaming and further rehydrated in Millipore water. Sections were then permeabilized with 0.1% Triton X-100 in PBS (1XPBS), blocked with 10% goat serum, and incubated with Abs against Kp, KISS1R, and α-SMA. Alexa Fluor 488 for α-SMA and Alexa Fluor 647 for Kp and KISS1R were used as secondary Abs with DAPI-AF408 counterstaining for nuclei. High-resolution Z-stack images were captured on a confocal microscope (Zeiss-LSM900 with Airyscan2) (87).



**Figure 10. Effect of Kp-10 on human ASM proliferative signaling pathways.** Western blot analysis showed PDGF-induced activation of p38 (A) Akt (B), and ERK1/2 (C) in human ASM cells. Pretreatment with Kp-10 significantly reduced the PDGF-induced phosphorylation of p38, Akt, and ERK1/2. Data are reported as a minimum to maximum of 6 individual samples from people without asthma and analyzed using 1-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$  vs. vehicle; # $P < 0.05$ , ## $P < 0.01$  vs. PDGF-exposed groups.

For immunofluorescence detection in human ASM cells, the cells were fixed with 4% paraformaldehyde in 1XPBS, pH 7.2, washed twice with PBS, and permeabilized using 0.05% Triton X-100 in PBS (the permeabilization step was omitted for KISS1R), washed twice with PBS, and blocked with 10% goat serum. After blocking, cells were incubated overnight at 4°C with polyclonal rabbit anti-KISS1R and monoclonal mouse anti-Kp Abs in different wells. Primary Abs were detected with Alexa Fluor 647 secondary Abs for Kp and KISS1R, using phalloidin as a smooth muscle marker, with DAPI-AF408 counterstaining for nuclei. Images were acquired with the Zeiss confocal microscope (88, 89).

### Cell treatments

Human ASM cells grown to confluence in T-75 flasks were trypsinized and mixed in 10% FBS-containing medium, counted, and seeded into 100 mm culture Petri plates (for RNA, protein, and flow cytometry) or 96-well plates (~7000 cells/well for proliferation studies). Cells were allowed to adhere overnight and were washed twice with PBS. After washing, serum medium was replaced with serum-free medium for 24 hours to synchronize cell growth. We used 1% serum medium as a vehicle to maintain the quiescent phase for the proliferation study. Initial studies showed that among the Kp fragments, Kp-10 was the most effective in modulating ASM proliferation; accordingly, we focused subsequent studies on this fragment. Efficacy and ASM toxicity of Kp-10 were determined using 3-log concentrations (0.1  $\mu$ M, 1  $\mu$ M, and 10  $\mu$ M), alone and in the presence of PDGF (2 ng/mL) (13, 14). ASM cells were treated with different concentrations of Kp-10 or KI (90, 91) to obtain initial optimal concentration, and all subsequent experiments followed a single concentration of Kp-10 or KI. The cytotoxicity of Kp-10 was measured by LDH-Cytotoxicity Colorimetric Assay Kit per the manufacturer's protocol. To measure the endogenous ASM Kp secretion, the media were collected after 48 hours of serum deprivation and concentrated equally to 500  $\mu$ L using Amicon Ultra-15 Centrifugal Filter Units (UFC901024). The endogenous Kp levels in the concentrated conditioned media were measured using the Kp ELISA kit per the manufacturer's protocol. Human ASM cells were exposed to Kp-10 or KI in the presence or absence of PDGF, added after 2 hours of preincubation with respective treatment groups, and incubated for a total of 6 hours (RNA) and 24 hours (proteins) (13).

### shRNA lentiviral particle transduction

ASM cells from individuals with and without asthma were cultured in 6-well plates to approximately 50% to 60% confluence. Transfection was achieved using a 20- $\mu$ L viral stock containing  $1 \times 10^5$  infectious units of the virus for control and KISS1R/GPR54 shRNA lentiviral particles. Once cells reached 60% to 70%, serum medium was replaced with fresh 5% serum medium (no antibiotics) and with polybrene (Santa Cruz Biotechnology; sc-134220). Lentiviral particles were thawed, added to the cells, and the cells were incubated overnight. The medium was replaced with a 5% serum medium containing 1% AbAm without polybrene and further incubated for 24 hours. After ensuring growth, the medium was replaced with a 10% serum

medium with antibiotics for 48 hours. For the selection of cells stably transfected with shRNA, the medium was replaced with a 10% serum medium containing 5 µg/mL puromycin and incubated for 48 to 72 hours. Cells were replenished with medium every 3 to 4 days until several puromycin-resistant colonies were identified. Once colonies reached 50% confluence, cells were expanded by transferring them into T-25 or T-75 cell culture flasks. Efficacy and successful shRNA transduction were verified by Western blot analysis.

### Cell proliferation assays

**BF cell count.** After 24 hours of respective treatments, total numbers of cells were counted in each of the 96-well plates, using a high-contrast, bright-field, direct cell counting on a Lionheart FX Automated Microscope (BioTek Instruments) (13).

**MTT cell proliferation assay.** After cell counting, the medium was aspirated carefully from the wells and replaced with 100 µL of 1% serum medium. For each well, 10 µL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL] reagent was then added and incubated at 37°C for 4 hours. The medium was aspirated, followed by the addition of 100 µL of DMSO, and cells were maintained with gentle shaking for 15 minutes at room temperature. Absorbance at 570 nm was then measured using a Synergy HTX Multi-Mode Plate Reader (BioTek Instruments).

### Flow cytometry

We seeded 12-well culture plates with ASM cells from individuals with and without asthma, and these were treated with appropriate treatments for 24 hours. Cells were trypsinized and fixed with ice-cold ethanol, followed by centrifugation at 2,000g. Pellets were washed and resuspended in PBS followed by PI (3 µM) staining for 15 minutes. PI-stained cells were analyzed using a BD Accuri C6 Flow cytometer (BD Biosciences). A minimum of 40,000 events were captured per sample and cells were analyzed for G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases using BD Accuri C6 Plus software (13).

### Western blot analysis

Human ASM cell lysates for individual treatment groups were prepared using cell lysis buffer as previously described (25). Briefly, cells were washed once with PBS and thereafter vortexed in lysis buffer supplemented with protease and phosphatase inhibitors, and subsequent supernatants were determined for protein content using a DC Protein Assay Kit (Bio-Rad). From respective treatment groups, 30 µg of equivalent protein was loaded on 4% to 15% gradient gels (Criterion Gel System; Bio-Rad) and transferred onto 0.22 µm PVDF membranes using a Bio-Rad Trans-Blot Turbo rapid transfer system. As a blocking buffer, we used 5% BSA in Tris-buffered saline for 1 hour at room temperature, and membranes were incubated overnight at 4°C with specific primary Abs of interest. After 3 washes for 8 min/wash with Tris-buffered saline containing 0.1% Tween, blots were incubated with LiCOR near-red conjugated secondary Abs at room temperature for 1 hour. β-Actin was used as a loading control. Protein expression was determined by imaging the membrane on a Li-Cor Odyssey XL system, and densitometry analysis was performed using Image Studio Lite software. Western blot analysis was performed by normalizing the raw values of the protein of interest to respective raw values of β-actin.

### qRT-PCR analysis

Cells were washed with RNA-grade PBS before trypsinization and then proceeded to RNA isolation. RNA cell isolation was performed using the Quick-RNA MiniPrep Kit (Zymo Research), and OneScript cDNA Synthesis Kit (Applied Biological Materials) was used for cDNA synthesis using a minimum of 500 ng of RNA for each sample. Genomic DNA contamination was avoided by using DNase I treatment. BrightGreen 2× qPCR Master Mix (Applied Biological Materials, Master-Mix-S-XL) was used on the QuantStudio 3 RT-PCR system following the manufacturer's instructions. The following primers were used for qRT-PCR analysis ("h" before a primer refers to human): hKISS1 (forward 5'-CAAGCCTCAAGGCACTTCTA-3'; reverse 5'-AAAGTGGGTGGCACAGAG-3'); hKISS1R (forward 5'-ATCGGAATTCACCATGCACACCGTGG-3'; reverse 5'-ATCATCTAGAACAGATAGCCGC-3'); hPCNA (forward 5'-GCCTGAATGGCGAATGGA-3'; reverse 5'-GAAGGGAAGAAAGCGAAAGGA-3'); hKi67 (forward 5'-GCTTACTCCGACGATTCT-3'; reverse 5'-GCCGATGCTTGCAATAGTTAG-3'); hC/EBP-α (forward 5'-ATTGGACCCAGAGAAGTTGAC-3'; reverse 5'-TCAGACCATTAAAGTCTTCAGAGAT-3'); hcyclin-D1 (forward 5'-GACGGG



TAGAACCTCAGTAATC-3'; reverse 5'-CTCGGGTGTTCCTATAATC-3'); hcyclin E (forward 5'-GTCCTGGCTGAATGTATACATGC-3'; reverse 5'-CCCTATTTTGTTCAGACATGGC-3'); and hS16 (forward 5'-CAATGGTCTCATCAAGGTGAACGG-3'; reverse 5'-CTGACGGATAGCATAAATCTGGGC-3'). Ct values of target mRNA were normalized to reference gene s16 (13, 92). Final graphs were plotted using a range of Ct values for each mRNA, and accuracy was confirmed by a single peak in the melt curves.

### Statistics

Human ASM cells from at least 5 donors (men and women; without asthma and with asthma) were used for all experiments. For expression studies, cell lysates for Western blot analysis and cDNA for qRT-PCR were obtained from at least 5 different, individual donor samples. Statistical analysis was performed using a 1-tailed unpaired *t* test or 1- or 2-way ANOVA followed by Tukey's post hoc multiple comparisons test using GraphPad Prism version 9.1.0 for Windows. Statistical significance was tested at a minimum level of  $P < 0.05$ . All values are expressed as mean  $\pm$  SEM.

### Study approval

Human bronchi from third to sixth generations were isolated from lung specimen incidental to donor thoracic surgeries at Mayo Clinic (focal, noninfectious indications; typically, lobectomies, rarely pneumonectomies). Normal lung areas were identified by a pathologist. Donors with no prior history of obstructive lung disease (including chronic obstructive pulmonary disease) were considered to have an otherwise normal lung function and classified as not having asthma (deidentified pulmonary conditions of the donors). The protocols were approved by the Mayo Clinic Institutional Review Board (IRB no. 08-002518).

### Author contributions

VS, CMP, and YSP conceptualized and designed the experimental plan. NAB, NSA, and RSRK performed experimental studies; NAB, NSA, RSRK, YSP, CMP, and VS performed analyses and interpretation; and NAB, NSA, RSRK, YSP, CMP, and VS wrote the drafts and final version of the manuscript. All authors reviewed and approved the final version.

### Acknowledgments

This research was supported by NIH grants R01-HL123494 and R01-HL146705 (to VS), R01-HL142061 (to CMP and YSP), and R01-HL088029 (to YSP). The authors also acknowledge the DaCCoTA-CTR confocal Microscopy Core Facility supported by NIGMS-U54GM128729 grant.

Address correspondence to: Venkatachalem Sathish, Department of Pharmaceutical Sciences, North Dakota State University, Fargo, North Dakota 58102, USA. Phone: 701.231.6371; Email: s.venkatachalem@ndsu.edu.

1. Borkar NA, Sathish V. Sex Steroids and Their Influence in Lung Diseases Across the Lifespan In: Silveyra P, Tigno XT, eds. *Sex-Based Differences in Lung Physiology*. Springer; 2021:39–72.
2. Ambhore NS, et al. Sex-steroid signaling in lung diseases and inflammation. *Adv Exp Med Biol*. 2021;1303:243–273.
3. Sathish V, Prakash Y. Sex Differences in Pulmonary Anatomy and Physiology: Implications for Health and Disease. In: Neigh GN, Mitzelfelt MM, eds. *Sex Differences in Physiology*. Elsevier; 2016:89–103.
4. Sathish V, et al. Sex steroid signaling: implications for lung diseases. *Pharmacol Ther*. 2015;150:94–108.
5. Saeed MA, et al. Association of dietary fiber on asthma, respiratory symptoms, and inflammation in the adult national health and nutrition examination survey population. *Ann Am Thorac Soc*. 2020;17(9):1062–1068.
6. James A. Remodelling of airway smooth muscle in asthma: what sort do you have? *Clin Exp Allergy*. 2005;35(6):703–707.
7. Townsend EA, et al. Sex differences and sex steroids in lung health and disease. *Endocr Rev*. 2012;33(1):1–47.
8. Ambhore NS, et al. Role of differential estrogen receptor activation in airway hyperreactivity and remodeling in a murine model of asthma. *Am J Respir Cell Mol Biol*. 2019;61(4):469–480.
9. Kalidhindi RSR, et al. Role of estrogen receptors  $\alpha$  and  $\beta$  in a murine model of asthma: exacerbated airway hyperresponsiveness and remodeling in ER $\beta$  knockout mice. *Front Pharmacol*. 2019;10:1499.
10. Kalidhindi RSR, et al. Androgen receptor activation alleviates airway hyperresponsiveness, inflammation, and remodeling in a murine model of asthma. *Am J Physiol Lung Cell Mol Physiol*. 2021;320(5):L803–L818.
11. An SS, et al. Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. *Eur Respir J*. 2007;29(5):834–860.
12. Prakash Y. Airway smooth muscle in airway reactivity and remodeling: what have we learned? *Am J Physiol Lung Cell Mol Physiol*. 2013;305(12):L912–L933.

13. Ambhore NS, et al. Estrogen receptor beta signaling inhibits PDGF induced human airway smooth muscle proliferation. *Mol Cell Endocrinol.* 2018;476:37–47.
14. Ambhore NS, et al. Differential estrogen-receptor activation regulates extracellular matrix deposition in human airway smooth muscle remodeling via NF- $\kappa$ B pathway. *FASEB J.* 2019;33(12):13935–13950.
15. Noble PB, et al. Airway smooth muscle in asthma: linking contraction and mechanotransduction to disease pathogenesis and remodelling. *Pulm Pharmacol Ther.* 2014;29(2):96–107.
16. Bhallamudi S, et al. Estrogen receptors differentially regulate intracellular calcium handling in human nonasthmatic and asthmatic airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* 2020;318(1):L112–L124.
17. Hirst SJ, et al. Phenotypic diversity and molecular mechanisms of airway smooth muscle proliferation in asthma. *Eur Respir J.* 2000;16(1):159–177.
18. Lazaar AL, et al. Airway smooth muscle: a modulator of airway remodeling in asthma. *J Allergy Clin Immunol.* 2005;116(3):488–495.
19. De S, et al. Role of phospholipase C and tyrosine kinase systems in growth response of human airway smooth muscle cells. *Am J Physiol.* 1996;270(5 pt 1):L795–L802.
20. Melgert BN, et al. Are there reasons why adult asthma is more common in females? *Curr Allergy Asthma Rep.* 2007;7(2):143–150.
21. Matteis M, et al. Effects of sex hormones on bronchial reactivity during the menstrual cycle. *BMC Pulm Med.* 2014;14(1):101–108.
22. Agarwal AK, Shah A. Menstrual-linked asthma. *J Asthma.* 1997;34(6):539–545.
23. Sakazaki F, et al. 17 $\beta$ -estradiol enhances expression of inflammatory cytokines and inducible nitric oxide synthase in mouse contact hypersensitivity. *Int Immunopharmacol.* 2008;8(5):654–660.
24. Myers JR, Sherman CB. Should supplemental estrogens be used as steroid-sparing agents in asthmatic women? *Chest.* 1994;106(1):318–319.
25. Aravamudan B, et al. Differential expression of estrogen receptor variants in response to inflammation signals in human airway smooth muscle. *J Cell Physiol.* 2017;232(7):1754–1760.
26. Keselman A, Heller N. Estrogen signaling modulates allergic inflammation and contributes to sex differences in asthma. *Front Immunol.* 2015;6:568.
27. Santoro N, et al. Characterization of reproductive hormonal dynamics in the perimenopause. *J Clin Endocrinol Metab.* 1996;81(4):1495–1501.
28. Terasawa E, et al. Kisspeptin and puberty in mammals. *Adv Exp Med Biol.* 2013;784:253–273.
29. Dungan HM, et al. The role of kisspeptin-GPR54 signaling in the tonic regulation and surge release of gonadotropin-releasing hormone/luteinizing hormone. *J Neurosci.* 2007;27(44):12088–12095.
30. Kauffman AS. Gonadal and nongonadal regulation of sex differences in hypothalamic Kiss1 neurones. *J Neuroendocrinol.* 2010;22(7):682–691.
31. Ohtaki T, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature.* 2001;411(6837):613–617.
32. Kotani M, et al. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem.* 2001;276(37):34631–34636.
33. Farrell PM, Avery ME. Hyaline membrane disease. *Am Rev Respir Dis.* 1975;111(5):657–688.
34. Guan-Zhen Y, et al. Reduced protein expression of metastasis-related genes (nm23, KiSS1, KAI1 and p53) in lymph node and liver metastases of gastric cancer. *Int J Exp Pathol.* 2007;88(3):175–183.
35. d'Anglemont de Tassigny X, Colledge WH. The role of kisspeptin signaling in reproduction. *Physiology (Bethesda).* 2010;25(4):207–217.
36. Seminara SB, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med.* 2003;349(17):1614–1627.
37. Masui T, et al. Metastin and its variant forms suppress migration of pancreatic cancer cells. *Biochem Biophys Res Commun.* 2004;315(1):85–92.
38. d'Anglemont de Tassigny X, et al. Mechanistic insights into the more potent effect of KP-54 compared to KP-10 in vivo. *PLoS One.* 2017;12(5):e0176821.
39. Halayko AJ, et al. Airway smooth muscle phenotype and function: interactions with current asthma therapies. *Curr Drug Targets.* 2006;7(5):525–540.
40. Johnson PR, et al. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med.* 2001;164(3):474–477.
41. Black JL, et al. Airway smooth muscle—its relationship to the extracellular matrix. *Respir Physiol Neurobiol.* 2003;137(2–3):339–346.
42. Chung KF. Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? *Eur Respir J.* 2000;15(5):961–968.
43. Ambhore N, et al. Estrogen regulates lamellipodial and focal adhesion dynamics in airway smooth muscle cell migration. *Am J Respir Crit Care Med.* 2021;203:A4482.
44. Raju KRS, et al. Salicylic acid derivatives as potential anti asthmatic agents using disease responsive drug delivery system for prophylactic therapy of allergic asthma. *Med Hypotheses.* 2016;87:75–79.
45. Banerjee P, et al. Network and co-expression analysis of airway smooth muscle cell transcriptome delineates potential gene signatures in asthma. *Sci Rep.* 2021;11(1):14386.
46. Bousquet J, et al. Asthma: a disease remodeling the airways. *Allergy.* 1992;47(1):3–11.
47. Bergeron C, et al. Remodeling in asthma. *Proc Am Thorac Soc.* 2009;6(3):301–305.
48. Halwani R, et al. Airway remodeling in asthma. *Curr Opin Pharmacol.* 2010;10(3):236–245.
49. Zhang WX, Li CC. Airway remodeling: a potential therapeutic target in asthma. *World J Pediatr.* 2011;7(2):124–128.
50. Seow CY, van Eeden S. Airway remodeling in asthma: tumor of the airway? *Respiration.* 2013;86(5):364–366.
51. Hirota N, Martin JG. Mechanisms of airway remodeling. *Chest.* 2013;144(3):1026–1032.
52. Kirby HR, et al. International Union of Basic and Clinical Pharmacology. LXXVII. Kisspeptin receptor nomenclature, distribution, and function. *Pharmacol Rev.* 2010;62(4):565–578.
53. Cho SG, et al. KiSS1 suppresses TNF $\alpha$ -induced breast cancer cell invasion via an inhibition of RhoA-mediated NF- $\kappa$ B activation. *J Cell Biochem.* 2009;107(6):1139–1149.

54. Yan C, et al. KiSS-1 represses 92-kDa type IV collagenase expression by down-regulating NF-kappa B binding to the promoter as a consequence of Ikappa Balpha -induced block of p65/p50 nuclear translocation. *J Biol Chem.* 2001;276(2):1164–1172.
55. Castano JP, et al. Intracellular signaling pathways activated by kisspeptins through GPR54: do multiple signals underlie function diversity? *Peptides.* 2009;30(1):10–15.
56. Yang G, et al. Baicalin inhibits PDGF-induced proliferation and migration of airway smooth muscle cells. *Int J Clin Exp Med.* 2015;8(11):20532–20539.
57. Seidel P, et al. DMF inhibits PDGF-BB induced airway smooth muscle cell proliferation through induction of heme-oxygenase-1. *Respir Res.* 2010;11(1):145.
58. Aravamudan B, et al. Brain-derived neurotrophic factor induces proliferation of human airway smooth muscle cells. *J Cell Mol Med.* 2012;16(4):812–823.
59. Dong L-H, et al. Baicalin inhibits PDGF-BB-stimulated vascular smooth muscle cell proliferation through suppressing PDGFRβ-ERK signaling and increase in p27 accumulation and prevents injury-induced neointimal hyperplasia. *Cell Res.* 2010;20(11):1252–1262.
60. Becker JA, et al. Activation of GPR54 promotes cell cycle arrest and apoptosis of human tumor cells through a specific transcriptional program not shared by other Gq-coupled receptors. *Biochem Biophys Res Commun.* 2005;326(3):677–686.
61. Wylam ME, et al. Mechanisms of cigarette smoke effects on human airway smooth muscle. *PLoS One.* 2015;10(6):e0128778.
62. Ruffell D, et al. A CREB-C/EBPβ cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A.* 2009;106(41):17475–17480.
63. Miglino N, et al. House dust mite extract downregulates C/EBPα in asthmatic bronchial smooth muscle cells. *Eur Respir J.* 2011;38(1):50–58.
64. Borger P, et al. Impaired translation of CCAAT/enhancer binding protein alpha mRNA in bronchial smooth muscle cells of asthmatic patients. *J Allergy Clin Immunol.* 2009;123(3):639–645.
65. Sun X, Kaufman PD. Ki-67: more than a proliferation marker. *Chromosoma.* 2018;127(2):175–186.
66. Zhai W, et al. Mitogen-activated protein kinase signalling pathways in IL-1β-dependent rat airway smooth muscle proliferation. *Br J Pharmacol.* 2004;143(8):1042–1049.
67. Gerthoffer WT, Singer CA. MAPK regulation of gene expression in airway smooth muscle. *Respir Physiol Neurobiol.* 2003;137(2):237–250.
68. Borkar NA, et al. Nicotinic α7 acetylcholine receptor (α7nAChR) in human airway smooth muscle. *Arch Biochem Biophys.* 2021;706:108897.
69. Hu KL, et al. Kisspeptin/kisspeptin receptor system in the ovary. *Front Endocrinol (Lausanne).* 2017;8:365.
70. Zhu N, et al. The KiSS-1/GPR54 system: essential roles in physiological homeostasis and cancer biology. *Genes Dis.* 2022;9(1):28–40.
71. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev.* 2004;18(16):1926–1945.
72. Garat CV, et al. Platelet-derived growth factor BB induces nuclear export and proteasomal degradation of CREB via phosphatidylinositol 3-kinase/Akt signaling in pulmonary artery smooth muscle cells. *Mol Cell Biol.* 2006;26(13):4934–4948.
73. Lipton JO, Sahin M. The neurology of mTOR. *Neuron.* 2014;84(2):275–291.
74. Harms JF, et al. KISS1 metastasis suppression and emergent pathways. *Clin Exp Metastasis.* 2003;20(1):11–18.
75. Balzano G, et al. Asthma and sex hormones. *Allergy.* 2001;56(1):13–20.
76. Kalidhindi RSR, et al. Androgen receptor-mediated regulation of intracellular calcium in human airway smooth muscle cells. *Cell Physiol Biochem.* 2019;53(1):215–228.
77. Bhallamudi S, et al. Estrogen receptor β attenuates contraction of human airway smooth muscle cells and ex vivo murine airways. *Am J Respir Crit Care Med.* 2021;203:A4481.
78. Riffo-Vasquez Y, et al. Role of sex hormones in allergic inflammation in mice. *Clin Exp Allergy.* 2007;37(3):459–470.
79. Carey MA, et al. It's all about sex: gender, lung development and lung disease. *Trends Endocrinol Metab.* 2007;18(8):308–313.
80. Dimitropoulou C, et al. Estrogen replacement therapy prevents airway dysfunction in a murine model of allergen-induced asthma. *Lung.* 2009;187(2):116–127.
81. Haggerty CL, et al. The impact of estrogen and progesterone on asthma. *Ann Allergy Asthma Immunol.* 2003;90(3):284–291.
82. Lieberman D, et al. Influence of estrogen replacement therapy on airway reactivity. *Respiration.* 1995;62(4):205–208.
83. Matsubara S, et al. Estrogen determines sex differences in airway responsiveness after allergen exposure. *Am J Respir Cell Mol Biol.* 2008;38(5):501–508.
84. Sathish V, et al. Cigarette smoke and estrogen signaling in human airway smooth muscle. *Cell Physiol Biochem.* 2015;36(3):1101–1115.
85. Abcejo AJ, et al. Brain-derived neurotrophic factor enhances calcium regulatory mechanisms in human airway smooth muscle. *PLoS One.* 2012;7(8):e44343.
86. Sathish V, et al. Caveolin-1 regulation of store-operated Ca(2+) influx in human airway smooth muscle. *Eur Respir J.* 2012;40(2):470–478.
87. Kalidhindi RSR, et al. Sex steroids skew ACE2 expression in human airway: a contributing factor to sex differences in COVID-19? *Am J Physiol Lung Cell Mol Physiol.* 2020;319(5):L843–L847.
88. Yarova PL, et al. Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma. *Sci Transl Med.* 2015;7(284):284ra60.
89. Rasmussen I, et al. Effects of F/G-actin ratio and actin turn-over rate on NADPH oxidase activity in microglia. *BMC Immunol.* 2010;11:44.
90. Takeda T, et al. Prognostic role of KiSS-1 and possibility of therapeutic modality of metastasis, the final peptide of the KiSS-1 gene, in urothelial carcinoma. *Mol Cancer Ther.* 2012;11(4):853–863.
91. Pasquier J, et al. Molecular evolution of GPCRs: kisspeptin/kisspeptin receptors. *J Mol Endocrinol.* 2014;52(3):T101–T117.
92. Loganathan J, et al. Laser-capture microdissection of murine lung for differential cellular RNA analysis. *Cell Tissue Res.* 2019;376(3):425–432.